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A protein interaction map for cell polarity development

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Many genes required for cell polarity development in budding yeast have been identified and arranged into a functional hierarchy. Core elements of the hierarchy are widely conserved, underlying cell polarity development in diverse eukaryotes. To enumerate more fully the protein–protein interactions that mediate cell polarity development, and to uncover novel mechanisms that coordinate the numerous events involved, we carried out a large-scale two-hybrid experiment. 68 Gal4 DNA binding domain fusions of yeast proteins associated with the actin cytoskeleton, septins, the secretory apparatus, and Rho-type GTPases were used to screen an array of yeast transformants that express ~90% of the predicted *Saccharomyces cerevisiae* open reading frames as Gal4 activation domain fusions. 191 protein–protein interactions were detected, of which 128 had not been described previously. 44 interactions implicated 20 previously uncharacterized proteins in cell polarity development. Further insights into possible roles of 13 of these proteins were revealed by their multiple two-hybrid interactions and by subcellular localization. Included in the interaction network were associations of Cdc42 and Rho1 pathways with proteins involved in exocytosis, septin organization, actin assembly, microtubule organization, autophagy, cytokinesis, and cell wall synthesis. Other interactions suggested direct connections between Rho1- and Cdc42-regulated pathways; the secretory apparatus and regulators of polarity establishment; actin assembly and the morphogenesis checkpoint; and the exocytic and endocytic machinery. In total, a network of interactions that provide an integrated response of signaling proteins, the cytoskeleton, and organelles to the spatial cues that direct polarity development was revealed.

Introduction

Cell polarity is an essential characteristic of virtually every cell type (Drubin, 2000). In response to a cue acting at a specific site on the cell cortex, a cascade of events involving receptors, signaling proteins, the cytoskeleton, and organelles results in an asymmetric distribution of cellular components (Drubin and Nelson, 1996). The budding yeast *Saccharomyces cerevisiae* has been critical for elucidation of proteins and mechanisms that underlie cell polarity development. Growth of the yeast cell is polarized to direct budding during cell division and projection formation during mating. As in other eukaryotic cells, polarized growth is mediated by a series of steps involving cortical landmarks, Rho GTPases, and a polarized actin cytoskeleton. Secretion is targeted to the bud or mating projection, allowing selective growth in

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that area (for reviews see Drubin and Nelson, 1996; Chant, 1999; Pruyne and Bretscher, 2000a,b).

Several Rho type GTPases function in the establishment and maintenance of cell polarity (Bender and Pringle, 1989; Johnson and Pringle, 1990; Matsui and Toh-e, 1992; Drgonová et al., 1996; Imai et al., 1996; Kamada et al., 1996; Robinson et al., 1999). One of these, Cdc42, is a crucial factor in the switch from isotropic to polarized growth that occurs when the cyclin-dependent protein kinase Cdc28 is activated by G1 cyclins (Adams et al., 1990; Ayscough et al., 1997). A decisive event for the establishment and maintenance of cell polarity is the recruitment of Cdc42 to the cell surface and its activation in response to positional cues and cell cycle signals (Chant, 1999). In budding cells, spatial markers left by previous cell divisions stimulate the local activation of the Ras-related Rsr1/Bud1 GTPase, which recruits and activates Cdc42 via interaction with the guanidine nucleotide exchange factor Cdc24 (Ruggieri et al., 1992; Bender, 1993; Zheng et al., 1995; Park et al., 1999). In haploid cells exposed to mating pheromone, the protein Far1 interacts with Cdc24 and recruits Cdc42 to the tip of the mating projection (Butty et al., 1998). The activated GTP-bound form of Cdc42 interacts with several proteins that are presumed to be effectors that transduce its signal to bring about polarization of the actin cytoskeleton (Cvrcková et al., 1995; Brown et al., 1997; Chen et al., 1997; Evangelista et al., 1997; Bi et al., 2000). Actin cables are proposed to serve as tracks for vesicle, organelle, and mRNA transport, whereas cortical actin patches are important for endocytosis (Pruyne and Bretscher, 2000a,b). Largely unknown are how cortical cues lead to localized activation of Rho GTPases, how their activation polarizes the spatial distribution of cytoskeletal proteins, the secretory apparatus, and other cellular constituents, and what mechanisms coordinate the many events that underlie cell polarity development. For example, at the site of bud formation, several Rho proteins function together with associated protein kinases and other effector proteins, and the cytoskeleton and secretory apparatus become organized around these signaling proteins. Bud morphogenesis requires spatial and temporal coordination of these events, but little is known of the coordinating mechanisms.

The yeast two-hybrid system (Fields and Song, 1989) is a powerful method for identifying pairs of proteins that associate with each other, and it can be used in a high-throughput manner (Uetz et al., 2000; Ito et al., 2001). Uetz et al. (2000) constructed an array of yeast transformants, each of which expresses one of the ~6,000 predicted yeast ORFs as a fusion to an activation domain (Hudson et al., 1997). This array was screened by a simple automated procedure in which protein–protein interactions responsible for positive responses were identified by the position within the array. A similar strategy was used for analysis of protein–protein interactions of vaccinia virus (McCraith et al., 2000). One advantage of the array-based approach is that each individual assay is compared with multiple identical assays, making it easier to distinguish bona fide interactions from background due to nonspecific activation of the reporter gene. Here we present the results of an array-based two-hybrid experiment designed to systematically detect protein–protein interactions involved in yeast cell polarity development. The proteins screened included Cdc42 and other Rho-type GTPases, their regulators and effectors, actin cytoskeleton–associated proteins, septin-associated proteins, and proteins involved in secretion. Our aims were to identify new links in the network of protein–protein associations controlling polarized growth and to provide biological context for ORFs of unknown functions, with the goal of understanding their functional roles. Owing to high conservation of cell polarity development pathways, this information should be useful for developing a deeper understanding of cell polarity development in all types of eukaryotic cells (Drubin and Nelson, 1996; Pruyne and Bretscher, 2000a,b).

Results and discussion

Overview and general considerations

68 proteins with various functions in cell polarity development were used as DNA binding domain hybrids for two-hybrid screens. These included Rho-type GTPases and their regulators and effectors, actin cortical patch components, septin-associated proteins, and proteins involved in secretion (Table I). The yeast ORF-Gal4 activation domain fusion array used in our experiments expresses ~85–90% of the predicted ORFs of *S. cerevisiae* (Hudson et al., 1997; Uetz et al., 2000). 14 proteins, Aip2, Bud5, Bud6, Bud7, Bud9, Cap2, Cdc3, Cdc10, Iqg1, Kin1, Msb1, Sec9, Snc1, and Snc2, showed no reproducible two-hybrid interactions when used as baits in our screens. Screens of the other 54 baits found from 1 to 13 interactions each. Overall, 196 reproducible two-hybrid positives were detected that describe 191 putative protein–protein interactions involving 110 proteins (Table I and Figs. 1–3). 128 interactions had not been described previously and 44 involve 20 proteins of unknown function. The results of this study clearly do not represent all of the detectable or probable interactions between the proteins examined. The lack of an interaction detected in this analysis is not necessarily meaningful, as some constructs in the array might not express the expected fusion proteins or might express them in a nonfunctional form due to the Gal4 fusion. Differences in fusion construction, construct expression, strain background, and selection stringency are also factors that may account for discrepancies between the set of interactions seen here and those found in other studies.

To observe the subcellular localization of the proteins of unknown function, we expressed 13 of them in yeast under control of their own promoters as fusions with yellow fluorescent protein, a variant of the *A. victoria* green fluorescent protein (Niedenthal et al., 1996; Miller et al., 1999). Results of the localization experiments are shown in Table II and Figs. 4–6. Of the 128 new interactions, many appear plausible on the basis of genetic or localization criteria. The significance of others remains unclear. The two-hybrid results derived from these screens should be considered as a set of putative interactions requiring further verification. It is also important to note that an interaction might be direct, or might be bridged by a protein or proteins that bind to both the bait and the prey protein.

As shown in Fig. 1, two-hybrid interactions were observed not only between proteins involved in the same polarity-related process, but also between proteins involved in distinct

Table I. **Summary of protein–protein interactions detected in two-hybrid screens**

Protein		Interacting protein(s)	
Abp1	Actin binding protein involved in cortical actin assembly, has SH3 domain	Cla4[a] (AD)	PAK kinase required for cytokinesis
		Rvs167 (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Srv2 (AD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
		Ynl094w[a] (AD)	Protein of unknown function
		Yor284w[a] (AD)	Protein of unknown function
Acf2	Protein involved in cortical actin assembly	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
Act1	Actin, involved in cell polarization, endocytosis, cytoskeletal function	Cof1 (BD)	Cofilin, actin binding and severing protein
		Las17 (BD)	WASP homologue involved in cortical actin assembly
		Pfy1 (BD)	Profilin, can act to prevent actin polymerization and to complex with monomeric actin
		Rvs167 (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Srv2 (AD,BD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
Aip1	Actin interacting protein involved in disassembly of actin filaments	Srv2 (BD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
Air1	RING finger protein that affects RNA processing	Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
Apg17	Protein essential for autophagy	Apg17[a] (AD,BD)	Protein essential for autophagy
		Exo84[a] (BD)	Subunit of the exocyst complex, required for exocytosis
		Myo1[a] (BD)	Myosin heavy chain (myosin II), involved in septation and cell wall organization
		Nip100[a] (BD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
		Rho1–GTP[a] (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Rho2–GTP[a] (BD)	GTP-binding protein, member of the rho subfamily of ras-like proteins
		Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
		Ykr083c[a] (AD)	Protein of unknown function
Apg7	Conjugation protein essential for autophagy	Sso2[a] (BD)	Syntaxin homologue (t-SNARE) involved in vesicle transport from Golgi to plasma membrane
Arp1	Actin-related protein of the dynactin complex, required for mitotic spindle orientation and nuclear migration	Nip100 (BD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
Bcy1	Regulatory subunit of cAMP-dependent protein kinases	Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
Bem1	Protein required for cell polarization and bud formation, has two SH3 domains	Boi2 (BD, AD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Cdc24 (AD,BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Cdc42–GDP (AD,BD)	Rho type GTPase involved in bud site assembly and cell polarity
		Cdc42–GTP (BD)	Rho type GTPase involved in bud site assembly and cell polarity
Bem1 (continued)	Protein required for cell polarization and bud formation, has two SH3 domains	Far1 (AD)	Inhibitor of CDK–cyclin complexes involved in cell cycle arrest for mating and polarized growth of mating projection
		Sec15[a] (AD)	Component of exocyst complex required for exocytosis
		Ste20 (AD)	PAK kinase of the pheromone pathway; also regulates polarized growth
		Swe1[a] (AD)	Serine/tyrosine dual-specificity protein kinase; able to phosphorylate Cdc28p on tyrosine and inhibit its activity
Bem3	GAP for Cdc42p and Rho1p	Bem4[a] (AD)	Bud emergence protein, interacts with Rho type GTPases
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Zds1[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds2
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Bem4	Bud emergence protein, interacts with Rho type GTPases	Bem3[a] (BD)	GAP for Cdc42p and Rho1p
		Cdc11[a] (AD)	Septin, involved in cytokinesis
		Cdc12[a] (AD)	Septin, involved in cytokinesis
		Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Cdc42–GDP (BD)	Rho type GTPase involved in bud site assembly and cell polarity
		Cdc42–GTP (BD)	Rho type GTPase involved in bud site assembly and cell polarity
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Nfi1[a] (AD)	Septin-interacting protein
		Rho1–GTP (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Rho2–GTP (BD)	GTP-binding protein, member of the rho subfamily of ras-like proteins
		Rho4–GTP (BD)	GTP-binding protein of the rho subfamily of ras-like proteins
		Rsr1–GDP[a] (BD)	GTP-binding protein of the ras superfamily involved in bud site selection
		Rsr1–GTP[a] (BD)	GTP-binding protein of the ras superfamily involved in bud site selection
		Spr28[a] (AD)	Septin-related protein expressed during sporulation
Bni1	Formin protein involved in cytoskeletal polarization and cytokinesis	Msb3[a] (BD)	Protein involved in bud emergence
		Pfy1 (BD)	Profilin, can act to prevent actin polymerization and to complex with monomeric actin
		Rho1–GTP (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Bni4	Protein linking chitin synthase III to septins of the neck filaments	Msb2[a] (BD)	Protein involved in bud emergence
Bnr1	Bni1p-related formin protein	Pfy1 (BD)	Profilin, can act to prevent actin polymerization and to complex with monomeric actin
Boi1	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi2 homologue	Ste20 (AD)	PAK kinase of the pheromone pathway; also regulates polarized growth
		Yer124c[a] (AD)	Protein of unknown function
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Boi2	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue	Bem1 (BD, AD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Cdc42–GTP (BD)	Rho type GTPase involved in bud site assembly and cell polarity, GTP bound form
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Mrs6[a] (AD)	Rab geranylgeranyltransferase regulatory component (component A) and rab GDI
		Msb1[a] (AD)	Protein involved in cell polarity and bud emergence
		Ste20 (AD)	PAK kinase of the pheromone pathway; also regulates polarized growth
		Yer124c[a] (AD)	Protein of unknown function
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Bub2	Protein required for cell cycle arrest in response to loss of microtubule function	Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
Bud2	GTPase-activating protein for Rsr1, involved in bud site selection	Cln2[a] (AD)	G1/S-specific cyclin
Bud8	Protein required for bipolar budding	Ste20[a] (AD)	PAK kinase of the pheromone pathway; also regulates polarized growth
		Ykl082c[a] (AD)	Protein of unknown function
Cap1	Actin-capping protein, α subunit	Cap2 (AD)	Actin-capping protein, β subunit
		Gic2[a] (AD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Ypr171w[a] (AD)	Protein of unknown function
Cap2	Actin-capping protein, β subunit	Cap1 (BD)	Actin-capping protein, α subunit
Cdc11	Septin, involved in cytokinesis	Bem4[a] (BD)	Bud emergence protein, interacts with Rho-type GTPases
		Cdc12 (AD,BD)	Septin, involved in cytokinesis
		Nfi1 (AD)	Septin-interacting protein
		Spr28[a] (AD)	Septin-related protein expressed during sporulation
		Yor084w[a] (AD)	Protein of unknown function
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Cdc12	Septin, involved in cytokinesis	Bem4[a] (BD)	Bud emergence protein, interacts with Rho-type GTPases
		Cdc11 (AD,BD)	Septin, involved in cytokinesis
		Cdc12 (AD,BD)	Septin, involved in cytokinesis
		Cla4[a] (BD)	PAK kinase required for cytokinesis
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		She3[a] (AD)	Protein required for mother cell-specific expression of HO
Cdc24	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection	Air1[a] (AD)	RING finger protein that affects RNA processing
		Bem1 (AD,BD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Bem4[a] (AD)	Bud emergence protein, interacts with Rho-type GTPases

