Mutational and hyperexpression-induced disruption of bipolar budding in yeast

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Analysis of bud-site selection in the yeast *Saccharomyces cerevisiae* has helped to identify many genes that are generally important for eukaryotic cell polarization. Colony morphology screens were used to identify factors relevant to the process of bipolar budding in yeast. Mutants defective in bipolar budding were identified by virtue of their inability to grow as pseudohyphae in a haploid *bud3* background. A mutant allele of the *MYO2* gene, encoding a class-V unconventional myosin was identified that perturbs bipolar budding without affecting axial budding and without grossly affecting the role of Myo2p in secretion and maintenance of the actin cytoskeleton. Several genes were also identified whose products, when overexpressed, are capable of disrupting bipolar budding. Among these are the actin-monomer-binding protein profilin and the Aip3p/Bud6p-interacting protein Atc1p. The results strongly support involvement of the actin cytoskeleton in the establishment of bipolar budding and in the maintenance of pseudohyphal growth.

Keywords: Polarity, actin, profilin, MYO2, myosin

INTRODUCTION

Cellular reproduction and organismal development are dependent upon the ability of cells to determine an axis of polarity. The budding yeast *Saccharomyces cerevisiae* displays distinct patterns of polarity with respect to its choices in bud position on the mother cell, organization of the newly emerging bud and in directing alternative developmental patterns, including pseudohyphal growth, mating, and sporulation (Chant, 1996; Gimeno *et al.*, 1992; Roemer *et al.*, 1996a). The choice of a site for bud emergence is strongly dependent upon the genetic composition of the mating-type locus, with those cells that are homozygous for mating type displaying axial budding (proximal pole with respect to the mother-bud junction) and cells heterozygous for mating type displaying bipolar budding (predominantly distal pole budding in the daughter cell and proximal or distal pole budding in the mother cell). The mechanisms regulating axial versus bipolar budding show distinct differences, such that independent mechanisms have been proposed for these bud-site selection pathways (Chant, 1996; Chant & Pringle, 1995; Zahner *et al.*, 1996).

Budding patterns are also influenced by nutrient availability in strains that are competent to grow as pseudohyphae. Upon appropriate nutrient deprivation, such strains undergo a specialized form of bipolar budding in which daughter cells bud distal and mother cells bud proximal to the mother-bud junction (Gimeno *et al.*, 1992). Further, mother and daughter cells become more elongated and have synchronous cell-division cycles, rather than displaying the normally observed lag in daughter cell division (Kron *et al.*, 1994). Such pseudohyphal growth is thought to allow cells to forage for limiting nutrient sources, and requires the ability to grow in a bipolar pattern. Pseudohyphal growth also requires the actin cytoskeleton to properly organize in a polarized fashion (Cali *et al.*, 1998). Indeed, mutations that perturb actin function can disrupt both bipolar budding and pseudohyphal growth, while having little or no adverse effect on axial budding (Haarer *et al.*, 1996; Yang *et al.*, 1997).

Due to the strong dependence of pseudohyphal growth and bipolar budding on the actin cytoskeleton, we have sought to learn more about these relationships, and thus about regulation of the actin cytoskeleton, by identifying conditions that perturb normal bipolar budding. We have screened for mutants that are defective in bipolar budding and pseudohyphal growth, and have also randomized budding through gene overexpression. We

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Abbreviations: PIP₂, phosphatidylinositol 4,5-bisphosphate

have found that mild mutation of a class-V myosin, encoded by *MYO2*, eliminates bipolar budding. We also found that overproduction of the actin-regulatory protein profilin perturbs bipolar budding, and that profilin's ability to interact with actin is important for this effect. These and other results confirm the essential nature of the actin cytoskeleton for normal polarization in *S. cerevisiae*.

METHODS

Yeast strains, plasmids and growth conditions. *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were generally grown on rich medium (YEPD) or synthetic defined medium (SD) containing appropriate nutritional supplements to maintain plasmid selection (Sherman *et al.*, 1986). For induction of pseudohyphal growth, strains were grown on the pseudohyphal growth medium SLAD (Gimeno *et al.*, 1992). Unless otherwise indicated, growth of yeast strains was at room temperature (24 °C) or 30 °C. Plasmid YCp(ACT1) was provided by D. Drubin and K. Wertman (Dept. Molecular and Cellular Biology, University of California, Berkeley, USA); YCpMYO2 (Lillie & Brown, 1994) was provided by S. Lillie (Dept. Cell and Molecular Biology, University of Michigan, Ann Arbor, USA). Plasmid YEp352(MYO4) has been described previously (Haarer *et al.*, 1994).

