A Synthetic Lethal Screen Identifies a Role for the Cortical Actin Patch/Endocytosis Complex in the Response to Nutrient Deprivation in *Saccharomyces cerevisiae*

Alison Care,[1] Katherine A. Vousden,[1,2] Katie M. Binley,[1,3] Pippa Radcliffe,[1,2] Janet Trevethick, Ilaria Mannazzu[4] and Peter E. Sudbery[5]

Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

Manuscript received July 28, 2003
Accepted for publication October 27, 2003

ABSTRACT

*Saccharomyces cerevisiae whi2*Δ cells are unable to halt cell division in response to nutrient limitation and are sensitive to a wide variety of stresses. A synthetic lethal screen resulted in the isolation of *siw* mutants that had a phenotype similar to that of *whi2*Δ. Among these were mutations affecting *SIW14*, *FEN2*, *SLT2*, and *THR4*. Fluid-phase endocytosis is severely reduced or abolished in *whi2*Δ, *siw14*Δ, *fen2*Δ, and *thr4*Δ mutants. Furthermore, *whi2*Δ and *siw14*Δ mutants produce large actin clumps in stationary phase similar to those seen in *prk1*Δ *ark1*Δ mutants defective in protein kinases that regulate the actin cytoskeleton. Overexpression of *SIW14* in a *prk1*Δ strain resulted in a loss of cortical actin patches and cables and was lethal. Overexpression of *SIW14* also rescued the caffeine sensitivity of the *slt2* mutant isolated in the screen, but this was not due to alteration of the phosphorylation state of Slt2. These observations suggest that endocytosis and the organization of the actin cytoskeleton are required for the proper response to nutrient limitation. This hypothesis is supported by the observation that *rvs161*Δ, *sla1*Δ, *sla2*Δ, *vrp1*Δ, *ypt51*Δ, *ypt52*Δ, and *end3*Δ mutations, which disrupt the organization of the actin cytoskeleton and/or reduce endocytosis, have a phenotype similar to that of *whi2*Δ mutants.

TO achieve balanced growth and proliferation it is necessary that cells cease division when there are insufficient nutrients. When cells of the budding yeast *Saccharomyces cerevisiae* are starved of nutrients, they arrest in the G_1 phase of the cell cycle in an unbudded, phase-bright state (Pringle and Hartwell 1981). A series of physiological changes occurs, which allows cells to survive adverse environmental conditions (Snow 1966; Schenberg-Frascino and Moustacchi 1972; Deutch and Parry 1973; Parry *et al.* 1976; Lillie and Pringle 1980). Cells carrying a *whi2* mutation fail to show this response (Sudbery *et al.* 1980; Saul *et al.* 1985). As *whi2*Δ cells approach stationary phase, cell division continues beyond the point where it ceases in wild-type cells and is accompanied by prolonged expression of the G_1 cyclins Cln1 and Cln2 (Radcliffe *et al.* 1997). As a result of continued division without growth, *whi2*Δ cells are abnormally small in stationary phase. Furthermore, they fail to acquire the stress resistance shown by wild-type cells (Saul *et al.* 1985). Exponentially growing *whi2*Δ cells also show increased sensitivity to certain environmental stresses, such as caffeine and 1.0 M NaCl (Binley *et al.* 1999).

Recently it has been shown that Whi2 acts in the stress-response pathway (Kaida *et al.* 2002). When *whi2*Δ cells are exposed to 37°, 0.4 M NaCl, or 0.4 mM H_2O_2, the expression of stress-response genes mediated by stress-response elements (STREs) in their promoters was reduced to 50% of wild-type levels (Kaida *et al.* 2002). STRE-mediated gene expression is also induced as wild-type cells enter stationary phase, but induction is delayed by several hours in *whi2*Δ cells and cell division continues (Kaida *et al.* 2002). Whi2 physically interacts with Psr1, one of a pair of redundant phosphatases located in the plasma membrane (Kaida *et al.* 2002). A *psr1*Δ *psr2*Δ mutant has phenotypes similar to those of *whi2*Δ with respect to sensitivity to Na^+ ions and small size in stationary phase. Furthermore, expression of STRE-mediated stress-response genes is also reduced in a *psr*Δ*1 psr2*Δ mutant. In addition to Psr1, Whi2 also physically interacts with Msn2, a transcription factor that plays a key role in the regulation of stress-response genes (Martinez-Pastor *et al.* 1996). Msn2 is hyperphosphorylated in *whi2*Δ or *psr1 psr2* mutants (Kaida *et al.* 2002) and overexpression of *MSN2* rescues the heat sensitivity of *whi2*Δ or *psr1 psr2* mutants (Kaida *et al.* 2002). While Psr1 and Psr2 are cell surface proteins (Siniossoglou *et al.* 2000), Msn2 shuttles between the nucleus and cyto-

[1]These authors contributed equally to this work.

[2]*Present address:* Cambridge Antibody Technology, Cambridge CB1 6GH, United Kingdom.

[3]*Present address:* Oxford Biomedica, Oxford OX4 4GA, United Kingdom.

[4]*Present address:* Dipartimento di Biotechnologie Agrarie, Ed Ambientali Universita' Di Ancona, Ancona 60100, Italy.

[5]*Corresponding author:* Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom. E-mail: p.sudbery@sheffield.ac.uk.

TABLE 1

Strains used in this study

Strain	Genotype	Source
Y763	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200*	M. Snyder
W303a	*MAT***a** *ade2-1, can1-100, his3-11,15 leu2-3,112 trp1-1 ura3[psi$^+$] GAL1 ssd1-d*	M. Tyers
PAR5	As Y763 *whi2ΔHIS3*	This work
PAR5-pPR14	As PAR5 containing pPR14	This work
PAR6	As Y763 *fen2^{siw1}* pPR14	This work
KTv3	As Y763 *fen2Δ::URA3*	This work
KTd5	As W303a *fen2Δ::URA3*	This work
siw9	As PAR5 *mpk^{siw9}* pPR14	This work
siw14	As PAR5 *siw14* pPR14	This work
KTv7	As Y763 *siw14Δ::URA3*	This work
KTd7	As W303a *siw14Δ::URA3*	This work
siw17	As PAR5 *prs3^{siw17}*	This work
KTd11	As W303a *prs3Δ::URA3*	This work
siw5	As PAR5 *alg9^{siw5}* pPR14	This work
IM1	As Y763 *alg9Δ::URA3* pPR14	This work
siw7	As PAR5 *zds1^{siw5}* pPR14	This work
IM2	As W303a *zds1Δ::URA3*	This work
Kad116	As Y763 *SLT2::3xHA*	This work
siw12	As *PAR 5 thr4^{siw12}*	This work
YAW17	As W303a *prk1ΔKanMX*	This work
YAW18	As W303a *ark1ΔKanMX*	This work
YAW24	As Y763 *thr4Δ::URA3*	This work
YAW25	As W303a *ypt52Δ::KanMX*	This work
YAW26	As W303a *ypt51Δ::kanMX*	This work
YAW27	As W303a *ypt53Δ::kanMX*	This work
DEY2934c	*MAT***a** *slt2Δ::TRP1 ade2-1 his3-11 leu2-3 ura3-1 can1-100*	D. Evans
KAY302	*MAT*α *trp1-1 leu2-3,112 lys2-801 his3-Δ200, ura3-52*	K. Ayscough
KAY378	As KAY302 *ark1Δ::HIS3 prk1Δ::URA3*	K. Ayscough
KAY0126	As KAY302 *abp1::LEU2*	K. Ayscough
KAY0254	*MAT***a** *ade2-101 his3Δ200 ura5-52 can1^r pan1::HIS3 CEN URA3* pan1-3	K. Ayscough
KAY300	As KAY302 *sla1Δ::LEU2*	K. Ayscough
KAY138	As KAY302 *sla2Δ::URA3*	K. Ayscough
BY472[a]	*MAT*α *his3Δ leu2Δ lys2Δ ura3Δ*	Research Genetics

[a] The following mutants were derived from BY472 by disruption with KanMX: *akr1Δ*, *ark1Δ*, *arl1Δ*, *clc1Δ*, *dnm1Δ*, *end3Δ*, *ent1Δ*, *ent2Δ*, *gaa1Δ*, *pkh1Δ*, *prk1Δ*, *swa2Δ*, *rvs161Δ*, *rvs167Δ*, *tlg2Δ*, *van1Δ*, *vps4Δ*, *vps34Δ*, *vrpΔ1*, *ypk1Δ*, *ypk2Δ*, and *slt2Δ* (Research Genetics).

plasm (Gorner *et al.* 1998). Whi2 interacts with both Msn2 and Psr1/2 and is located throughout the cell (Kaida *et al.* 2002). These observations suggest that Whi2 and Psr1/2 are functional partners that regulate the activity of Msn2 through its state of phosphorylation (Kaida *et al.* 2002).

In this article, we describe the use of a colony-sectoring assay to isolate mutants that have a more severe effect on fitness when combined with a *whi2Δ* mutation. Seven independent mutants that affect a variety of cell functions were recovered. None of the mutations were strictly colethal with *whi2Δ*, so we have called the mutants *siw* (synthetic interaction with *whi2Δ*). We show that *whi2Δ* and several of the *siw* mutants have defects in actin organization and endocytosis. Further, we demonstrate a genetic interaction between *SIW14* and *ARK1* and *PRK1*, which encodes a pair of redundant protein kinases that regulate the stability of actin cortical patches. Finally, we show that mutations known to cause defects in the actin cytoskeleton and in endocytosis fail to show cell cycle arrest upon nutrient deprivation. Thus, we conclude that the normal function of the actin cytoskeleton and endocytosis are required to coordinate cell proliferation with nutrient availability.