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Cdc24 (continued)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection	Cdc42-GDP (BD)	Rho-type GTPase involved in bud site assembly and cell polarity
		Ent2[a] (AD)	Epsin homologue required for endocytosis
		Far1 (AD)	Inhibitor of CDK-cyclin complexes involved in cell cycle arrest for mating and polarized growth of mating projection
		Rsr1-GDP (BD)	GTP-binding protein of the ras superfamily involved in bud site selection
		Sec15[a] (AD)	Component of exocyst complex required for exocytosis
		Ste20 (AD)	PAK kinase of the pheromone pathway; also regulates polarized growth
		Swe1[a] (AD)	Serine/tyrosine dual-specificity protein kinase; able to phosphorylate Cdc28p on tyrosine and inhibit its activity
		Ygr221c[a] (AD)	Protein of unknown function
Cdc42–GDP	Rho type GTPase involved in bud site assembly and cell polarity, GDP-bound form	Bem1 (AD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Bem4 (AD, BD)	Bud emergence protein, interacts with Rho type GTPases
		Cdc24 (AD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
Cdc42–GTP	Rho type GTPase involved in bud site assembly and cell polarity, GTP bound form	Bem1 (AD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Bem4 (AD)	Bud emergence protein, interacts with Rho type GTPases
		Boi2 (AD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Cla4 (AD)	PAK kinase required for cytokinesis
		Gic1 (AD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2 (AD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Rgal (AD)	Rho-type GTPase activating protein (GAP) for Cdc42
		Ste20 (AD)	Serine/threonine protein kinase of the pheromone pathway; also participates in the pathway regulating filamentous growth
Chs4	Protein that stimulates chitin synthase III activity	Yil007c[a] (AD)	Protein of unknown function
Cka1	Casein kinase II catalytic (α) subunit	Rho3–GTP[a] (BD)	GTP-binding protein involved in control of actin cytoskeleton and exocytosis, member of the rho subfamily of ras-like proteins
Cka2	Casein kinase II catalytic (α′) subunit	Rho3–GTP[a] (BD)	GTP-binding protein involved in control of actin cytoskeleton and exocytosis, member of the rho subfamily of ras-like proteins
Ckb1	Casein kinase II regulatory (β) subunit	Rho3–GTP[a] (BD)	GTP-binding protein involved in control of actin cytoskeleton and exocytosis, member of the rho subfamily of ras-like proteins
Cla4	PAK kinase required for cytokinesis	Abp1[a] (BD)	Actin binding protein involved in cortical actin assembly, has SH3 domain
		Bem3[a] (BD)	GAP for Cdc42p and Rho1p
		Boi2[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Cdc12[a] (AD)	Septin, involved in cytokinesis

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Cla4 (continued)	PAK kinase required for cytokinesis	Cdc42–GTP (BD)	Rho type GTPase involved in bud site assembly and cell polarity
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Msb2[a] (BD)	Protein involved in bud emergence
		Rga1 (BD)	Rho type GTPase-activating protein (GAP) for Cdc42p
		Sla2[a] (BD)	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Cln2	G1/S-specific cyclin	Bud2[a] (BD)	GTPase-activating protein for Rsr1, involved in bud site selection
Cof1	Cofilin, actin binding and severing protein	Act1 (AD)	Actin, involved in cell polarization, endocytosis, cytoskeletal functions
Crn1	Coronin, actin-bundling protein	Svl3[a] (AD)	Protein involved in vacuolar uptake of endocytosed vital dyes
		Ynl094w[a] (AD)	Protein of unknown function
Dfg5	Protein required for cell polarity, apical growth, and pseudohyphal growth	Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
Ent2	Epsin homologue required for endocytosis	Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
Exo84	Subunit of the exocyst complex, required for exocytosis	Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Sec15 (AD)	Component of exocyst complex required for exocytosis
Far1	Inhibitor of CDK-cyclin complexes involved in cell cycle arrest for mating and polarized growth of mating projection	Bem1 (BD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Cdc24 (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
Gic1	Effector of Cdc42p, important for bud emergence, Gic2 homologue	Bem4[a] (AD)	Bud emergence protein, interacts with Rho-type GTPases
		Bub2[a] (AD)	Protein required for cell cycle arrest in response to loss of microtubule function
		Cdc12[a] (AD)	Septin, involved in cytokinesis
		Cdc42-GTP (BD)	Rho-type GTPase involved in bud site assembly and cell polarity
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Dfg5[a] (AD)	Protein required for cell polarity, apical growth, and pseudohyphal growth
		Gic1 (AD,BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2 (AD,BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Hof1[a] (AD)	Protein involved in cytokinesis, has an SH3 domain
		Ste50[a] (AD)	Protein required for feedback control of pheromone-induced signal transduction
		Ykl082c[a] (AD)	Protein of unknown function
		Ycr086w[a] (AD)	Protein of unknown function
Gic1 (continued)	Effector of Cdc42p, important for bud emergence, Gic2 homologue	Zds1[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds2
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Gic2	Effector of Cdc42p, important for bud emergence, Gic1 homologue	Cap1[a] (AD)	Actin-capping protein, α subunit
		Cdc12[a] (AD)	Septin, involved in cytokinesis
		Cdc42-GTP (BD)	Rho-type GTPase involved in bud site assembly and cell polarity
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Gic1 (AD,BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Rga1[a] (BD)	Rho-type GTPase-activating protein (GAP) for Cdc42p
		Ste50[a] (AD)	Protein required for feedback control of pheromone-induced signal transduction
		Ycr086w[a] (AD)	Protein of unknown function
		Ykl082c[a] (AD)	Protein of unknown function
		Zds1[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds2
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Hof1	Protein involved in cytokinesis, has an SH3 domain	Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
Hsl7	Negative regulatory protein of the Swe1p protein kinase	Ynl094w (AD)	Protein of unknown function
Kin3	Serine/threonine protein kinase, unknown function	Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
Las17	WASP homologue involved in cortical actin assembly	Act1 (AD)	Actin, involved in cell polarization, endocytosis, cytoskeletal functions
		Rvs167 (AD, BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1 (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
		Yhr133c[a] (AD)	Protein of unknown function
		Ypl246c[a] (AD)	Protein of unknown function
Mrs6	Rab geranylgeranyltransferase regulatory subunit and rab GDI	Boi2[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
Msb1	Protein involved in cell polarity and bud emergence	Boi2[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
Msb2	Protein involved in bud emergence	Bni4[a] (AD)	Protein linking chitin synthase III to septins of the neck filaments
		Cla4[a] (AD)	PAK kinase required for cytokinesis
Msb3	Protein involved in bud emergence	Bni1[a] (AD)	Formin protein involved in cytoskeletal polarization and cytokinesis
		Spa2[a] (AD)	Protein involved in cell polarity and cell fusion during mating
Msb4	Protein involved in bud emergence	Spa2[a] (AD)	Protein involved in cell polarity and cell fusion during mating
Myo1	Myosin heavy chain (myosin II), involved in septation and cell wall organization	Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Spr3[a] (AD)	Sporulation-specific septin
		Ypr188c[a] (AD)	Protein of unknown function

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Nfi1	Septin-interacting protein	Bem4[a] (BD)	Bud emergence protein, interacts with Rho type GTPases
		Cdc11[a] (BD)	Septin, involved in cytokinesis
		Nip100[a] (BD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
Nip100	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle	Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Arp1 (AD)	Actin-related protein of the dynactin complex, required for mitotic spindle orientation and nuclear migration
		Nfi1[a] (AD)	Septin-interacting protein
		Nip100 (AD,BD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
		Pac11 (AD)	Protein with similarity to rat dynein intermediate chain; required in the absence of Cin8p, member of WD (WD-40) repeat family
		Rho1–GTP[a] (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Rho2–GTP[a] (BD)	GTP-binding protein, member of the rho subfamily of ras-like proteins
		Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
Pac11	Protein with similarity to rat dynein intermediate chain	Nip100 (BD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
Pfs1	Protein required for sporulation	Yil007c[a] (BD)	Protein of unknown function
Pfy1	Profilin, can act to prevent actin polymerization and to complex with monomeric actin	Act1 (AD)	Actin, involved in cell polarization, endocytosis, cytoskeletal functions
		Bni1 (AD)	Formin protein involved in cytoskeletal polarization and cytokinesis
		Bnr1 (AD)	Bni1p-related formin protein
		Srv2 (AD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
Pkc1	Protein kinase C, regulates MAP kinase cascade involved in regulating cell wall metabolism	Rho1–GTP (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Ygr221C[a] (BD)	Protein of unknown function
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Rga1	Rho type GTPase-activating protein (GAP) for Cdc42p	Cdc42 (BD)	Rho-type GTPase involved in bud site assembly and cell polarity
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Gic2[a] (AD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Rho1 (AD,BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
Rho1–GTP	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins, GTP-bound form	Rga1 (AD,BD)	Rho type GTPase-activating protein (GAP) for Cdc42 and Rho1
		Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Bem4 (AD)	Bud emergence protein, interacts with Rho type GTPases
		Bni1 (AD)	Formin protein involved in cytoskeletal polarization and cytokinesis
		Nip100[a] (AD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
		Pkc1 (AD)	Protein kinase C, regulates MAP kinase cascade involved in regulating cell wall metabolism
		Shc1[a] (AD)	Protein involved in cell wall chitin synthesis or deposition
		Yil007c[a] (AD)	Protein of unknown function
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Rho2–GTP	GTP-binding protein, member of the rho subfamily of ras-like proteins, GTP bound form	Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Bem4 (AD)	Bud emergence protein, interacts with Rho type GTPases
		Nip100[a] (AD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
Rho3–GTP	GTP-binding protein, member of the rho subfamily of ras-like proteins, GTP bound form	Cka1[a] (AD)	Casein kinase II catalytic (α) subunit
		Cka2[a] (AD)	Casein kinase II catalytic (α′) subunit
		Ckb1[a] (AD)	Casein kinase II regulatory (β) subunit
Rho4–GTP	GTP-binding protein of the rho subfamily of ras-like proteins, GTP bound form	Bem4 (AD)	Bud emergence protein, interacts with Rho type GTPases
		Yil007c[a] (AD)	Protein of unknown function
Rpn4	Subunit of the regulatory particle of the proteasome	Yil007c[a] (BD)	Protein of unknown function
Rsr1-GDP	GTP-binding protein of the ras superfamily involved in bud site selection, GDP-bound form	Bem4[a] (AD)	Bud emergence protein, interacts with Rho type GTPases
		Cdc24 (AD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Sec15[a] (AD)	Component of exocyst complex required for exocytosis
Rsr1–GTP	GTP-binding protein of the ras superfamily involved in bud site selection, GTP-bound form	Bem4[a] (AD)	Bud emergence protein, interacts with Rho type GTPases
Rvs161	Protein required for viability after N, C, or S starvation, for internalization step of endocytosis, and for cell fusion during mating; roles in endocytosis and in cell fusion are independent of one another	Rvs167 (AD,BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
Rvs167	Protein that affects actin distribution and bipolar budding, has an SH3 domain	Abp1 (AD)	Actin binding protein involved in cortical actin assembly, has SH3 domain
		Acf2[a] (AD)	Protein involved in cortical actin assembly
		Act1 (AD)	Actin, involved in cell polarization, endocytosis, cytoskeletal functions
		Las17 (AD, BD)	WASP homologue involved in cortical actin assembly
		Rvs161 (AD, BD)	Protein required for viability after N, C, or S starvation, for internalization step of endocytosis, and for cell fusion during mating; roles in endocytosis and in cell fusion are independent of one another
		Rvs167 (AD, BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1 (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
		Sla2 (AD)	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis
		Srv2 (AD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Rvs167 (continued)	Protein that affects actin distribution and bipolar budding, has an SH3 domain	Ybr108c (AD)	Protein of unknown function
		Yjr083c[a] (AD)	Protein of unknown function
		Ygr268c[a] (AD)	Protein of unknown function
		Ynl086w[a] (AD)	Protein of unknown function
		Ynl094w[a] (AD, BD)	Protein of unknown function
		Yor284w[a] (AD)	Protein of unknown function
		Ypr171w[a] (AD)	Protein of unknown function
		Ysc84[a] (AD)	Protein of unknown function, has an SH3 domain
Sec15	Component of exocyst complex required for exocytosis	Bem1[a] (BD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Exo84 (BD)	Subunit of the exocyst complex, required for exocytosis
		Rsr1–GDP[a] (BD)	GTP-binding protein of the ras superfamily involved in bud site selection
Shc1	Protein involved in cell wall chitin synthesis or deposition	Rho1–GTP[a] (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
She3	Protein required for mother cell-specific expression of HO	Cdc12[a] (BD)	Septin, involved in cytokinesis
Sla1	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains	Las17 (AD)	WASP homologue involved in cortical actin assembly
		Rvs167 (AD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Srv2 (AD)	Adenylate cyclase–associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
		Ygr268c[a] (AD)	Protein of unknown function
		Ynl094w[a] (AD)	Protein of unknown function
		Ypr171w[a] (AD)	Protein of unknown function
		Ysc84[a] (AD)	Protein of unknown function, has an SH3 domain
Sla2	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis	Cla4[a] (AD)	PAK kinase required for cytokinesis
		Rvs167 (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Ynl094w[a] (AD)	Protein of unknown function
		Yor284w[a] (AD)	Protein of unknown function
		Ysc84[a] (AD)	Protein of unknown function, has an SH3 domain
Spa2	Protein involved in cell polarity and cell fusion during mating	Msb3[a] (BD)	Protein involved in bud emergence
		Msb4[a] (BD)	Protein involved in bud emergence
Spr28	Septin-related protein expressed during sporulation	Bem4[a] (BD)	Bud emergence protein, interacts with Rho type GTPases
		Cdc11[a] (BD)	Septin, involved in cytokinesis
Spr3	Sporulation-specific septin	Myo1[a] (BD)	Myosin heavy chain (myosin II), involved in septation and cell wall organization
Spr6	Sporulation-specific protein	Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Sro77	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase	Apg17[a] (AD)	Protein essential for autophagy response to nutritional stress
		Bcy1[a] (AD)	Regulatory subunit of cAMP-dependent protein kinases
		Ent2[a] (AD)	Epsin homologue required for endocytosis
		Kin3[a] (AD)	Serine/threonine protein kinase, unknown function