DNA manipulations and yeast genetic techniques. Standard procedures were used for recombinant DNA manipulation and *Escherichia coli* transformation (Sambrook *et al.*, 1989). Yeast transformation was by the lithium acetate procedure (Ma *et al.*, 1987).

Mutant screen and assessment of budding patterns. Haploid *bud3* strains TFY147 and TFY149, which bud bipolarly and are capable of pseudohyphal growth, were mutagenized with EMS and plated on YEPD at room temperature. Colonies were replica plated on SLAD media and examined with a dissecting microscope after 3 d. Potential pseudohyphal-defective mutants were restreaked on YEPD, then replica plated on SLAD media to confirm the mutant phenotype. Screening of ~40000 colonies yielded 146 pseudohyphal-defective mutants, of which 49 were defective in bipolar budding. The bud-site selection pattern was assayed by staining exponential-phase cells from liquid YEPD cultures with the chitin-specific dye calcofluor white (Pringle *et al.*, 1989). Bud-site selection was scored in cells having more than one calcofluor white-stained bud scar. Cells having scars at both poles, but not in the middle, were scored as bipolar, while

cells having at least one scar not at either pole were scored as random. Six random-budding mutants with the strongest phenotypes were chosen for further analysis. Of these, genetic crosses showed that five were the result of single-locus mutations. Attempts to clone the responsible genes by complementation of the pseudohyphal-defective phenotype proved unproductive, as the frequency of reversion (presumably through second-site mutations) was quite high. Therefore, the randomizing mutations were introduced into the 'rough colony' background of strain TFY209 (see below) and candidate genes were obtained by screening for a smooth to rough colony transition upon transformation with yeast genomic DNA libraries.

Complementation tests of bipolar-defective mutants. Mutations that disrupted bipolar budding were tested against each other and against mutations in known genes in the following complementation assay. Strains of opposite mating type, of which one strain was *ura3 HIS4* and the other was *URA3 his4*, were mixed together on SLAD plates lacking uracil and histidine. The resulting colonies, which should arise from *ura3/URA3 HIS4/his4* diploids, were then examined for pseudohyphal growth. The absence of pseudohyphal growth was considered to be non-complementation and the corresponding mutations were placed in the same complementation group. Conversely, demonstration of pseudohyphal growth resulted in placement of the mutations in separate complementation groups. Most pairwise complementation tests were repeated by using strains of the opposite mating type for the given mutations. In three instances, one cross failed to complement, while the reciprocal cross yielded a positive complementation result. In these cases, the mutations were considered to represent separate complementation groups. Mutations that fell into unknown complementation groups were tested for bipolar specificity by generating the corresponding *BUD3* haploid strains.

Overproduction screen. A multicopy genomic yeast DNA library in plasmid YEp13 was transformed into the bipolar-budding *bud3* strain TFY209, which exhibits a rough colony morphology. While not well characterized, this rough colony phenotype arises from cells that are budding in a bipolar pattern. Axial or random budding results in a smooth colony morphology. In this screen, 323 of ~14000 transformed colonies exhibited a reduction of the rough colony morphology. Of these transformants, 12 budded in an axial pattern (due to acquisition of *BUD3*-containing plasmids) and 30 showed random budding. The remaining 281 transformants exhibited normal bipolar budding and were not examined further. Plasmids were isolated from each bipolar-defective transformant and retested. Subcloning and sequence analysis

Table 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source
TFY147	<i>MATa bud3::TRP1 ura3</i>	This study
TFY148	<i>MATα bud3::TRP1 his4 ura3</i>	This study
TFY149	<i>MATα bud3::TRP1 his4 ura3</i> + plasmid YCp50	This study
TFY209	<i>MATα bud3Δ::URA3 ura3 leu2 trp1</i>	This study
TFY211	<i>MATa bud3Δ::URA3 ura3 leu2 trp1</i>	This study
TFY250	<i>MATa/MATα myo2-117/myo2-117</i>	This study
TFY648	<i>MATa/MATα leu2/leu2 trp1/trp1 ura3/ura3 can1/can1</i>	J. Chant*

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revealed the genes responsible for perturbing bipolar budding, as summarized in Table 3.

RESULTS

Mutations in *MYO2* disrupt bipolar but not axial budding

Under specific nitrogen-starvation conditions, certain *S. cerevisiae* strains are induced to switch from the yeast form to the filament-like pseudohyphal form (Gimeno *et*

al., 1992). Cells become highly elongated, remain loosely attached to each other and appear as long chains. Bipolar budding is one requirement for pseudohyphal growth (Gimeno *et al.*, 1992), as wild-type haploid a or α cells, which bud axially, are not competent to grow pseudohyphally. Wild-type diploid a/α cells, which bud in a bipolar pattern, grow as pseudohyphae when cultured on appropriate nitrogen-limited media. However, a/α cells that are unable to bud in a bipolar fashion (e.g. *bud1*, *bud2* and *bud5* mutants) are incapable of

Fig. 1. Bipolar-budding defective mutants fail to undergo pseudohyphal growth. Shown are (a) strain TFY149 and (b) representative random-budding mutant TFY55; growth was on SLAD medium at 25 °C for 5 d.

