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. *MAT***a** and *MAT*α cells bud in an axial pattern, and *MAT***a**/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 *MAT***a** and *MAT*α cells. In this study, we determined the budding patterns of cells carrying mutations in the actin gene or in genes encoding actin-associated proteins: *MAT***a**/α cells were defective in the bipolar budding pattern, but *MAT***a** and *MAT*α cells still exhibit a normal axial budding pattern. We also observed that *MAT***a**/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 *MAT***a**/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 *MAT***a**/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 *mad2-1* 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. *MAT***a** or *MAT*α 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. *MAT***a**/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 *MAT***a**/α daughter cell is almost always placed at the distal pole, opposite the birth scar (the proximal pole) (Freifelder, 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), Bud3p, 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,

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1. *Abbreviations used in this paper*: *mad*, mitotic arrest defective; *MAT*, mating type; PH, pseudohyphal.

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Table I. Yeast Strains Used in This Study

Name	Genotype	Source
Haploid conditional-lethal actin mutations*		
DDY354	*MAT***a** *ACT1::HIS3 cry1*	1
DDY338	*MAT***a** *act1-101::HIS3 cyr1 can1-1*	1
DDY356	*MAT*α *act1-105::HIS3 can1-1*	1
DDY337	*MAT***a** *act1-108::HIS3 cry1 can1-1*	1
DDY342	*MAT*α *act1-113::HIS3 can1-1*	1
DDY346	*MAT***a** *act1-119::HIS3 ade2-101 cry1*	1
DDY347	*MAT***a** *act1-120::HIS3 cry1 can1-1*	1
DDY655	*MAT***a** *act1-122::HIS3 can1-1 ade2-101*	1
DDY349	*MAT*α *act1-124::HIS3 can1-1*	1
DDY351	*MAT*α *act1-129::HIS3 can1-1*	1
DDY336	*MAT***a** *act1-133::HIS3 cry1 can1-1*	1
Diploid conditional-lethal actin mutations‡		
DDY440	*MAT***a**/α *ACT1::HIS3/ACT1::HIS3 can1-1/can1-1 ade4/+*	1
DDY439	*MAT***a**/α *act1-101::HIS3/act1-101::HIS3 ade4/+*	1
DDY980	*MAT***a**/α *act1-105::HIS3/act1-105::HIS3 can1-1/+*	2
DDY438	*MAT***a**/α *act1-108::HIS3/act1-108::HIS3 can1-1/1 ade4/+*	1
DDY441	*MAT***a**/α *act1-113::HIS3/act1-113::HIS3 can1-1/can1-1 ade4/+*	1
DDY430	*MAT***a**/α *act1-119::HIS3/act1-119::HIS3 ade4/+*	1
DDY433	*MAT***a**/α *act1-120::HIS3/act1-120::HIS3 can1-1/can1-1 ade4/+*	1
DDY1051	*MAT***a**/α *act1-122::HIS3/act1-122::HIS3 can1-1/can1-1 ade4/+*	2
DDY434	*MAT***a**/α *act1-124::HIS3/act1-124::HIS3 can1-1/1 ade4/+*	1
DDY431	*MAT***a**/α *act1-129::HIS3/act1-129::HIS3 can1-1/1 ade4/+*	1
DDY437	*MAT***a**/α *act1-133::HIS3/act1-133::HIS3 ade4/+*	1
Haploid pseudo–wild-type actin mutants*		
DDY339	*MAT***a** *act1-102::HIS3 cry1 can1-1 ade2-101*	1
DDY340	*MAT*α *act1-104::HIS3 can1-1*	1
DDY343	*MAT*α *act1-115::HIS3 can1-1 ade2-101*	1
DDY344	*MAT*α *act1-116::HIS3 can1-1*	1
DDY345	*MAT***a** *act1-117::HIS3 cry1 ade2-101*	1
DDY348	*MAT***a** *act1-123::HIS3 cry1 can1-1 ade2-101*	1
DDY353	*MAT***a** *act1-135::HIS ade2-101*	1
Diploid pseudo–wild-type actin mutants‡		
DDY974	*MAT***a**/α *act1-102::HIS3/act1-102::HIS3 cry1/1 can1-1 ade4/+*	2
DDY975	*MAT***a**/α *act1-104::HIS3/act1-1-4::HIS3 can1-1/can1-1*	2
DDY976	*MAT***a**/α *act1-115::HIS3/act1-115::HIS3 can1-1/1 ade4/+*	2
DDY977	*MAT***a**/α *act1-116::HIS3/act1-116::HIS3 can1-1/can1-1*	2
DDY978	*MAT***a**/α *act1-117::HIS3/act1-117::HIS3 cry1/+ can 1-1/+ ade4/+*	2
DDY979	*MAT***a**/α *act1-123::HIS3/act1-123::HIS3 cry1/+ can1-1/can1-1 ade2/ade2*	2
DDY477	*MAT***a**/α *act1-135::HIS3/act1-135::HIS3 cry1/+ can1-1/+ ade4/+*	1

1991; Chant et al., 1991; Park et al., 1993) result in a random budding pattern in both haploids and diploids.

