Polarized Growth Controls Cell Shape and Bipolar Bud Site Selection in *Saccharomyces cerevisiae*

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We examined the relationship between polarized growth and division site selection, two fundamental processes important for proper development of eukaryotes. Diploid *Saccharomyces cerevisiae* cells exhibit an ellipsoidal shape and a specific division pattern (a bipolar budding pattern). We found that the polarity genes *SPA2*, *PEA2*, *BUD6*, and *BNI1* participate in a crucial step of bud morphogenesis, apical growth. Deleting these genes results in round cells and diminishes bud elongation in mutants that exhibit pronounced apical growth. Examination of distribution of the polarized secretion marker Sec4 demonstrates that *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants fail to concentrate Sec4 at the bud tip during apical growth and at the division site during repolarization just prior to cytokinesis. Moreover, cell surface expansion is not confined to the distal tip of the bud in these mutants. In addition, we found that the p21-activated kinase homologue Ste20 is also important for both apical growth and bipolar bud site selection. We further examined how the duration of polarized growth affects bipolar bud site selection by using mutations in cell cycle regulators that control the timing of growth phases. The *grr1Δ* mutation enhances apical growth by stabilizing G₁ cyclins and increases the distal-pole budding in diploids. Prolonging polarized growth phases by disrupting the G₂/M cyclin gene *CLB2* enhances the accuracy of bud site selection in wild-type, *spa2Δ*, and *ste20Δ* cells, whereas shortening the polarized growth phases by deleting *SWE1* decreases the fidelity of bipolar budding. This study reports the identification of components required for apical growth and demonstrates the critical role of polarized growth in bipolar bud site selection. We propose that apical growth and repolarization at the site of cytokinesis are crucial for establishing spatial cues used by diploid yeast cells to position division planes.

Cellular morphogenesis and division plane selection are two fundamental processes common to both unicellular and multicellular organisms. Morphological changes enable cells to establish unique shapes and specialized functions. Polarized cell division allows cells to assume higher-order organization, which is essential for the development of complex multicellular organs and organisms (reviewed in references 12, 15, and 71). Coordination of cell division with changes in cell shape is likely to be important for ensuring the proper segregation of cytoplasmic components. How cells undergo morphogenesis, determine division patterns, and coordinate the position of the division plane with cellular morphogenesis is not well understood.

The budding yeast *Saccharomyces cerevisiae* is an excellent model for studying cell shape and division plane selection. *S. cerevisiae* exists in different forms, each with a specific cell morphology and division pattern (12, 15, 47, 55, 61). During growth in rich media, both haploid and diploid yeast cells are ellipsoid and select budding sites according to their *MAT* locus and pedigree (10, 23, 33, 68). Haploid *MATa* and *MATα* cells use an axial budding pattern in which cells form new buds adjacent to the preceding site of cytokinesis. Diploid *MATa/MATα* cells exhibit a bipolar budding pattern: mother cells bud either distal or proximal to the birth site, whereas daughter cells bud almost exclusively at distal poles. It is thought that future bud sites are marked by bud site tags that serve as cues for the initiation of new buds (9, 10, 22, 69). When nitrogen sources are limited, diploid cells undergo pseudohyphal differ-

entiation: cells become extremely elongated and preferentially bud distal to their birth site (26, 60). A similar mode of growth has also been observed in certain haploid strains grown on solid media (59). These features are thought to help cells spread efficiently across a surface to gain access to nutrients (26, 47).

The morphology of yeast cells is determined during bud growth. Buds emerge at late G₁ of the yeast cell cycle. Early bud growth is restricted to the bud tip and is termed apical growth (42, 73). As the bud enlarges, growth enters a second step, the isotropic phase, during which growth is still restricted to the bud but deposited over the entire bud surface. After nuclear division, a repolarization phase begins, and new materials are directed toward the mother-bud neck to prepare for cytokinesis (septation). Understanding apical growth and its regulation and identifying the molecules that participate in this growth process will provide insight into how morphogenesis is controlled and coordinated with other cellular events.

A number of components important for bud growth have been identified and characterized (12, 15, 32, 55). The actin cytoskeleton is essential for bud growth and is thought to mediate directional transport of secretory vesicles (1, 15, 35, 49, 52, 57). Both cortical actin patches and components of the secretory apparatus localize to sites of growth (1, 21, 35).