MATERIALS AND METHODS

Strains and culture conditions: Strains used in this article are described in Table 1. They were routinely cultured at 30° on YEPD [1% Difco yeast extract (Becton Dickinson, Sparks, MD), 2% Difco peptone, plus 2% glucose]. Plates were solidified by the addition of 2% Difco agar. Cells were grown to stationary phase as follows: 2.5 ml of YEPD in a 50-ml conical flask was inoculated with yeast from a fresh YEPD plate culture and incubated for 48 hr at 26° on a rotary shaker at a speed of 150 rpm. By this time, wild-type cells have arrested as unbudded cells that have a bright appearance when examined by

TABLE 2

Oligonucleotides used in this study

Oligonucleotide	Use
GCCACTATCGACTACGCGATCA	Sequencing insert in YCp50
CACGATGCGTCCGGCGTAGA	Sequencing insert in YCp50
AACGATGAATTGCAAGGCGGTAACCATTAGTTTATTACTGTTG TTTTAGTTTTGCTGGCCGCATC	*ALG9* disruption forward
CACCGTCGATGAATGCAGCACATGCCAGCTTATTCCAGTTATC CACAATGATGGGTAACAAGAGC	*ALG9* disruption reverse
ATGCTGCTGACTACATCAAGCGATAAGGCGATCGCTAGTCAA AGGTTAGTTTTGCTGGCCGCATA	*ZDS1* disruption forward
TATGTACGTGTGATGTGTATATGTCTATGTATGCAGCGCTGAA GCCAATGATGGGTAACAAGAGC	*ZDS1* disruption reverse
TTATTTGTAACCTCCTATCTTTAGTTGAACTGATCCAAAAACA CGGATCCCCGGGTTAATTAA	*PRK1* disruption forward
CTTTTAATATTACATAGTCTATTATGTGTGAGAGCAAGTTGAA TTCGAGCTCGTTTAAAC	*PRK1* disruption reverse
TACTCTGCATAATTAGGTATTTTAAGCAACCAGATAAATCAAC CGGATCCCCGGGTTAATTAA	*ARK1* disruption forward
CCTCTTCAGAGATCGATCCGGTTCTGTTGAGCCAAATACGAAT TCGAGCTCGTTTAAAC	*ARK1* disruption reverse
CAGTCACTTCCATAAAGTTGGTACTGTTGGGTGAGGCAGCCG GATCCCCGGGTTAATTAA	*VPS21* disruption forward
CCCCTCTCTAACAACTGCAAGCACTGTTTGCGCTGGTCCCGAA TTCGAGCTCGTTTAAAC	*VPS21* disruption reverse
GGTTTAACTTATTTGGAGTAAACGTATATATTATTAACAGCG GATCCCCGGGTTAATTAA	*YPT52* disruption forward
GCAACTTCTGTTGTTTTTCCTCTAAAACACAAATCATAGGGAA TTCGAGCTCGTTTAAAC	*YPT52* disruption reverse
CAGCATCGAAGTTAAGTAGAATGGATAAACATACAGCAGCCG GATCCCCGGGTTAATTAA	*YPT53* disruption forward
CGCTAAGACGCTGAATATGCCGCGTTTGCTGTTGTTTTGCGAA TTCGAGCTGCTTTAAAC	*YPT53* disruption reverse
CAACCGATACTGATACATAGTAATAGAGGCAAACATAGAACG	Mutagenesis of SIW14: C214S forward
CGTTCTATGTTTGCCTCTATTACTATGTATCAGTATCGGTTG	Mutagenesis of SIW14: C214S reverse
CTGATACATTGTAATAGAGCCAAACATAGAACGGGGTGTTTG	Mutagenesis of SIW14: G217A forward
CAAACACCCCGTTCTATGTTTGGCTCTATTACAATGTATCAG	Mutagenesis of SIW14: G217A reverse
GTAATAGAGGCAAACATAAAACGGGGTGTTTGATTGGTTG	Mutagenesis of SIW14: R220K forward
CAACCAATCAAACACCCCGTTGTATGTTTGCCTCTATTAC	Mutagenesis of SIW14: R220K reverse
GCGAAATGTTGGCAGAATGG	Cloning *sklt2*^{siw9} 5′ region forward
CCATACGTCAATCGCCTTGG	Cloning *sklt2*^{siw} 5′ region reverse
TTATTCGGAGAATCCTGTCG	Cloning *sklt2*^{siw} 3′ region forward
CGAGCTACAACAAGAGCACG	Cloning *sklt2*^{siw} 3′ region reverse

phase-contrast microscopy. The high ratio of flask size to culture volume is important for the development of the *whi2*Δ phenotype, which is manifested only under conditions of vigorous aeration (Rahman *et al.* 1988). Cell volume was measured with a ZB1 Coulter Counter and Channelyser as described previously (Sudbery *et al.* 1980). Values reported are the median of the volume distribution.

Cloning and characterization of *SIW* genes: Because of the similarity of the *siw* mutants to mutants affecting the *PKC1/SLT2* mitogen-activated protein (MAP) kinase pathway, *siw* mutants were transformed with plasmids carrying wild-type copies of all the genes in this pathway. The mutant originally designated *siw9* was complemented by a centromeric plasmid carrying the *SLT2* gene, suggesting that *siw9* is an allele of *SLT2*. This was confirmed by the observation that *siw9* failed to complement an *slt2*Δ mutation. This mutation is referred to as *slt2*^{siw9}. The remaining mutants were transformed with a genomic library constructed in YCp50, a centromeric *URA3* vector (Rose *et al.* 1987; purchased from the ATCC, http://www.atcc.org/). Ura$^+$ transformants, representing approximately five-genome equivalents, were replica plated onto YEPD plates containing 5 mm caffeine, and transformants that grew were retained for further study. Normally the clones that grew produced red sectors, indicating that there was no longer selection against the loss of plasmid pPR14 (*ADE2 WHI2*) used in the colony-sectoring screen. The dependency of caffeine-resistant growth on the presence of a plasmid from the library was examined by growing transformants on media containing 5′-fluroorotic acid (5′-FOA) that selects for cells that have lost the YCp50-based plasmid containing the *URA3* gene (Boeke *et al.* 1984). Colonies that grew on 5′-FOA were no longer able to grow on a YEPD plate containing 5 mm caffeine. Plasmids were recovered from independent colonies by transformation of *Escherichia coli* with total yeast DNA preparations. Recovered plasmids were transformed into the original *siw* mutant. Plasmids that both induced sectoring of pPR14 and

conferred wild-type levels of caffeine resistance were retained for further study. Two primers (Table 2) were used to sequence ~400 bp at either end of the genomic insert in the plasmids recovered. These sequences were compared to the *S. cerevisiae* genome database (http://genome-www.stanford.edu/Saccharomyces/) and the intervening sequence was retrieved. Subcloning was carried out using the derived restriction map of the insert to identify the minimum complementing region.

Gene deletions were carried out in two different strains: the Y763 strain used as the parent for the colony-sectoring assay (Costigan *et al.* 1992) and W303a, which is known to be *ssd1-1d* (Cvrckova *et al.* 1995). Deletions were carried out as follows. *FEN2* (*SIW1*) was deleted using a *URA3*-based disruption cassette kindly supplied by G. Lucchini. The deletion was verified by Southern hybridization. For *ALG9* (*SIW5*), *ZDS1* (*SIW7*), *THR4* (*SIW12*), and *SIW14* (YNLO32w), pairs of PCR primers were designed (Table 2) to amplify a *URA3* template so that the resulting DNA molecule consisted of the *URA3* gene flanked by 45 bp of DNA homologous to the sequence immediately upstream of the start codon and downstream of the stop codon of the gene to be deleted. In the case of *SIW14* (YNLO32w), *Bam*HI and *Hin*dIII restriction sites were incorporated into the primers between the YNLO32w and *URA3* sequences to aid subsequent Southern analysis of putative disruptants. PCR amplification of the *URA3* template was performed for 35 cycles with an annealing temperature of 57° and the resulting PCR fragment was used to transform yeast strains Y763 and W303a, both of which are *ura3*. Deletion of the target open reading frame was verified by Southern hybridization or PCR analysis.

In all cases, the deleted strain was more sensitive to caffeine than was the parent. A diploid was formed by crossing the disruptant and the original mutant. Allelism was demonstrated by noncomplementation of mutant phenotypes in the diploid and 4:0 segregation of caffeine sensitivity in tetrads dissected after sporulation. The deleted strain was also crossed to a strain with the wild-type *SIW* allele and tetrads were dissected. In all cases, caffeine sensitivity cosegregated with the auxotrophic marker used to engineer the deletion.

Characterization of the *slt2^{siw9}* mutation: PCR primers (Table 2) were designed to amplify the locus from the *slt2^{siw9}* mutant in two segments with an overlapping region spanning the *Sac*I site at nucleotide 600, each fragment containing a *Bgl*II or *Hin*dIII site present in the respective 5′ and 3′ flanking regions. The resulting PCR products were digested with either *Bgl*II and *Sac*I (5′ fragment) or *Hin*dIII and *Sac*I (3′ fragment) and ligated to pUC19 digested with *Hin*dIII and *Bgl*II. The full-length sequence of the resulting *slt2^{siw9}* clone was determined and compared to the wild-type sequence.

Mutagenesis of *SIW14*: Mutagenesis of *SIW14* cloned with its own promoter into the multicopy plasmid pYES2 (Invitrogen, San Diego) was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used are listed in Table 2. Following mutagenesis, the entire *SIW14* gene was resequenced for each mutant allele generated.

Western blotting: Cells were disrupted with glass beads and total soluble protein was prepared as described (Boyne *et al.* 2000). Proteins were fractionated by SDS/polyacrylamide gel electrophoresis and electroblotted to a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Primary antibody was rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204; Cell Signaling Technology, Beverly, MA) used at 1:1000 dilution. Two rounds of antibody binding were used to amplify the weak signal that resulted from this primary antibody. Secondary antibody was mouse anti-rabbit immunoglobulins (Jackson Immunoresearch, Cambridge, UK) used at 1:3500 dilution. Tertiary antibody was goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (Dako A/S, Glostrup, Denmark) diluted 1:5000. Binding of tertiary antibody was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) and recorded by a Gene Gnome chemiluminescence recording system (Syngene, Cambridge, UK).