Fig. 2. Bud-site selection in *myo2* mutant strains. Shown are calcofluor white-stained cells from control strains TFY147 (a) and TFY149 (b), and from strains of genotype *myo2-117 bud3* (c); *myo2-117/myo2-117* (d); *myo2-117 bud3 bud4*, grown at 37 °C (e); *myo2-117 bud3*, containing plasmid YCpMYO2 (f); *myo2-66 bud3* (g); and *myo2-66* (h). Unless otherwise indicated, growth was at 25 °C in YEPD.

Fig. 3. Actin distribution in *myo2-117* cells. Control strain TFY147 (a), *myo2-117 bud3* strain TFY14 (b), and *myo2-117/myo2-117 bud3/bud3* strain TFY250 (c) stained with rhodamine-conjugated phalloidin.

Table 2. Partial suppression of the *myo2-117/myo2-117* bipolar budding defect by actin overexpression

Strain TFY250 was used in all cases. The following criteria were used to assess budding patterns of cells having two or more bud scars: unipolar, all scars at one pole of the cell only; bipolar, scars at both poles of the cell, but not in the middle; random, at least one scar not at either pole. Values are shown as percentages and represent the mean of two experiments; $n = 150$ – 200 cells for each transformant per replicate.

Plasmid*	Budding Pattern		
	Random	Bipolar	Unipolar
YEp352	70	20	10
YCp(ACT1)	55	37	8
YEp(MYO4)	70	19	11

*YEp352, control vector; YCp(ACT1), low-copy-number plasmid carrying the yeast actin gene; YEp352(MYO4), high-copy-number plasmid carrying the *MYO2*-related *MYO4* gene.

growing as pseudohyphae (Gimeno *et al.*, 1992). Conversely, haploid *bud3* or *bud4* mutants, which bud in a bipolar pattern, are capable of pseudohyphal growth (Sanders & Herskowitz, 1996) (see Fig. 1a).

Zhaner *et al.* (1996) have previously used a *bud3* strain to microscopically screen for mutants that are defective for bipolar budding. Using a similar haploid *bud3* strain, we carried out a screen for mutants that are unable to grow pseudohyphally. Of 146 such mutants (see Fig. 1b), 49 were found to display random budding patterns. The random-budding mutations were tested for complementation (see Methods) of each other and of known bipolar-defective mutations in *BUD1/RSR1*, *BUD2*, *BUD5*, *SPA2*, *RVS161*, *RVS167*, *SUR4* and *SUR5* (Bauer *et al.*, 1993; Bender & Pringle, 1989; Chant *et al.*, 1991, 1995; Chant & Herskowitz, 1991; Crouzet *et al.*, 1991; Durrens *et al.*, 1995; Park *et al.*, 1993; Powers *et al.*, 1991; Revardel *et al.*, 1995; Snyder, 1989). The complementation data suggested that we obtained mutations in *BUD1* (one isolate), *BUD2* (nine isolates), *BUD5* (five isolates) and *SPA2* (one isolate). In addition,

one mutation failed to complement *bud1* and *bud2* mutations, and thus is likely to correspond to one of these genes. Oddly enough, the remaining 23 mutations fall into separate complementation groups (nine mutations convey a mating defect and were not included in the complementation test). Thus, there is either a high incidence of interallelic complementation among these mutations, or there are a great number of mutations that will generate bipolar budding defects (at least in this genetic background; see Discussion).

Six mutant strains were chosen from this collection as having the greatest randomizing effect on bipolar budding. A complementing plasmid clone was identified for one of these strains and was found to contain the *MYO2* gene, which encodes a type-V myosin previously shown to be essential for polarized secretion (Govindan *et al.*, 1995; Johnston *et al.*, 1991; Lillie & Brown, 1994). Subsequent crosses to a *myo2-66* mutant confirmed that the random-budding mutation is a novel allele of *MYO2* and will hereafter be referred to as *myo2-117*. Complementation genes for the remaining mutations have not yet been identified.