Creating a bud involves first marking the appropriate site with a spatial cue, and then the orienting cellular components towards the site of the cue. Cytoskeletal elements are well suited for positioning spatial cues since they play 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 comprised 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 late 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

Table I. (continued)

Name	Genotype	Source
Haploid actin cytoskeleton mutants		
DDY186	*MATα ura3-52 leu2-3, 112*	1
DDY263	*MAT***a** *abp1Δ::LEU2 leu2-3,112 ura3-52 lys2-801*	1
DDY771	*MATα sac6Δ::URA3 ura3-52 his4*	1
DDY817	*MATα srv2Δ::HIS3 his3Δ200 ura3-52 leu2-3, 112*	1
DDY332	*MAT***a** *sla1Δ::URA3 ura3-52 his3Δ200*	1
DDY667	*MATα sla2Δ::URA3 ura3-52 leu2-3, 112*	1
DDY1053	*MATα sla2-6 ura3 leu2 trp1*	2
Diploid actin cytoskeleton mutants		
DDY288	*MAT***a**/α *ura3-52/ura3-52 leu2-3, 112/+ his4-619/+*	1
DDY1007	*MAT***a**/α *abp1Δ::LEU2/abp1Δ::LEU2 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3/+ his4/+*	
DDY772	*MAT***a**/α *sac6Δ::URA3/sac6Δ::URA3 ura3-52/ura3-52/ura3-52 leu2-3, 112/+ his4/+*	1
DDY773	*MAT***a**/α *srv2Δ::HIS3/srv2Δ::HIS3 his3 Δ200/his3Δ200 ura3-52/+ leu2-3,112/leu2-3, 112 lys2/+*	1
DDY1085	*MAT***a**/α *sla1Δ::URA3/sla1Δ::URA3 ura3/ura3/ura3 his3/his3*	
DDY540	*MAT***a**/α *sla2Δ::URA3/sla2Δ::uURA3 ura3-52/ura3-52 leu2-3, 112/1 his4-619+*	1
DDY1054	*MAT***a**/α *sla2-6/sla2-6 ura3/ura3 leu2/leu2 trp1/trp1 ade2/1 ade3/+ his3/+ lys2/+*	2
Strains capable of undergoing pseudohyphal growth		
DDY1056	*MAT***a** *ura3-52* (*pseudohyphal growth+*)	3
DDY1060	*MAT***a**/α *ura3-52/ura3 his3/his3 leu2/+ trp1/+*	2
DDY1061	*MAT***a**/α *sla2Δ::HIS3/sla2Δ::HIS3 ura3-52/ura3-52 his3/his3 leu2/+ trp1/+*§	2
DDY1064	*MAT***a**/α *sla2-6/sla2-6 ura3-52/ura3-52*	2
Unipolar budding and actin cytoskeleton mutants		
DDY1173	*MAT***a**/α *bu8Δ::TRP1/bu8Δ::TRP1 trp1/trp1 his3/his3 leu2/leu2 lys2/lys2 ura3/ura3*	5
DDY1174	*MAT***a**/α *bud9Δ::HIS3/bud9Δ::HIS3 his3/his3 trp1/trp1 leu2/leu2 lys2/lys2 ura3/ura3*	5
DDY1114	*MAT***a**/α *bud8Δ::TRP1/bud8Δ::TRP1 sla2-6/sla2-6 trp1/trp1 his3/his3 leu2/leu2 lys2/lys2 ura3/ura2 ade2/+*	2
DDY1117	*MAT***a**/α *bud9Δ::HIS3/bud9Δ::HIS3 sla2-6/sla2-6 trp1/trp1 his3/his3 leu2/leu2 lys2/lys2 ura3/ura2*	2
DDY1120	*MAT***a**/α *bud9Δ::HIS3/bud9Δ::HIS3 act1-119::HIS3/act1-119::HIS3 his3/his3 leu2/leu2 ura3/ura2 lys2/+ trp1/+ade2/+ tub2-201/tub2-201*	2
DDY1123	*MAT***a**/α *bud8Δ::TRP1/bud8Δ::TRP1 act1-119::HIS3/act1-119::HIS3 his3/his3 trp1/trp1 leu2/leu2 ura3/ura2 lys2/lys2 tub2-201/tub2-201*	2
Δbud4 and *sla2-6* mutants		
DDY964	*MATα bud4Δ::TRP1 trp1 ura3 his4 can1*	3
DDY1070	*MAT***a**/α *bud4Δ::TRP1/bud4Δ::TRP1/trp1 leu2/leu2 his3/+ lys2/+*	2
DDY1065	*MAT***a** *bud4Δ::TRP1 sla2-6 trp1 leu2 lys2*	2
DDY1067	*MAT***a**/α *bud4Δ::TRP1/bud4Δ::TRP1 sla2-6/sla2-6 trp1/trp1 leu2/leu2 lys2/lys2 his3/+*	2
mad2-1 mutants		
DDY986	*MAT***a**/α *mad2-1/mad2-1 ade2-1/ade2-1 his3-11/his3-11 leu2-3,112 leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100*	4
DDY1049	*MAT***a** *mad2-1 leu2 ura3 trp1*	2
DDY1048	*MATα mad2-1 leu2 his3 lys2 ade2 ura3*	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*.
‡These strains also contain: *his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 tub2-201/tub2-201 ade2/+* (except for DDY979, which contains *ade2/ade2*).
§Isogenic with DDY1060.