Glycogen staining: Cells from 1 ml of stationary-phase cultures were collected by centrifugation and resuspended in 1 ml of 0.2% w/v iodine and 0.4% w/v potassium iodide. After 5 min, cells were collected by centrifugation and washed 3× in distilled water and finally resuspended in 0.2 ml of water. Each sample was placed in a well of a 96-well microtiter plate and photographed with transmitted light. Cells producing glycogen stained dark purple, while cells not producing glycogen remained light brown.

Actin staining: Cultures were stained with phalloidin conjugated to tetramethylrhodaminyl-isothiocyanate (TRITC) conjugated (Sigma, St. Louis) as previously described (Adams and Pringle 1983).

Heat shock: Stationary-phase cultures of 500 μl were heated using a Hybaid thermocyler for 10 min. Samples were diluted in triplicate and plated on YEPD and the mean number of colonies that grew after 3 days was recorded. For control cultures, unheated samples of the same cultures were diluted and plated in triplicate. Viability is expressed as percentage viability of the heated cultures relative to control.

Plate dilution tests: Cells were harvested from a freshly grown YEPD plate and resuspended in YEPD to an OD_{600} of 8.0. This suspension was then serially diluted and 5 μl of the undiluted and 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions were spotted onto the surface of the agar plate and cultures were incubated for 3 days at 30°. These dilutions resulted in ~20 cells in the spot from the most diluted suspension. All growth tests were carried out using YEPD media supplemented as indicated except for the 37° growth test in which minimal medium was used.

Lucifer yellow uptake assays: Lucifer yellow assays were carried out as described (Dulic *et al.* 1991).

Immunocytofluorescence: Immunocytofluorescence was carried out as described (Ayscough and Drubin 1998). Mouse anti-actin antibody was purchased from Sigma and used at a dilution of 1:200. Rabbit polyclonal anti-Cdc11 was purchased from Santa Cruz and used at 1:250 dilution. Mouse polyclonal antisera to Cof1, Sac6, and Abp1 were a kind gift from Kathryn Ayscough.

Microscopy: For differential interference contrast (DIC) and fluorescence microscopy, cells were examined with a Leica DMLB fluorescence microscope. Digital images were recorded by a low-temperature CCD camera (model RTE Princeton Instruments, Princeton, NJ) controlled by an Apple Macintosh G4 computer running Open Lab software, version 2.2.5 (Improvison, Warwick, UK). Images were exported as tif files and edited for contrast and brightness in Adobe Photoshop, version 5.5. Composite figures were assembled using Microsoft Power Point 2000.

RESULTS

A colony-sectoring assay identifies seven genes that interact with *whi2*Δ: To identify genes that may interact with *WHI2*, we carried out a colony-sectoring assay for mutations that either are colethal or show an enhanced growth defect with the *whi2*Δ allele. For this screen, we applied the methodology used by Costigan and Snyder (Costigan *et al.* 1992). Briefly, the screen is based on a *whi2*Δ *ade2-1* host that harbors an unstable plasmid

TABLE 3

Sensitivity of Y763, *whi2*Δ, and *siw* mutants to various conditions

Strain	% heat-shock survival (relative to wild type)	0.05 mM Calcofluor		5 mM caffeine			MM at 37°			1.0 M NaCl			6.6 mM $MnCl_2$		0.7 M $CaCl_2$		YEP glycerol	
		H	L	H	L	+S	H	L	+S	H	L	$+Ca^{2+}$	H	L	H	L	H	L
Y763	9.0 (1.0)	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
*whi2*Δ	0.4 (0.04)	+[a]	−	+[b]	−	+	+[b]	+[b]	+	+[2]	−	+	−	−	+	+	+[b]	+[b]
*fen2*Δ	ND	−	−	−	−	+	+	+	+	+	−	+	−	−	−	−	+	+
*alg9*Δ	11.7 (1.3)	+	+	+[c]	−	+	+	−	+	+	−	+	−	−	+	−	+	+
*zds1*Δ	6.9 (0.77)	+	−	−	−	−	+	−	+	+	−	+	−	−	+	+	+	−
slt2^{siw9}	ND	+	−	−	−	+	−	−	+	−	−	ND	−	−	+	−	−	−
siw12	ND	+	+	−	−	+	+	−	+	+	−	+	−	−	−	−	+	+
*siw14*Δ	0.4 (0.044)	+	+	−[d]	−	+	+	+[e]	+	+	+	+	+	+	+	+	−	−
*prs3*Δ	10 (1.1)	+	+	−	−	+	+	−[f]	ND	+	−[g]	ND	+	+	+	+	+	+

Five-microliter aliquots of serial dilutions were plated as described in MATERIALS AND METHODS. Growth is recorded at the highest cell densities (H)—$\sim 2 \times 10^5$ cells/drop—and at the lowest cell density (L)—$\sim$20 cells/drop. MM, minimal medium. +S, 1.0 M sorbitol was added to the medium and growth was recorded at the lowest plating density. $+Ca^{2+}$, 5 mM $CaCl_2$ was added to the medium.

[a] Sensitivity of *whi2* mutants to Calcofluor white is strain dependent; the most sensitive strain is *Iso34*, which harbors the original *whi2* mutation (SAUL and SUDBERY 1985).

[b] Sensitivity of *whi2* mutants to these treatments is dependent on the allelic status of the *SSD1* locus. In strain W303a (*ssd1-d*), growth of a *whi2*Δ mutant is completely blocked even at the highest cell densities. This increased sensitivity is rescued by *SSD1-1V* on a multicopy plasmid. Growth of Y763 *whi2*Δ strains is prevented at high cell densities by 7 mM caffeine.

[c] Growth is blocked by 6 mM caffeine.

[d] Inhibition of growth of the original mutant and an *siw14*Δ allele in the W303a background requires 7 mM caffeine.

[e] Growth of the original *siw14* mutant was temperature sensitive, but the deletion allele was not, regardless of strain background.

[f] Growth of the original mutant was not temperature sensitive.

[g] Growth of the original mutant was not inhibited by 1.0 M NaCl.

carrying a wild-type copy of *WHI2*. Mutations that have a more severe phenotype in conjunction with *whi2*Δ result in selection against loss of the *WHI2* plasmid and therefore produce homogeneous white colonies that lack red sectors. In this way we isolated seven mutants, which subsequent analysis showed affected the following genes: *ALG9*, *FEN2*, *PRS3*, *SIW14*, *SLT2* (*MPK1*), *THR4*, and *ZDS1* (see MATERIALS AND METHODS).

SIW14 encodes a protein that contains the sequence IHCNRGKHRTGCL, which is an exact match to the canonical sequence for tyrosine phosphatases, [LIVMF]HC xxGxxx[STC][STAG]x[LIVMFY], listed in the Prosite database (http://ca.expasy.org/prosite/). *SLT2* (*MPK1*) encodes the MAP kinase of the PKC1 MAP kinase pathway (TORRES *et al.* 1991; LEE *et al.* 1993). We refer to the allele isolated in our screen as *slt2*^{siw9}. We cloned *slt2*^{siw9} by PCR and showed that the mutation was E225K, an amino acid substitution in the conserved region IX of the kinase domain. *FEN2* was first identified in a screen for mutants resistant to fenproprimorph, an inhibitor of ergosterol biosynthesis (MARCIREAU *et al.* 1996) and it was subsequently shown that Fen2 is the membrane pantothenate transporter (STOLZ and SAUER 1999). *PRS3* is one of a family of five genes that encodes phosphoribosyl pyrophosphate synthetase, an enzyme that catalyzes the first step in a variety of biosynthetic pathways, including amino acid and nucleotide biosynthesis (CARTER *et al.* 1994, 1995). We have previously published a detailed characterization of *prs3*Δ (BINLEY *et al.* 1999). *ALG9* encodes a mannosyl transferase that transfers core oligosaccharides from the lipid carrier dolichol pyrophosphate to the asparagine residues of secreted proteins in the endoplasmic reticulum (BURDA *et al.* 1996). *THR4* encodes threonine synthetase, required for threonine biosynthesis. *ZDS1* has been isolated in many genetic screens, hence its name, which is derived from "zillion different screens" (BI and PRINGLE 1996; YU *et al.* 1996). Deletion of both *ZDS1* and *ZDS2* genes results in a mitotic delay and elongated buds with constrictions where cytokinesis has been attempted but not completed (YU *et al.* 1996; MIZUNUMA *et al.* 1998).

Deletion phenotype of genes identified in the colony-sectoring assay: *ALG9*, *FEN2*, *PRS3*, *SIW14*, *THR4*, and *ZDS1* were deleted in two strain backgrounds: Y763, the host strain for the synthetic lethal screen, and W303a, a strain carrying the *ssd1-1d* allele in which we found that the stress sensitivity of the *whi2*Δ phenotype is enhanced (see Table 3). Strains with a *slt2*Δ allele in two different backgrounds were obtained elsewhere (Table 1). We carried out appropriate crosses to congenic strains containing a *whi2*Δ allele to determine whether the deletion alleles were colethal with *whi2*Δ. In each case, viable double mutants could be constructed, showing that the mutations were not colethal with *whi2*Δ. However, upon reintroduction of the unstable *WHI2*-bearing plasmid into each of the double mutants, it was either absolutely