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Sro77 (continued)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase	Nip100[a] (AD)	Mitotic spindle positioning protein, dynactin complex protein associated with the spindle
		Yap1801[a] (AD)	Protein homologous to clathrin assembly polypeptide AP180; interacts with Pan1p
		Yip1[a] (AD)	Protein involved in vesicular transport; interacts with transport GTPases Ypt1p and Ypt31p at the Golgi membrane
		Ynl094w[a] (AD)	Protein of unknown function
		Yor197w[a] (AD)	Protein of unknown function
Srv2	Adenylate cyclase-associated protein (CAP) that may provide a link between growth signals and the cytoskeleton	Abp1(BD)	Actin binding protein involved in cortical actin assembly, has SH3 domain
		Act1 (AD,BD)	Actin, involved in cell polarization, endocytosis, cytoskeletal functions
		Aip1 (AD)	Actin interacting protein involved in disassembly of actin filaments
		Pfy1 (BD)	Profilin, can act to prevent actin polymerization and to complex with monomeric actin
		Rvs167 (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1 (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
		Srv2 (AD,BD)	Adenylate cyclase-associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
		Yhr070w[a] (AD)	Protein of unknown function
Sso1	Syntaxin homologue (t-SNARE) involved in vesicle transport from Golgi to plasma membrane	Yap1801 (AD)	Protein homologous to clathrin assembly polypeptide AP180; interacts with Pan1p
Sso2	Syntaxin homologue (t-SNARE) involved in vesicle transport from Golgi to plasma membrane	Apg7[a] (AD)	Protein essential for autophagy response to nutritional stress
		Yap1801 (AD)	Protein homologous to clathrin assembly polypeptide AP180; interacts with Pan1p
Ste20	Serine/threonine protein kinase of the pheromone pathway; also participates in the pathway regulating filamentous growth	Bem1 (AD)	Protein required for cell polarization and bud formation, has two SH3 domains
		Bem4[a] (AD)	Bud emergence protein, interacts with Rho-type GTPases
		Boi1 (AD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi2 homologue
		Boi2 (AD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Bud8[a] (BD)	Protein required for bipolar budding
		Cdc42-GTP (BD)	Rho-type GTPase involved in bud site assembly and cell polarity
Ste50	Protein required for feedback control of pheromone-induced signal transduction	Gic1 (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2 (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
Svl3	Protein involved in vacuolar uptake of endocytosed vital dyes	Crn1[a] (BD)	Coronin, actin-bundling protein
Swe1	Serine/tyrosine dual-specificity protein kinase; able to phosphorylate Cdc28p on tyrosine and inhibit its activity	Bem1[a] (BD)	Protein required for cell polarization and bud formation, has two SH3 domains

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Swe1 (continued)	Serine/tyrosine dual-specificity protein kinase; able to phosphorylate Cdc28p on tyrosine and inhibit its activity	Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Ynl094w[a] (AD)	Protein of unknown function
Yal004w	Protein of unknown function	Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Yap1801	Protein homologous to clathrin assembly polypeptide AP180; interacts with Pan1p	Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
		Sso1 (BD)	Syntaxin homologue (t-SNARE) involved in vesicle transport from Golgi to plasma membrane
		Sso2 (BD)	Syntaxin homologue (t-SNARE) involved in vesicle transport from Golgi to plasma membrane
Ybr108c	Protein of unknown function	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
Ycr086w	Protein of unknown function	Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
Yel023	Protein of unknown function	Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Yer124c	Protein of unknown function	Boi1[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Boi2[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Ygr221c	Protein of unknown function	Cdc24[a] (BD)	GEF for Cdc42, involved in bud emergence, bud site selection, growth of mating projection
		Pkc1[a] (AD)	Protein kinase C; regulates MAP kinase cascade involved in regulating cell wall metabolism
Ygr268c	Protein of unknown function	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1[a] (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
Yhr070w	Protein of unknown function	Srv2[a] (BD)	Adenylate cyclase-associated protein (CAP) that may provide a link between growth signals and the cytoskeleton
Yhr133c	Protein of unknown function	Las17[a] (BD)	WASP homologue involved in cortical actin assembly
Yhr149C	Protein of unknown function	Zds1[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds2
		Zds2[a] (AD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Yil007C	Protein of unknown function	Chs4[a] (AD)	Protein that stimulates chitin synthase III activity
		Pfs1[a] (AD)	Protein required for sporulation
		Rho1–GTP[a] (BD)	GTP-binding protein required to activate the PKC1 pathway and β-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Rho4–GTP[a] (BD)	GTP-binding protein of the rho subfamily of ras-like proteins
		Rpn4[a] (AD)	Subunit of the regulatory particle of the proteasome
Yip1	Protein involved in vesicular transport; interacts with transport GTPases Ypt1p and Ypt31p at the Golgi membrane	Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Yjr083c	Protein of unknown function	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
Ykl082c	Protein of unknown function	Bud8[a] (BD)	Protein required for bipolar budding
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Zds2[a] (BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Ykr083c	Protein of unknown function	Apg17[a] (BD)	Protein essential for autophagy response to nutritional stress
Ynl086w	Protein of unknown function	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
Ynl094w	Protein of unknown function	Abp1[a] (BD)	Actin binding protein involved in cortical actin assembly, has SH3 domain
		Crn1[a] (BD)	Coronin, actin-bundling protein
		Hsl7 (BD)	Negative regulatory protein of the Swe1p protein kinase
		Rvs167[a] (AD,BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1[a] (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
		Sla2[a] (BD)	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis
		Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
		Swe1[a] (BD)	Serine/tyrosine dual-specificity protein kinase; able to phosphorylate Cdc28p on tyrosine and inhibit its activity
Yor084w	Protein of unknown function	Cdc11[a] (BD)	Septin, involved in cytokinesis
Yor197w	Protein of unknown function	Sro77[a] (BD)	Protein that functions together with Sec9p in exocytosis downstream of the Rho3p GTPase
Yor284w	Protein of unknown function	Abp1[a] (BD)	Actin binding protein involved in cortical actin assembly, has SH3 domain
		Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla2[a] (BD)	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis
Ypl246c	Protein of unknown function	Las17[a] (BD)	WASP homologue involved in cortical actin assembly
Ypr171w	Protein of unknown function	Cap1[a] (BD)	Actin-capping protein, α subunit
		Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1[a] (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
Ypr188c	Protein of unknown function	Myo1[a] (BD)	Myosin heavy chain (myosin II), involved in septation and cell wall organization
Ysc84	Protein of unknown function, has an SH3 domain	Rvs167[a] (BD)	Protein that affects actin distribution and bipolar budding, has an SH3 domain
		Sla1[a] (BD)	Protein involved in assembly of cortical actin cytoskeleton, has three SH3 domains
		Sla2[a] (BD)	Talin-like protein involved in membrane cytoskeleton assembly and required for cell polarization; also required for the internalization phase of endocytosis

Table I. **Summary of protein–protein interactions detected in two-hybrid screens** (Continued)

Protein		Interacting protein(s)	
Zds1		Bem3[a] (BD)	GAP for Cdc42p and Rho1p
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Yhr149c[a] (BD)	Protein of unknown function
		Zds2[a] (AD,BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1
Zds2	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1	Bem3[a] (BD)	GAP for Cdc42p and Rho1p
		Bni1[a] (AD)	Formin protein involved in cytoskeletal polarization and cytokinesis
		Boi1[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi2 homologue
		Boi2[a] (BD)	Bem1p-binding protein, involved in bud formation, has an SH3 domain, Boi1 homologue
		Cdc11[a] (AD)	Septin, involved in cytokinesis
		Cla4[a] (AD)	PAK kinase required for cytokinesis
		Gic1[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic2 homologue
		Gic2[a] (BD)	Effector of Cdc42p, important for bud emergence, Gic1 homologue
		Pkc1[a] (AD)	Protein kinase C; regulates MAP kinase cascade involved in regulating cell wall metabolism
		Rho1-GTP[a] (BD)	GTP-binding protein required to activate the PKC1 pathway and beta-1,3-glucan synthase, member of the rho subfamily of ras-like proteins
		Spr6[aa] (AD)	Sporulation-specific protein
		Yal004w[a] (AD)	Protein of unknown function
		Yel023c[a] (AD)	Protein of unknown function
		Yer124c[a] (AD)	Protein of unknown function
		Yhr149c[a] (BD)	Protein of unknown function
		Ykl082c[a] (AD)	Protein of unknown function
		Zds1[a] (AD,BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds2
		Zds2[a] (AD,BD)	Protein with effects on cell polarity and transcriptional silencing, homologue of Zds1

A total of 191 reproducible two-hybrid interactions involving 110 proteins were detected. Proteins are listed in alphabetical order. Each pairwise interaction appears twice in the table, once under the bait protein and once under the interacting prey protein. Entries in the second column are noted as BD (binding domain) or AD (activation domain) to signify the direction of the two-hybrid interaction.
[a]Interactions not previously identified.

processes. These two classes of interaction can, respectively, provide novel insights into the biochemical mechanisms responsible for each process and into the regulatory mechanisms that coordinate the different processes spatially and temporally within a cell. Interaction of a protein with others involved in a process distinct from the one it was originally implicated in might reflect an underlying regulatory mechanism linking the two processes, or it might indicate that one

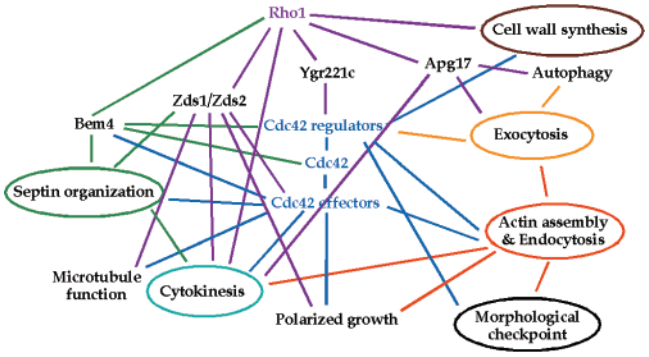

Figure 1. **Schematic overview of connections between processes involved in cell polarity development.** Major processes are color coded in this and the following figures: blue, Cdc42-signaling pathways; purple, Rho1-signaling pathways; green, septin organization; red, actin organization and endocytosis; yellow, exocytosis; brown, cell wall synthesis; turquoise, cytokinesis. Only individual proteins that appear to be branchpoints or major nodal connections between different processes are depicted. Bem4, for example, shows interactions with both Rho1 and Cdc42 GTPase pathways and with the septins. Zds1 and Zds2 link Rho1 with Cdc42 effectors and downstream processes. Ygr221c also shows interactions with both Cdc42 and Rho1 pathways. Apg17 shows interactions with proteins involved in cytokinesis, exocytosis, and Rho1 function.

of the interacting proteins has a previously unrecognized function. Additionally, it is not possible to know the directionality of the flow of information through the protein interaction network. Finally, further studies are required to determine when, where, and why two proteins interact. Here we discuss some interactions that appear particularly significant or provocative.