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 neck filaments during the period of the cell cycle when a new bud site is selected, and this localization is dependent upon the presence of the neck filaments (Chant et al., 1995; Sanders and Herskowitz, 1996).

In this study, we determined whether the actin and microtubule cytoskeletons function in the generation of bud-site selection patterns of *MAT***a** or *MAT*α and *MAT***a**/α cells. Cells carrying mutations in *ACT1*, the actin gene, or in genes encoding actin-associated proteins exhibited a normal axial budding pattern in *MAT***a** or *MAT*α haploids. In *MAT***a**/α diploids, however, mother cells budded randomly while daughters correctly positioned their first bud at the distal pole of the cell. Thus, the actin cytoskeleton is required for proper selection of bud sites in mother cells. As the actin mutations tested cover the entire actin molecule, we were also able to identify an area on the actin molecule that might be involved specifically in localizing the bipolar 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-

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 appropiate deletion plasmid (Holtzman et al., 1993) to generate the mutant strain.

The original *mad2-1* mutation (Li and Murray, 1991) was in a strain background that did not show axial budding in *MAT***a** or *MAT*α cells. Therefore, to examine axial budding in a *mad2-1* strain, strain DDY981 (previously called AFS99) containing this mutation was crossed with a strain (DDY194) wild-type for axial budding. Haploid cells that contained the *mad2-1* 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 100× 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.

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 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 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.I. 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 *mad2-1* 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.

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 *MAT***a**/α diploids and *MAT***a** or *MAT*α 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: *MAT***a** or *MAT*α cells exhibited the normal axial pattern, but *MAT***a**/α cells showed a random budding pattern instead of a bipolar pattern (Fig. 1 *A*).

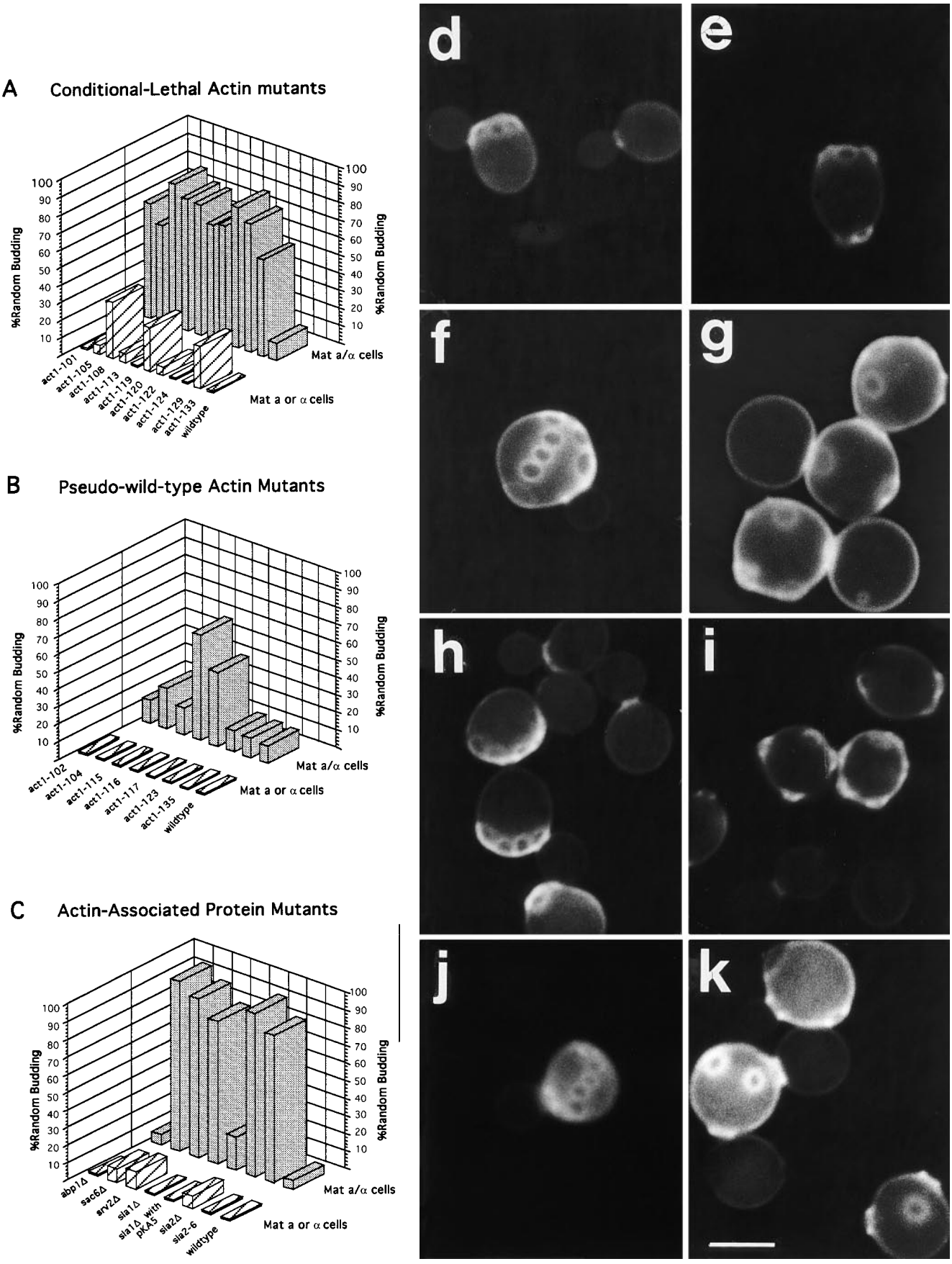
In addition to the bipolar defect, some of the actin mutants, such as *act1-108*, also exhibited a slight defect in the axial budding pattern in *MAT***a** or *MAT*α cells (Fig. 1 *A*). These usually were the less healthy mutants. We speculate that the poor growth and general pleiotropic nature of these 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 actin mutant *MAT***a**/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

