A Role for the Actin Cytoskeleton of *Saccharomyces cerevisiae* in Bipolar Bud-Site Selection

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Abstract. *Saccharomyces cerevisiae* cells select bud sites according to one of two predetermined patterns. *MATα* and *MATα* cells bud in an axial pattern, and *MATα/α* cells bud in a bipolar pattern. These budding patterns are thought to depend on the placement of spatial cues at specific sites in the cell cortex. Because cytoskeletal elements play a role in organizing the cytoplasm and establishing distinct plasma membrane domains, they are well suited for positioning bud-site selection cues. Indeed, the septin-containing neck filaments are crucial for establishing the axial budding pattern characteristic of *MATα* and *MATα* cells. In this study, we determined the budding patterns of cells carrying mutations in the actin gene or in genes encoding actin-associated proteins: *MATα/α* cells were defective in the bipolar budding pattern, but *MATα* and *MATα* cells still exhibit a normal axial budding pattern. We also observed that *MATα/α* actin cytoskeleton mutant daughter cells correctly position their first bud at the distal pole of the cell, but mother cells position their buds randomly. The actin cytoskeleton therefore functions in generation of the bipolar budding pattern and is required specifically for proper selection of bud sites in mother *MATα/α* cells. These observations and the results of double mutant studies support the conclusion that different rules govern bud-site selection in mother and daughter *MATα/α* cells. A defective bipolar budding pattern did not preclude an *sla2-6* mutant from undergoing pseudohyphal growth, highlighting the central role of daughter cell bud-site selection cues in the formation of pseudohyphae. Finally, by examining the budding patterns of *mad2-I* mitotic checkpoint mutants treated with benomyl to depolymerize their microtubules, we confirmed and extended previous evidence indicating that microtubules do not function in axial or bipolar bud-site selection.

The budding yeast *Saccharomyces cerevisiae*, like many other organisms (e.g., *Caenorhabditis elegans* (Hyman and White, 1987; Hird and White, 1993) or the water fern *Azolla* (Gunning, 1982)), has programmed patterns of cell division. The bud site, from which a daughter cell grows, specifies both the region of polarized cell surface growth and the plane of cell division. Bud-site selection occurs by one of two patterns, depending on the genotype of the mating type (*MAT*)¹ locus. *MATα* or *MATα* cells (normal haploids) exhibit an axial pattern in which the new bud is placed immediately adjacent to the bud site used during the preceding cell cycle. *MATα/α* cells (normal diploids) exhibit a bipolar pattern in which the new bud can be placed either near the last bud site, or at the opposite pole of the cell. Additionally, the first bud of a *MATα/α* daughter cell is almost always placed at the distal pole, opposite the birth scar (the proximal pole) (Felder, 1960; Chant and Pringle, 1995). The bipolar and axial bud-site selection patterns seem to be the result of two separate pathways because either pattern can be disrupted without affecting the other pattern (Chant et al., 1991; Bauer et al., 1993; Zahner et al., 1996).

Several proteins necessary for bud-site selection have been identified. These proteins can be classified into three groups based on their budding pattern phenotypes. Mutations in Cdc10p, Cdc11p, Cdc12p (Flescher et al., 1993; Chant et al., 1995), Bud5p, Bud4p (Chant and Herskowitz, 1991), Axl1p (Fujita et al., 1994), and Axl2p (Halme et al., 1996; Roemer et al., 1996) result in a bipolar budding pattern in haploids but do not affect the budding pattern in diploids. In contrast, mutations in Rvs161p, Rvs167p (Bauer et al., 1993; Sivadon et al., 1995), Sur4p, Fen1p (Durrens et al., 1995), Spa2p, Bni1p, Bud6p, Bud7p, Bud8p, and Bud9p (Zahner et al., 1996) perturb the bipolar pattern in diploids but do not affect the axial pattern in haploids. While the first two groups of proteins are important for selecting a bud site according to one of the two patterns, a third group, consisting of Rsr1p, Bud2p, and Bud5p, appears to decode the spatial cues for both axial and bipolar budding patterns. Thus, mutations in Rsr1p, Bud2p, and Bud5p (Bender and Pringle, 1989; Chant and Herskowitz, 1995).
comprised of septins (encoded by GIP2, GIP3, GIP4, and CIC1) that localize toward, and concentrated at, the mother-bud neck throughout the cell cycle and are necessary for nuclear migration and cytokinesis (Jacobs et al., 1984). These microtubules are well-placed for a role in organizing the cytoplasm and establishing distinct plasma membrane domains.

Three types of cytoskeletal proteins have been described in yeast. The neck filaments, which are mainly made up of septins (encoded by CDC3, 10, 11, and 12), form a ring at the presumptive bud site that persists at the mother-bud neck throughout the cell cycle and are necessary for cytokinesis (for review see Longtine et al., 1996). The actin cytoskeleton is thought to spatially organize growth of the cell surface. Cortical patches composed of filamentous actin first become localized around the presumptive bud site when the cell initiates a cell cycle, remain polarized at the growing bud surface during much of the cell cycle, and later in the cell cycle become reoriented toward, and concentrated at, the mother-bud neck, where they presumably play a role in septum formation and cytokinesis. Cytoplasmic actin cables seem to extend from the cortical actin patches in the bud (or at the presumptive bud site) into the cytoplasm (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Mulholland et al., 1994). Microtubules are necessary for nuclear migration and division but not bud growth (Huffaker et al., 1988; Jacobs et al., 1988). The spindle pole body, the microtubule organizing center of a yeast cell, and the cytoplasmic microtubules that emanate from it are oriented toward the bud from an early point in the cell cycle (Byers and Goetsch, 1975; Byers, 1981; Adams and Pringle, 1984; Kilmartin and Adams, 1984). These microtubules are well-placed for a role in bud-site selection but do not appear to function in this process (Jacobs et al., 1988; Chant and Pringle, 1995).