Cdc42 effectors

Activation of the Cdc42 GTPase is a key event in establishment and maintenance of cell polarity. Yeast cells deficient in Cdc42 function grow isotropically and are unable to form buds, mating projections, or pseudohyphae. They are unable to properly organize the actin cytoskeleton, septins, or the secretory pathway. Cdc42 interacts with several effector proteins that transduce its signal to bring about several processes, including polarization of the actin cytoskeleton (Cvrcková et al., 1995; Brown et al., 1997; Chen et al., 1997; Evangelista et al., 1997; Bi et al., 2000; Jaquenod and Peter, 2000). Protein–protein interactions detected in our two-hybrid screens suggest connections between Cdc42, its regulatory and effector proteins, and proteins involved in several different processes required for cell polarity development (Figs. 1–3 and Table I).

Screening with mutant Cdc42 baits locked in the GDP or GTP state, we found interactions between Cdc42 and several of its known regulators and effector proteins: Cdc24, Rga1, Bem1, Bem4, Cla4, Ste20, Gic1, and Gic2. Two-hybrid interactions were observed between Cdc42 GAPs and Cdc42 effectors. The GAP Rga1 interacted with Gic2, and the GAP Bem3 interacted with Cla4. It is possible that these interactions were bridged by the Cdc42 protein itself (Kozminski et al., 2000). However, if these interactions are direct, they might reflect a feedback mechanism for Cdc42 regulation.

Table II. **Cellular localization of YFP-tagged proteins encoded by novel ORFs**

Protein	Null mutant phenotype	Two-hybrid interactions	Homologues[a]	YFP fusion location	Figure
Ykl082c	Lethal[b]	Bud8, Zds2, Gic1, Gic2	*D. melanogaster* CG13648 (21%); CG9274 (22%) *C. elegans* ZK354.3 (25%); C17F3.3 (27%) *S. pombe* Spac8c9.10cp (24%); Spbc1861.01cp (20%)	Nucleolus	Fig. 4, A and B
Ycr086w	Viable, benomyl sensitive[c]	Gic1, Gic2	None known	Punctate localization at nuclear periphery, nuclear envelope	Fig. 4, C and D
Ygr221c	Viable, sensitive to nonhydrolyzable GDP analogues[c]	Cdc24, Pkc1	*S. cerevisiae* Yhr149c (32%); Muc1(23%)	Bud tip, bud neck	Fig. 4, E and F
Yhr149c	Viable[b]	Zds1, Zds2	*S. cerevisiae* Ygr221c (32%)	Bud tip, bud neck	Fig. 4, O and P
Yil079c/Air1	Viable[b]	Cdc24	*H. sapiens* ZNF9 (26%) *M. musculus* CNBP (26%) *D. melanogaster* CG9715 (28%); CG3800 (28%) *C. elegans* GLH-4 (25%)	Nucleolus	Fig. 4, G and H
Yer124c	Viable[b]	Boi1, Boi2, Zds2	*D. melanogaster* DS02740.2:BG:DS02740.2 (32%)	No detectable signal	Data not shown
Yil007c	Viable[b]	Rho1, Rho4, Pfs1, Chs4, Rpn4	*H. sapiens* PSMD9 (35%) *D. melanogaster* CG9588 (28%) *C. elegans* C44B7.1 (31%); Y42H9AR.F (30%) *S. pombe* Spac2h10.02cp (27%)	Cytoplasm	Data not shown
Ylr423c/Apg17	Viable[b]	Rho1, Rho2, Apg17, Exo84, Myo1, Nip100, Sro77, Ykr083c	*S. cerevisiae* Ynl047p (24%)	Punctate localization in cytoplasm	Fig. 4, I and J
Ypr171w	Viable[b]	Cap1, Rvs167, Sla1	*H. sapiens* USP8 (23%) *D. melanogaster* CG13648 (22%) *S. cerevisiae* Crp1 (25%)	Actin patches	Figs. 4, K and L, and 5
Ygr268c	Viable[b]	Rvs167, Sla1	*S. pombe* Spac17a5.10p (31%)	Cytoplasm	Data not shown
Yor284w	Viable[b]	Abp1, Rvs167, Sla2	None known	2–5 fast moving dots around cell periphery	Figs. 4, M and N, and 6
Yjr083c	Viable[b]	Rvs167	None known	No detectable signal	Data not shown
Ynl094w	Viable[b]	Abp1, Crn1, Hsl7, Rvs167, Sla1, Sla2, Sro77, Swe1	*S. pombe* Spbc29b5.04cp (26%)	Actin patches	Unpublished data

Proteins are listed in the order in which they are discussed in the text. Percentages refer to amino acid identity between homologues.
[a]Information on homologues from other species is taken from the Yeast Proteome Database at http://www.proteome.com.
[b]Information on these phenotypes of null mutants is taken from Winzeler et al. (1999).
[c]Information on these phenotypes of null mutants is taken from Rieger et al. (1999).

The Cla4 p21-activated protein kinase (PAK)* showed two-hybrid interactions with several proteins. Its interaction with the septin Cdc12 suggests that a direct interaction might underlie the role of this PAK in regulation of septin filament organization and cytokinesis (Benton et al., 1997; Weiss et al., 2000). The relevance of this interaction is supported by the observation that a *cla4 cdc12* double mutant is a synthetic lethal (Cvrcková et al., 1995). Two-hybrid interactions between Cla4 and the cortical patch proteins Sla2 and Abp1 suggest a previously unrecognized regulatory role associated with cortical actin patches. Both Abp1 and Sla2 have functions in cortical patch assembly and in endocytosis, a process that is intimately linked to cortical patches (Lila and Drubin, 1997; Wesp et al., 1997). Sla2 is required to nucleate actin assembly in permeabilized yeast cells (Li et al., 1995; Ayscough et al., 1997) and it mediates polarization of actin cortical patches in a Cdc42-dependent process (Yang et al., 1999). The *cla4* null mutant, like an *sla2* mutant, is defective in actin nucleation in permeabilized yeast cells (Eby et al., 1998). Colocalization of Sla2 with actin is most evident in unbudded and small-budded cells, suggesting that its activity might be most important early in the cell cycle (Yang et al., 1999). The kinase activity of Cla4 also appears to be required at an early stage of the cell cycle, as inhibition of the Cla4 kinase in unbudded cells, but not at later stages, leads to hyperpolarized bud growth and defects in cytokinesis (Weiss et al., 2000). Perhaps Cla4 regulates the polarity of cortical patches via an interaction with Sla2. The NH_2-terminal region of Cla4, which appears to have a function in maintaining cell polarity (Bi et al., 2000), contains proline-rich motifs which might be binding sites for the SH3 domain of Abp1 (Weiss et al., 2000). Both Sla2 and Abp1 have vertebrate homologues, and it will be important to test these for interactions with and regulation by PAK kinases (Engqvist-Goldstein et al., 1999; Kessels et al., 2000). Interestingly, PAK family protein kinase was implicated previously in the regulation of yeast class I myosins, and Abp1, Sla2, and class I myosins are each implicated in separate mechanisms to activate the Arp2/3 complex (Wu et al., 1996, 1997; Evangelista et al., 2000; Lechler et al., 2000; Goode et al., 2001; M. Duncan, J. Cope, and D. Drubin, personal communication).

Multicopy expression of *MSB2* suppresses the defects of a *cdc24* mutant (Bender and Pringle, 1992), but the function of the Msb2 protein is unknown. A two-hybrid interaction between Cla4 and Msb2 suggests that Msb2 is also part of the Cdc42 regulatory pathway. We found that Msb2 interacts with Bni4, a protein that targets chitin deposition to sites of polarized growth by linking chitin synthase to septins (DeMarini et al., 1997). Msb2 might coordinate cell wall

*Abbreviation used in this paper: PAK, p21-activated protein kinase.

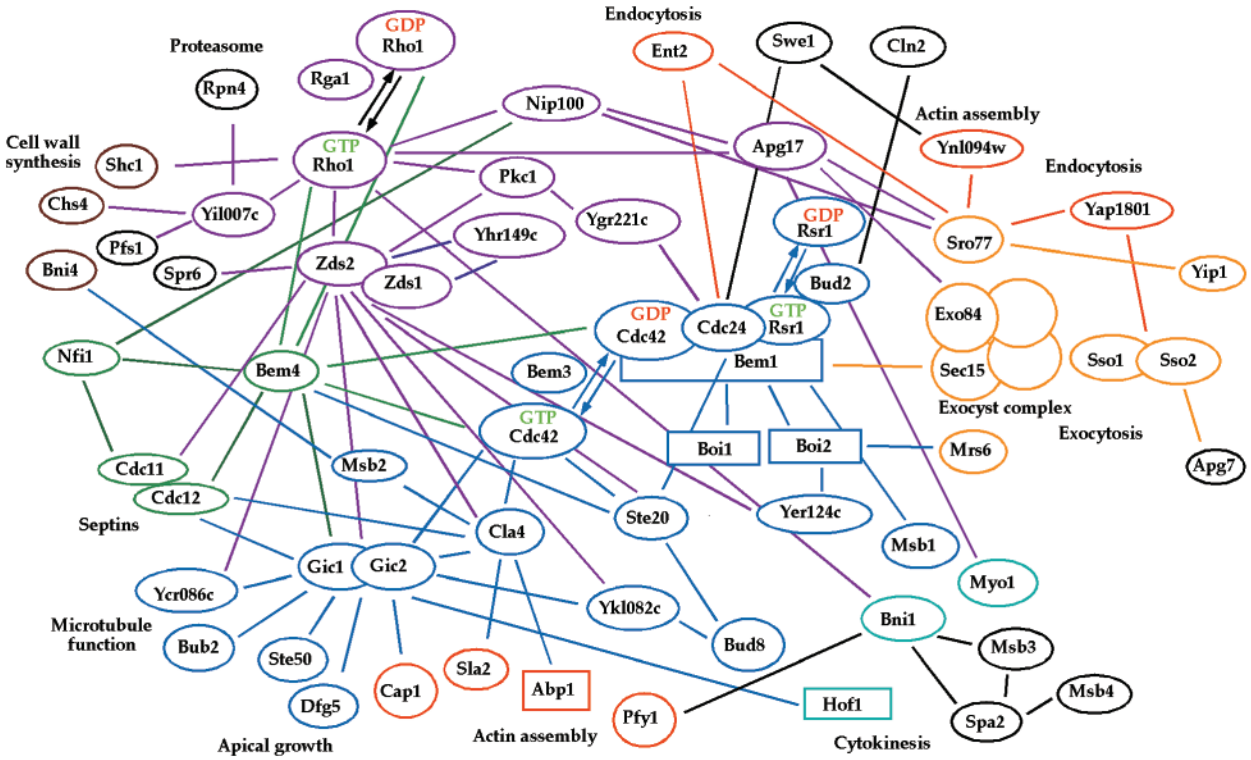

Figure 2. **Interaction map for proteins involved in Cdc42- and Rho1-regulated processes and in other cell polarity development pathways.** Proteins that regulate Cdc42 function or that transduce signals from activated Cdc42 are shown in blue. Rho1 and its effectors are shown in purple. Cdc42-regulated pathways show interactions with proteins involved in septin organization via interactions with Bem4; with Rho1 via interactions with Zds2 and Ygr221c; and with proteins involved in cell cycle control, endocytosis, and polarized exocytosis. Cdc42 effector proteins show interactions with proteins involved in cytokinesis, microtubule stability, polarized growth, actin assembly, polarized secretion, and cell wall synthesis. Proteins involved in septin organization may interact with Rho1 via Bem4. Rho1 involvement in nuclear migration, actin/myosin ring contraction, and septum formation (exocytosis) during cytokinesis is suggested by Apg17-mediated connections between proteins involved in these processes. Other interactions suggest connections between late exocytic and early endocytic processes, between early and late steps in secretory pathways, and between exocytosis and autophagy.

growth with other Cdc42-regulated processes. Cla4 also showed two-hybrid interactions with the Cdc42 effectors Gic1 and Gic2 and with Zds2, a protein that might be a regulator of Cdc42 and Rho1 (Bi and Pringle, 1996). As discussed below, Zds2 and its homologue Zds1 showed interactions with Rho1 and several proteins downstream of Cdc42 and may therefore connect the Rho1 and Cdc42 pathways.

The homologous Cdc42 effectors Gic1 and Gic2 (Brown et al., 1997; Chen et al., 1997; Jaquenod and Peter, 2000) also showed interactions with Ste50, a protein that positively regulates the Ste11 kinase in the pheromone response pathway (Xu et al., 1996), in the Hog1 osmotic stress pathway (Posas et al., 1998), and during pseudohyphal growth (Ramezani Rad et al., 1998). Ste50 and the pheromone response pathway have been implicated recently in maintenance of cell wall integrity in budding cells (Cullen et al., 2000). Dfg5, another protein required for polarized and pseudohyphal growth (Mösch and Fink, 1997), also interacts with Gic1. These interactions may be involved in maintaining polarized growth during budding and mating and in reestablishing polarity after osmotic stress (Brewster and Gustin, 1994). An interaction between Gic2 and the Cap1 subunit of the actin filament capping protein suggests a possible role in regulating actin assembly and, therefore, a potential novel link between Cdc42 and the actin cytoskeleton.