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 ($n = 200$) in various haploid (*front rows*) and diploid (*back rows*) strains. The wild-type strains shown in *A* and *B* are DDY354 (*MAT***a**) and DDY440 (*MAT***a**/α). The wild-type strains shown in *C* are DDY186 (*MAT*α) and DDY288 (*MAT***a**/α). Only cells containing three or more bud scars were scored. (*d–k*) Representative wild-type and mutant cells stained with Calcofluor. *MAT***a** or *MAT*α cells are pictured in *d*, *f*, *h*, and *j* and *MAT***a**/α cells are pictured in *e*, *g*, *i*, and *k.* Wild-type cells are shown in *d* (DDY354) and *e* (DDY440). *act1-124* 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.


A
Conditional-Lethal Actin mutants
%Random Budding
100
90
80
70
60
50
40
30
20
10
act1-101
act1-105
act1-108
act1-113
act1-119
act1-120
act1-122
act1-124
act1-129
act1-133
wildtype
Mat a or α cells
Mat a/α cells
B
Pseudo-wild-type Actin Mutants
act1-102
act1-104
act1-115
act1-116
act1-117
act1-123
act1-135
wildtype
C
Actin-Associated Protein Mutants
abp1Δ
sac6Δ
srv2Δ
sla1Δ
sla1Δ with pKA5
sla2Δ
sla2-6
d
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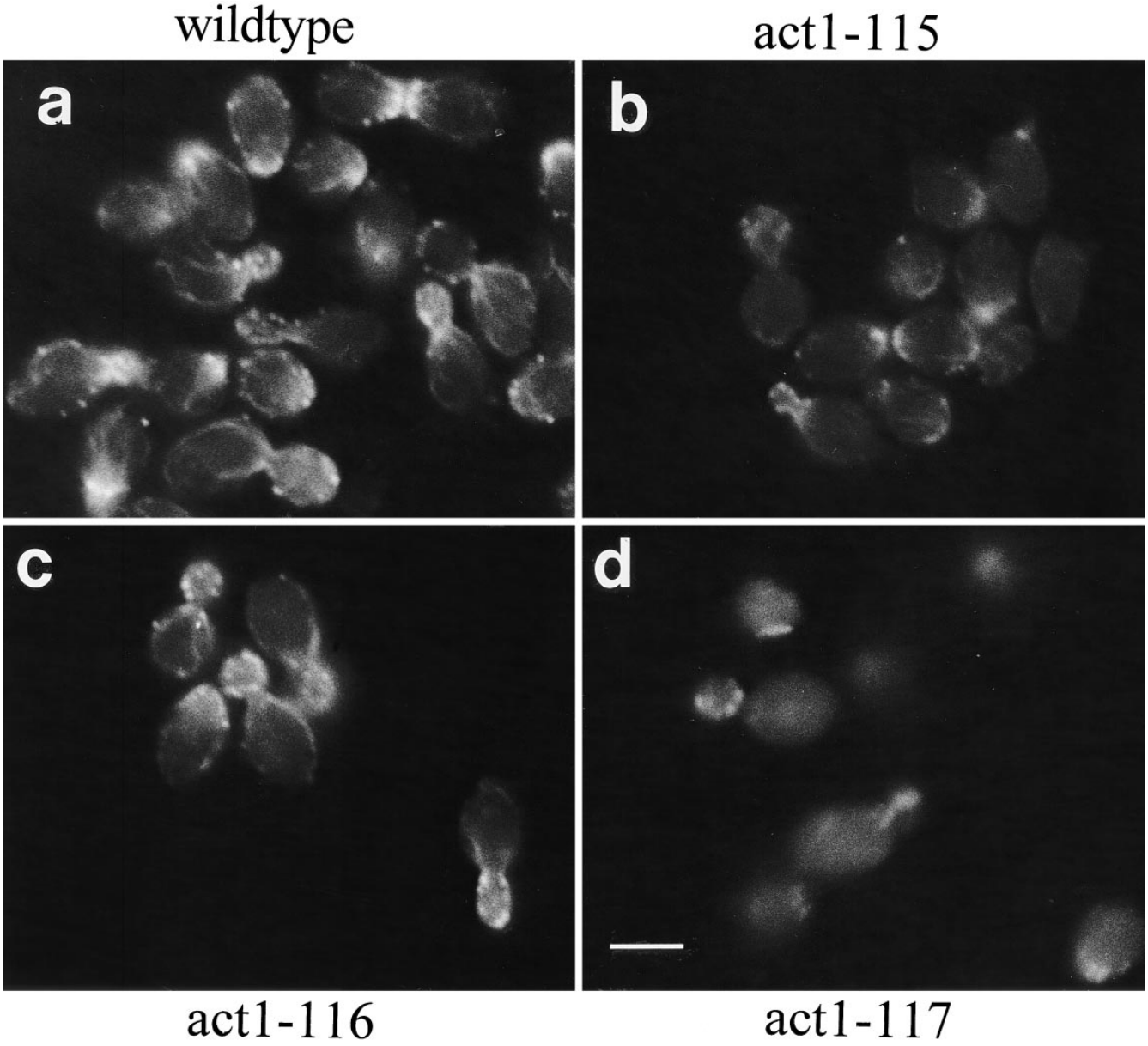

Figure 2. Pseudo–wild-type actin mutants stained with rhodamine-phalloidin to visualize the actin cytoskeleton. (*a*) DDY440, wild-type cells; (*b*) DDY976, *act1-115* mutant cells; (*c*) DDY977, *act1-116* mutant cells; and (*d*) DDY978, *act1-117* mutant cells. *act1-116* and *act1-117* mutants show a bipolar budding defect (with *act1-116* showing the more pronounced defect), while *act1-115* mutants do not show a bipolar budding defect. The same exposure and printing times were used for each panel. Bar, 5 μm.