For axial budding, a remnant of the division site most likely marks the bud site because new bud sites always contact physically the scar left behind after a bud separates from its mother. The neck filaments are thought to provide a scaffold upon which the axial bud-site cue localizes (for review see Longtine et al., 1996). Bud3p and

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**Table I. Yeast Strains Used in This Study**

| Name                        | Genotype                                      | Source |
|-----------------------------|-----------------------------------------------|--------|
| **Haploid conditional-lethal actin mutations** |                                               |        |
| DDY354                      | MATa ACT1::HIS3 cryl                           | 1      |
| DDY338                      | MATa act1-101::HIS3 cryl can1-1               | 1      |
| DDY356                      | MATa act1-105::HIS3 can1-1                    | 1      |
| DDY337                      | MATa act1-108::HIS3 cryl can1-1               | 1      |
| DDY342                      | MATa act1-113::HIS3 can1-1                    | 1      |
| DDY346                      | MATa act1-119::HIS3 ade2-101 cryl             | 1      |
| DDY347                      | MATa act1-120::HIS3 cryl can1-1               | 1      |
| DDY349                      | MATa act1-124::HIS3 can1-1                    | 1      |
| DDY351                      | MATa act1-129::HIS3 can1-1                    | 1      |
| DDY336                      | MATa act1-133::HIS3 cry1 can1-1               | 1      |
| **Diploid conditional-lethal actin mutations** |                                               |        |
| DDY440                      | MATa/α ACT1::HIS3 ACT1::HIS3 can1-1/α can1-1 ade4+ | 1      |
| DDY439                      | MATa/α act1-101::HIS3 act1-101::HIS3 ade4+    | 1      |
| DDY980                      | MATa/α act1-105::HIS3 act1-105::HIS3 can1-1+  |        |
| DDY438                      | MATa/α act1-108::HIS3 act1-108::HIS3 can1-1 ade4+ | 1      |
| DDY441                      | MATa/α act1-113::HIS3 act1-113::HIS3 can1-1 ade4+ | 1      |
| DDY430                      | MATa/α act1-119::HIS3 act1-119::HIS3 ade4+   |        |
| DDY433                      | MATa/α act1-120::HIS3 act1-120::HIS3 can1-1 ade4+ | 1      |
| DDY1051                     | MATa/α act1-122::HIS3 act1-122::HIS3 can1-1 ade4+ | 2      |
| DDY434                      | MATa/α act1-124::HIS3 act1-124::HIS3 can1-1 ade4+ | 1      |
| DDY431                      | MATa/α act1-129::HIS3 act1-129::HIS3 can1-1 ade4+ | 1      |
| DDY437                      | MATa/α act1-133::HIS3 act1-133::HIS3 ade4+  |        |
| **Haploid pseudo–wild-type actin mutants** |                                               |        |
| DDY339                      | MATa act1-102::HIS3 cry1 can1-1 ade2-101     | 1      |
| DDY340                      | MATa act1-104::HIS3 can1-1                   | 1      |
| DDY343                      | MATa act1-115::HIS3 can1-1 ade2-101          | 1      |
| DDY344                      | MATa act1-116::HIS3 can1-1                   | 1      |
| DDY345                      | MATa act1-117::HIS3 cry1 ade2-101            | 1      |
| DDY348                      | MATa act1-123::HIS3 cry1 can1-1 ade2-101     | 1      |
| DDY353                      | MATa act1-135::HIS3 ade2-101                 | 1      |
| **Diploid pseudo–wild-type actin mutants** |                                               |        |
| DDY974                      | MATa/α act1-102::HIS3 act1-102::HIS3 cry1/1 can1-1 ade4+ | 2      |
| DDY975                      | MATa/α act1-104::HIS3 act1-1-4::HIS3 can1-1 can1-1 | 2      |
| DDY976                      | MATa/α act1-115::HIS3 act1-115::HIS3 can1-1 ade4+ | 2      |
| DDY977                      | MATa/α act1-116::HIS3 act1-116::HIS3 can1-1+ can1-1 | 2      |
| DDY978                      | MATa/α act1-117::HIS3 act1-117::HIS3 cry1/+ can1-1 ade4+ | 2      |
| DDY979                      | MATa/α act1-123::HIS3 act1-123::HIS3 cry1/+ can1-1 ade2/ade2 | 2      |
| DDY977                      | MATa/α act1-135::HIS3 act1-135::HIS3 cry1/+ can1-1 ade2/ade2 | 2      |
Bud4p, which are required for the axial budding pattern but not the bipolar pattern, are thought to be components of the axial bud-site cue. Bud3p and Bud4p localize with the bipolar pattern, are thought to be components of the axial bud-site cue. Finally, we confirmed and extended previous evidence indicating that microtubules are not involved in axial or bipolar bud-site selection.

### Materials and Methods

#### Strains and Growth Conditions

Yeast strains used in this study are listed in Table I. Standard yeast genetic procedures and media were used (Rose et al., 1990). Except where noted, yeast strains were grown at 25°C in rich YPD medium.

To initiate pseudohyphal (PH) growth, yeast cells were streaked onto synthetic limiting ammonium dextrose histidine (SLADH) nitrogen-limiting plates, as described previously (Gimeno et al., 1992). Our laboratory strains (S288C background) were not able to undergo PH growth. There-

| Name | Genotype | Source |
|------|----------|--------|
| DDY186 | MATa ura3-52 leu2-3, 112 | 1 |
| DDY263 | MATa abp1Δ::LEU2 leu2-3, 112 ura3-52 lys2-801 | 1 |
| DDY771 | MATa sac6Δ::URA3 ura3-52 his4 | 1 |
| DDY187 | MATa svr2Δ::HIS3 his3Δ200 ura3-52 leu2-3, 112 | 1 |
| DDY332 | MATa sla1Δ::URA3 ura3-52 his3Δ200 | 1 |
| DDY667 | MATa sla2Δ::URA3 ura3-52 leu2-3, 112 | 1 |
| DDY1053 | MATa sla2-6 ura3 leu2 trp1 | 2 |
| DDY288 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY1007 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY772 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY777 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY1085 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY1054 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 1 |
| DDY1056 | MATa ura3-52 (pseudohyphal growth+) | 3 |
| DDY1060 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY1061 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY1064 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY112 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY964 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY1070 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY1087 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY986 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |
| DDY1049 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ | 2 |

For each strain, the strain collection number, genotype, and source are indicated. Sources: 1, Drubin lab; 2, this study; 3, Herskowitz lab (University of California, San Francisco, San Francisco, CA); 4, Murray lab (University of California San Francisco); 5, Pringle Lab (University of North Carolina, Chapel Hill, NC).