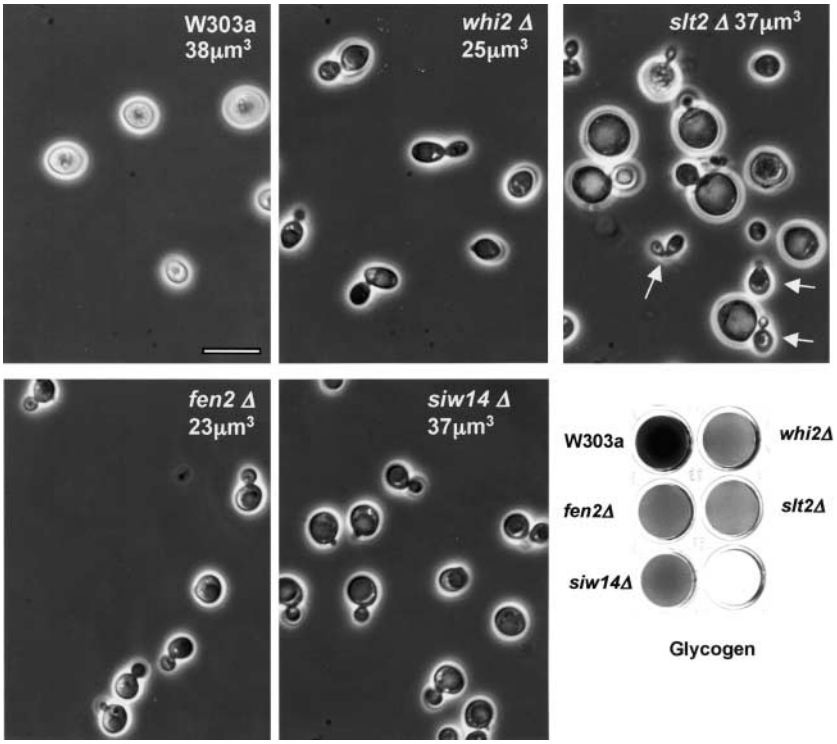

FIGURE 1.—Stationary-phase appearance of wild-type, *whi2*Δ, and *siw*Δ mutants. Cells with the indicated genotype were grown in YEPD for 2 days at 30°. The median cell volumes were determined and the appearance of cells was recorded using phase-contrast optics with a ×100 objective. Arrows show small cells in the *slt2*Δ strain. In the glycogen test, a positive result is indicated by a dark purple color while lack of accumulation results in light brown; these colors reproduce in the image shown here as dark gray/black and light gray, respectively. The strains grown were congenic to the wild-type W303a strain shown here. Y763, the parent strain for the synthetic lethal screen, and the respective *siw*Δ deletions in the Y763 background, had a similar appearance. Bar, 10 μm.

stable or lost at a very low frequency compared to a wild-type host. Often, where a red sector appeared it clearly grew more slowly than the rest of the colony (data not shown). Moreover, in some cases enhanced phenotypes were observed in the double mutant. For example, a *fen2*Δ *whi2*Δ mutant was unable to grow on minimal medium and an *slt2*Δ whi2Δ strain had a grossly abnormal morphology (data not shown). Thus, although not colethal, these mutations have a more severe phenotype when combined with *whi2*Δ. For this reason we designated these mutants *siw* (synthetic interaction with *whi2*Δ).

Most of the *siw* mutants showed stress sensitivities that were similar to those of a *whi2*Δ strain. Most were hypersensitive to caffeine and 1 M NaCl (Table 3). In addition, *alg9*Δ, *slt2*^{siw9}, and *zds1*Δ mutants were temperature sensitive on minimal medium; *fen2*Δ and *slt2*^{siw9} mutants were hypersensitive to 0.7 M $CaCl_2$; the *fen2*Δ mutant was sensitive to Calcofluor white, a characteristic of cell wall mutants; s*iw14*Δ and *slt2*^{siw9} were unable to utilize glycerol; and, finally, *siw14*Δ cells were resistant to 6.6 mM $MnCl_2$, which inhibits the growth of wild-type cells.

When grown to stationary phase in YEPD, *siw14*Δ, *fen2*Δ, and *slt2*Δ arrested as phase-dark, budded cells, which failed to accumulate glycogen (Figure 1). Moreover, *siw14*Δ cells were hypersensitive to a 53° heat shock (Table 3). Thus, like *whi2*, these mutants failed to have the normal physiological responses to nutrient limitation. There was no reduction in cell size. However, many of the mutants showed very large vacuoles and it is possible that the cytoplasmic mass is less than that of a wild-type cell. This is particularly marked in the case of the *slt2*Δ mutant where, interestingly, some very small cells are evident (arrows in Figure 1). In contrast, *alg9*Δ, *thr4*Δ, and *zds1*Δ mutants showed the normal responses to nutrient limitation (data not shown). However, the *alg9* and *zds1* alleles isolated in the colony-sectoring screen did arrest as phase-dark budded cells in stationary phase (data not shown). Thus, it is possible that the mutant alleles isolated in the screen have a different phenotype from that of the deletion alleles. However, the genetic complexity of the original mutants makes such a conclusion uncertain and we did not pursue this matter further.

Cytoskeletal abnormalities in stationary-phase *whi2*Δ and *siw14*Δ cells: We previously reported that *whi2*Δ cells contain abnormal actin clumps in stationary phase (BINLEY *et al.* 1999). We examined the *siw* mutants for any abnormalities in the actin cytoskeleton. While the actin distribution of growing cells was normal, we observed that stationary-phase *siw14*Δ cells had a single intensely staining clump of actin in >95% of the cells examined (Figure 2A). The clumps also contained other actin-associated proteins such as Abp1, Sac6, and Cof1 (Figure 2B). This abnormality was present in all strain backgrounds containing the *whi2*Δ and *siw14*Δ mutations. We also observed that *siw14*Δ stationary-phase cells contained abnormal clumps of the septin Cdc11, which is normally located in a ring structure at the bud neck (Figure 2C).

Endocytosis is defective in *whi2*Δ, *fen2*Δ, *thr4*Δ, and *siw14*Δ mutants: Defects in the cortical actin cytoskeleton are often associated with defects in endocytosis. To

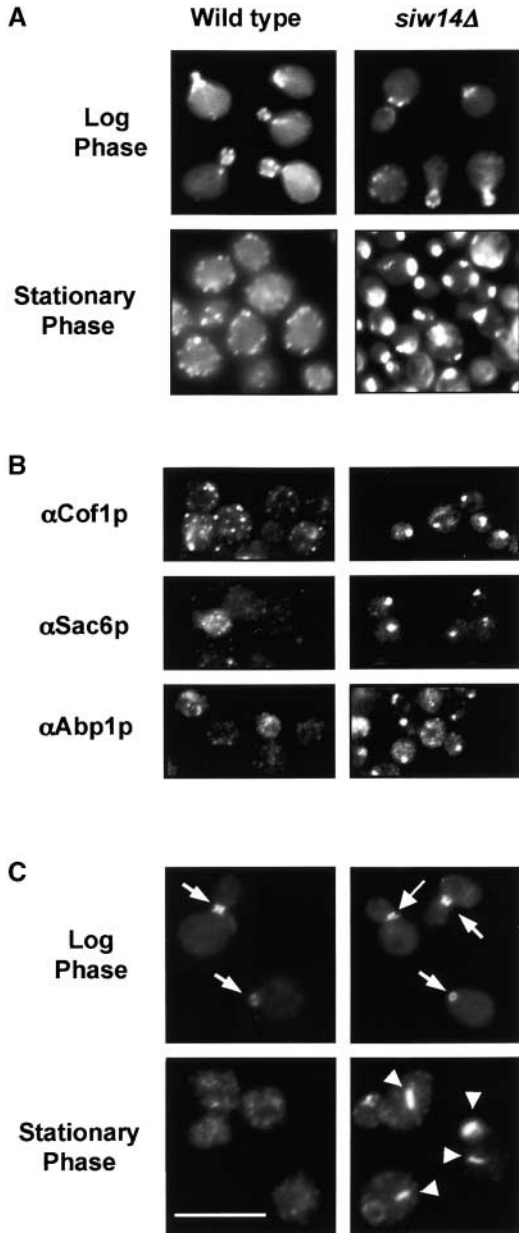

FIGURE 2.—*Siw14*Δ mutants in stationary phase show defects in the actin and septin cytoskeletons. Wild-type (W303a) and *siw14*Δ cells were grown in exponential or stationary phases as indicated. (A) Actin visualized by immunocytofluorescence using a mouse anti-actin antibody. (B) The indicated proteins visualized by immunocytofluorescence using mouse antisera (a kind gift from Kathryn Ayscough). (C) The septin cytoskeleton visualized by immunocytofluorescence using a polyclonal antibody to Cdc11. Arrows show normal septin ring structures. Arrowheads show abnormal septin bars in stationary-phase cells. Bar, 10 μm.

determine if fluid-phase endocytosis was functioning normally in *whi2*Δ and *siw* mutants, we carried out lucifer yellow uptake assays. The *whi2*Δ, *fen2*Δ, *thr4*Δ, and *siw14*Δ mutants were defective in fluid-phase endocytosis (Figure 3). The defect in *fen2*Δ mutants was particularly profound.

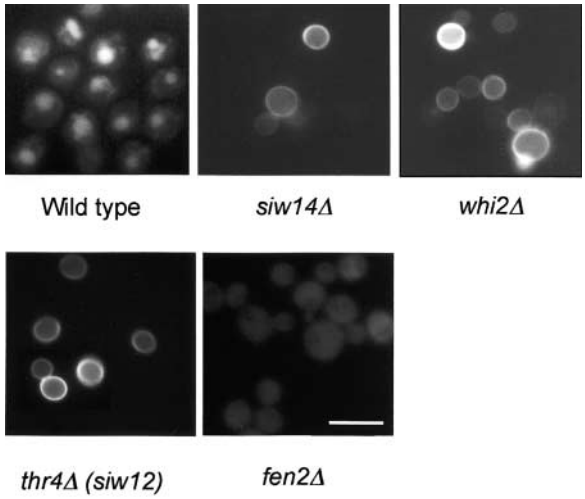

FIGURE 3.—*Whi2*Δ, *siw14*Δ, *fen2*Δ, and *thr4*Δ are defective in fluid-phase endocytosis. Wild-type (W303a) cells and the indicated mutants were tested for fluid-phase endocytosis using the lucifer yellow uptake assay. Bar, 10 μm.