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

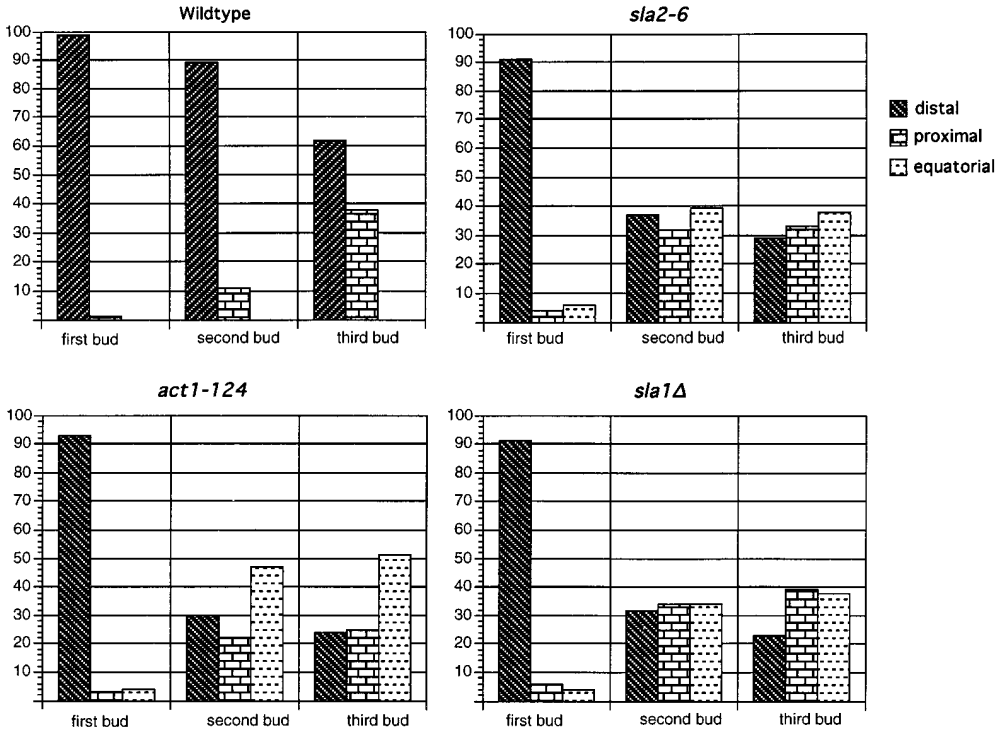

Figure 3. Placement of first three buds in wild-type, *act1-124*, *sla2-6*, and *sla1Δ* mutants. Bud scars were scored as either distal (opposite the birth scar), proximal (near the birth scar), or equatorial. The placement of the first bud was scored by counting only cells that displayed either no bud 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.

pattern as mutations in *ACT1*. Thus, we determined the budding patterns of mutants defective in *ABP1*, *SAC6*, *SRV2*, *SLA1*, and *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). 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. *abp1Δ* mutants have normal actin organization and show no readily observable phenotype (Drubin et al., 1990). Null mutations in *SAC6*, *SRV2*, and *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, *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.

abp1Δ mutants show normal budding patterns in *MAT***a**, *MAT*α, and *MAT***a**/α cells. However, *sac6Δ*, *srv2Δ*, *sla1Δ*, and *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 *SLA1* and *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 *sla2-6* is synthetic-lethal with both *abp1Δ* and *sac6Δ* mutants, but, unlike the *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 *sla2Δ* mutant (Fig. 1 *C*, *j*, and *k*). On the other hand, an *sla1* deletion mutant construct that lacks one SH3 domain (*sla1ΔSH3#3*, pKA5), when transformed into *sla1Δ* mutants, does not rescue the abnormal actin or-

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

		Overall budding pattern				Placement of first bud		
Strain	MAT**a**/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.

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 *MAT***a**/α 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 *MAT***a**/α *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-

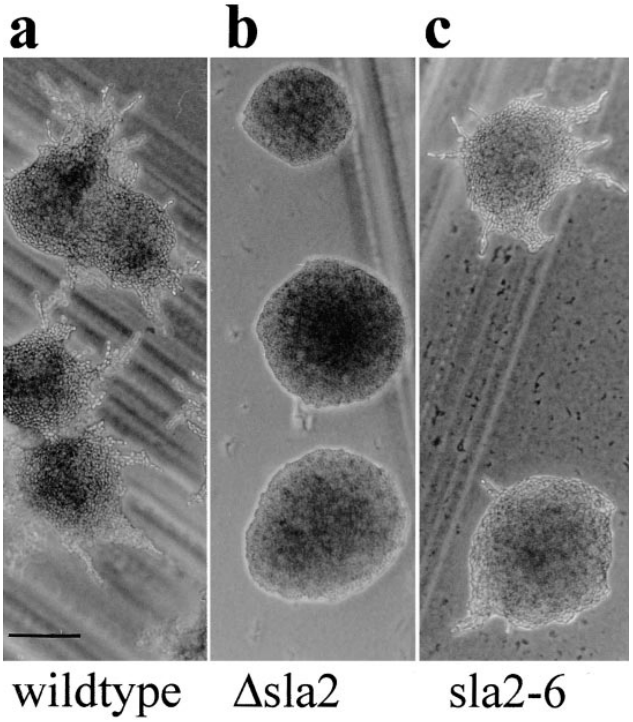

Figure 4. Pseudohyphal growth in *sla2* mutants. Cells were streaked onto SLADH plates (see Materials and Methods) to assay for their ability to undergo pseudohyphal growth. (*a*) DDY1060, wildtype; (*b*) DDY1061, *sla2Δ*; and (*c*) DDY1064, *sla2-6.* Cells were grown at 25°C for 2 d. Bar, 25 μm.

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 *bud4Δ sla2-6* double mutant haploid to further test this conclusion. (*sla2-6* was used because this mutation has little effect on cell growth but nevertheless causes a high degree of random budding in *MAT***a**/α cells.) 90% of *MAT***a** or *MAT*α *bud4Δ* mutant cells exhibit bipolar budding, and 99% of *MAT***a** or *MAT*α *sla2-6* mutant cells exhibit axial budding (Table III). 88% of *MAT***a** or α *bud4Δ sla2-6* double mutants were found to exhibit random budding, a number comparable to that of the *MAT***a**/α *sla2-6* mutant. *MAT***a**/α *bud4Δ 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 (*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 *MAT* **a**/α *mad2-1* cells were pulse-labeled with FITC-ConA and then shifted to medium containing 40 μg/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 are 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 *mad2-1* mutant cells formed three to four buds in the absence of microtubules. *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. *MAT***a** or *MAT*α *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 *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).