These strains also contain: his3Δ200 ura3-52 leu2-3, 112 tub2-201/201.

| Name | Genotype | Source |
|------|----------|--------|
| DDY979 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ (except for DDY979, which contains ade2/ade2). | 2 |
| DDY1060 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ (except for DDY979, which contains ade2/ade2). | 2 |
| DDY1060 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ (except for DDY979, which contains ade2/ade2). | 2 |
| DDY1060 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ (except for DDY979, which contains ade2/ade2). | 2 |
| DDY1060 | MATa ura3-52/ura3-52 leu2-3, 112/+ his4-619/+ (except for DDY979, which contains ade2/ade2). | 2 |

For each strain, the strain collection number, genotype, and source are indicated. Sources: 1, Drubin lab; 2, this study; 3, Herskowitz lab (University of California, San Francisco, San Francisco, CA); 4, Murray lab (University of California San Francisco); 5, Pringle Lab (University of North Carolina, Chapel Hill, NC).

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These strains also contain: his3Δ200 ura3-52 leu2-3, 112 tub2-201/201.

These strains also contain: his3Δ200 ura3-52 leu2-3, 112 tub2-201/201.
fore, for PH growth experiments, the sla2-6 mutation was crossed into the PH background to obtain a sla2-6 PH+ strain. For the sla2Δ mutants, a PH+ strain was transformed with the appropriate deletion plasmid (Holtzman et al., 1993) to generate the mutant strain. The original mzd2-1 mutation (Li and Murray, 1991) was in a strain background that did not show axial budding in MATα or MATα cells. Therefore, to examine axial budding in a mzd2-1 strain, strain DDY981 (previously called AF599) containing this mutation was crossed with a strain (DDY194) wild-type for axial budding. Haploid cells that contained the mzd2-1 mutation in an axial-budding-competent background were identified among the segregants from this cross.

**Calcofluor and FITC-ConA Staining; Quantitation of Budding Patterns**

Cells that had been grown exponentially for at least 12 doubling times in liquid medium were fixed by the addition of formaldehyde to 5% for at least 1 h. The cells were briefly sonicated and resuspended in PBS and 0.1% Calcofluor (from Sigma Chemical Co., St. Louis, MO) as described previously (Pringle, 1991). Stained cells were observed by epifluorescence using a fluorescence microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) and a 100x neofluor objective. Only cells with three or more bud scars were scored, and all such cells in the field were scored. Cells that contained a chain of bud scars that originated at the birth scar, with each scar physically contacting at least one other bud scar, were scored as axial. Cells that contained bud scars at both poles of the cell, or only at the distal pole, were scored as bipolar. Cells that showed neither of these patterns were scored as random. For FITC-ConA staining, living cells were incubated for at least 45 min at 25°C. A 100-μm suspension in 50 μl PBS was incubated with FITC-ConA as described above and then either fixed with formaldehyde to 5% for at least 1 h. Cells were then washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.3) and resuspended in 50 μl PBS with 0.2% Triton X-100 for 10 min. Cells were then incubated for at least 45 min at 25°C with 6 U of rhodamine phalloidin (Molecular Probes, Eugene, OR) that had been added directly to the cell suspension. Cells were then washed once with PBS. Cells were mounted as described (Pringle et al., 1991) and observed by fluorescence microscopy.

**Rhodamine Phalloidin Staining**

1 ml of exponentially growing culture was fixed by the addition of formaldehyde to 5% for at least 1 h. Cells were then washed twice in PBS (157 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, 50 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.3) and resuspended in 50 μl PBS with 0.2% Triton X-100 for 10 min. Cells were then incubated for at least 45 min at 25°C with 6 U of rhodamine phalloidin (Molecular Probes, Eugene, OR) that had been added directly to the cell suspension. Cells were then washed once with PBS. Cells were mounted as described (Pringle et al., 1991) and observed by fluorescence microscopy.

**Benomyl Treatment of Cells**

For experiments involving benomyl treatment, cells were first grown exponentially in YPD for at least 12 doubling times. Cells were then labeled with FITC-Con A as described above and then either fixed with formaldehyde or shifted to YPD medium with 40 μg/ml of benomyl (E.L. du Pont de Nemours, Wilmington, DE), a concentration that completely depolymerizes microtubules within 10 min and causes wild-type cells to arrest as large budded cells in the first cell cycle. Cells were grown in the benomyl-containing medium for up to 7 h. Aliquots were removed and fixed at 3, 5, and 7 h. (Immunofluorescence performed on the different timepoints confirmed the absence of microtubules. Also, wild-type cells grown in parallel with the mzd2-1 mutant cells were arrested as large-budded cells at all time points.) After fixation, cells were stained with Calcofluor as described above, and the bud-scar pattern was scored. Only bud scars formed before the shift to benomyl-containing medium were stained with FITC-ConA, whereas all bud scars were stained with Calcofluor. As expected, all cells exhibited some bud scars that stained with Calcofluor but did not stain with FITC-ConA.

**Figure 1.** Mutations in actin or actin-associated proteins cause a specific defect in the bipolar budding pattern. (A–C) Histograms showing the percent random budding (front rows) and diploid (back rows) strains. The wild-type strains shown in A and B are DDY334 (MATα) and DDY440 (MATαα). The wild-type strains shown in C are DDY186 (MATαα) and DDY288 (MATααα). Only cells containing three or more bud scars were scored. (d–k) Representative wild-type and mutant cells stained with Calcofluor. MATα or MATαα cells are pictured in d, f, h, and j and MATααα cells are pictured in e, g, i, and k. Wild-type cells are shown in d (DDY334) and e (DDY440). act1-116 mutant cells are shown in f (DDY349) and g (DDY434). act1-116 mutant cells are shown in h (DDY344) and i (DDY977). sla2-6 mutant cells are shown in j (DDY1053) and k (DDY1064). Bar, 5 μm.