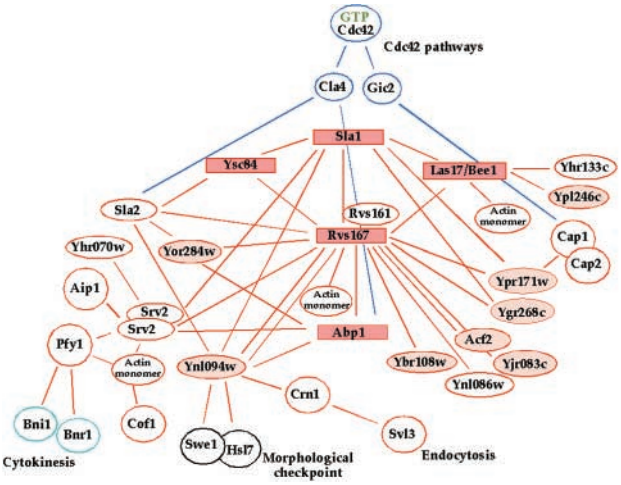

Figure 3. **Protein interactions involved in actin assembly and actin functions in endocytosis, cytokinesis, and morphogenesis.** Cdc42 effectors show interactions with proteins involved in endocytosis and cytokinesis. Interactions between Ynl094w, several actin cytoskeleton proteins, and Swe1 and Hsl7 may underlie the morphogenesis checkpoint that monitors actin assembly. Several interactions between SH3 domain–containing proteins (shaded rectangles) and proteins containing proline-rich putative SH3 binding sites (shaded ovals) are shown.

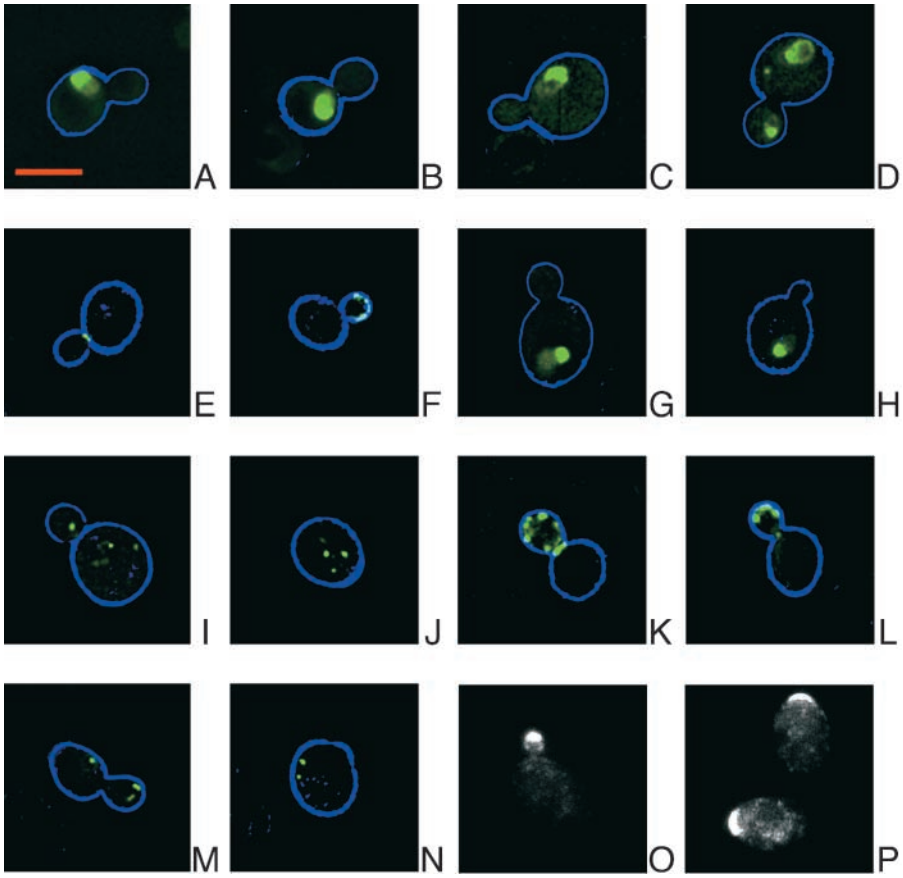

Figure 4. **Fluorescence micrographs of proteins tagged at the COOH terminus with YFP (A–N).** YFP signal is shown in green. Cells were outlined by staining with Alexa fluor 633 conjugated to concanavalin A (blue). (A and B) Ykl082c-YFP; (C and D) Ycr086w-YFP; (E and F) Ygr221c-YFP; (G and H) Yil079c/Air1-YFP; (I and J) Ylr423c/Apg17-YFP; (K and L) Ypr171w-YFP; (M and N) Yor284w-YFP. (O and P) Immunofluorescence micrographs of Yhr149c tagged with a 13Myc epitope. GFP-tagged Yhr149 exhibits the same localization although the GFP signal is extremely weak. Bar, 5 μm.

Apical bud growth appears to stimulate establishment of a distal bud site landmark that functions in the bipolar budding pattern seen in diploids. Several interactions may shed new light on this process. Gic1 and Gic2 both interact with Zds2, and all three proteins showed interactions with Ykl082c, an essential protein of unknown function (Winzeler et al., 1999). Ykl082c also showed interactions with Bud8, a protein that appears to be a component of the distal bud site tag (Zahner et al., 1996; Harkins et al., 2001). Recent findings also suggest that Ste20 PAK kinase, a Cdc42 effector, regulates the pattern of diploid bud site selection via Bud8 (Sheu et al., 2000). Ste20 was found to affect bipolar bud site selection through its regulation of apical growth in the bud. The decreased period of polarized bud growth seen in *ste20* mutants reduced the accuracy of bud site selection in diploid cells and produced a unipolar budding pattern like that of the *bud8* mutant. Interestingly, we found that Bud8 interacts with Ste20. The Ykl082c protein may also be involved in polarized growth and participate in this process. We found that haploid cells containing the genomic Ykl082c-YFP fusion were slow growing and temperature sensitive. Heterozygous diploids appeared to have a cell cycle delay in late mitosis, suggesting a possible defect in nuclear migration (data not shown). Curiously, YFP-tagged Ykl082c localized to the nucleolus (Table II and Fig. 4, A and B). Nucleolar localization may be connected to its interaction with Zds2, which in addition to its effects on cell polarity also has a role in gene silencing and interacts with the nucleolar protein Sir2 (Roy and Runge, 2000). Nucleolar sequestration via association with a multiprotein complex containing Sir2 has been found to control the functions of regulatory proteins, including the protein phosphatase Cdc14 that regulates mitotic exit (Shou et al., 1999; Visintin and Amon, 2000).

The double mutant *gic1 gic2* has depolarized microtubules as well as a disorganized actin cytoskeleton (Brown et al., 1997). We found two novel interactions for Gic1 and Gic2 that suggest that these proteins may directly affect microtubule polarization and nuclear migration during mitosis. The first is an interaction between Gic1 and Bub2, which functions in the microtubule/spindle checkpoint (Hoyt et al., 1991). The second is with an uncharacterized protein, Ycr086w. The *ycr086w* null mutant is benomyl sensitive and has impaired nuclear migration (Rieger et al., 1999). YFP-tagged Ycr086w localizes to the nuclear periphery in a punctate pattern (Table II and Fig. 4, C and D). Other interactions of Gic1 and Gic2 with the septin Cdc12 and with Hof1/Cyk2, an SH3-domain containing protein involved in cytokinesis (Kamei et al., 1998; Lippincott and Li, 1998a; Vallen et al., 2000), suggest that Gic1 and Gic2 might regulate cytokinesis, particularly septum formation. In total, these interactions suggest that Gic1 and Gic2 have the potential to regulate microtubule polarity and to coordinate nuclear migration and division with cytokinesis (Pereira et al., 2000). Gic1 also interacts with Bem4, which is interesting because Bem4 also interacts with the septins Cdc11 and Cdc12 and with several GTPases, including Cdc42 (and Rsr1, see below), and is thought to have a role in GTPase localization or regulation (Hirano et al., 1996; Mack et al., 1996). It is tempting to speculate that these Bem4 interactions might target Cdc42 and other GTPases to the bud neck to regulate septation.

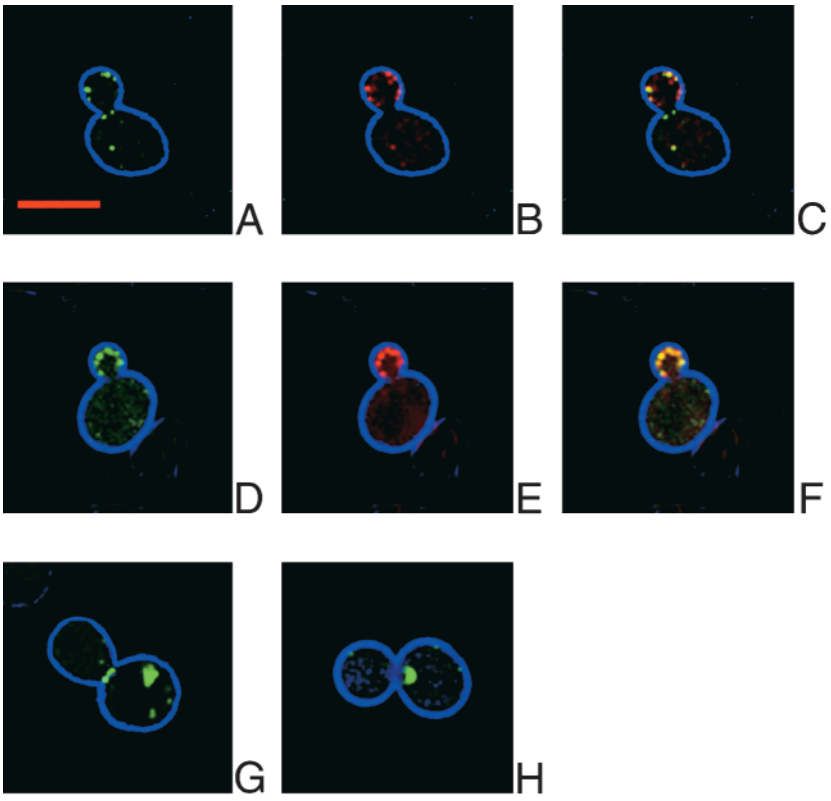

Figure 5. **Fluorescence micrographs of Ypr171w-YFP showing localization to actin cortical patches.** Cells were outlined by staining with Alexa fluor 633 conjugated to concanavalin A (blue). (A and D) Ypr171w-YFP (green); (B and E) Abp1-CFP (red); (C and F) merged image. (G and H) Ypr171w-YFP (G) and Abp1-CFP (H) in an *ark1 prk1* deletion strain. Bar, 5 μm.

Cdc42 regulators

The guanidine nucleotide exchange factor Cdc24 is required for activation of Cdc42 (Zheng et al., 1994). We found novel interactions between Cdc24 and several other proteins. One of the most interesting is Ygr221c, a protein of unknown function. Consistent with a function for Ygr221c as a positive GTPase regulator, the *ygr221c* null mutant is sensitive to a GDP analogue that inhibits G protein activation by GTP (Rieger et al., 1999). Ygr221c also interacted with the yeast protein kinase C (Pkc1), which functions downstream of the Rho1 GTPase. Therefore, Ygr221c may provide a regulatory connection between Rho1- and Cdc42-regulated pathways. In support of the in vivo relevance of these two-hybrid interactions, YFP-tagged Ygr221c localizes to sites of cell growth, including the nascent bud site, bud tips, and bud necks, similar to the pattern observed for Cdc42 (Table II and Fig. 4, E and F; S. Tcheperegine and E. Bi, personal communication). A homologue of Ygr221c, Yhr149c, was identified as a dosage suppressor of a *cdc42-118* polarized growth defect (M. Lau, S. Gadde, and K. Kozminski, personal communication). Like Ygr221c, Yhr149c localizes to sites of cell growth (Fig. 4, Q and R; S. Tcheperegine and E. Bi, personal communication). Yhr149c was used as a bait in a two-hybrid screen and found to interact with Zds1 and Zds2, suggesting that it may be involved in coordinating Rho1- and Cdc42-regulated pathways (see below).

Cdc24 localizes to the nucleus during the G1 phase of the cell cycle (Toenjes et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000). We detected interactions between Cdc24 and Yil079c. Yil079c was found recently to have a role in regulation of nuclear RNA processing and named Air1 (Inoue et al., 2000). YFP-tagged Yil079c localized to the nucleolus (Fig. 4, G and H). This result is particularly intriguing in light of the fact that the human Cdc42 was recently found to stimulate RNA splicing (Wilson and Cerione, 2000; Wilson et al., 2000).