Table III. Budding Patterns of Δbud4 sla2-6 Mutants

Strain	Percent axial	Percent Bipolar	Percent Random
DDY964 *MAT*α *bud4Δ*		88	12
DDY1053 *MAT*α *sla2-6*	99		1
DDY1065 *MAT***a** *bud4Δ sla2-6*	2	10	88
DDY1070 *MAT***a**/α *bud4Δ*		96	4
DDY1054 *MAT***a**/α *sla2-6*		17	83
DDY1067 *MAT***a**/α *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.

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 microtu-

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

	No benomyl		40 μg/ml benomyl	
Yeast strain	Percent axial	Percent Bipolar	Percent axial	Percent bipolar
DDY1049 or DDY048 (*MAT***a** or *MAT*α)	99		96	
DDY986 (*MAT***a**/α)		91		85

To determine the budding pattern of *mad2-1* cells grown in the presence of benomyl, exponentially growing *mad2-1* haploid (DDY1048, 1049) or *mad2-1/mad2-1* diploid (DDY986) cells were labeled with FITC-ConA and then shifted to YPD containing 40 μg/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 FITConA. 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.

bules 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 placement of budding cues and did not arise because of nonspecific secondary defects. First, mutations in the 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 (*sla1ΔSH3#3*) 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 Ca^{2+}-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 *MAT***a**/α 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 *spa2*. (*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 *MAT***a** and *MAT*α cells (which show an axial budding pattern) nor *MAT***a**/α *rsr1* null mutants (which show a random budding pattern) can undergo PH growth (Gimeno et al., 1992). However, *MAT***a**/α *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 conjuction with the unipolar budding mutants, *bud8Δ* and *bud9Δ*. *MAT***a**/α *bud9Δ* mutants place buds only at the distal pole. *MAT***a**/α *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-