**Calcofluor and FITC-ConA Staining; Quantitation of Budding Patterns**

Cells that had been grown exponentially for at least 12 doubling times in liquid medium were fixed by the addition of formaldehyde to 5% for at least 1 h. The cells were briefly sonicated and resuspended in PBS and 0.1% Calcofluor (from Sigma Chemical Co., St. Louis, MO) as described previously (Pringle, 1991). Stained cells were observed by epifluorescence using a fluorescence microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) and a 100x neofluor objective. Only cells with three or more bud scars were scored, and all such cells in the field were scored. Cells that contained a chain of bud scars that originated at the birth scar, with each scar physically contacting at least one other bud scar, were scored as axial. Cells that contained bud scars at both poles of the cell, or only at the distal pole, were scored as bipolar. Cells that showed neither of these patterns were scored as random. For FITC-ConA staining, living cells were incubated in YPD containing 0.1 mg/ml FITC-ConA for 5 min as described by Chant and Pringle (1995). Cells were washed once with YPD and then either fixed with formaldehyde or resuspended into fresh medium to permit growth to resume.

**Results**

**Bud-Site Selection Pattern in Conditional-lethal Actin Mutants**

A systematic charged-amino-acid-to-alanine mutagenesis of the yeast actin gene generated 36 mutations (Wertman et al., 1992). 11 were recessive-lethal, two were putatively dominant-lethal, sixteen were conditional-lethal, and seven had no readily observable phenotype and were therefore designated “pseudo–wild-type.” Previously, eight of the conditional-lethal mutants were found to be defective in the bipolar bud-site selection pattern (Drubin et al., 1993). However, the mutants were not assayed for defects in the axial budding pattern. Here, we examined the bud-site selection pattern of 17 nonlethal charged-to-alanine act1 mutants in both MATαα diploids and MATα or MATαα haploids. The results are plotted in Fig. 1, A and B, and representative mutants are pictured in Fig. 1, d–i.

We scored the budding patterns of 10 of the 16 conditional-lethal act1 mutants at the permissive temperature (25°C). The other six mutants, which had the most pronounced growth defects, could not be scored because of increased and irregular chitin deposition that led to high background staining with Calcofluor. All of the 10 scored mutants showed a bipolar-specific defect; MATα or MATαα cells exhibited the normal axial pattern, but MATααα cells showed a random budding pattern instead of a bipolar pattern (Fig. 1 A).

In addition to the bipolar defect, some of the actin mutants, such as act1-108, also exhibited a slight defect in the axial budding pattern in MATα or MATαα cells (Fig. 1 A). These usually were the less healthy mutants. We speculate that the poor growth and general pleiotropic nature of these alleles result in a nonspecific axial budding defect. The axial pattern has been reported to be sensitive to perturbation of cell physiology, while the bipolar pattern is more robust, a feature that makes the bipolar-specific phenotype less prone to nonspecific effects (Chant and Pringle, 1995).

The actin mutants that showed wild-type levels of axial budding in haploids varied in the amount of random budding in diploids. The mutant MATαα α diploids exhibited 55 to 80% random budding, compared to 10% in the isogenic wild-type diploid strain (Fig. 1 A).

**Bud-Site Selection Pattern in Pseudo–Wild-Type Mutants**

Two of the seven pseudo–wild-type actin mutants, act1-116 and act1-117, exhibit a bipolar-specific budding defect (Fig. 1 B). As these mutants show normal growth over a wide range of temperatures and salt concentrations (Wertman et al., 1992), growth perturbation is not responsible for the budding defect. The amino acids altered in these
two mutant alleles are located near each other on the same face of an α-helix in actin-subdomain 4 (see Fig. 5). act1-115, another pseudo–wild-type allele that contains amino acid changes proximal to those changed in act1-116 and act1-117 in the atomic actin structure (see Fig. 5), did not show an effect. Because act1-116 and act1-117 had been previously characterized as pseudo–wild-type ACT1 alleles that showed wild-type growth characteristics, it was important to determine whether actin organization was defective in these mutants. Rhodamine-phalloidin staining of the mutants showed that their overall actin organization is normal (Fig. 2, c and d, compare to a). However, there appears to be less F-actin in the mutants as compared to wild type. For example, it is difficult to visualize actin cables in act1-117 by rhodamine-phalloidin staining (Fig. 2 d) or by anti-actin immunofluorescence (not shown). Significantly, this apparent decrease in F-actin levels does not correlate with defective bipolar budding, because other pseudo–wild-type act1 mutants with even fainter F-actin staining still show wild-type budding. For example, act1-116 (Fig. 2 c), the pseudo–wild-type allele with the most dramatic effect on bipolar budding, appears to have more pronounced staining of F-actin structures than act1-115 (Fig. 2 b), which shows nearly wild-type budding.

**Bud-Site Selection in Actin-associated Protein Mutants**

We also wanted to test whether mutations in actin-associated proteins would have the same effect on the budding
pattern as mutations in \textit{ACT1}. Thus, we determined the budding patterns of mutants defective in \textit{ABP1}, \textit{SAC6}, \textit{SRV2}, \textit{SLA1}, and \textit{SLA2}. All of these genes encode proteins that localize to cortical actin patches (Drubin et al., 1988; Freeman et al., 1996; Ayscough, K., T. Lila, and S. Yang, unpublished results). \textit{Sac6p} (fimbrin) is an actin-bundling protein (Adams et al., 1991), Abp1p binds filamentous actin (Drubin et al., 1988), and Srv2p can bind actin monomers (Freeman et al., 1995). Mutations in these genes have varying effects on the actin cytoskeleton. \textit{abp1Δ} mutants have normal actin organization and show no readily observable phenotype (Drubin et al., 1990). Null mutations in \textit{SAC6}, \textit{SRV2}, and \textit{SLA2}, result in delocalization of cortical actin patches even at 25°C, a temperature permissive for growth (Adams et al., 1991; Vojtek et al., 1991; Holtzman et al., 1993). In contrast, at 25°C, \textit{sla1Δ} mutants show abnormally large cortical “chunks” of actin that are still localized to the bud (Holtzman et al., 1993). These mutants also show various degrees of temperature sensitivity for growth.

\textit{abp1Δ} mutants show normal budding patterns in \textit{MATa}, \textit{MATa}, and \textit{MATa/α} cells. However, \textit{sac6Δ}, \textit{srv2Δ}, \textit{sla1Δ}, and \textit{sla2Δ} mutants show a normal axial pattern in haploid cells but show a markedly elevated incidence (~90%) of random, as opposed to bipolar, budding in diploid cells at 25°C (Fig. 1 C).