Cdc24 was also found to interact with the epsin Ent2 (Wendland et al., 1999). Ent2 and other epsins are clathrin-binding proteins that function during the internalization step of endocytosis (Chen et al., 1998). They are also essential for normal cortical actin patch assembly (Wendland et al., 1999; Tang et al., 2000). Cortical actin patch proteins are essential for the internalization step of endocytosis (Kübler and Riezman, 1993), and the cortical patches are concentrated proximal to sites of rapid exocytosis (Pruyne and Bretscher, 2000b). The interaction between Cdc24 and an epsin might target the endocytic pathway to bud tips, where it would be in proximity with the exocytic pathway. As each process retrieves components necessary for the other, both may be made more efficient by this proximity.

Zds1 and Zds2 are homologous proteins. The double mutant *zds1 zds2* has abnormally elongated buds, abnormal septin localization, and a cytokinesis defect (Bi and Pringle, 1996). Zds1 localizes to bud tips in small- and medium-budded cells. Based on genetic interactions, Zds1 and Zds2 seem to be negative regulators of the polarized growth and septation processes initiated by Cdc42 activation (Bi and Pringle, 1996). Two-hybrid interactions of Zds1 and Zds2 with the Cdc42 effectors Gic1, Gic2, and Cla4, and with other proteins that are likely to function downstream of these effectors (Table I and Figs. 1 and 2), provide support for a role in regulation of Cdc42-dependent pathways. Intriguingly, Zds2 also showed interactions with Rho1 and its downstream effectors Pkc1 and Bni1, suggesting either a mechanism to coordinate Cdc42 and Rho1

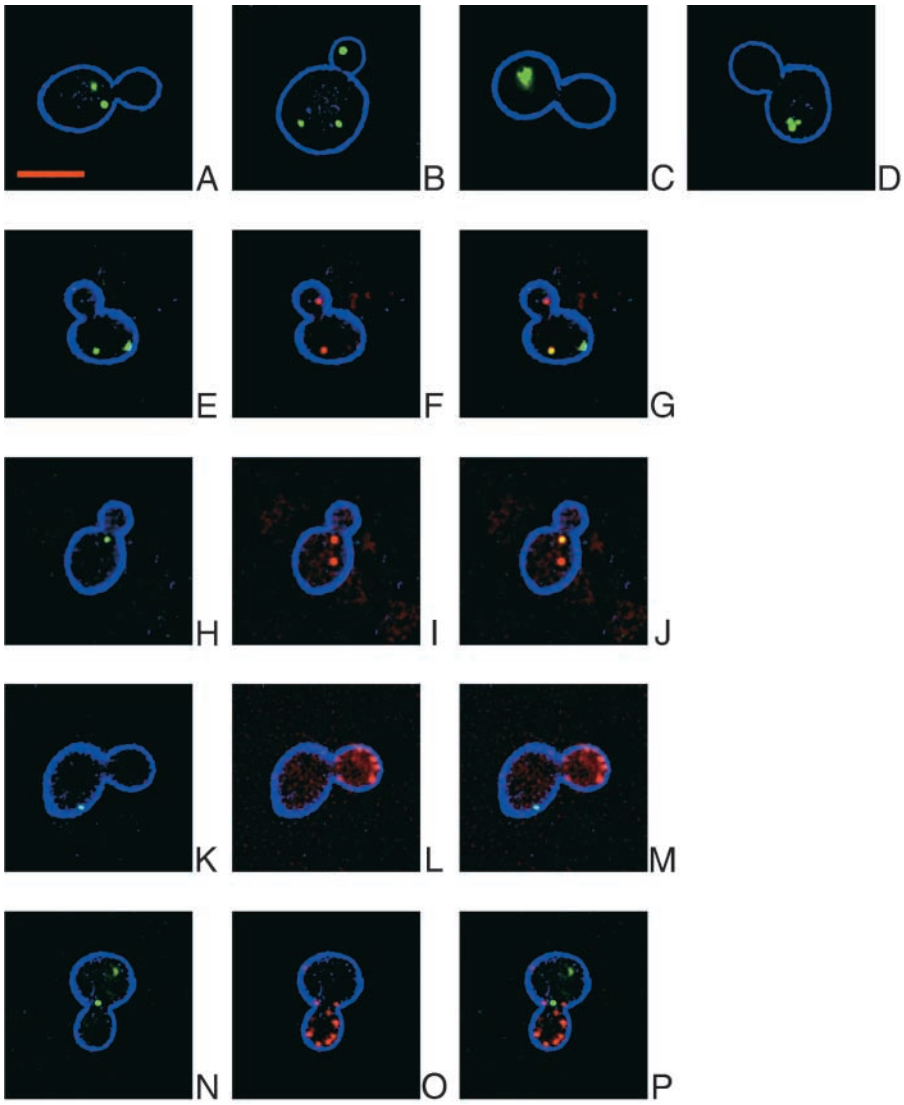

Figure 6. **Fluorescence micrographs of Yor284w-YFP showing the effect of actin depolarization (A–D), localization to the spindle pole body (E–J), and localization relative to the actin cytoskeleton (K–P).** Cells were outlined by staining with Alexa fluor 633 conjugated to concanavalin A (blue). (A and B) Yor284w-YFP; (C and D) Yor284w-YFP in an *ark1 prk1* deletion strain. (E and H) Yor284w-YFP (green); (F and I) Spc29-CFP (red); (G and J) merged image. (K and N) Yor284w-YFP (green); (L and O) Abp1-CFP (red); (M and P) merged image. Bar, 5 μm.

pathways, or that Zds2 has distinct roles in the two pathways. Zds2 also interacts with the septin Cdc11, a sporulation-specific protein, Spr6 (Kallal et al., 1990), and three proteins of unknown function, Yer124c, Yal004w, and Yel023c.

Cell polarity develops in response to cortical cues. In budding yeast, cortical markers left by previous cell divisions result in recruitment and local activation of the Rsr1/Bud1 GTPase. Rsr1 is linked to Cdc42 via interaction with Cdc24 (Ruggieri et al., 1992; Bender, 1993; Zheng et al., 1995; Chant, 1999; Park et al., 1999) and via the scaffold protein Bem1, whose two-hybrid interactions are described in a subsequent paragraph. We detected a novel interaction between Rsr1 and Bem4, a protein mentioned above as interacting with Gic1 and the septins Cdc11 and Cdc12. Bem4 interacts with both the GTP- and GDP-bound forms of Rsr1 (Table I), as it does in its interactions with Rho-type GTPases (Hirano et al., 1996; Mack et al., 1996). As suggested above, Bem4 might bring multiple GTPases to the bud neck.

The activity of the Cdc28 cyclin–dependent kinase is required to recruit Cdc24 to the plasma membrane in G1 to coordinate cell polarity development with the nuclear division cycle (Jaquenod and Peter, 2000). We found that Bud2, the GTPase-activating protein that regulates Rsr1 (Bender, 1993; Park et al., 1993, 1999), interacts with the cyclin Cln2, which activates Cdc28 in G1. Bud2 might be a target of the Cln2-Cdc28 kinase to regulate polarity in G1. The observation that a *bud2 cln2* double mutant is a synthetic lethal provides support for this possibility (Benton et al., 1993; Cvrcková and Nasmyth, 1993).

Bem1 interacts with several proteins involved in Cdc42 activation and is thought to act as a scaffold for proteins involved in cell polarity development (Chant, 1999; Moskow et al., 2000). Here, Bem1 and Cdc24 were found to interact with Swe1. Swe1 is a protein kinase that phosphorylates and inhibits Cdc28 in a morphogenesis checkpoint response that monitors actin perturbation (McMillan et al., 1998) and septin assembly (Barral et al., 1999; McMillan et al., 1999; Shulewitz et al., 1999; Lew, 2000; Longtine et al., 2000). Interactions between Swe1, Bem1, and Cdc24 raise the possibility that Swe1 might also monitor assembly of the Bem1–Cdc24–Cdc42 complex at the bud site. Alternatively, these interactions may reflect a role suggested for Swe1 in adaptation to defects in polarity establishment (Weiss et al., 2000).

The Bem1 protein, via its two SH3 domains, interacts with Boi1 and Boi2 (Bender et al., 1996; Matsui et al., 1996). Boi1 and Boi2 are homologous proteins and they themselves con-

tain SH3 domains. We found that Bem1, Boi1, and Boi2 participate in several previously unreported protein–protein interactions. Both Boi1 and Boi2, as well as Zds2, showed interactions with Yer124c, an uncharacterized protein. Expression of Yer124c appears to be cell cycle regulated, with transcript levels peaking in G1 (Spellman et al., 1998). The protein contains a motif that is a potential SH3 domain ligand and which binds specifically to the Boi1 SH3 domain (unpublished data). The Boi1 bait was an activator, so that two-hybrid positives could not be easily detected, but several other new interacting partners were identified in the Boi2 screen. One of these is Msb1, which has been implicated genetically in Cdc42-regulated processes. *MSB1* is a multicopy suppressor of *cdc24*, *cdc42*, and *bem4* mutants (Bender and Pringle, 1989; Mack et al., 1996), and the *msb1* null mutant is synthetic lethal with a *bem1* null (Bender and Pringle, 1991).

Connections between Cdc42-regulated pathways and the secretory pathway

Actin cables and a class V myosin are required for accumulation of secretory vesicles at the growing tips of yeast cells (Pruyne and Bretscher, 2000a,b). However, an unresolved question is how vesicle fusion at the plasma membrane is properly targeted. Therefore, it is noteworthy that Rsr1, Bem1, and Cdc24 were all found to interact with the exocyst component Sec15. The exocyst is a multiprotein complex thought to dock secretory vesicles at sites of polarized growth (Ter Bush and Novick, 1995; Ter Bush et al., 1996). An interaction between Sec15 and the activated Sec4 Rab GTPase induces formation of the exocyst complex and vesicle docking on the plasma membrane (Guo et al., 1999b). Recently, Rho1 was shown to interact with Sec3 and be required for localization of the exocyst to the bud tip (Guo et al., 2001). A connection between the exocyst and the GTPases that function during bud initiation may allow Rsr1-, Cdc42-, and Rho1-regulated processes to be coordinated with exocytosis to initiate bud growth. One or more of these GTPases might regulate exocyst assembly.

An additional link between the polarity-regulating Rho GTPases and exocytosis was suggested by the observation that Boi2 interacts with the yeast Rab escort protein Mrs6. Mrs6 is required for Sec4-dependent transport of secretory vesicles to the plasma membrane (Fujimura et al., 1994; Jiang and Ferro-Novick, 1994; Bauer et al., 1996; Alory and Balch, 2000). This interaction, like those between Sec15, Bem1, and Rsr1, suggests a link between regulators of bud initiation and the secretory apparatus. The possibility that Boi1 and Boi2 affect secretion is supported by the finding that the budding defect of a *boi1 boi2* double mutant is suppressed by overproduction of the Rho3 GTPase (Bender et al., 1996; Matsui et al., 1996). Rho3 specifically regulates vesicle transport and fusion during exocytosis (Adamo et al., 1999; Robinson et al., 1999).

Rho1 effectors

The Rho1 GTPase is a major regulator of cell polarity and cell wall synthesis in *S. cerevisiae* (Yamochi et al., 1994; Drgonová et al., 1996; Kamada et al., 1996; Qadota et al., 1996). The GTP-bound form of Rho1 activates 1,3-β-glucan synthase, which catalyzes the synthesis of the major structural component of the cell wall, and Pkc1, which controls a mitogen-activated protein kinase cascade–regulating cell wall metabolism and actin polarity (Paravicini et al., 1992; Delley and Hall, 1999). We found that Rho1 interacts with Bem4, Rga1, Pkc1, and Bni1. The formin protein Bni1 is thought to be a cortical anchor that directs actin polarity and nuclear migration (Imamura et al., 1997; Fujiwara et al., 1998, 1999; Vallen et al., 2000), in part via an interaction with profilin (Pfy1). An interaction of Bni1 with Spa2 localizes Bni1 to the bud growth sites, where it mediates reorganization of the actin cytoskeleton and concentration of polarized growth to bud tips during apical growth (Fujiwara et al., 1998; Sheu et al., 2000). Bni1 is also connected to Cdc42 pathways (Evangelista et al., 1997; Jaquenod and Peter, 2000).

New interactions for Rho1 and its associated proteins suggest possible roles in cell wall synthesis during sporulation, starvation-induced autophagy, and cytokinesis. A role for Rho1 in cell wall synthesis during sporulation was suggested previously by the finding that a *bem2* Rho1-GAP mutant has a sporulation defect due to loss of cell wall integrity (Cid et al., 1998). We found that Bem4 interacted with the sporulation-specific septin Spr28 (De Virgilio et al., 1996; Fares et al., 1996), as well as with the septins involved in vegetative growth. Rho1 also interacted with Shc1, a protein required for maintenance of cell wall integrity under osmotic stress (Hong et al., 1999). *SHC1* expression is upregulated during sporulation and it is involved in the chitin synthase III–dependent formation of the spore wall chitosan layer (Bulawa, 1993). Shc1 has homology to the Chs4 protein (Cid et al., 1995; Trilla et al., 1997), which stimulates chitin synthase III activity (Bulawa, 1993; DeMarini et al., 1997; Trilla et al., 1997). Rho1 also interacted with a novel protein, Yil007c, which may have a function in regulating cell wall synthesis and other processes during sporulation. When used as bait in two-hybrid screens, Yil007c interacted with Chs4 and with Pfs1, a protein required for sporulation (Deng and Saunders, 2001). Pfs1 is a homologue of the *S. pombe* protein tea1, which regulates polarized growth (Mata and Nurse, 1997) and contains kelch repeats, structures thought to mediate binding interactions with actin filaments (Mata and Nurse, 1997). Yil007c is homologous to a human proteasome regulatory subunit (Watanabe et al., 1998), but had not been implicated as functioning with the yeast proteasome (Russell et al., 1999). However, we found an interaction between Yil007c and the proteasome subunit Rpn4, supporting such a role and suggesting a novel function in cell wall synthesis regulation. A Yil007c-YFP fusion localized diffusely in the cytoplasm (data not shown).