The nonessential polarity proteins Spa2, Pea2, Bud6, and Bni1 also participate in yeast morphogenesis (2, 19, 25, 36, 68, 69, 74, 78). *spa2Δ* cells appear rounder than wild-type cells and have defects in cytokinesis, mating projection formation, and pseudohyphal growth (25, 48, 60, 68, 69). Similar phenotypes have also been reported for *pea2*, *bud6*, and *bni1* mutants (2, 19, 36, 48, 74, 78). At the onset of apical growth, Spa2, Pea2, Bud6, and Bni1 concentrate at the incipient bud site and remain at the bud tip during the apical growth phase (2, 19, 25,

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68, 69, 74). In large budded cells undergoing isotropic growth, they either become more dispersed or are not detectable. These abundant proteins have been proposed to function together as a complex (the polarisome) that regulates the actin cytoskeleton and/or concentrates cortical actin at the bud tip, thereby restricting growth to that site (24, 66).

Another protein that concentrates at the bud tip is Ste20 (40, 54). Ste20 is homologous to the mammalian p21-activated kinase (39, 64), and it functions upstream of a mitogen-activated protein kinase (MAPK) signaling pathway required for both mating and pseudohyphae formation (38, 44, 51, 59). Both of these morphogenetic processes also involve Spa2 function (25, 48, 60). Like Spa2, Ste20 is at the tips of both emerging and small buds and later disperses as buds enlarge (40, 54). The temporal and spatial localization patterns of Spa2, Pea2p, Bud6, Bni1, and Ste20 raise the possibility that they may function in apical growth. However, direct evidence of such a role for these proteins has not been investigated.

The timing of the apical growth phase is controlled by the cell cycle machinery (42). The onset of apical growth and actin polarization is induced by the G₁ cyclins Cln1 and Cln2, which activate the cyclin-dependent kinase Cdc28 (13, 50). Later, activation of Cdc28 by the G₂ cyclins Clb1 and Clb2 triggers the switch from apical to isotropic growth and the redistribution of the cortical actin patches over the entire bud surface (42, 58, 72). Finally, repolarization of growth materials and the actin cytoskeleton to the mother-bud neck prior to cytokinesis requires inactivation of Cdc28 through destruction of the G₂ cyclins. How the cell cycle machinery affects the localization of growth is unclear.

Many genes that function in cell morphogenesis in yeast are also required for bipolar bud site selection. Deletion of *SPA2*, *PEA2*, *BUD6*, or *BNI1* and some mutations that affect the actin cytoskeleton and secretory pathway cause a random-budding defect that specifically affects the bipolar budding program but not the axial pattern (7, 16, 20, 29, 67, 68, 74, 77, 78). Despite the large number of proteins that have been identified, it is still not clear how these gene products can simultaneously affect cell morphogenesis and bipolar bud site selection.

Here we demonstrate that *SPA2*, *PEA2*, *BUD6*, *BNI1*, and *STE20* are crucial for the apical growth phase of bud growth. Mutations in any of these genes lead to rounder cells (68, 78) (see below). These mutants also have bipolar bud site selection defects, suggesting that the altered budding pattern in these mutants is related to the apical growth defect. Indeed, we found that the fidelity of bipolar bud site selection strongly correlates with the length of the apical growth phase. Moreover, bud site selection defects resulting from inappropriate apical growth, as observed in *spa2* and *ste20* mutants, can be partially suppressed by extending the duration of apical growth. We propose that directional growth processes are crucial for establishing bipolar bud site selection tags at distal and proximal poles. This model explains why many mutants known to affect morphogenesis (such as cell cycle regulators, signaling proteins, components of the exocytic pathway, proteins involved in polarized growth, etc.) also affect bipolar bud site selection.

MATERIALS AND METHODS

Yeast strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1. All strains are congenic derivatives of S288C. Growth media and genetic manipulation were as described previously (28, 65).