Analysis of budding patterns in partial loss-of-function alleles of \textit{SLA1} and \textit{SLA2} demonstrated further that the severity of the defect in actin organization or assembly did not correlate with the severity of the budding-pattern defect (Fig. 1 C). The allele \textit{sla2-6} is synthetic-lethal with both \textit{abp1Δ} and \textit{sac6Δ} mutants, but, unlike the \textit{sla2Δ} mutant, grows well at all temperatures and shows polarized actin (data not shown). Despite the robust growth of the mutant and its lack of apparent defects in actin organization, this mutant exhibits a bipolar budding defect, similar to that of the \textit{sla2Δ} mutant (Fig. 1 C, j, and k). On the other hand, an \textit{sla1} deletion mutant construct that lacks one SH3 domain (\textit{sla1ΔSH3#3}, pKA5), when transformed into \textit{sla1Δ} mutants, does not rescue the abnormal actin or-

### Table II. Budding Patterns of Actin Cytoskeleton and Unipolar Budding Mutants

| Strain     | MATa/α | Percent bipolar | Percent distal | Percent proximal | Percent random | Percent distal | Percent Proximal | Percent equatorial |
|------------|--------|----------------|----------------|------------------|----------------|----------------|-----------------|--------------------|
| DDY288     | wild-type | 86             | 9              | 0                | 5              | 99             | 1               | 0                  |
| DDY1123    | bud8Δ act1-119 | 4              | 0              | 28               | 68             | 22             | 64              | 14                 |
| DDY1114    | bud8Δ sla 2-6  | 6              | 0              | 21               | 73             | 17             | 61              | 22                 |
| DDY1120    | bud9Δ act1-119 | 3              | 19             | 0                | 78             | 87             | 10              | 3                  |
| DDY1117    | bud9Δ sla 2-6  | 2              | 29             | 0                | 69             | 99             | 1               | 0                  |
| DDY1173    | bud8Δ         | 4              | 0              | 94               | 2              | 1              | 98              | 1                  |
| DDY1174    | bud9Δ         | 4              | 90             | 0                | 6              | 100            | 0               | 0                  |

Strains are homozygous for the indicated mutant alleles. For determining the overall budding pattern, only cells containing three or more bud scars were scored. Numbers are derived from the analysis of >200 cells for each strain. For determining the placement of the first bud, cells containing only one bud scar or one bud (and no bud scar) were scored. Numbers were derived from the analysis of >100 cells for each strain.

Figure 3. Placement of first three buds in wild-type, \textit{act1-124}, \textit{sla2-6}, and \textit{sla1Δ} mutants. Bud scars were scored as either distal (opposite the birth scar), proximal (near the birth scar), or equatorial. The placement of the first bud was scored by counting only cells that displayed either no bud scars and one bud, or only one bud scar. The placement of the second bud was scored by observing cells that displayed one bud scar and an attached bud. The placement of the third bud was scored by counting cells that displayed two bud scars and an attached bud. The strains used for counting were DDY288, DDY434, DDY1064, and DDY1085. Numbers are derived from the analysis of 100 cells for each strain.
ganization or temperature-sensitivity defects (Ayscough, K., and D. Drubin, manuscript in preparation). Nevertheless, this mutant allele does restore the bipolar budding pattern in diploid sla1Δ mutant cells. Although this mutant has a defective actin cytoskeleton, it is able to bud in a bipolar pattern. Thus, simply perturbing actin cytoskeleton organization does not always result in defects in the bipolar budding pattern.

Analysis of Bud-Site Selection at the Distal Pole of Daughter Cells in Actin Cytoskeleton Mutants

In wild-type MATa/a cells, >99% of daughter cells form their first bud at the distal pole, opposite the birth scar (Chant and Pringle, 1995) (Fig. 3). We observed that the actin cytoskeleton mutant cells that exhibited a largely random budding pattern nevertheless almost always correctly chose the distal pole for the first bud site in daughter cells. We quantitated this phenomenon for three of the mutants by determining the location of the placement of the first, second, and third buds (see Fig. 3 legend). In sla2-6, act1-124, and sla1Δ mutants, >90% of the cells (n = 100) placed the first bud at the distal pole. However, the second and third bud sites become more evenly distributed over the cell, resulting in a randomized bud scar pattern (Fig. 3).

Mutations in Bud8 and Bud9 (Zahner et al., 1996) result in unipolar budding patterns. bud8 mutants bud only from the proximal pole, and bud9 mutants bud only from the distal pole. We wanted to analyze the budding patterns of cells containing both an actin cytoskeleton mutation and a unipolar budding mutation. We generated MATa/a bud8Δ sla2-6, bud8Δ act1-119, bud9Δ sla2-6, and bud9Δ act1-119 double mutants, and assayed both their overall budding pattern and the placement of the first bud in daughter cells (Table II). In all of the double mutants, the majority of the cells exhibited a random budding pattern. Some of the double mutant cells still exhibited a unipolar budding pattern. The percentage showing a unipolar budding pattern was similar to that of act1-119 or sla2-6 single mutant cells showing a bipolar budding pattern (Table II and Fig. 1), suggesting that the cells with unipolar budding patterns reflected the incomplete penetrance of the act1-119 and sla2-6 alleles.

The bud9Δ act1-119 and bud9Δ sla2-6 double mutant daughter cells placed their first buds at the distal pole. However, both the bud8Δ act1-119 and bud8Δ sla2-6 double mutant daughter cells placed their first bud at the proximal pole in a majority (60%) of cells (Table II). While this is less than the 90%+ distal pole bias seen in wild-type cells (or the actin cytoskeleton mutant cells), it is still significantly higher than the percentage expected if the bud site was selected randomly. Thus, the bud8Δ act1-119 and bud8Δ sla2-6 double mutants, like bud8Δ single mutants, exhibit an initial bias for placing the bud at the proximal pole.

Effects of sla2 Mutations on Pseudohyphal Growth

S. cerevisiae, when starved for nitrogen, can undergo PH growth (Gimeno et al., 1992). The ability to position a budding cue at the distal pole of cells is necessary for PH growth. PH growth is characterized by formation of long, rod-shaped cells connected in tandem (as opposed to single ellipsoidal cells). After cytokinesis, daughter cells remain connected to mother cells, thereby forming chains of cells that grow away from the founding mother cell. PH cells bud in a unipolar manner, with only the distal pole used for new bud sites (Kron et al., 1994). rsr1 mutant diploids, which show random budding, cannot form long PH filaments, though these cells can develop an elongate morphology (Gimeno et al., 1992).