Rho1 also interacted with Ylr423c/Apg17, a protein that was shown recently to regulate autophagy by interacting with and activating the Apg1 kinase (Kamada et al., 2000). Autophagy is a poorly understood starvation-induced process by which cytoplasm is surrounded by a double membrane which then fuses with the vacuole or lysosome. Autophagy is induced by downregulation of the phosphatidylinositol kinases Tor1 and Tor2 (Ohsumi, 1999; Kamada et al., 2000). The interaction between Rho1 and Apg17 suggests that autophagy may be regulated in part by Rho1. In addition to its role in regulation of protein synthesis, Tor2 has an essential function in cell cycle–depen-

dent organization of the actin cytoskeleton (Helliwell et al., 1998). Overexpression of *PKC1*, *RHO1*, or *ROM2*, a gene that encodes a Rho1 guanidine nucleotide exchange factor, suppresses the actin organization defect of a *tor2* mutant. Perhaps Apg17 acts as an effector of Rho1 in a Tor2-dependent pathway that modulates cell polarity and autophagy.

Apg17 interacted with several proteins in addition to Rho1, including Rho2, Myo1, Nip100, Exo84, and Sro77, and it had been reported previously to make additional two hybrid interactions (Ito et al., 2001). Interactions with Exo84 and Sro77 suggest a connection to exocytosis. Exo84 is an exocyst component (Guo et al., 1999a), and Sro77 and its homologue Sro7 regulate vesicle docking and membrane fusion at the plasma membrane (Lehman et al., 1999). Interactions with Nip100 and Myo1 suggest that Apg17 may have a role in cytokinesis or that Myo1 and Nip100 have roles in autophagy. Nip100 is a yeast dynactin component involved in nuclear division and migration (Kahana et al., 1998; Fujiwara et al., 1999). Myo1 is a type II myosin that functions in the contractile ring (Bi et al., 1998; Lippincott and Li, 1998b). Sro77 and Sro7 have been found to form a complex with Myo1 (Kagami et al., 1998). The *Drosophila* homologue of Sro77 and Sro7, the *lethal(2) giant larvae* gene product, also interacts with myosins (Strand et al., 1994). During cytokinesis, targeted exocytosis at the site of cell division is coordinated with, and possibly guided by, contraction of the actinomyosin ring (Hales et al., 1999; Vallen et al., 2000). Perhaps a Myo1–Sro77 complex couples septum formation to contraction of the actomyosin ring, with Myo1 playing a specialized role in vesicle targeting to the bud neck (Schott et al., 1999). Interactions between Apg17 and Nip100, Myo1, Sro77, and Exo84 might be part of a mechanism coordinating nuclear migration, actomyosin ring contraction, and exocytosis during cytokinesis or autophagy (Kahana et al., 1998; Fujiwara et al., 1999; Hales et al., 1999). YFP-tagged Ylr423c/Apg17 localized in punctate patches in the cytoplasm (Fig. 4, I and J). Perhaps these patches play a role in autophagocytic vesicle formation.

Connections between Rho1 and Cdc42 pathways

As discussed above, Ygr221c interacts with both Cdc24 and the Rho1 effector Pkc1. This interaction is quite interesting as a potential means for Pkc1 to regulate Cdc42 function and cell polarity. Cell wall stress induces hyperactivation of Rho1, which in turn results in a transient loss of actin polarity in order to depolarize cell wall synthesis and repair widespread cell wall damage (Delley and Hall, 1999). An undefined Pkc1-dependent pathway controls actin depolarization. Depolarization is dependent on Pkc1 but not on the Pkc1-activated mitogen-activated protein kinase cascade, which is necessary for repolarization. The interactions between Pkc1, Ygr221c, and Cdc24, together with the proposed function of Ygr221c as a GTPase regulatory protein (Rieger et al., 1999), suggest that Pkc1 could affect the actin cytoskeleton by inhibiting Cdc42 function through an interaction with Ygr221c. Zds1 and Zds2 might also monitor and regulate Cdc42 in response to Rho1. Zds2 interacts with both Rho1 and the Rho1 effectors Pkc1 and Bni1, as well as with several proteins in Cdc42-regulated pathways. Interestingly, when Yhr149c, the homologue of the Cdc24-interacting protein Ygr221c, was used as bait in a two-hybrid screen, Zds1 and Zds2 were both found to interact with Yhr149c. These interactions suggest that Yhr149c may also be involved in coordinating Rho1- and Cdc42-regulated pathways.

Actin cortical patch assembly, the morphogenesis checkpoint, and endocytosis

Actin cortical patches are one of the major cytoskeletal structures in yeast and are essential for normal endocytosis, cell growth, and morphology (Botstein et al., 1997; Pruyne and Bretscher, 2000b). Patches are associated with invaginations of the plasma membrane (Mulholland et al., 1994) and are found in polarized clusters at regions of cell growth in budding cells. Numerous cortical patch proteins have been identified. How these proteins function in patch assembly and endocytosis are largely unknown. Patch assembly probably begins with the association of assembly factors recruited to the plasma membrane by Cdc42-associated proteins, and is then followed by nucleation of actin filaments and actin-dependent association of proteins regulating filament assembly and stability (Ayscough et al., 1997; Botstein et al., 1997; Pruyne and Bretscher, 2000b). Our results confirm several interactions between patch proteins, identify new interactions, and suggest roles for several uncharacterized proteins in patch assembly or patch-mediated endocytosis (Fig. 3).

Sla2 (related to mammalian Hip1), Sla1, Las17/Bee1 (related to mammalian WASp/SCAR), Rvs167 (related to mammalian amphiphysin), and Abp1 (related to mammalian Abp1) all function in actin nucleation and assembly (Holtzman et al., 1993; Amberg et al., 1995; Li et al., 1995; Li, 1997; Lila and Drubin, 1997; Wesp et al., 1997; Ayscough et al., 1999; Balguerie et al., 1999; Yang et al., 1999; Goode et al., 2001). Las17/Bee1 and Abp1 both localize with and activate the Arp2/3 complex to nucleate actin filament assembly (Madania et al., 1999; Winter et al., 1999; Goode et al., 2001). Sla1, Rvs167 and Abp1 might share functions in actin cortical patch assembly because they interact with overlapping sets of proteins and show synthetic–lethal genetic interactions (Holtzman et al., 1993; Lila and Drubin, 1997). The actin monomer binding adenylate cyclase regulatory subunit Srv2 can, for example, apparently interact with all three proteins to mediate the association of monomeric actin with the actin-nucleating complex (Freeman et al., 1996; Lila and Drubin, 1997).

Sla1, Rvs167, and Abp1 contain SH3 domains that are important for their function (Lila and Drubin, 1997; Ayscough et al., 1999), and several of the proteins with which they interact contain proline-rich regions that are potential SH3 domain binding sites. Sla1 and Rvs167 both showed interactions with Las17/Bee1 (Li, 1997) and Ysc84 (Madania et al., 1999). Ysc84 itself contains an SH3 domain that might make other protein contacts. Two proteins of unknown function, Ygr268c and Ypr171w, also interacted with Sla1 and Rvs167. As noted in Fig. 3, Ygr268c and Ypr171w contain proline-rich motifs that are potential SH3 domain binding sites. Ypr171w also interacted with Cap1, the α subunit of the yeast actin filament capping protein, which regulates the growth of actin filaments (Amatruda and Cooper, 1992; Amatruda et al., 1992). Thus, Ypr171w

may function as a link between actin-nucleating complexes and the actin-capping protein, perhaps coordinating the nucleation and elongation of actin filaments. YFP-tagged Ypr171w was observed at growth sites in the bud in a punctate pattern and at the bud neck of large-budded cells (Fig. 4, K and L). Coexpression of Ypr171w-YFP with CFP-tagged Abp1, an actin cortical patch component, demonstrated that Ypr171w colocalizes with some, but perhaps not all, Abp1-containing actin cortical patches (Fig. 5, A–F). The punctate localization pattern is, like that of Abp1, disturbed in the *ark1 prk1* double deletion strain (Fig. 5, G and H), which has abnormal actin clumps (Cope et al., 1999). However, Ypr171w localization to the bud neck is still seen in the *ark1 prk1* mutant (Fig. 5 G). A Ygr268c-YFP fusion localized diffusely in the cytoplasm (data not shown).

Another uncharacterized protein, Yor284w, interacted with cortical patch components Sla2, Rvs167, and Abp1. Sla2 localizes to sites of polarized growth independently of actin (Ayscough et al., 1997) and mediates an early step in the polarization of actin cortical patches (Yang et al., 1999). Rvs167 also affects patch polarization (Balguerie et al., 1999), perhaps via the interaction observed here with Sla2. Colocalization of Sla2 with actin is most visible in unbudded and small-budded cells, implying that its patch assembly activity is most important early in the cell cycle (Yang et al., 1999). Expression of Yor284w peaks in the G1 phase of the cell cycle (Spellman et al., 1998), supporting the idea that it plays a role in this process. The Yor284w-YFP fusion localized to a few distinct, mobile punctate structures in the cell (Figs. 4, M and N and 6, A and B). In many cases these dots were observed moving rapidly around the cell periphery. The relationship of these structures to cortical patches is unclear. Yor284w appears in a stationary, diffuse cytoplasmic clump in the *ark1 prk1* double deletion strain (Fig. 6, C and D), suggesting that its localization is dependent on actin polarization. However, coexpression of Yor284w-YFP with CFP-tagged Spc29 spindle pole body protein (Fig. 6, E–J) and CFP-tagged Abp1 (Fig. 6, K–P) showed that the Yor284w-containing dots do not localize to cortical patches, but that a subset of them colocalize with the spindle pole body.

Four other proteins that interact with Rvs167 contain proline-rich potential SH3 domain binding sites. One is Acf2, a protein implicated in cortical actin assembly (Lechler and Li, 1997), whereas the others, Ybr108w, Yjr083c, and Ynl094w, are proteins of unknown function. Ybr108w has been found to interact with both Rvs167 and Rvs161 (Bon et al., 2000), a protein that forms a complex with Rvs167 (Navarro et al., 1997). A Yjr083c-YFP fusion localized diffusely in the cytoplasm (data not shown).

Ynl094w showed interactions with five cortical patch proteins. It interacted with Sla2 and with three SH3 domain proteins involved in actin nucleation, Sla1, Rvs167, and Abp1. Ynl094w also interacted with the actin-bundling protein Crn1 (Goode et al., 1999) and with Sro77, a protein which functions in polarized secretion (Kagami et al., 1998; Lehman et al., 1999). These interactions suggest that Ynl094w may link actin nucleation to exocytosis. Other interactions suggest that Ynl094w might function in the morphogenesis checkpoint response. Because this response is poorly understood, two-hybrid interactions involving the checkpoint proteins might provide important insights into the mechanisms for monitoring the cytoskeleton. Ynl094w interacted with two proteins involved in regulation of the morphogenesis checkpoint, Swe1 and Hsl7 (Shulewitz et al., 1999). In addition to monitoring the actin cytoskeleton (McMillan et al., 1998; Lew, 2000), Swe1 may monitor septin organization via an interaction with Hsl7 and Hsl1 at the bud neck (Barral et al., 1999; McMillan et al., 1999; Shulewitz et al., 1999; Lew, 2000; Longtine et al., 2000). The fact that Ynl094w showed interactions with Swe1 and Hsl7 and with five cortical patch proteins suggests that Swe1 and Hsl7 might monitor actin assembly via an interaction with Ynl094w. Ynl094w has been found to localize to actin cortical patches (L. Tseng, M. Schulewitz, and J. Thorner, personal communication).

Interactions of the secretory apparatus

Polarized growth and budding require the delivery of proteins and lipids to specific sites on the plasma membrane. Under cell cycle control, exocytosis first becomes localized to regions of cell growth at the presumptive bud site. As a bud emerges, exocytosis initially localizes to a small region at the bud tip, then becomes delocalized in the bud, and finally it becomes localized to the bud neck, mirroring cortical actin cytoskeleton organization at each stage (Pruyne and Bretscher, 2000b). The mechanisms that insure this continual coordination between the exocytic machinery and the actin cytoskeleton have yet to be fully elucidated. The Rho3 GTPase interacts with elements of the exocytic machinery to control transport of secretory vesicles and vesicle docking and fusion at the plasma membrane. Vesicle transport is dependent on function of the class V myosin Myo2 (Schott et al., 1999). Vesicle fusion occurs through an interaction with Exo70 (Adamo et al., 1999; Robinson et al., 1999), a component of the exocyst complex. Rho3 can also affect organization of the actin cytoskeleton (Imai et al., 1996). We found that Rho3 interacts with three subunits of casein kinase II. This observation is interesting because one function of casein kinase II is to maintain actin cytoskeleton polarity (Rethinaswamy et al., 1998). Our results suggest that casein kinase II might therefore function as an effector of Rho3 to regulate actin cytoskeleton organization, and that Rho3 might coordinate secretory and actin cytoskeletal organization.