The *myo2-117* allele causes normally bipolar-budding cells to bud in a random fashion, but does not affect axial budding. This is true both for haploid *myo2-117 bud3* cells (Fig. 2c) and diploid *myo2-117/myo2-117 bud3* cells (Fig. 2d). While the *myo2-117* mutation does not result in the severe temperature sensitivity caused by the *myo2-66* mutation, *myo2-117* cells do display a partial cell-separation defect upon prolonged incubation at 37 °C (Fig. 2e). Zymolyase treatment of fixed *myo2-117* cells that were grown at 37 °C results in dissociation of daughter cells, suggesting that the defect is in cell-wall separation, rather than in cytokinesis (data not shown). The defects associated with the *myo2-117* allele are recessive and are efficiently rescued by plasmid-borne *MYO2* (Fig. 2f). Our observations of *myo2-117*-containing strains caused us to re-examine the previously described *myo2-66* allele (Johnston *et al.*, 1991; Lillie & Brown, 1994) for similar effects. At permissive temperatures, haploid *myo2-66 bud3* strains bud in a random pattern (Fig. 2g), while corresponding *myo2-66* strains bud in the normal axial pattern (Fig. 2h). As with *myo2-117* strains, homozygous *myo2-66* diploid strains bud randomly (data not shown). Interestingly, a *myo2-*

Fig. 4. Randomization of bipolar budding by gene overexpression. Calcofluor white-stained cells of strain TFY209 carrying control plasmid YEp351 (a), or library clones 414 (*BUD10*) (b); 422 (*PFY1*) (c); or 75 (*ATC1*) (d).

117/*myo2-66* diploid is partially restored for bipolar budding, suggesting partial interallelic complementation between these mutant *myo2* genes.

To determine if the random budding of *myo2* strains is the result of an inability to properly localize potential bud-site determinants, we examined the distribution of several proteins known to be important for normal polarized budding. Rhodamine-phalloidin staining showed that actin is properly localized in *myo2-117 bud3* haploid and *myo2-117/myo2-117* diploid strains (Fig. 3). Use of green fluorescent protein fusions further indicated that septins (Cdc3p) and the axial budding determinant Bud10p/Axl2p, which colocalize to the mother-bud neck, are positioned normally in *myo2-117* mutant cells (data not shown). Despite the normal appearance of the actin cytoskeleton, we did find that mild overexpression of yeast actin partially suppresses the bipolar budding defect of a *myo2-117/myo2-117* strain (Table 2), while overexpression of another type V myosin (Myo4p) has no effect.

Lillie & Brown (1992) have shown that increased expression of *SMY1*, which encodes a kinesin-related protein, can partially rescue the temperature sensitivity of strains carrying the *myo2-66* mutation. Expression of *SMY1* from a high-copy-number plasmid fails to suppress the bipolar budding defect of a *myo2-117 bud3* strain. Moreover, a *smy1/smy1* homozygous diploid buds in a normal bipolar pattern, and a haploid *smy1 myo2-117* strain buds in a normal axial pattern (data not shown). These results suggest that Smy1p activity is more relevant to the essential role of Myo2p and may have little to do with the participation of Myo2p in organizing budding polarity.

Multicopy randomization of bipolar budding

To complement our mutational studies, we also carried out a screen to identify genes that, when overexpressed, disrupt bipolar budding in a *bud3* haploid strain. In this screen, we utilized strain TFY209, which carries a *bud3* deletion mutation and exhibits a rough colony mor-

phology. This rough colony morphology is similar to pseudohyphal growth, in that it requires bipolar budding and can occur in a haploid *bud3* or *bud4* background. However, unlike pseudohyphal growth, the rough colony morphology occurs under non-starvation conditions and thus does not require specialized growth media. We transformed strain TFY209 with a high-copy yeast genomic library and screened for smooth colonies arising on selectable media. Candidate colonies were retested to confirm the smooth colony phenotype and cells were stained with calcofluor white to determine if the bipolar budding pattern was disrupted (representative transformants are shown in Fig. 4). Of 323 smooth colonies, 30 budded in a random pattern and 12 budded in an axial pattern. Plasmid clones were isolated from bipolar-defective transformants and were retested for their ability to promote the smooth-colony phenotype. Clones from the 12 axial budding transformants were found to contain the *BUD3* gene; clones from the random budding transformants are discussed below.

Sequence was obtained from the ends of yeast DNA inserts of plasmid clones and compared to the yeast genome database to identify the genes contained on each plasmid (Table 3). In many cases, further subcloning unambiguously identified individual genes that disrupt bipolar budding in the *bud3* strain. To further examine the nature of this perturbation, we tested the plasmids in other genetic backgrounds, including *bud3/bud3* and Bud⁺ diploids (Table 3). Our results indicate that most of the clones disrupt bipolar budding only when *BUD3* function is also perturbed. However, we have not ruled out strain background effects as contributing to these results (see Discussion).