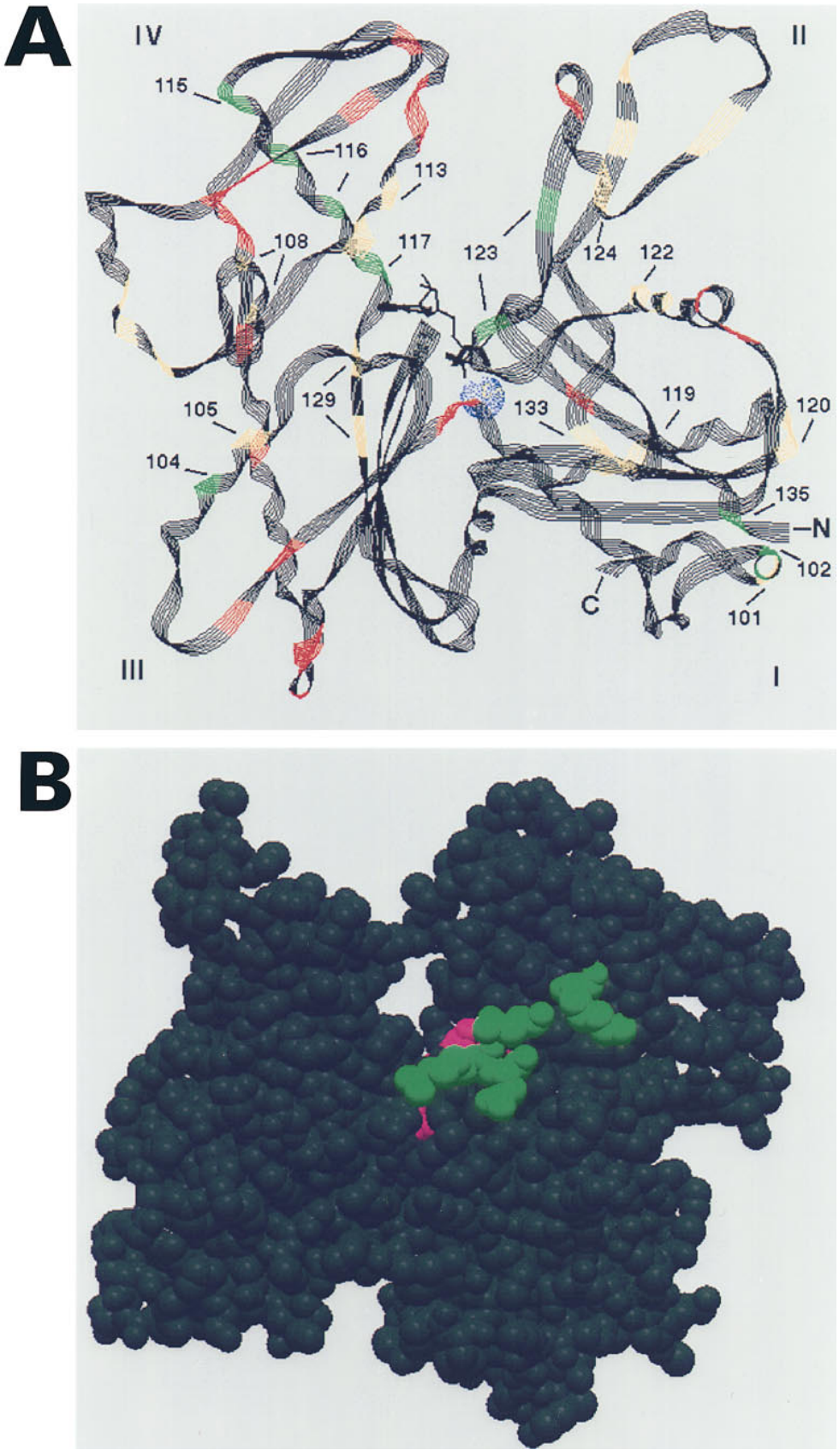

Figure 5. Location of mutations on the actin atomic model. (*A*) Ribbon diagram of the actin monomer. Red represents amino acids that when changed to alanine produce a lethal phenotype; yellow represents amino acids that when changed to alanine produce a conditional-lethal phenotype; and green represents amino acids that when changed to alanine produce a pseudo–wild-type phenotype. 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.

tants, adding further support to the conclusion that actin does not function in the placement of the distal pole cue in daughter cells. *MAT***a**/α *bud8Δ* mutants place buds only at the proximal pole. *MAT***a**/α *bud8Δ act1-119* and *bud8Δ sla2-6* double mutants exhibited an overall random pattern, similar to the *MAT***a**/α *bud9Δ act1-119* and *bud9Δ sla2-6* double mutants. However, *MAT***a**/α *bud8Δ act1-119* and *bud8Δ sla2-6* daughter cells exhibited a proximal pole bias (~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 (~40%) of *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, *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, *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, *Mutant*, *2*) and is also required for its maintenance. For all models, the observation that *bud8Δ act1-119* and *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.

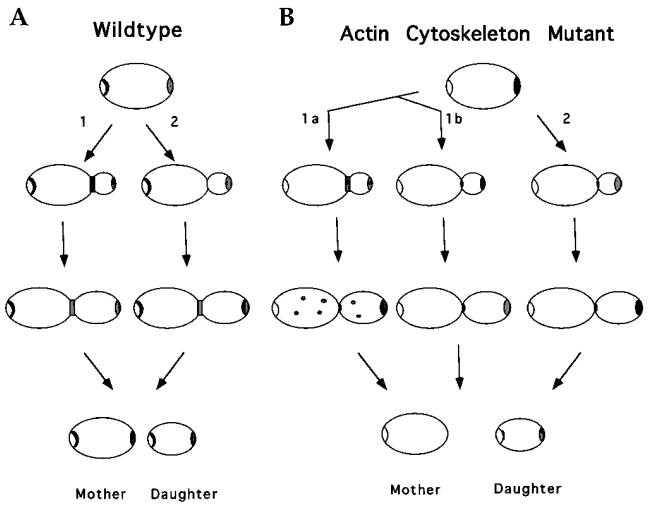