While diploid sla2 mutants exhibit budding-pattern defects, daughter cells still correctly place their first bud at their distal tip. Thus, we wanted to determine whether the sla2 mutants could undergo PH growth. We crossed sla2-6 and sla2Δ mutations into a PH+ strain background and tested for PH formation on limiting nitrogen. sla2-6 mutants, which as diploids grown with plentiful nitrogen exhibit random budding in mother cells while correctly positioning daughter cell buds (see above), still exhibited PH growth, though less pronounced than that of wild-type strains. However, sla2Δ mutants were unable to form PH filaments (Fig. 4) or undergo the morphological change to rodlike cells (data not shown).

The Axial and Bipolar Patterns Appear to be Generated through Two Separate Pathways

We have shown that mutations that perturb the actin cytoskeleton lead to randomization of the bipolar budding pattern but do not affect the axial pattern. Mutations in the gene Bud4 disrupt only the axial pattern, exhibiting a bipolar pattern in haploids and diploids (Chant and Herskowitz, 1991). This suggests that the axial and bipolar budding patterns are mediated through two separate path-

![Figure 4](image)

**Figure 4.** Pseudohyphal growth in sla2 mutants. Cells were streaked onto SLADH plates (see Materials and Methods) to assay for their ability to undergo pseudohyphal growth. (a) DDY1060, wildtype; (b) DDY1061, sla2Δ; and (c) DDY1064, sla2-6. Cells were grown at 25°C for 2 d. Bar, 25 μm.
ways, a conclusion also reached in earlier studies (Chant and Pringle, 1995; Durrens et al., 1995; Zahner et al., 1996).

We determined the budding phenotype of a \textit{bud4Δ sla2-6} double mutant haploid to further test this conclusion. \textit{(sla2-6} was used because this mutation has little effect on cell growth but nevertheless causes a high degree of random budding in MAT\textit{a} or MAT\textit{a} cells.) 90% of MAT\textit{a} or MAT\textit{a} \textit{bud4Δ} mutant cells exhibit bipolar budding, and 99% of MAT\textit{a} or MAT\textit{a} \textit{sla2-6} mutant cells exhibit axial budding (Table III). 88% of MAT\textit{a} or \textit{α} \textit{bud4Δ} \textit{sla2-6} double mutants were found to exhibit random budding, a number comparable to that of the MAT\textit{a} or \textit{α} \textit{sla2-6} mutant. MAT\textit{a}/\textit{α} \textit{bud4Δ} \textit{sla2-6} double mutants also exhibit random budding. These results suggest that the positioning of the axial and bipolar budding pattern cues are independent events mediated by different sets of proteins.

**Bud-Site Selection in a Benomyl-treated Mitotic Checkpoint Mutant**

Because the spindle pole body and the cytoplasmic microtubules that emanate from it are oriented toward the incipient bud site from an early point in the cell cycle (Byers and Goetsch, 1975; Snyder and McIntosh, 1976; Byers, 1981; Adams and Pringle, 1984), we wished to test the possibility that microtubules participate in bud-site selection. Jacobs et al. (1988) previously showed that in the absence of microtubules, cells select the first bud site correctly. However, in this experiment, the placement of only one bud could be analyzed, as the lack of microtubules causes a cell cycle arrest. Thus, this experiment did not rule out a role for microtubules in bud-site selection if the bud-site cue is, for example, placed early in mitosis, before the arrest point triggered by microtubule disruption, or if, as our data indicate, there is a fundamental difference between the selection of the first bud in daughter cells and the selection of subsequent buds.

To assess bud-site selection in cells undergoing multiple rounds of cell division in the absence of microtubules, we examined budding patterns in the mitotic arrest defective \textit{(mad2-1)} mutant during growth in the presence of the microtubule depolymerizing drug benomyl. This mutant is defective in a mitotic checkpoint and therefore does not arrest at mitosis when treated with benomyl (Li and Murray, 1991). These cells continue to divide and form buds for several cycles despite the absence of any microtubules. Exponentially growing \textit{MAT a/α mad2-1} cells were pulse-labeled with FITC-ConA and then shifted to medium containing 40 \textmu g/ml benomyl for 7 h. Cells were then fixed, stained with Calcofluor, and scored to determine their bud-scar pattern. Using this procedure, all bud scars were stained with Calcofluor, but only bud scars present before growth in benomyl are stained with FITC-ConA (Chant and Pringle, 1995). In this experiment, the \textit{mad2-1} mutant cells formed three to four buds in the absence of microtubules. \textit{mad2-1} cells fixed before growth in benomyl showed 91% bipolar budding. After 7 h in benomyl, the cells still showed 85% bipolar budding (Table IV).

We also used the same procedure to determine whether microtubules are required for axial budding. \textit{MAT a/α} or \textit{MAT a/α} \textit{mad2-1} cells were grown in the presence of benomyl and then scored to determine their bud-scar pattern. As with the bipolar budding of \textit{MAT a/α} cells, we found that the absence of microtubules had no effect on the axial bud-site selection pattern of haploids (Table IV).

**Discussion**

A complete understanding of the molecular basis for yeast budding patterns requires identification of the spatial cues that mark the prospective bud site, the mechanisms for positioning the cues, and the mechanisms for decoding the cues. Cytoskeletal elements are well suited for positioning spatial cues; indeed, the septin-containing neck filaments are thought to provide a scaffold upon which the axial bud-site cue localizes. We wanted to determine if the other cytoskeletal elements, specifically actin filaments and microtubules, are also involved in bud-site selection.

**Microtubules Do Not Appear to Function in Establishment of the Axial or the Bipolar Budding Pattern**

Microtubules have been postulated to have a role in bud-site selection because the spindle pole body and cytoplasmic microtubules are oriented toward the bud-site from an early point in the cell cycle (Byers and Goetsch, 1975; Byers, 1981; Kilmartin and Adams, 1984; Snyder et al., 1991). Previously, Jacobs et al. (1988) showed that cells correctly select their first bud site in the absence of microtubules. This result implies that microtubules play no role in bud-site selection in at least the first round of cell division in the absence of microtubules (also discussed in Chant and Pringle, 1995). Here, we have shown that cells undergoing multiple rounds of cell division in the absence of microtubules do not exhibit axial budding in the absence of microtubules.