Overproduction of profilin or Atc1p disrupts bipolar budding

The quantitatively strongest inhibitor of bipolar budding that we obtained was *PFY1* (see Fig. 4c), which is the sole profilin gene in yeast. Profilin is an actin-monomer-binding protein that is important for vegetative growth and essential for normal polarized

Table 3. Multicopy randomization of bipolar budding

Clone	Relevant gene(s) [ORF(s)] on clone*	Independent Isolates	Random budding in selected yeast strains (%)		
			<i>MATα</i> <i>bud3Δ</i>	<i>MATa/MATα</i> <i>bud3Δ/bud3Δ</i>	<i>MATa/MATα</i> <i>BUD3/BUD3$\dagger$</i>
422	<i>PFY1</i> (YOR122c)	1	57	67	BP
170, 414	<i>BUD10</i> (YIL140w)	5	36	38	A/BP
75	<i>ATC1</i> (YDR184c)	2	41	62	BP
118	<i>PDE2</i> (YOR360c)	3	53	33	ND
73	<i>MLP1</i> (YKR095w)	1	26	34	17
241	[<i>SAP4</i> (YGL229c)]‡	1	66	21	BP
106	<i>MEC3</i> (YLR288c)	1	37	11	BP
78	[YPL067c], YPL068c, <i>BTS1</i> (YPL069c), YPL070w, [YPL071c]	1	35	23	7
114	YDR333c	1	43	32	2
420	[<i>HYP2</i> (YEL034w)], YEL033w	1	39	31	BP
87	[YMR247c]§	1	29	16	BP
143	[YOR011w]	1	60	15	1
418	[<i>SRM1</i> (YGL097w)], YGL096w, delta element	1	20	31	BP

* In cases where the effect has not been narrowed down to a single gene, all candidate genes are indicated. If previously characterized, the gene name is given first, with the yeast database ORF designation in parentheses; square brackets indicate an incomplete gene/ORF on clone.

† Strains used were TFY209 (*MAT α bud3 Δ*), TFY209 $\times$ TFY211 (*MATa/MAT α bud3 Δ /bud3 Δ*), and TFY648 (*MATa/MAT α Bud $^+$*). For each data point, $n \geq 100$ cells; BP designates little or no departure from normal bipolar budding; A indicates an axial appearance of some cells in a population of cells that are overexpressing Bud10p; ND, not determined.

‡ Predicted to express the first 410 of 818 codons of *SAP4*.

§ Predicted to express the first 1309 of 1562 codons of YMR247c.

|| Predicted to express the first 1148 of 1394 codons of YOR011w.

budding in yeast (Haarer *et al.*, 1990; Sohn & Goldschmidt-Clermont, 1994). Deletion of *PFY1* results in randomized budding in haploids (Haarer *et al.*, 1990), while various *PFY1* point mutations result in randomized budding in diploids, but do not affect the normal axial budding of haploid strains (Haarer *et al.*, 1996; T. Freedman and B. Haarer, unpublished data). These results suggest that bipolar budding requires stringent controls on the levels of profilin expressed in the cell. This is in contrast to the relaxed cellular requirements regarding profilin quantity and function necessary to permit relatively normal vegetative growth (Haarer *et al.*, 1993, 1996; Magdolen *et al.*, 1993).

Profilin is able to interact with various substrates, including actin, phosphatidylinositol 4,5-bisphosphate (PIP₂) (Sohn & Goldschmidt-Clermont, 1994) and the proteins Bni1p and Bnr1p, which are related to each other in structure and have been implicated in actin and cell polarization (Evangelista *et al.*, 1997; Imamura *et al.*, 1997). Interaction of profilin with Bni1p and Bnr1p is through multiple proline residues, and requires profilin's characterized polyproline-binding activity (Evangelista *et al.*, 1997; see also Haarer *et al.*, 1993). To better understand profilin's perturbing effects on bipolar bud-site selection, we assessed the budding randomization effects of various mutant profilins that

Table 4. Effects of profilin mutation on multi-copy randomization of bud-site selection

Strain TFY148 was used in all cases. See Table 2 legend for budding pattern criteria. For each sample, $n = 250$ –300. Values shown are percentages.

Plasmid*	Budding Pattern		
	Random	Bipolar	Unipolar
YEpl352	20	52	28
<i>PFY1</i>	44	51	5
<i>pfy1-111</i>	26	62	12
<i>pfy1-112</i>	51	30	19
<i>pfy1-115</i>	28	47	25
<i>pfy1-ΔC3</i>	49	40	11

* YEpl352, control vector; Wild-type (*PFY1*) or mutant (*pfy1-111*, *pfy1-112*, *pfy1-115*, *pfy1 Δ C3*) profilin alleles were expressed from high-copy-number *URA3*-based plasmids.

are defective in one or more of these binding activities (Table 4). The *pfy1-111* and *pfy1-115* alleles produce profilins that exhibit greatly reduced interaction with actin, while *pfy1-112* profilin is only moderately affected in this activity (Haarer *et al.*, 1993); *pfy1 Δ C3* profilin

(–3PFY in Haarer *et al.*, 1993) is largely defective in binding polyproline and fails to interact with Bni1p (Evangelista *et al.*, 1997; S. Palmieri and B. Haarer, unpublished data). Based on these data (Table 4), the ability of profilin to randomize budding correlates with its actin-binding activity and is independent of its ability to interact with proline-rich substrates. While we cannot rule out an interaction with PIP₂ as contributing to this effect, it is unlikely to be the sole activity responsible for bud-site randomization, as *pfy1-111* profilin is defective in PIP₂ binding, while *pfy1-115* profilin is not (Haarer *et al.*, 1993).