The null alleles *bni1Δ::HIS3*, *bud3Δ::TRP1*, *clb2Δ::TRP1*, *pea2Δ::HIS3*, and *spa2Δ::HIS3* lack the entire protein-coding regions and were constructed using the strategy described by Baudin et al. (6). The null alleles *bud6Δ::HA*, *ste7Δ::HA*, *ste11Δ::HA*, and *ste20Δ::HA* were created by replacing the entire protein-coding sequence on the chromosome by a cassette encoding three copies

of the hemagglutinin epitope (62). For alleles *spa2(1-2, 116-1466)::HA* and *spa2(1-410, 531-1466)::HA*, a region of the *SPA2* gene on the chromosome was deleted and replaced with the hemagglutinin epitope by using the same method. The *spa2Δ::URA3* allele was created by replacing the *SacI-SphI* fragment of the *SPA2* coding sequence with the *URA3* gene. This construct removes residues 41 to 1205 of Spa2. The *ste20Δ::URA3* and *ste20Δ::TRP1* alleles were generated using the disruption plasmids pEL45 (38) and pDH104 (76), respectively. *swe1Δ::LEU2* and *grr1Δ::hisG* were as described previously (43, 46). All deletions were confirmed by PCR. The *cdc12-1* and *cdc34-2* allele have been described previously (27, 30).

Examination of cell morphology. Mid-log-phase cells were treated as indicated and fixed for 1 h with 3.7% formaldehyde. Fixed cells were then washed and resuspended in phosphate-buffered saline (PBS) solution. Cells were examined by phase-contrast and Nomarski microscopy. In some cases, the samples were stained with fluorescent brightener 28 (2 μg/ml; Calcofluor White; Sigma), which stains chitin in the cell wall, and examined by fluorescence microscopy.

To measure the length and the width of yeast cells, random fields of cells for each sample were recorded using a photometric Sensys charge-coupled device camera and cell diameters were measured (in pixels) by using Imagepoint Lab Spectrum software (Signal Analytics Corporation). All unbudded and budded mother cells and unseparated daughters larger than one-half the size of mother cells were measured. The length is the distance of a cell's long axis, determined relative to the position of its birth pole (illustration in Table 2). The width is defined as the maximum measurement perpendicular to the long axis. Indicated numbers of samples were selected for further analysis according to the order of entry (Table 2). For distribution graphs, the length/width ratios of these samples were sorted in an ascending order and plotted (Fig. 1B; see Fig. 5B).

FITC-ConA labeling. Log phase cultures (1.5 ml) were collected, washed once with a solution containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl, and sonicated to separate clumps. Fluorescein isothiocyanate (FITC)-concanavalin A (ConA) (Polysciences Inc., Warrington, Pa.), which binds tightly to glycoproteins of the cell wall, was added to the sonicated cell suspension to a final concentration of 100 μg/ml, and cells were incubated in the dark for 10 min at 25°C. Cells were then washed once and resuspended in 3 ml of yeast extract-peptone-adenine-dextrose (YPAD) medium to resume growth. After 45 min at 30°C, cells were fixed in 3.7% formaldehyde at 30°C, washed with PBS, and resuspended in PBS solution. Labeled cells were observed using fluorescence microscopy. The percentages of buds or new cells with apical and isotropic growth patterns were determined. Buds or new cells with weaker staining at their tips than at their bases were categorized as undergoing apical growth, while those with uniform staining were scored as undergoing isotropic growth. New cells were distinguished by the lack of FITC-ConA labeling at their birth scars.

Indirect immunofluorescence of Sec4. Mid-log-phase cells from wild-type and mutant cultures were prepared for indirect immunofluorescence microscopy as described previously (75). Cells were fixed in 3.7% formaldehyde at 30°C for 1 h, spheroplasted, and attached to polylysine-coated multiwell microslides. Cells were then treated with 0.5% sodium dodecyl sulfate in PBS for 5 min, washed, and blocked with 0.1% bovine serum albumin (BSA) in PBS (PBS-BSA) for 30 min at room temperature. Cells were then incubated with a 1:4 dilution of monoclonal antibody against Sec4 (a generous gift from P. Novick's laboratory) overnight at 4°C. After washes with PBS-BSA, cells were incubated with a 1:200 dilution of preabsorbed Cy3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) at room temperature for 1.5 h, washed, mounted, and visualized by fluorescence microscopy.