**Table III. Budding Patterns of \textit{Δbud4 sla2-6} Mutants**

| Strain         | Percent axial | Percent Bipolar | Percent Random |
|----------------|---------------|-----------------|----------------|
| DDY7964 MATa bud4Δ |               |                 | 88             | 12             |
| DDY1053 MATa sla2-6 | 99             |                 |               | 1              |
| DDY1065 MATa bud4Δ sla2-6 | 2             | 10              | 88             |
| DDY1070 MATa/sla2-6 | 96             |                 |               | 4              |
| DDY1054 MATa/sla2-6 | 17             |                 | 83             |
| DDY1067 MATa bud4Δ sla2-6 | 9             |                 | 91             |

Only cells containing three or more bud scars were scored. Numbers are derived from the analysis of >200 cells for each strain.

**Table IV. Budding Pattern of mad2-1 Cells in the Presence and Absence of Benomyl**

| Yeast strain          | No benomyl | 40 μg/ml benomyl |
|-----------------------|------------|------------------|
|                       | Percent    | Percent          |
|                       | axial      | bipolar          |
|                       |            |                  |
| DDY1049 or DDY048 (MATa or MATα) | 99      | 96               |
| DDY986 (MATa/α)       | 91         | 85               |

To determine the budding pattern of \textit{mad2-1} cells grown in the presence of benomyl, exponentially growing \textit{mad2-1} haploid (DDY1048, 1049) or \textit{mad2-1/mad2-1} diploid (DDY986) cells were labeled with FITC-ConA and then shifted to YPD containing 40 \textmu g/ml benomyl. After 7 h, cells were fixed with formaldehyde and stained with Calcofluor. All bud scars stained with Calcofluor while only bud scars present before the shift to benomyl were stained with FITC-ConA. Cells contained three to four bud scars that did not stain with FITC-ConA. Percentages were derived from the analysis of >200 cells for each strain.
bulles still correctly select bud positions for both the axial and the bipolar patterns. Thus, the orientation of microtubules towards the bud site occurs in response to the cue establishing an axis of cell polarity and is not responsible for positioning the cue.

**The Actin Cytoskeleton Is Required for the Bipolar Bud-Site Selection Pattern**

We have shown that mutations in the genes encoding either actin or actin-associated proteins perturb the bipolar bud-site selection pattern. Several lines of evidence support the conclusion that the budding defects in actin cytoskeleton mutants reflect a direct role of the actin cytoskeleton in the actin cytoskeleton in placement of budding cues and did not arise because of nonspecific secondary defects. First, mutations in actin cytoskeleton affect only the bipolar budding pattern and show no effect on the axial budding pattern. This is especially striking since the axial budding pattern, but not the bipolar budding pattern, is very sensitive to perturbation of cell growth and cell physiology. This sensitivity is probably due to the dependence of the axial budding pattern upon a transient marker (Flescher et al., 1993; Chant and Pringle, 1995). Second, some pseudo–wild-type mutations in the actin cytoskeleton (act1-116, act1-117, sla2-6) that have little or no effect on cell growth nevertheless perturb the bipolar budding pattern. Thus, the bipolar budding pattern defect in these mutants is not likely to result from general effects on cell physiology. Third, at least one mutant with markedly abnormal actin organization (sla1ΔSH3Δ83) nevertheless shows normal bipolar budding. This mutation may not perturb the specific aspect of actin function necessary for its role in bipolar budding. Thus, the actin cytoskeleton appears to be directly involved in establishing the bipolar budding pattern, presumably by placing or maintaining the bipolar bud-site cue.

**A Particular Region of Actin Is Critical for Its Role in Bipolar Bud-Site Selection**

As mutations in any of several genes (RSR1, BUD2-5) can affect budding patterns without impairing growth (Chant and Herskowitz, 1991), mutations in actin that only perturb placement of the bud-site marker are also predicted to show wild-type growth. Thus, analysis of pseudo–wild-type actin mutants might allow us to pinpoint the regions of the actin monomer involved in positioning the bipolar bud-site cue. Two pseudo–wild-type mutants, act1-116 and act1-117, exhibit a bipolar budding defect, with act1-116 exhibiting the more dramatic defect. The amino acids altered in these two mutants are located near each other on the same face of an α-helix in actin subdomain 4 (Fig. 5) and are exposed on the surface of the actin filament. While these mutations are located close to the ATP- and Ca2+-binding sites on actin, they are unlikely to have a gross effect on actin structure and cell growth because overall actin organization in these mutants is normal. These mutants, especially act1-117, appear to have less F-actin, or possibly less bundled F-actin, than wild-type cells. However, act1-116 shows as much if not more F-actin than act1-115, which does not show a bipolar budding defect. Thus, the effects of act1-116 and act1-117 on the bipolar budding pattern are most likely due to disruption of a specific interaction between actin and the proteins necessary for positioning the bipolar bud-site cue, or perhaps the cue itself.

**Other Genes Necessary for the Bipolar Budding Pattern**

Other genes have also been shown to disrupt the bipolar, but not the axial, budding pattern. Mutations in some of these genes, RVS161, RVS167 (Bauer et al., 1993; Sivadon et al., 1995), END3 (Bénédetti et al., 1994), and BUD6/ AIP3 (Zahner et al., 1996; Amberg et al., 1996) also affect the actin cytoskeleton, consistent with the conclusion that the actin cytoskeleton plays a role in the placement of bipolar budding cues. Less is presently known about the other genes implicated in bipolar budding. For example, sur4 and fen1 (Desfarges et al., 1993; Revardel et al., 1995) mutants, which also exhibit a bipolar budding pattern–specific defect, have altered phospholipid content and are thought to be involved in membrane lipid metabolism. One possible explanation for the effects of these mutations on bipolar budding would be that proteins resident in the plasma membrane function in selection of bipolar budding sites.

Mutations in two other genes required only for the bipolar budding pattern, BUD8 and BUD9 (Zahner et al., 1996), result in a more specific defect, a unipolar budding pattern. bud8 mutants bud only from the proximal pole and bud9 mutants bud only from the distal pole. These phenotypes suggest that different mechanisms are used for placing the cue at the two poles and/or that different cues are used to mark the distal and proximal poles.