Overproduction of another gene implicated in actin regulation also results in a partial disruption of bipolar budding. The product of the *ATC1* gene was identified via its interaction with Bud6p/Aip3p in the two-hybrid system (D. Amberg, personal communication; and *Saccharomyces* Genome Database, <http://genome-www.stanford.edu/Saccharomyces/>). Bud6p/Aip3p is an actin-binding protein that is essential for normal bipolar budding (Amberg *et al.*, 1995, 1997; Zahner *et al.*, 1996). Evangelista *et al.* (1997) have shown that Bud6p/Aip3p binds to the formin Bni1p and is probably recruited to sites of Cdc42p or Rho1p activation through this interaction. Thus, overproduction of Atc1p may exert its effects on bipolar budding by perturbing the normal localization or binding activity of Bud6p/Aip3p. Interestingly, this putative signalling complex also includes profilin, which is recruited by Bni1p. As indicated above, profilin overproduction also disrupts bipolar budding, perhaps through perturbation of this signalling complex or simply by decreasing the pool of actin monomers available to contribute to actin filament formation or binding to Bud6p/Aip3p. We have recently found that overproduction of the Bni1p homologue Bnr1p can largely overcome the budding defects caused by profilin overproduction (T. Freedman and B. Haarer, unpublished). This suppression may occur via enhanced signalling through Bnr1p, or may simply be due to sequestration of excess profilin.

Overexpression of *BUD10/AXL2* and *BUD1/RSR1* disrupt bipolar budding

The most frequently obtained multicopy disrupter of bipolar budding in our screen was *BUD10/AXL2* (see Fig. 4b), a gene previously identified via its requirement for axial budding (Halme *et al.*, 1996; Roemer *et al.*, 1996b). This is particularly interesting, as mutation of *BUD10/AXL2* results in bipolar budding in strains that would otherwise bud in an axial pattern. While Bud10p/Axl2p overproduction tends to randomize bud-site selection, a low percentage of cells bud in an axial pattern (Table 3). Further examination of this phenomenon will be presented in a separate study (T. Freedman and J. Chant, unpublished).

A plasmid clone containing the gene for the Ras-related GTPase Bud1p/Rsr1p was also identified as a disrupter of bipolar budding, although it is much less effective at perturbing bud-site selection and only had a significant

effect in the *bud3* haploid background (data not shown). *BUD1/RSR1* is essential for maintaining proper axial and bipolar budding polarity (Bender & Pringle, 1989; Chant & Herskowitz, 1991), as are its GTPase-activating protein and guanine nucleotide exchange factor, Bud2p and Bud5p, respectively (Bender, 1993; Chant *et al.*, 1991; Park *et al.*, 1993; Powers *et al.*, 1991). Further, an activated allele of the Bud1/Rsr1 GTPase fails to rescue the bud-site selection defects of a *bud1/rsr1* deletion strain (Ruggieri *et al.*, 1992). Thus, it is not surprising that extra copies of the wild-type *BUD1/RSR1* gene disrupt bipolar budding in our strain.

Additional multicopy disrupters of bipolar budding

Several other multicopy disrupters of bipolar budding were obtained in this study, as indicated in Table 3. Space constraints preclude extensive discussion regarding the possible effects these genes may have on polarity determinants; however, of particular note to the issue of bipolar budding and pseudohyphal growth is the gene *PDE2*. *PDE2* encodes a high-affinity phosphodiesterase that is responsible, along with the product of the *PDE1* gene, for decreasing cAMP levels. This is crucial for reversing the effects of the Ras-stimulated adenylate cyclase pathway in yeast, which is responsible for growth stimulation in response to nutrient availability. Ward *et al.* (1995) have demonstrated that *PDE2* inhibits pseudohyphal growth. Further, Ras is necessary for pseudohyphal growth, and activated *RAS2* alleles can drive pseudohyphal growth under non-starvation conditions (Gimeno *et al.*, 1992). The G-protein alpha subunit encoded by *GPA2* also stimulates pseudohyphal growth through elevation of cAMP levels (Lorenz & Heitman, 1997). Given these data, it is not surprising that *PDE2* overexpression suppresses the pseudohyphal response by decreasing cAMP levels. Beyond these specific effects on the pseudohyphal pathway, our results demonstrate that overexpression of *PDE2* is also capable of disrupting bipolar budding, presumably in the absence of upstream activation of the pseudohyphal pathway (i.e. without *GPA2* or *RAS2* activation).