Budding pattern analysis. The cells used for budding pattern analysis were maintained in log phase for at least six consecutive generations. Mid-log-phase cells were then fixed in 3.7% formaldehyde at 30°C, washed with PBS, and resuspended in PBS. Calcofluor White was added to a final concentration of 2 μg/ml, and bud scars and birth scars were visualized by fluorescence microscopy (31). Birth scars are chitin-poor regions in the cell wall where cells separated from their mother. Bud scars are chitin-rich ring structures that mark the region where mother cells separated from their daughter cells. A bud site is defined as proximal when a bud or a bud scar resides in the third (portion) of the cell closest to the birth scar, as distal when it is in the third of the cell opposite to the birth scar and as medial when it is in the middle third of the cell (22, 78). Bud positions for first, second, and third bud sites were scored as described elsewhere (78). A total of 200 to 600 cells were scored for each division event of each sample.

To quantify the fidelity of bipolar bud site selection, only cells that had experienced at least three budding events were examined; cells with medial budding sites were scored as random budding. The percentage of random budding cells was then determined for each sample. For each sample, 100 cells were counted from at least four independent fields.

RESULTS

Spa2, Pea2, Bud6, and Bni1 are important for apical growth. Wild-type diploid cells are normally ellipsoidal. However, microscopic examination of *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* cells as well as of two other *spa2* mutants, *spa2(1-2, 116-1466)* and *spa2(1-410, 531-1466)*, which lack two con-

TABLE 1. Yeast strains used in this study

Strain(s)	Genotype	Source
Y2017, Y2018, Y2019	<i>MATa/MATα leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y555	<i>MATa/MATα spa2Δ::TRP1/spa2Δ::TRP1 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This laboratory
Y2020	<i>MATa/MATα spa2(1-2, 116-1466)::HA/spa2(1-2, 116-1466)::HA leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y2021	<i>MATa/MATα spa2(1-410, 531-1466)::HA/spa2(1-410, 531-1466)::HA leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y2022	<i>MATa/MATα pea2Δ::HIS3/pea2Δ::HIS3 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y2023	<i>MATa/MATα bud6Δ::HA/bud6Δ::HA leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y2024	<i>MATa/MATα bni1Δ::HIS3/bni1Δ::HIS3 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 TRP1/trp1-Δ1</i>	This study
Y2025	<i>MATa/MATα swe1Δ::LEU2/swe1Δ::LEU2 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y2026	<i>MATa/MATα clb2Δ::TRP1/clb2Δ::TRP1 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y2027	<i>MATa/MATα spa2Δ3::URA3/spa2Δ3::URA3 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y2028	<i>MATa/MATα clb2Δ::TRP1/clb2Δ::TRP1 spa2Δ3::URA3/spa2Δ3::URA3 leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y270	<i>MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This laboratory
Y2029	<i>MATa/MATα ste20Δ::URA3/ste20Δ::TRP1 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y2030	<i>MATa/MATα clb2Δ::TRP1/clb2Δ::TRP1 ste20Δ::URA3/ste20Δ::HA leu2Δ98/leu2Δ98 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1</i>	This study
Y2031	<i>MATa/MATα grr1Δ::hisG/grr1Δ::hisG ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200</i>	This study
Y2032	<i>MATa cdc34-2 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2033	<i>MATa cdc34-2 spa2Δ3::URA3 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2034	<i>MATa cdc34-2 pea2Δ::HIS3 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2035	<i>MATa cdc34-2 bud6Δ::HA leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2036	<i>MATa cdc34-2 bni1Δ::HIS3 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2037	<i>MATa cdc34-2 ste20Δ::URA3 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2038	<i>MATa cdc34-2 ste11Δ::HA leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2039	<i>MATa cdc34-2 ste7Δ::HA leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	This study
Y2040	<i>MATa cdc12-1 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	B. Santos
Y2041	<i>MATa cdc12-1 spa2Δ3::URA3 leu2Δ98 ura3-52 lys2-801 ade2-101 his3-Δ200</i>	B. Santos
Y2042	<i>MATa bud3Δ::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study
Y2043	<i>MATa bud3Δ::TRP1 ste20Δ::URA3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study
Y2044	<i>MATa bud3Δ::TRP1 ste11Δ::HA ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study
Y2045	<i>MATa bud3Δ::TRP1 ste7Δ::HA ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study
Y2046	<i>MATa bud3Δ::TRP1 spa2Δ::HIS3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study
Y2047	<i>MATa bud3Δ::TRP1 spa2Δ::HIS3 ste20Δ::URA3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200</i>	This study