***SIW14* interacts with *ARK1* and *PRK1*:** Ark1 and Prk1 are two homologous protein kinases that are thought to regulate the association of the actin cortical patch complex with the endocytic machinery by phosphorylating Pan1 and Sla1 (COPE *et al.* 1999; ZENG and CAI 1999; ZENG *et al.* 2001). The actin clumps in *whi2*Δ and *siw14*Δ mutants are strikingly similar to the actin clumps seen in *ark1*Δ *prk2*Δ cells (COPE *et al.* 1999). Because of this phenotypic similarity, we searched for interactions between *ARK1* and *PRK1* with *WHI2* and *SIW14*. We found that overexpression of *SIW14* from a *GAL1* promoter on a multicopy plasmid was lethal in a *prk1*Δ strain and reduced growth in an *ark1*Δ strain (Figure 4A). A high proportion of cells with no visible actin patches or cables accumulated within 4 hr upon induction of *SIW14* in a *prk1*Δ strain (Figure 4B).

Mutants in endocytosis and the actin cytoskeleton are defective in cell cycle arrest in stationary phase: The inability of *whi2*Δ and the *siw* mutants to arrest the cell cycle upon nutrient limitation could be a consequence of the defects in the actin cytoskeleton and endocytosis. To test this hypothesis, we determined whether mutations in genes known to function in endocytosis and the actin cytoskeleton have defects in cell cycle arrest. We monitored the effect on cell size and appearance in the stationary phase of deletion alleles of the following genes that have defects in actin organization or are listed in the Saccharomyces Genome Database as having defects in endocytosis: *AKR1*, *ARK1*, *ARL1*, *CLC1*, *DNM1*, *END3*, *ENT1*, *ENT2*, *PKH1*, *PRK1*, *SWA2*, *RVS161*, *RVS167*, *SLA1*, *SLA2*, *TLG2*, *VAN1*, *VPS 4*, *VPS34*, *VRP1*, *YPK1*, *YPK2*, *VPS21* (*YPT51*), *YPT52*, and *YPT53*. Of these, we found that *end3*Δ, *rvs161*Δ, *sla1*Δ, *vrp1*Δ, *sla2*Δ, *vps21*Δ (*ypt51*Δ), and *ypt52*Δ arrested in stationary phase as phase-dark budded cells, instead of the phase-bright unbudded ap-

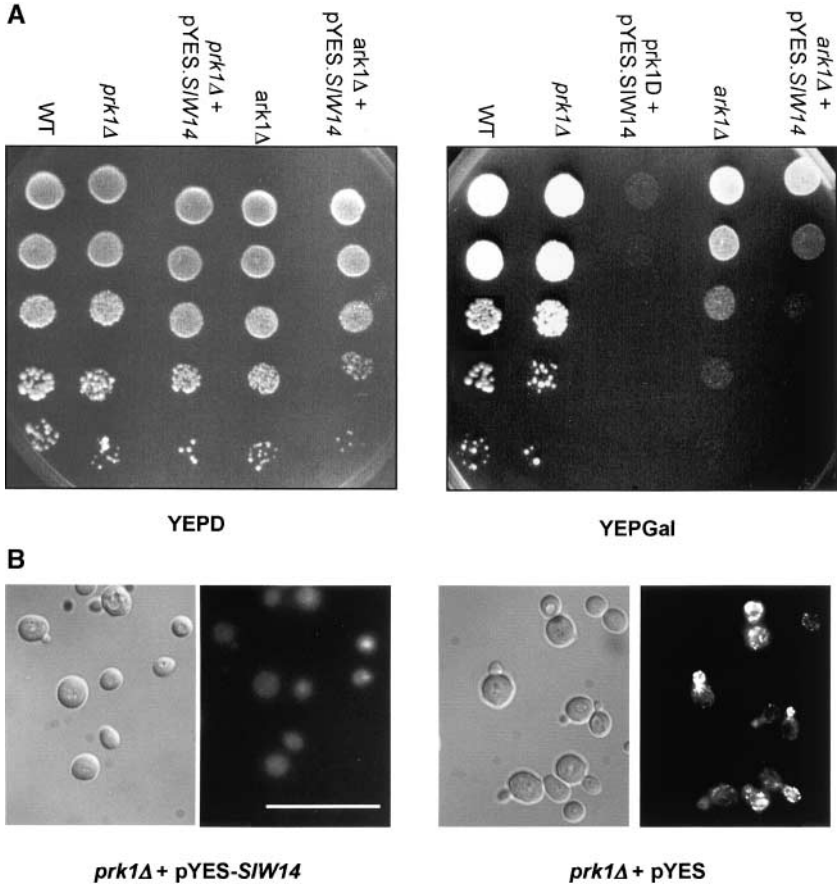

FIGURE 4.—Overexpression of *SIW14* is lethal in a *prk1*Δ mutant. (A) Wild-type (BY742) *prk1*Δ and *ark1*Δ mutants were transformed with either the multicopy pYES2 vector or pYES2 containing *SIW14* under the control of the *GAL1* promoter. Dilutions were plated on YEPD or YEPGal to induce the Gal1 promoter. (B) *Prk1*Δ cells transformed with either multicopy *SIW14* under the control of the *GAL1* promoter in pYES2 or the pYES2 vector alone. Transformed cells were grown to mid-exponential phase on YEP-raffinose to which 1% galactose was then added and the cells were incubated for another 4 hr. Actin was visualized using phalloidin-TRITC staining. In each case, the DIC image shows the same field of cells as those stained with phalloidin. Note that the majority of pYES2-*SIW14*-transformed cells in the DIC field do not stain with phalloidin.

pearance of wild-type cells (Figure 5). Furthermore, *vrp1*Δ and *rvs161*Δ cells were much smaller than wild-type cells: 52 and 65%, respectively, of the size of wild-type cells. The *ark1*Δ and *prk1*Δ mutants showed some indication that cell cycle arrest was not normal, but this was not so marked and an *ark1*Δ *prk1*Δ double mutant did not show a more severe defect in nutrient arrest (data not shown). All the other mutants listed had a similar appearance and size to wild-type cells except for the *akr*Δ mutant, which as noted before has an unusual "peanut" shape (PRYCIAK and HARTWELL 1996).

We examined *end3*Δ, *rvs161*Δ, *sla1*Δ, *sla2*Δ, *vrp1*Δ, *vps21*Δ (*ypt51*Δ), and *ypt52*Δ mutants to see if they also showed clumps of actin similar to that seen in *whi2*Δ and *siw14*Δ cells. Figure 6 shows that *end3*Δ, *sla1*Δ, *prk1*Δ, and *ypt52*Δ did indeed show such clumps. Thus, this type of actin disorganization is a feature of cells that are unable to respond appropriately to nutrient deprivation. Clumps of actin in *sla1*Δ mutants have been reported previously (HOLTZMAN *et al.* 1993).

***SIW14* shows a genetic interaction with *SLT2*:** The phenotypes of *whi2* and *siw* mutants and mutants affecting the PKC1/Slt2 MAP kinase share the following phenotypic abnormalities: (a) they are sensitive to caffeine and other stresses, and these sensitivities are rescued by 1 M sorbitol and exacerbated by a *ssd1-1d* allele; (b) they fail to show a normal response to nutrient limitation; and (c) they display defects in the actin cytoskeleton, which in *slt2*Δ mutants is marked by loss of polarity and the appearance of actin bars in the cytoplasm (COSTIGAN *et al.* 1992, 1994; MAZZONI *et al.* 1993). Moreover, we isolated an allele of *SLT2* in the synthetic lethal screen. We therefore searched for interactions between the *siw* mutants and *Slt2*. We found that *SIW14* on a multicopy plasmid could rescue the caffeine (Figure 7), but not the temperature sensitivity of the $slt2^{siw9}$ mutation (data not shown). The minimum inhibitory concentration of caffeine of $slt2^{siw9}$ is 4 mM, whereas that of a congenic *slt2*Δ strain is 2 mM. It is therefore likely that the $slt2^{siw9}$ kinase retains some activity. Interestingly, multicopy *SIW14* exacerbated the caffeine sensitivity of the congenic *slt2*Δ strain, so that growth on 1 mM caffeine was prevented (Figure 7). Thus, multicopy *SIW14* interacts with *SLT2* in an allele-specific fashion: it rescues the caffeine sensitivity of the $slt2^{siw9}$ allele but enhances the caffeine sensitivity of an *slt2*Δ allele. To determine whether the putative tyrosine phosphatase active site was required for the interaction, we generated the following point mutations in three conserved residues in the putative tyrosine phosphatase active site: C214S, G217A, and R220K. The C214S mutation changes a critical cysteine residue that participates in catalysis and is known to abolish tyrosine phosphatase activity (JOHNSON *et al.* 1992). Multicopy plasmids car-