Figure 6. Models for the placement of the spatial cues for the bipolar budding pattern. The placement of bipolar budding cues (represented by the shaded areas), starting from a daughter cell. (Models 1 and 2 are adapted from Chant and Pringle [1995] and Zahner et al. [1996]). The left pole represents the proximal pole with the birth scar being represented by a thin curved line. (*A*) Wild-type diploid cells. For model 1, the spatial budding cue is concentrated at the presumptive bud site at the beginning of the cell cycle. As the bud emerges, the cue is partitioned between the growing tip and the mother–daughter neck. For model 2, the cue is first positioned at the bud tip by the cell surface growth apparatus as the bud emerges and grows. When the growth apparatus is reoriented toward the mother–daughter neck for septation late in the cell cycle, the cue is placed at the neck region and is partitioned between the mother and daughter at cytokinesis. These models do not attempt to explain why daughter cells have an extremely strong bias toward forming their first bud at the distal pole. (*B*) Based on the two models in *A* several possible explanations exist for the bipolar budding defect in diploid mother cells with an altered actin cytoskeleton. In all of the models, the bipolar budding cue is initially placed correctly at the distal pole, and to a lesser degree at the proximal pole, in daughter cells. For model 1a, the cue might become properly partitioned, but there is a defect in retention of the cue at the region where septation ocurred. For model 1b, the cue might not get properly partitioned as the bud emerges. For model 2, the actin cytoskeleton might function properly in cytokinesis, but might be defective in placement and/or maintenance of the cue at the septal region.

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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