served domains, SHD-I and -II (60), respectively, reveals that these cells are rounder than wild-type cells (Fig. 1A). To quantify this defect, we measured the length and the width of vegetatively grown wild-type and mutant cells relative to their birth sites (see Materials and Methods and illustration in Table 2). The ratio of length to width is expected to be 1 for round cells and >1 for both ellipsoidal and elongated cells. The length/width ratios, plotted by ascending value, for 100 randomly selected cells from three independent wild-type strains and several mutants are shown in Fig. 1B. Unlike wild-type cells, which have an average length/width ratio of 1.4, the average length/width ratios for the polarity mutants *spa2Δ*, *spa2(1-2, 116-1466)*, *spa2(1-410, 531-1466)*, *pea2Δ*, *bud6Δ*, and *bni1Δ* are closer to 1.0 (Table 2). Thus, all three *spa2* mutants and *pea2Δ*, *bud6Δ*, and *bni1Δ* cells are quantitatively rounder than wild-type cells. Since yeast cell elongation occurs primarily during the apical growth phase, these results suggest roles for Spa2, Pea2, Bud6, and Bni1 in apical growth.

To investigate further the importance of *SPA2*, *PEA2*, *BUD6*, and *BNI1* in apical growth, we used several yeast strains that form highly elongated buds due to pronounced apical growth. *CDC34* encodes a component of the ubiquitin-depen-

dent degradation machinery that targets the cyclin-dependent kinase inhibitor Sic1 for destruction (27, 63). Sic1 inhibits the activation of Clb-Cdc28p kinase (63); hence its stabilization results in failure to switch from apical to isotropic growth. After prolonged incubation at the restrictive temperature (37°C), temperature-sensitive *cdc34* mutant cells form multiple buds that are thin and elongated (Fig. 2A). *cdc34 spa2Δ* cells also form multiple buds at 37°C. However, the buds are never as pointed and elongated as those of *cdc34* single mutants; instead, they are much more rounded. Similar results are obtained for *cdc34 pea2Δ*, *cdc34 bud6Δ*, and *cdc34 bni1Δ* cells (Fig. 2A). *cdc34 bni1Δ* cells display the strongest defect, whereas *cdc34 bud6Δ* cells exhibit a less-severe defect in apical growth. Thus, bud elongation in *cdc34* mutants requires the functions of *SPA2*, *PEA2*, *BUD6*, and *BNI1*.

We also demonstrated a role for Spa2 in bud and cell elongation using two other mutant strains. Clb2 is the major cyclin that controls the switch from apical to isotropic growth and prevents actin repolarization to the mother-bud neck prior to cytokinesis (42). Deletion of *CLB2* yields elongated cells as a result of a delayed apical-to-isotropic switch. As reported previously (42, 58, 72), we found that *clb2Δ* cells are more elon-

TABLE 2. Length/width ratios of yeast strains

a = Length
b = Width

Strain	Length/width ratio (mean $\pm$ SD) ^a
<i>n</i> = 100	
Wild type	1.43 $\pm$ 0.15
Wild type	1.36 $\pm$ 0.15
Wild type	1.36 $\pm$ 0.13
<i>spa2Δ</i>	1.07 $\pm$ 0.10
<i>spa2(1-2, 116-1466)::HA</i>	1.07 $\pm$ 0.11
<i>spa2(1-410, 531-1466)::HA</i>	1.11 $\pm$ 0.10
<i>pea2Δ</i>	1.08 $\pm$ 0.11
<i>bud6Δ</i>	1.08 $\pm$ 0.11
<i>bni1Δ</i>	1.00 $\pm$ 0.11
<i>n</i> = 150	
Wild type	1.42 $\pm$ 0.15
<i>swe1Δ</i>	1.25 $\pm$ 0.11
<i>clb2Δ</i>	1.66 $\pm$ 0.29
<i>spa2Δ</i>	1.07 $\pm$ 0.09
<i>clb2Δ spa2Δ</i>	1.14 $\pm$ 0.14
<i>n</i> = 125	
Wild type	1.37 $\pm$ 0.13
<i>ste20Δ</i>	1.23 $\pm$ 0.10
<i>clb2Δ</i>	1.73 $\pm$ 0.32
<i>clb2Δ ste20Δ</i>	1.37 $\pm$ 0.20