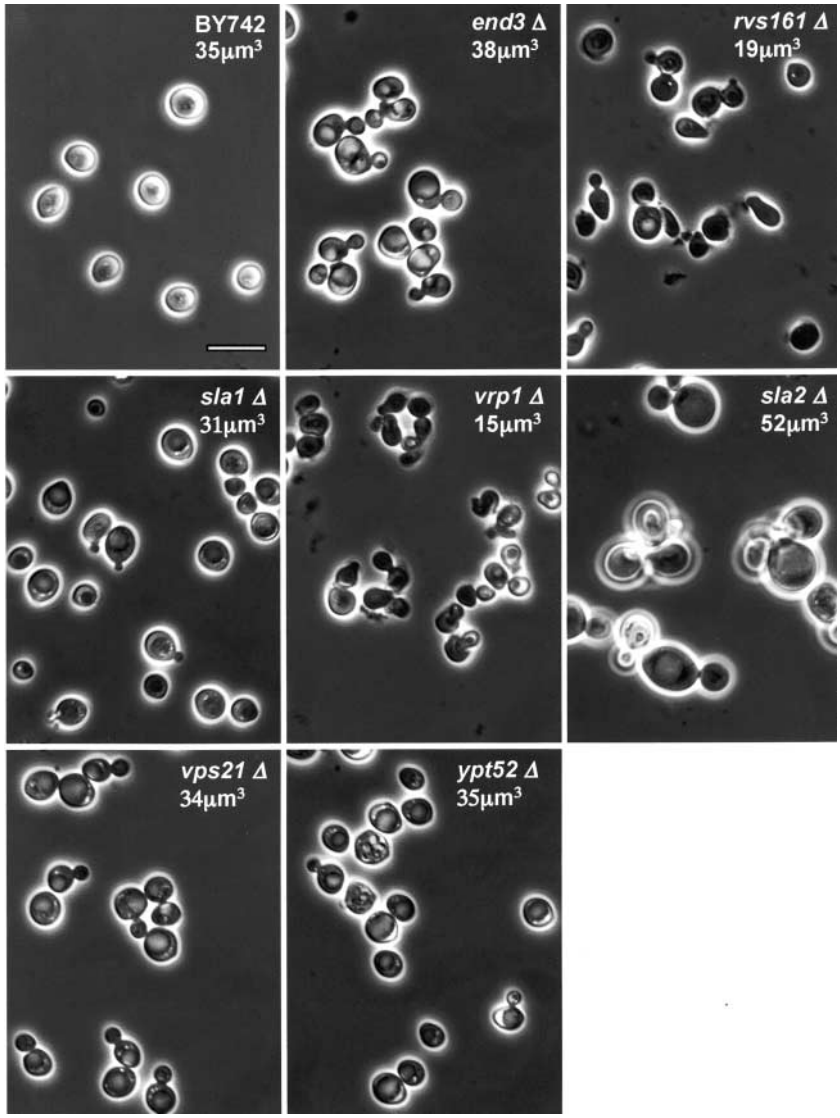

FIGURE 5.—Actin cortical patch/endocytosis mutants fail to show the normal response to nutrient deprivation. Cells with the indicated genotype were grown to stationary phase, photographed, and median cell volumes were determined as described in Figure 1. The wild-type strain shown here is BY742 since it is the parent for all strains except *vps51*Δ and *ypt52*Δ, which are congenic to W303a. W303a has an identical appearance to BY742 (see Figure 1).

rying these catalytically dead versions of Siw14 were unable to rescue the caffeine sensitivity of *slt2*^{siw9} and failed to enhance the caffeine sensitivity of *slt2*Δ (Figure 7). Thus, the interaction between Siw14 and Slt2 depends on a functioning tyrosine phosphatase active site. Surprisingly, the mutants were still able to rescue the caffeine sensitivity of an *siw14*Δ mutation (Figure 7), indicating that the tyrosine phosphatase active site is not required for at least some of the normal functions of Siw14.

Slt2 is activated by dual phosphorylation on threonine 190 and tyrosine 192 (LEE *et al.* 1993). Since Siw14 is a putative tyrosine phosphatase, it is possible that multicopy *SIW14* affects the phosphorylation state of Slt2. To determine if this were the case, we used an antibody specific to the activated form of Slt2 to monitor the phosphorylation state of Slt2 (MARTIN *et al.* 2000). Phosphorylation of Slt2 increased upon exposure to caffeine as reported previously (Figure 8; MARTIN *et al.* 2000). Although the level of activating phosphorylation was reproducibly reduced in an *slt2*^{siw9} mutant upon caffeine exposure, perhaps explaining its reduced activity, the level of phosphorylation was unaffected by multicopy *SIW14*. Furthermore, the level of phosphorylation of wild-type Slt2 was also unaffected by deletion or overexpression of *SIW14*. Thus, Siw14 does not act directly to dephosphorylate Slt2.

DISCUSSION

***Whi2*Δ cells are defective in the organization of the actin cytoskeleton and endocytosis:** We have shown here that the effect of a *whi2*Δ allele is pleiotropic. In addition to the failure to respond to nutrient limitation by ceasing cell division, *whi2*Δ cells are sensitive to a wide range of stresses, have a disorganized actin cytoskeleton upon nutrient limitation, and are defective in fluid-phase endocytosis. Overexpression of *WHI2* has been reported to rescue the stress sensitivity of an *rsp5* mutation (KAIDA *et al.* 2002). Rsp5 is a ubiquitin protein ligase that is

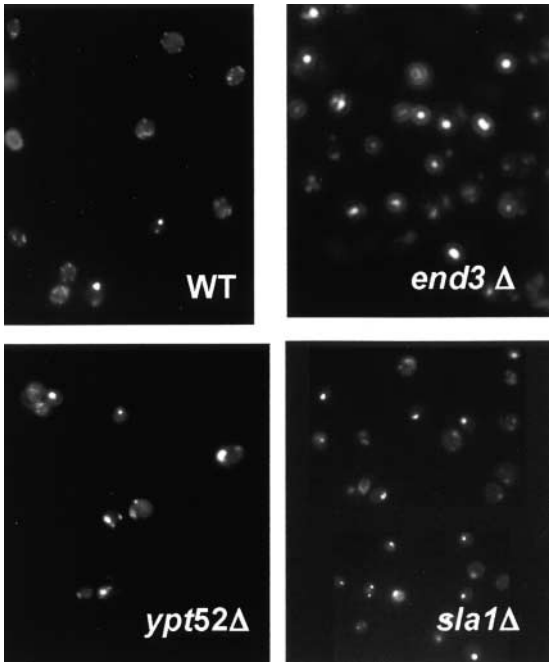

FIGURE 6.—Actin cortical patch/endocytosis mutants accumulate actin clumps in stationary phase. Wild-type (WT) and the indicated mutants were grown to stationary phase as described in Figure 1 and actin was visualized by rhodamine-phalloidin staining.

thought to regulate the endocytic machinery and is required for normal stress response (reviewed in ROTIN *et al.* 2000). It is responsible for the mono-ubiquitination and consequent internalization of many membrane proteins such as permeases, transporters, and receptors, including the Ste2 α-factor receptor. Rsp5 interacts with Pan1, a protein that links the endocytic machinery to the plasma membrane (ZOLADEK *et al.* 1997), and its location to the plasma membrane and perivacuolar structures is Sla2 dependent (WANG *et al.* 2001). The suppression of the *rsp5* phenotype by *WHI2* overexpression suggests that Whi2 may be a positive regulator of endocytosis, which would be consistent with the defects in endocytosis reported here in a *whi2*Δ mutant.

A synthetic lethal screen isolated further mutants with the same pleiotropic defects as *whi2*Δ: Using a colony-sectoring assay, we isolated a number of other mutants that had some or all of the phenotypes of a *whi2*Δ mutant. Of these *prs3*Δ, *fen2*Δ, and *siw14*Δ were particularly interesting since they showed sensitivity to stresses such as caffeine and 1 M NaCl and failed to respond by halting cell proliferation in response to nutrient depletion.

The amino acid sequence of Siw14 suggests that it is a member of a small family of tyrosine phosphatases. There are two homologs of *SIW14* in the yeast genome: *OCA1* (33% identity, 51% similarity) and YNL056w (35% identity and 58% similarity). Oca1 is required for cell cycle arrest in response to acid linoleic hydroperoxide, a lipid peroxidation product that accumulates during oxygen stress (ALIC *et al.* 2001), so other members of this family are also concerned with stress response. We mutated the active site of Siw14 and found that catalytically dead alleles still complement the caffeine sensitivity of the *siw14*Δ mutation. Clearly, Siw14 has a function that is not dependent on tyrosine phosphatase activity. However, it is likely that Siw14 does have tyrosine phosphatase activity, because the catalytically dead mutants are unable to suppress the caffeine sensitivity of the $slt2^{siw9}$ allele or to enhance the sensitivity of the *slt2*Δ allele. Thus, Siw14 appears to be a bifunctional protein.

The inability of *slt2*Δ mutants to arrest upon nutrient deprivation and an abnormal distribution of actin in *slt2*Δ cells has been reported previously (MAZZONI *et al.* 1993; COSTIGAN and SNYDER 1994). Null alleles of *SIW14* also show cytoskeletal defects. When depleted of nutrients, *siw14*Δ cells contain a single large clump of actin and associated proteins such as Abp1, Cof1, and Sac6. Furthermore, the septin cytoskeleton shows a similar phenotype, as Cdc11 accumulates in large bars. This phenotype is strikingly similar to that of an *ark1*Δ *prk1*Δ mutant. Ark1 and Prk1 are two protein kinases that regulate the actin cytoskeleton (COPE *et al.* 1999; ZENG and CAI 1999; ZENG *et al.* 2001). In an *ark1*Δ *prk1*Δ double mutant, actin was found in large clumps, similar to the appearance of actin clumps that we observe in

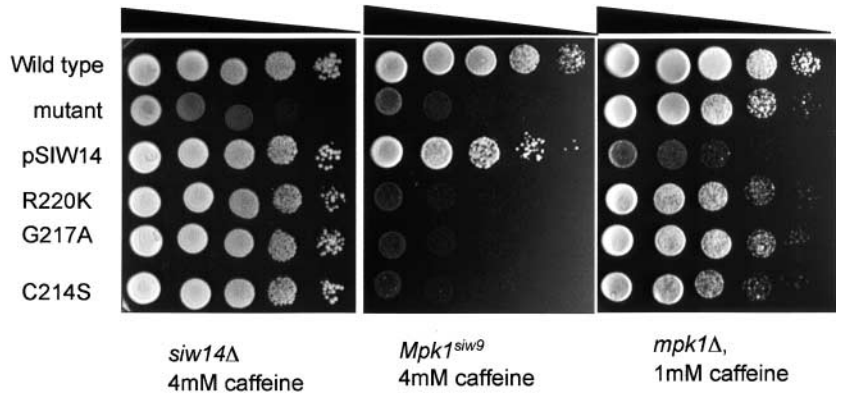

FIGURE 7.—Effect of overexpressing wild-type and mutant *SIW14* on the caffeine sensitivity of different *slt2* mutants. The top two rows show dilutions of wild-type (W303a) or mutant strains. The concentration of caffeine and the identity of the mutant are indicated at the bottom. The remaining rows show dilutions of the particular mutant strain transformed with wild type or the indicated mutants of *SIW14*, cloned with its own promoter into the 2-μm-based pYES2 plasmid.