DISCUSSION

Use of colony morphology as an indicator of bipolar budding and cytoskeletal function

In this study, we have used the bipolar budding requirement for pseudohyphal growth and colony morphology to screen for factors that, when mutated or overproduced, can perturb this aspect of cell polarization. While the *bud3* strain background used for these studies is similar to that utilized by Zahner *et al.* (1996), the use of colony morphology as an indicator of budding pattern, rather than direct microscopic examination, has greatly streamlined the process of identifying factors that affect bipolar budding. Furthermore, the preponderance of bipolar budding defects arising from mutations in genes that relate to actin cytoskeletal function (see Ayscough *et al.*, 1997; Haarer *et al.*, 1996; Palmieri & Haarer, 1998; Yang *et al.*, 1997, and

references therein) suggested that such an approach could identify additional actin regulatory genes and may help to explain the involvement of such factors in the maintenance of bipolar budding.

It is apparent that our screens have identified factors that are involved in one or more of the following morphogenetic processes: bipolar budding, pseudohyphal determination, pseudohyphal growth or actin polarization. Indeed, it may not be possible to completely separate these functions, as bipolar budding and pseudohyphal growth require normal actin polarization (Cali *et al.*, 1998; Mosch & Fink, 1997; Yang *et al.*, 1997), pseudohyphal growth generally requires unipolar budding, which is likely to be a specialized form of bipolar budding (Gimeno *et al.*, 1992; Kron *et al.*, 1994; Zahner *et al.*, 1996) and bipolar budding may require some of the same activators that are necessary for pseudohyphal determination (Mosch & Fink, 1997). Put more simply, the induction of pseudohyphal growth by nutritional signals requires the ability to bud bipolarly, which in turn requires normal actin polarization and regulation.

Contribution of strain background to bipolar budding screens

In our screen for multi-copy disrupters of bipolar budding, we noted that many of the clones identified had their most significant effect in *bud3* mutant strains, most notably in the case of diploid strains. At present, we do not know if this effect is specific to *bud3* or if there are subtle genetic differences between these strains that are contributing to the observed results. If the effect is indeed *bud3*-specific, this would imply a potential, albeit minor, role for Bud3p in bipolar budding. Attenuation of this function apparently makes yeast cells especially susceptible to additional perturbations in bipolar budding, thus enhancing their utility in such screens.

Involvement of Myo2p in bud-site selection

The relative lack of morphological abnormalities in strains expressing the *myo2-117* allele suggests that the mutant myosin is able to provide the bulk of its normal cellular functions, particularly those relevant to secretion and cytoskeletal organization (Govindan *et al.*, 1995; Johnston *et al.*, 1991). Therefore, its inability to support bipolar growth suggests that the mutant protein is lacking in some non-essential function, presumably involving either an inability to interact with a protein that normally relies on Myo2p for its proper cellular distribution or an inability to properly position itself in the cell to deliver bipolar determinants. Catlett & Weisman (1998) have identified an allele of *MYO2* that is defective in vacuolar inheritance, bipolar bud-site selection and normal localization of the mutant Myo2 protein, but that is not grossly defective in bud growth or actin polarization. Thus, the role of Myo2p in bud-site selection may require the efficient delivery of polarity determinants to the plasma membrane, perhaps via the secretory pathway. We suspect that Myo2p may help to

position the proposed bipolar determinant(s) at presumptive budding sites, and that the observed effects are due to reduced efficiency of binding (directly or indirectly) to a polarity-determining cargo.

Myo2p has been strongly implicated in directing normal secretion in yeast (Govindan *et al.*, 1995; Johnston *et al.*, 1991; Lillie & Brown, 1992, 1994), a process that is also dependent upon the actin cytoskeleton (Ayscough *et al.*, 1997; Haarer *et al.*, 1996; Novick & Botstein, 1985). Surprisingly, Myo2p is able to polarize, albeit in a delayed fashion, independently of an intact actin cytoskeleton (Ayscough *et al.*, 1997), which would suggest that it is able to directly respond to the global polarizing signal provided by Cdc42p. In contrast, Schott *et al.* (1999) found that Myo2p localization is dependent on actin. Consistent with this finding is the partial suppression of *myo2-117*-induced budding defects by overproduction of yeast actin (Table 2). Since perturbation of the actin cytoskeleton through mutation of various actin-accessory factors is likely to affect the timing and efficiency of Myo2p localization, it is possible that the general bipolar budding defects caused by such mutations (Yang *et al.*, 1997) actually arise due to attenuation of Myo2p function and consequent mislocalization of a crucial polarity determinant. Thus, the situation may be analogous to the asymmetric delivery of the *ASH1* mRNA and protein exclusively to daughter cells. This polarized delivery is strictly dependent upon the type-V myosin Myo4p and is inhibited by factors that partially disrupt the actin cytoskeleton (Bobola *et al.*, 1996; Jansen *et al.*, 1996; Long *et al.*, 1997; Takizawa *et al.*, 1997).