**Possible Mechanisms for Actin’s Function in Bipolar Bud-Site Selection**

While mutations affecting the actin cytoskeleton cause an overall randomization of the budding pattern in MATαα cells, we observed that the daughter cells almost always correctly placed their first bud at the distal pole. Subsequent bud sites were then selected randomly. Zahner et al. (1996) also observed this phenomenon for some of their bipolar budding mutants, specifically bud6 and sla2-6 (BUD6 is identical to AIP3, an actin-interacting protein [Amberg et al., 1996]). We also tested the ability of some of the actin cytoskeleton mutants to undergo PH growth. Cells undergoing PH growth use a unipolar budding pattern, with buds forming only at the distal pole (Kron et al., 1994). Neither wild-type MATαα and MATαα cells (which show an axial budding pattern) nor MATαα rsl1 null mutants (which show a random budding pattern) can undergo PH growth (Gimeno et al., 1992). However, MATαα sla2-6 cells, which exhibit random budding, can undergo PH growth. These results further support the conclusion that at least initially, the distal pole is selected and marked properly in the sla2-6 mutant.

To further analyze the role of actin in determining the bipolar budding pattern, we examined the budding pattern of actin cytoskeleton mutants in conjunction with the unipolar budding mutants, bud8Δ and bud9Δ. MATαα bud9Δ mutants place buds only at the distal pole. MATαα bud9Δ act1-119 and bud9Δ sla2-6 double mutants exhibited an overall random budding pattern. However, double mutant daughter cells placed their first buds at the distal pole, similar to the phenotype of the actin cytoskeleton single mu-
Figure 5. Location of mutations on the actin atomic model. (A) Ribbon diagram of the actin monomer. Red represents amino acids that when changed to alanine produce a lethal phenotype; yellow represents amino acids that when changed to alanine produce a conditional-lethal phenotype; and green represents amino acids that when changed to alanine produce a pseudo–wild-type phenotype. $\text{Ca}^{2+}$ is represented by the blue dot, and ATP is shown as a stick figure (black). The location of the alleles tested for their effects on the budding pattern (i.e., the actin alleles shown in Fig. 1, a and b) are indicated. (B) Space filling model of the actin monomer. The back side of the actin monomer (as compared to A) is shown. The amino acids mutated in act1-116 and act1-117 are colored green. ATP is colored magenta.
of the proximal pole. \textit{MATa/bud8Δ act1-119} and \textit{bud8Δ sla2-6} double mutants exhibited an overall random pattern, similar to the \textit{MATa/bud9Δ act1-119} and \textit{bud9Δ sla2-6} double mutants. However, \textit{MATa/bud8Δ act1-119} and \textit{bud8Δ sla2-6} daughter cells exhibited a proximal pole bias ( \textasciitilde 60\%) for the placement of the first bud. These double mutant results imply that either bud-site selection is fundamentally different in mother and daughter cells, with bud-site selection in daughter cells occurring through an actin-independent process, or that actin is mainly necessary for the maintenance of bipolar budding cues. In the latter scenario, the actin cytoskeleton plays no role in the placement of the distal budding cue as discussed above. Instead, the observation that a substantial percentage ( \textasciitilde 40\%) of \textit{bud8Δ} actin cytoskeleton double mutants do not bud from their proximal pole implies that the budding cue is not as efficiently localized or maintained at the proximal pole in actin cytoskeleton mutants, and therefore actin might stabilize cues in the cell cortex.

Two possible models for the establishment of the bipolar budding pattern were proposed by Chant and Pringle (1995) and by Zahner et al. (1996) (Fig. 6 A). Below, we interpret our results in the context of these models. In model 1 (Fig. 6, \textit{wildtype}, 1), the bipolar cue present at the bud-site is partitioned between the tip of the newly emerging bud and the mother-bud neck as the bud emerges. At cytokinesis, the cue remaining at the mother-bud neck is further divided, this time between the mother cell and the daughter cell. In model 2 (Fig. 6, \textit{wildtype}, 2), the bipolar cue is present at the presumptive bud site and remains associated with the distal tip of the bud as it grows. Late in the cell cycle, when the cell surface in the neck region grows during septation, a cue is positioned in the septal region and is divided between the mother cell and the daughter cell at cell division. Thus, in model 2, the cell surface growth apparatus functions in placement of the bipolar cue first at the distal bud tip, then in the septal region.

We propose that actin is the cytoskeletal structure necessary for the maintenance, and perhaps also the placement, of the presently unidentified bipolar bud-site cues. There are several possibilities for how the actin cytoskeleton functions in establishing the bipolar budding pattern. In model 1 (Fig. 6, in which a cue is partitioned as a bud emerges, the cue might be partitioned normally, but actin helps retain the cue at the septal (but not the distal pole) region (Fig. 6, B, 1a). Alternatively, the actin cytoskeleton might also be involved in the actual partitioning of the cue during bud emergence (Fig. 6, B, 1b). In model 2, the cue is positioned at the distal pole independent of the actin cytoskeleton. Several proteins, such as Spa2p, are still able to localize to the tip of cells in the absence of the actin cytoskeleton (Ayscough, K., and D. Drubin, manuscript submitted for publication). Although not absolutely required, the actin cytoskeleton helps mediate the deposition of the cue at the mother-bud neck during cytokinesis late in the cell cycle (Fig. 6, \textit{Mutant}, 2) and is also required for its maintenance. For all models, the observation that \textit{bud8Δ act1-119} and \textit{bud8Δ sla2-6} daughter cells have a strong bias toward proximal budding, however, suggests that the proximal cue is present in daughters but is less stably associated with the cortex since subsequent budding is random. Additionally, while actin is not required for placement of the distal pole cue, it might be required for maintenance of the cue at the distal-pole after one cell division because the second bud formed from a daughter cell is positioned randomly. The bipolar cues are not predicted to associate stably with actin because actin is not concentrated at the relevant regions (i.e., the distal or the proximal pole) when the new bud site is selected.

Several key questions about the bipolar budding pattern remain to be answered. Chief among these is the identity of the bipolar budding cues. Identification of the cues is required to elucidate both the mechanisms by which they are positioned at the cell cortex and the mechanism by which these cues are decoded by the cell.

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