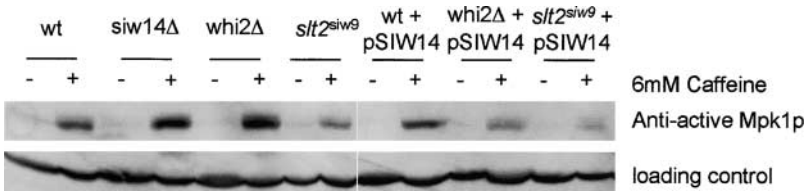

FIGURE 8.—Siw14 does not affect the phosphorylation state of Slt2. Exponentially growing wild-type (wt; W303a), *whi2*Δ, *siw14*Δ, and *slt2*^{siw9} strains, with or without a multicopy, p*SIW14* as indicated, were incubated in YEPD for 1 hr in the presence or absence of 6 mM caffeine. Total protein extracts were prepared and probed by Western blotting using polyclonal anti-active Slt2 antisera. The loading control is a nonspecific band that does not alter its intensity upon caffeine treatment.

stationary-phase *whi2*Δ and *siw14*Δ cells (COPE *et al.* 1999). Overexpression of either Prk1 or Ark1 results in a different type of actin abnormality: the formation of large cytoplasmic bars. We showed that overexpression of *SIW14* in a *prk1*Δ results in an apparent loss of all forms of filamentous actin and is lethal. The similar actin cytoskeletal defects in both *siw14*Δ and *ark1*Δ *prk1*Δ mutants as well as the genetic interaction between *prk1*Δ and *SIW14* overexpression suggest that *SIW14* may influence budding during nutrient deprivation through the actin cytoskeleton.

The actin cytoskeleton participates in endocytosis, and *siw14*Δ mutants are defective in fluid-phase endocytosis. Siw14 has been shown to physically interact with Ypt53, a Rab5-like GTPase required for vesicle-mediated transport (UETZ *et al.* 2000). *YPT53* shares homology with *YPT52* and *VPS21* (*YPT51*). It is thought that Vps21, Ypt52, and Ypt53 are required for the early steps in endocytosis (SINGER-KRUGER *et al.* 1994; PRESCIANOTTO-BASCHONG and RIEZMAN 2002). A null allele of *VPS21* causes an impairment of endocytosis that is more severe when combined with *ypt52*Δ and *ypt53*Δ alleles. We found that, like *siw14*Δ mutants, *vps21*Δ and *ypt52*Δ mutants upon nutrient limitation show a failure to arrest cells and have a disorganized actin cytoskeleton. We also found that *vps21*Δ and *ypt52*Δ mutants are sensitive to caffeine and 1 M NaCl and display actin clumps in stationary phase. We also examined a *ypt53*Δ strain but failed to see any phenotypic abnormalities. This may be because the function of Ypt53 is redundant with Vps21 and Ypt52. However, taken together, these observations support the conclusion that a normal response to nutrient and other stresses involves actin organization and endocytosis.

FEN2 was first identified in a search for mutants resistant to fenproprimorph, an inhibitor of ergosterol biosynthesis (MARCIREAU *et al.* 1996). Cells harboring a *fen2*Δ mutation were shown to have a threefold decrease in ergosterol levels. Subsequently, it was shown that Fen2 is a membrane pantothenate transporter (STOLZ and SAUER 1999). Pantothenate is essential for the biosynthesis of coenzyme A, which is a carrier of activated C_2 units in sterol biosynthesis. Thus, the reduction in ergosterol levels in a *fen2* mutant is due to reduced availability of pantothenate. Ergosterol is required for the proper fluidity and function of cell membranes and for endocytosis (HONGAY *et al.* 2002). Thus, the reduction in endocytosis in a *fen2* mutant that we observed can be satisfactorily explained by the known function of Fen2.

We isolated a *thr4* mutation in the synthetic lethal screen and we found that a *thr4*Δ mutant was sensitive to 1 M NaCl and was defective in endocytosis. The involvement of threonine synthetase was initially puzzling. However, a *thr4*Δ mutation was recovered in a screen for mutations that are colethal with *sec13*Δ, which is defective in the transport of the general amino acid permease from the Golgi to the cell surface (ROBERG *et al.* 1997). Thus, it is possible that Thr4 does have an unexpected role in protein trafficking.

Mutations known to affect the organization of the actin cytoskeleton and reduce endocytosis decouple growth and cell division: Although the molecular functions of the Whi2, Siw14, Fen2, and other Siw genes are diverse, they share the common property that mutations result in a disorganized actin cytoskeleton and/or reduce endocytosis. This observation led us to examine the phenotype of other mutants known to be defective in the organization of the actin cytoskeleton and endocytosis. Many mutants defective in endocytosis did not show any obvious deficiencies in response to nutrient stress. However, we found that *rvs161*Δ, *sla1*Δ, *sla2*Δ, *vrp1*Δ, *vps21*Δ, *ypt52*Δ, *ypt53*Δ, and *end3*Δ had the same phenotype as the original *whi2*Δ mutation. The phenotypes of *vrp1*Δ and *rvs161*Δ were particularly strong, resulting in a marked reduction in cell size compared to wild-type cells. It is not clear why only some mutants studied here show a reduction in cell size. However, one important point to be considered is that we have measured cell volume, which includes both the cytoplasmic and vacuolar compartments. Many of the mutants clearly result in enlarged vacuoles, which could be masking a reduction in the size of the cytoplasmic compartment. It is also important to note that the sizes measured here refer to stationary-phase cells. We observed no size reduction in exponentially growing cells.

Recently, two systematic surveys have yeast deletion sets to identify genes that regulate cell size in *S. cerevisiae*. The work of JORGENSEN *et al.* (2002) focused on exponentially growing cells and thus is not directly comparable to the experiments reported here. ZHANG *et al.* (2002) initially screened the deletion collection for mu-

tants that had an abnormally small cell size in stationary-phase cultures and then tested for size abnormalities in exponentially growing cells. This work identified *whi2*Δ as one of the 20 mutations that caused abnormally small size in stationary phase. None of the other mutations identified here that cause a reduction in cell size were present in the list of Zhang *et al.* (2002). However, *FEN2* (YCR028c) was listed in a table of mitochondrial mutants that showed a reduction in cell size. The criterion for mitochondrial mutations was a failure to grow on glycerol. As discussed above, the molecular defect has been identified in *fen2* and it seems likely that it is misclassified as a mitochondrial mutant. *VPR1* was also present in a list of genes whose deletion resulted in small cell size, but that for various reasons failed a quality control test. The *rvs*161Δ allele also showed a size reduction, but was also omitted for quality control reasons (B. Schneider, personal communication). Thus, the results presented here are broadly consistent with the results of the systematic gene deletion survey.

The proteins that are required for the proper nutrient response function either in the first steps of endocytosis or in the actin complex that mediates endocytosis. Taken together, these observations suggest that the actin/endocytosis complex is required for cells to cease cell division in response to nutrient stress. We suggest two possible explanations. First, endocytosis could result in the internalization of membrane proteins required for growth and budding. Second, fluid-phase endocytosis could be required for the uptake of small molecules from the medium used to sense cell density. Such molecules may accumulate to form the signal to cease cell division in the high cell densities found in stationary-phase culture. A precedent for this may be the way that *Candida albicans* cells undergo only the yeast-to-hypha transition at low cell densities. It has recently been shown that the accumulation of farnesol provides the signal that is used by *C. albicans* cells to sense cell density (Hornby *et al.* 2001; Oh *et al.* 2001).

We thank Mike Snyder, Kevin Costigan, Kathryn Ayscough, Mike Tyers, and Giovanna Lucchini for strains and plasmids and Kathryn Ayscough for critical reading of the manuscript. This work was supported by a project grant from the Biotechnology and Biological Sciences Research Council (BBSRC). A.C. received a BBSRC Cooperative Award in Science and Engineering studentship. K.B. and P.R. were supported by BBSRC research training studentships.

LITERATURE CITED

Adams, A. E. M., and J. R. Pringle, 1983 Relationship of actin and tubulin distribution to bud growth in wild type and morphogenetic-mutant *Saccharomyces cerevisiae*. J. Cell Biol. **98:** 934–945.

Alic, N., V. J. Higgins and I. W. Dawes, 2001 Identification of a *Saccharomyces cerevisiae* gene that is required for G1 arrest in response to the lipid oxidation product linoleic acid hydroperoxide. Mol. Biol. Cell **12:** 1801–1810.

Ayscough, K. R., and D. G. Drubin, 1998 Immunofluorescence microscopy of yeast cells, pp. 477–486 in *Cell Biology: A Laboratory Handbook*, edited by J. E. Celis. Academic Press, San Diego/London.

Bi, E., and J. R. Pringle, 1996 *ZDS1* and *ZDS2*, genes whose products may regulate Cdc42p in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 5264–5275.

Binley, K. M., P. A. Radcliffe, J. Trevethick, K. A. Duffy and P. E. Sudbery, 1999 The yeast *PRS3* gene is required for cell integrity, cell cycle arrest upon nutrient deprivation, ion homeostasis and the proper organization of the actin cytoskeleton. Yeast **15:** 1459–1469.

Boeke, J. D., F. Lacroute and G. R. Fink, 1984 A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast-5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:** 345–346.

Boyne, J. R., H. M. Yosuf, P. Bieganowski, C. Brenner and C. Price, 2000 Yeast myosin light chain, Mlc1p, interacts with both IQGAP and class II myosin to effect cytokinesis. J. Cell Sci. **113:** 4533–4543.

Burda, P., S. te Heesen, A. Brachat, A. Wach, A. Dusterhoft *et al.*, 1996 Stepwise assembly of the lipid-linked oligosaccharide in the endoplasmic reticulum of *Saccharomyces cerevisiae:* identification of the *alg9* gene, encoding a putative mannosyl transferase. Proc. Natl. Acad. Sci. USA **93:** 7160–7166.