The vesicle-docking protein Sro77, homologous to the *Drosophila* protein *lethal(2) giant larvae* gene product, showed numerous interactions implicating this protein in regulatory, cytoskeletal, and endocytic roles (Table I). As mentioned above, Sro77 showed interactions with Apg17 and Nip100. It also showed interactions with the epsin Ent2 and with Yap1801, both clathrin-binding proteins that function during the internalization step of endocytosis (Chen et al., 1998; Wendland and Emr, 1998; Wendland et al., 1999). Sro77 and the related protein Sro7 might therefore also function in the early steps of endocytosis, or they may coordinate endocytosis and exocytosis. The v-SNAREs Snc1 and Snc2 appear to be involved in both exocytic and endocytic transport at the plasma membrane. Snc1 and Snc2 can interact with endosomal t-SNAREs and *snc* mutants are defective in endocytosis as well as exocytosis (Gurunathan et al., 2000). Perhaps the plasma membrane t-SNAREs and

their regulators, such as Sro7/Sro77, are also able to participate in both processes. Similiarly, an interaction we observed between Sro77 and Yip1, which recruits the Ypt1 and Ypt31 transport GTPases to the Golgi apparatus, suggests similar possible relationships between proteins involved in early and late steps of exocytosis and/or endocytosis.

Sro77 also showed an interaction with Bcy1, the regulatory subunit of cAMP-dependent protein kinases (Cannon et al., 1990). In epithelial cells, exocytosis is stimulated by the cAMP-dependent protein kinase PKA (Takuma, 1990; Koh et al., 2000), which appears to regulate SNARE complex formation (Foster et al., 1998). Perhaps SNARE assembly in *S. cerevisiae* is also regulated in response to cAMP-dependent protein kinase activity.

Further Sro77 interactions were observed with several uncharacterized proteins, including Ynl094w, which may be a link between the secretory apparatus and actin organization (see above), and Kin3, a protein kinase of unknown function.

Sso1 and Sso2 are syntaxin homologue t-SNAREs that mediate vesicle targeting to the plasma membrane during exocytosis (Aalto et al., 1993). Sso2 interacted with Apg7, a regulator of autophagy, revealing another possible connection between exocytic and autophagocytic processes. Apg7 has homology to the E1 family of ubiquitin-activating enzymes and mediates a novel protein conjugation reaction required for autophagy (Mizushima et al., 1998; Kim et al., 1999; Tanida et al., 1999). Perhaps Apg7-mediated modification of plasma membrane t-SNAREs recruits them for formation of autophagocytic vesicles or for processes related to exocytosis.

MSB3 and *MSB4* are multicopy suppressors of bud emergence mutations and appear to link Cdc42 to the actin cytoskeleton (Bach et al., 2000; Bi et al., 2000). Surprisingly, Msb3 and Msb4 were also found to have GAP activity towards the Rab GTPases Sec4 and Ypt6, respectively (Albert and Gallwitz, 1999, 2000). Therefore, Msb3 and Msb4 may have roles in exocytosis. Sec4 is required for formation of the exocyst complex and for vesicle docking at the plasma membrane (Walworth et al., 1992; Guo et al., 1999b), whereas Ypt6 is required for vesicle transport from the endoplasmic reticulum to the Golgi apparatus (Lupashin et al., 1996). In our screens, both Msb3 and Msb4 interacted with Spa2, a protein that concentrates polarized growth to bud and mating projection tips during apical growth, and to the septum during cytokinesis (Fujiwara et al., 1998; Sheu et al., 2000). Sec4 is partially delocalized in *spa2* mutants (Sheu et al., 2000), and Spa2 is required for normal localization of secretory vesicles to the cell fusion zone during mating (Gammie et al., 1998). These genetic observations and the interactions with Msb3 and Msb4 provide support for a role for Spa2 in polarized exocytosis.

Conclusions

We have reported here the results of a two-hybrid study of proteins involved in cell polarity development, a highly complex process involving cortical cues, signaling proteins, the cytoskeleton, and the secretory apparatus, each of which is itself characterized by considerable complexity. Elucidation of the mechanisms that underlie cell polarity development is a daunting task, due to the vast number of proteins involved. The result of our studies is a protein interaction map that helps define the scope of the problem of understanding cell polarity development and can be used to guide further genetic and biochemical studies. Because most of the proteins and processes included in this study are highly conserved, this map should prove useful for studies of polarity development in diverse cell types. Among interactions identified in this map are some that implicate new proteins in polarity development, and others that suggest modes for coordinating distinct processes involved in polarity development. Our localization of previously uncharacterized proteins implicated here in polarity development is only the first step in verification of the biological relevance of each interaction. Future studies must address when, where, and why each interaction occurs and how these interactions are regulated. Despite years of genetic analysis of cell polarity development in budding yeast, we were able to implicate uncharacterized proteins in this process, and to suggest novel functions for proteins studied previously. Genetic studies may have missed much of this information, due to factors including redundancy, lack of appropriate alleles, homeostasis mechanisms, and checkpoints that mask the underlying mutant defect by arresting a process before an informative phenotype develops. In total, the large number of interactions that we identified among proteins involved in diverse aspects of cell polarity development suggests a high level of integration in the functioning of these proteins.

Materials and methods

Generation of DNA binding domain hybrids

Transformants containing Gal4 DNA binding domain hybrids were constructed in the α mating type of the yeast strain PJ694 (*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (James et al., 1996) as described (McCraith et al., 2000; Uetz et al., 2000). Recombination (Ma et al., 1987) of the linearized vector pOBD2 (McCraith et al., 2000) with PCR fragments corresponding to each of the yeast ORFs was used to generate the hybrids (Hudson et al., 1997). Transformation was carried out using the lithium acetate procedure (Ito et al., 1983). After transformation, cells were plated on synthetic trp media. Yeast media were prepared as described (Sherman et al., 1986). Cdc3, Cdc11, Cdc12, and Bud8 baits were made in the vector pGDBU and transformants selected on ura media (James et al., 1996).

Several GTPase baits contained cysteine-to-serine amino acid substitutions to prevent prenylation and facilitate nuclear entry, and other substitutions that affect GTP binding and hydrolysis to favor either the GTP- or GDP-bound form: Rho1-GTP (Q68H, C206S), Rho2-GTP (Q 65H, C188S), Rho3-GTP (G25V, C228S), Rho4-GTP (Q70H, C228S), Cdc42-GTP (G12V, C188S), Cdc42-GDP (D57Y, C188S), Rsr1-GTP (G12V), and Rsr1-GDP (K16N). Gic1 and Gic2 fusions were truncations lacking 30 residues at the COOH terminus because the full-length protein was an activator. The Sso1(1–515), Sso2(1–266), and fusions were truncations that removed predicted transmembrane domains at their COOH termini. The Msb2 fusion contained a truncation, Msb2(1186–1306) that removed predicted transmembrane domains at the NH_2 and COOH termini. The Bud8 and Bud9 fusions also contained truncations, Bud8(534–578) and Bud9(480–521), that removed predicted transmembrane domains.

Two-hybrid screens

Screens of the yeast ORF activation domain fusion array were performed in a manner similar to that described previously (McCraith et al., 2000; Uetz et al., 2000). This array was expressed in the **a** mating type of strain PJ694 (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (James et al., 1996). To screen for protein–protein interactions, we mated a transformant containing one of the DNA binding domain hybrids to all of the transformants of the array, selecting diploids using markers carried on the two-hybrid plasmids. The diploids were then transferred to selective plates deficient in histidine, and colonies positive for the two-hybrid reporter *HIS3* gene were identified by their positions in the array.

Two-hybrid screens can generate significant numbers of false positives that are not reproducible in duplicate screens. Random generation of histidine-positive colonies can result from overexpression of a fusion protein that affects transcription or cell metabolism, rearrangements or deletions of the DNA-binding domain plasmid, recombinational events between plasmids, or genomic rearrangements of the host strain. To identify reproducible two-hybrid interactions rapidly, we duplicated the array so that each ORF-Gal4 activation domain fusion is represented twice on a microassay plate. The entire array is contained in duplicate on 16 microassay plates of 768 colonies each. Only protein combinations that resulted in histidine prototrophy for both duplicate colonies of a given activation domain fusion were scored as two-hybrid interactions. We estimate that 10–20% of the yeast ORF-Gal4 activation domain fusions are not expressed due to errors in the PCR amplification of the ORFs. In addition, there are likely to be other constructs that are expressed poorly or in a nonfunctional form due to improper folding.

All pinning steps were carried out using a Biomek 2000 robot (Beckman Coulter) and a 768-pin replicating tool (UW Scientific Instruments, Machine/Optical Division). Strains containing the Gal4-DNA binding-domain hybrids were first tested on plates of synthetic his media with different concentrations of 3-aminotriazole to determine the level of stringency needed to eliminate background activation of the *HIS3* reporter gene. Strains were grown overnight at 30°C in 25 ml of trp synthetic medium, centrifuged at 1,000 rpm for 2 min and resuspended in 5–7 ml of medium. The cell suspension was transferred to 16 plates of solid YEPD medium. The yeast ORF activation domain fusion array was then replica-pinned onto the plates. Plates were incubated at 30°C for 2–4 d to allow mating, and then cells were replica-pinned to synthetic trp leu medium. After 2–4 d, cells were replica-pinned onto his synthetic media containing the appropriate concentration of 3-aminotriazole. Plates were incubated at 30°C for 7–10 d and then scored for two-hybrid positives.

Generation of ORFs tagged with CFP or YFP

A PCR-based method was used to integrate a gene encoding either YFP or CFP at the 3′ end of the targeted yeast ORF such that each fusion protein is expressed under the control of its native promoter (Wach et al., 1997). Detailed protocols are described at http://depts.washington.edu/~yeastrc/fm_home3.htm. The template for integration of YFP was the plasmid pDH6, which contains the YFP ORF followed by the kan^r gene. pDH6 was made by replacing the AvaI–AscI fragment encoding GFP in pFA6a-GFP(S65T)KanMX6 (Wach et al., 1997) with the AvaI–AscI fragment encoding YFP from pDH5. pDH5 was derived from pFA6a-GFP(S65T)HIS3MX6 by site-directed mutagenesis using the QuikChange method as described by the supplier (Stratagene). The mutations in YFP as compared with the original GFP are: S65G, V68L, Q69K, S72A, Q80R, and T203Y. The template for integration of CFP was plasmid pDH3, which contains the CFP ORF followed by the kan^r gene. pDH3 was derived from pFA6a-GFP(S65T)KanMX6 by site-directed mutagenesis using the QuikChange method. The mutations in CFP as compared with the original GFP are: F64L, S65T, Y66W, Q80R, N146I, M153T, V163A, and N164H. Integrations were checked by PCR.

Fluorescence microscopy

Cells containing YFP or CFP fusion proteins were grown on solid YPD medium overnight at 30°C and then resuspended in PBS containing 1.3 μg/ml concanavalin A tagged with Alexa 633 (Molecular Probes) and incubated for 30 min at 30°C. Cells were washed twice with PBS and resuspended in S medium or SD complete medium (1.7 g/liter Difco yeast nitrogen base without amino acids or ammonium sulfate, 5 g/liter ammonium sulfate, 0.1% casamino acids, 25 μg/ml uracil, 50 μg/ml adenine, 100 μg/ml tryptophan, 2% glucose). Cells were mounted in one of two ways. An aliquot of cells (3 μl) was mixed on the slide with an equal volume of SD complete medium containing 1.2% SeaPlaque low melting temperature agarose (FMC BioProducts) at 40°C. A coverslip (No. 1.5) was quickly added and pressed firmly. Alternatively, a cushion of 1.2% SeaKem LE agarose (FMC BioProducts) in SD complete medium was poured into 0.5-mm concavity slides (PGC Scientific) and pressed flat with another slide. Once the agar was solidified, an aliquot (15 μl) of cells was pipetted onto the cushion and covered with a coverslip (No. 1.5). The latter method is preferable for examination of actin cytoskeletal components.

Fluorescence microscopy was performed on a DeltaVision microscope with a Photometrics Quantix camera. The filter sets were from Omega. The images were sharpened by two dimensional deconvolution using the SoftWorX software (Applied Precision, Inc.).

To localize Yhr149c, the COOH terminus was tagged with a 13Myc epitope using the PCR-based method of Longtine et al. (1998) in the strain DDY1102 (MAT**a**/MATα ade2-1/ADE2 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 LYS2/lys2-801) (Kozminski et al., 2000). Log phase cells were processed for indirect immunofluorescence microscopy and images were acquired as described by Kozminski et al. (2000). Mouse anti-Myc antibody (9E10; Santa Cruz Biotechnology, Inc.) and FITC-conjugated donkey anti–mouse antibody (Jackson ImmunoResearch Laboratories) were diluted 1:50 and 1:100, respectively.

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