The inability of *SMY1* overproduction to suppress the bud-site selection defects of a *myo2-117* strain argues against such defects resulting solely from reduced secretory efficiency; i.e. the proposed polarity determinant(s) may require direct interaction with Myo2p for proper positioning, such that simply increasing secretory efficiency would be insufficient to properly localize the determinant(s). It is also noteworthy that *myo2-117/myo2-66* heteroallelic diploids show an intermediate bipolar phenotype, whereas corresponding homoallelic diploids are incapable of supporting bipolar budding. Since the *myo2-66* allele generates a change at the actin-binding face of the motor domain (Lillie & Brown, 1994), it is possible that heterodimers of the two mutant myosins compensate by providing a single fully functional motor domain and a single wild-type binding site required for delivery of the hypothesized bipolar determinant(s).

Actin-regulatory factors and bud-site selection

Recent reports have stressed the importance of the actin cytoskeleton for various aspects of cell polarization, including bipolar bud-site selection and pseudohyphal growth (Ayscough *et al.*, 1997; Cali *et al.*, 1998; Haarer *et al.*, 1996; Yang *et al.*, 1997; Zahner *et al.*, 1996). This may reflect a requirement of the actin cytoskeleton (and Myo2p) for properly placing or maintaining one or

more positional cues that normally mark or determine bipolar budding sites. Potential candidates for such positional cues are the proteins Bud8p and Bud9p, defects of which result in loss of distal- or proximal-pole budding, respectively (Yang *et al.*, 1997; Zahner *et al.*, 1996), and Aip3p/Bud6p, an actin-interacting protein that is generally required for bipolar budding (Amberg *et al.*, 1997; Zahner *et al.*, 1996). Actin-mediated placement of these (or other) positional cues is likely to be indirect via effects on polarized secretion or myosin-based delivery of positional markers to polarized sites. Candidates for contributing to the delivery of positional cues include Sec3p and other components of the exocyst complex (Finger *et al.*, 1998; Finger & Novick, 1997; Haarer *et al.*, 1996), in addition to Myo2p (see above). Defects in a subset of these secretory pathway components result in a randomization of bipolar budding, probably by reducing the efficiency with which positional cues achieve their polarized localization at or near the plasma membrane. Interestingly, normal polarization of Aip3p requires the secretory pathway and functional Myo2p (Jin & Amberg, 2000).

As mentioned in the Results, profilin and Atc1p have been linked to Cdc42p and Rho protein-based signalling complexes through interactions with formins or formin-associated proteins (for a recent review, see Palmieri & Haarer, 1998). It is likely that such signalling complexes serve to arrange the actin cytoskeleton into organized networks, which is critical for directing polarized secretion and for the distribution of polarity determinants. Presumably, the disruption of bipolar budding in strains that express various mutated profilins (Haarer *et al.*, 1996; T. Freedman and B. Haarer, unpublished data) arises by significantly affecting the efficiency of these signalling complexes. Most likely, attenuated profilin function retards the rate of actin filament assembly at sites of Cdc42/Rho activation by reducing the rate of conversion of ADP-actin to ATP-actin or by failing to properly deliver bound actin monomers to the growing ends of actin filaments. While profilin may normally act in complexes that require its interaction with proline-rich proteins, its ability to disrupt bipolar budding upon overproduction probably rests in its ability to sequester actin monomers (Table 4). Thus, although profilin may normally stimulate actin polymerization in yeast (Yeh & Haarer, 1996), its overproduction may serve to reduce the pool of actin available for polymerization (see Magdolen *et al.*, 1993, for example).

As discussed by Yang *et al.* (1997), a better understanding of the connections between actin cytoskeletal function and bipolar budding will come with identification of the bipolar positional cue(s) and a determination of how such molecules achieve their polarized distribution. Our results (this study and Haarer *et al.*, 1996), suggest that these cues achieve polarization through an actin-dependent, Myo2p-based and probably secretory pathway-based, delivery to the cell surface. Continued analysis of factors that affect cytoskeletal organization and cell polarization, particularly

bipolar budding, will help establish the identity and activities of such positional determinants.

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