Carter, A. T., A. Narbad, B. M. Pearson, K. F. Beck, M. Logghe *et al.*, 1994 Phosphoribosylpyrophosphate synthetase (*PRS*)—a new gene family in *Saccharomyces cerevisiae*. Yeast **10:** 1031–1044.

Carter, A. T., F. Beiche, A. Narbad, B. Hove-Jensen, L. M. Schweizer *et al.*, 1995 Are all four yeast *PRS* genes essential? Biochem. Soc. Trans. **23:** 621S.

Cope, M. J. T. V., S. Yang, C. Shang and D. G. Drubin, 1999 Novel protein kinases Ark1p and Prk1p associate with and regulate the cortical actin cytoskeleton in budding yeast. J. Cell Biol. **144:** 1203–1218.

Costigan, C., and M. Snyder, 1994 *SLK1*, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutrient sensing. Mol. Gen. Genet. **243:** 243–296.

Costigan, C., S. Gehrung and M. Snyder, 1992 A synthetic lethal screen identifies *SLK1*, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. Mol. Cell. Biol. **12:** 1162–1178.

Costigan, C., D. Kolodrubetz and M. Snyder, 1994 NHP6A and NHP6B, which encode HMG1-like proteins, are candidates for downstream components of the yeast SLT2 mitogen-activated protein kinase pathway. Mol. Cell. Biol. **14:** 2391–2403.

Cvrckova, F., C. De Virgilio, E. Manser, J. R. Pringle and K. Nasmyth, 1995 Ste20-like protein-kinases are required for normal localization of cell-growth and for cytokinesis in budding yeast. Genes Dev. **9:** 1817–1830.

Deutch, C. E., and J. M. Parry, 1973 Sphaeroplast formation in yeast during the transition from exponential phase to stationary phase. J. Gen. Microbiol. **80:** 259–268.

Dulic, V., M. Egerton, I. Elguindi, S. Raths, B. Singer *et al.*, 1991 Yeast endocytosis assays. Methods Enzymol. **194:** 697–710.

Gorner, W., E. Durchschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer *et al.*, 1998 Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. **12:** 586–597.

Holtzman, D. A., S. Yang and D. G. Drubin, 1993 Synthetic-lethal interactions identify two novel genes, *SLA1* and *SLA2*, that control membrane cytoskeleton assembly in *Saccharomyces cerevisiae*. J. Cell Biol. **122:** 635–644.

Hongay, C., N. Jia, M. Bard and F. Winston, 2002 Mot3 is a transcriptional repressor of ergosterol biosynthetic genes and is required for normal vacuolar function in Saccharomyces cerevisiae. EMBO J. **21:** 4114–4124.

Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke *et al.*, 2001 Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. **67:** 2982–2992.

Johnson, P., H. L. Ostergaard, C. Wasden and I. S. Trowbridge, 1992 Mutational analysis of CD45. J. Biol. Chem. **267:** 8035–8041.

Jorgensen, P., J. L. Nishikawa, B. J. Breitkreutz and M. Tyers, 2002 Systematic identification of pathways that couple cell growth and division in yeast. Science **297:** 395–400.

Kaida, D., H. Yashiroda, A. Toh-e and Y. Kikuchi, 2002 Yeast Whi2 and Psr1-phosphatase form a complex and regulate STRE-mediated gene expression. Genes Cells **7**: 543–552.

Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki *et al.*, 1993 A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. **13:** 3067–3075.

Lillie, S. H., and J. R. Pringle, 1980 Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J. Bacteriol. **143:** 1384–1394.

Marcireau, C., J. Joets, D. Pousset, M. Guilloton and F. Karst, 1996 *FEN2*—a gene implicated in the catabolite repression-mediated regulation of ergosterol biosynthesis in yeast. Yeast **12:** 531–539.

Martin, H., J. M. Rodriguez-Pachon, C. Ruiz, C. Nombela and M. Molina, 2000 Regulatory mechanisms for modulation of signalling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **275:** 1511–1519.

Martinez-Pastor, M. T., G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis *et al.*, 1996 The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). EMBO J. **15:** 2227–2235.

Mazzoni, C., P. Zarzov, A. Rambourg and C. Mann, 1993 The *SLT2* (*MPK1*) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. J. Cell Biol. **123:** 1821–1833.

Mizunuma, M., D. Hirata, K. Miyahara, E. Tsuchiya and T. Miyakawa, 1998 Role of calcineurin and Mpk1 in regulating the onset of mitosis in the budding yeast. Nature **392:** 303–306.

Oh, K. B., H. Miyazawa, T. Naito and H. Matsuoka, 2001 Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in Candida albicans. Proc. Natl. Acad. Sci. USA **98:** 4664–4668.

Parry, J. M., P. Davies and W. E. Evans, 1976 The effect of 'cell age' upon the lethal effects of physical and chemical mutagens in yeast. Mol. Gen. Genet. **146:** 27–35.

Prescianotto-Baschong, C., and H. Riezman, 2002 Ordering of compartments in the yeast endocytic pathway. Traffic **3:** 37–49.

Pringle, J. R., and L. H. Hartwell, 1981 The *Saccharomyces cerevisiae* cell cycle, pp. 97–142 in *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, edited by J. N. Strathern, E. W. Jones and J. R. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Pryciak, P. M., and L. H. Hartwell, 1996 AKR1 encodes a candidate effector of the G beta gamma complex in the *Saccharomyces cerevisiae* pheromone response pathway and contributes to control of both cell shape and signal transduction. Mol. Cell. Biol. **16:** 2614–2626.

Radcliffe, P. A., J. Trevethick, M. Tyers and P. Sudbery, 1997 Deregulation of *CLN1* and *CLN2* in the *Saccharomyces cerevisiae whi2* mutant. Yeast **13:** 707–715.

Rahman, D. R. J., P. E. Sudbery, S. Kelly and I. W. Marison, 1988 The effect of dissolved-oxygen concentration on the growth physiology of *Saccharomyces cerevisiae whi2* mutants. J. Gen. Microbiol. **134:** 2241–2248.

Roberg, K. J., S. Bickel, N. Rowley and C. A. Kaiser, 1997 Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by *SEC13, LST4, LST7* and *LST8*. Genetics **147:** 1569–1584.

Rose, M. D., P. Novick, J. H. Thomas, D. Botstein and G. R. Fink, 1987 A *Saccharomyces cerevisiae* genomic gene bank based on a centromere containing shuttle vector. Gene **60:** 237–243.

Rotin, D., O. Staub and R. Haguenauer-Tsapis, 2000 Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. J. Membr. Biol. **176:** 1–17.

Saul, D. J., and P. E. Sudbery, 1985 Molecular-cloning of *whi2*, a gene involved in the regulation of cell-proliferation in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **131:** 1797–1806.

Saul, D. J., E. F. Walton, P. E. Sudbery and B. L. A. Carter, 1985 *Saccharomyces cerevisiae whi2* mutants in stationary phase retain the properties of exponentially growing cells. J. Gen. Microbiol. **131:** 2245–2251.

Schenberg-Frascino, A., and E. Moustacchi, 1972 Lethal and mutagenic effects of elevated temperature on haploid yeast. 1. Variations in sensitivity during the cell cycle. Mol. Gen. Genet. **115:** 243–257.

Singer-Kruger, B., H. Stenmark, A. Dusterhoft, P. Philippsen, J. S. Yoo *et al.*, 1994 Role of three Rab5-like GTPases, Ypt51p, Ypt52p, and Ypt53p, in the endocytic and vacuolar protein sorting pathways of yeast. J. Cell Biol. **125:** 283–298.

Siniossoglou, S., E. C. Hurt and H. R. B. Pelham, 2000 Psr1p/Psr2p, two plasma membrane phosphatases with an essential DXDX(T/V) motif required for sodium stress response in yeast. J. Biol. Chem. **275:** 19352–19360.

Snow, R., 1966 An enrichment method for auxotrophic yeast mutants using 'Nystatin'. Nature **211:** 206–207.

Stolz, J., and N. Sauer, 1999 The fenpropimorph resistance gene FEN2 from Saccharomyces cerevisiae encodes a plasma membrane H+-pantothenate symporter. J. Biol. Chem. **274:** 18747–18752.

Sudbery, P. E., A. R. Goodey and B. L. C. Carter, 1980 Genes that control cell proliferation in the yeast *Saccharomyces cerevisiae*. Nature **288:** 401–404.

Torres, L., H. Martin, M. I. Garcia-Saez, J. Arroyo, M. Molina *et al.*, 1991 A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae LYT2* mutants. Mol. Microbiol. **5:** 2845–2854.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature **403:** 623–627.

Wang, G. L., J. M. McCaffery, B. Wendland, S. Dupre, R. Haguenauer-Tsapis *et al.*, 2001 Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. Mol. Cell. Biol. **21:** 3564–3575.

Yu, Y., Y. W. Jiang, R. J. Wellinger, K. Carlson, J. M. Roberts *et al.*, 1996 Mutations in the homologous *ZDS1* and *ZDS2* genes affect cell-cycle progression. Mol. Cell. Biol. **16:** 5254–5263.

Zeng, G., and M. J. Cai, 1999 Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p. J. Cell Biol. **144:** 71–82.

Zeng, G., X. W. Yu and M. J. Cai, 2001 Regulation of yeast actin cytoskeleton-regulatory complex Pan1p/Sla1p/End3p by serine/threonine kinase Prk1p. Mol. Biol. Cell **12:** 3759–3772.

Zhang, J., C. Schneider, L. Ottmers, R. Rodriguez, A. Day *et al.*, 2002 Genomic scale mutant hunt identifies cell size homeostasis genes in S. cerevisiae. Curr. Biol. **12:** 1992–2001.

Zoladek, T., A. Tobiasz, G. Vaduva, M. Boguta, N. C. Martin *et al.*, 1997 *MDP1*, a *Saccharomyces cerevisiae* gene involved in mitochondrial/cytoplasmic protein distribution, is identical to the ubiquitin-protein ligase gene *RSP5*. Genetics **145:** 595–603.

Communicating editor: B. Andrews