^a The measurement of the length/width ratios was performed as described in Materials and Methods.

gated, with an average length/width ratio of about 1.7 (Table 2), than wild-type cells. In contrast, *spa2Δ clb2Δ* double mutants are not elongated and have an average length/width ratio of 1.1, slightly higher than that of the *spa2Δ* mutant (Fig. 2B and Table 2). Thus, *SPA2* is required for the elongated cell shape of the *clb2Δ* cells. At nonpermissive temperatures, *cdc12* mutants form elongated buds due to the activation of a septin-dependent checkpoint that prevents the switch from apical to isotropic growth (5). As shown in Fig. 2C, *SPA2* is required for the elongated bud phenotype of the *cdc12* mutant at 37°C; *cdc12 spa2Δ* cells form buds that are much less elongated. In summary, analysis of cell morphology and the genetic studies using *cdc34*, *cdc12*, and *clb2Δ* mutants strongly indicate that *SPA2*, *PEA2*, *BUD6*, and *BNI1* are important for apical growth.

***spa2Δ* mutants fail to confine cell wall expansion to a small region at the bud tip during apical growth.** We also analyzed apical growth and isotropic growth directly by using FITC-ConA labeling experiments (42, 73). Vegetatively growing cells were collected, labeled briefly over their entire surface with FITC-ConA, and then returned to growth in the absence of FITC-ConA. In wild-type cells, buds undergoing apical growth lack FITC-ConA staining at the bud tip but maintain strong staining at the base (Fig. 3A). This pattern indicates a tightly confined area of cell surface growth at the apical tip of the bud. Buds undergoing isotropic growth expand uniformly and have a uniformly faded staining on their surface. We found that the percentages of buds or cells with an apical growth pattern are very similar in wild-type, *spa2Δ*, *pea2Δ*, and *bud6Δ* cells (48, 45, 46, and 47%, respectively [Fig. 3B and data not shown]). However, the quality of apical growth in *spa2Δ* mutants is different

from that in wild-type cells. Approximately 60% of *spa2Δ* buds with apical growth patterns show a gradient of FITC-ConA staining that is stronger at the base and gradually diminishes at the tip; the staining at these bud tips is weak but apparent (Fig. 3A, arrowheads). Only about 30% of wild-type buds undergoing apical growth show this pattern. The ConA-staining patterns for *pea2Δ*, *bud6Δ*, and *bni1Δ* cells are similar to that of *spa2Δ* cells (data not shown). Interestingly, *bni1Δ* cells also appear to have less apical growth (28%) than *spa2Δ*, *pea2Δ*, and *bud6Δ* cells; *bni1Δ* mutants have most severe polarity defects among these polarity mutants. In conclusion, the staining pattern in *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants suggests that, in these cells, apical growth is not as tightly confined at bud tips as in wild-type cells.

Sec4 is more dispersed in *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants. If apical growth is defective in *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* cells, we might expect polarized secretion to be disturbed in these mutants. To address this possibility, the localization of a polarized marker, Sec4, which is involved in late secretory steps, was examined in *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants. In wild-type cells, Sec4 is tightly concentrated at bud tips during apical growth and at the mother-bud neck

FIG. 1. *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* polarity mutants are rounder than wild-type cells. (A) Cell shapes of wild-type, *spa2Δ*, *spa2(1-2, 116-1466)* (i.e., *spa2^{SHD-1Δ}*), *pea2Δ*, *bud6Δ*, and *bni1Δ* cells in mid-log phase. Abnormal cell wall protrusions in mutant cells are indicated with arrowheads. (B) The length/width ratios of 100 individual mid-log-phase cells from wild-type (WT) *spa2Δ*, *spa2(1-2, 116-1466)*, *spa2(1-410, 531-1466)* (i.e., *spa2^{SHD-1Δ}*), *pea2Δ*, *bud6Δ*, and *bni1Δ* cultures. The ratios for individual cells are plotted in ascending order.

FIG. 2. Spa2, Pea2, Bud6, and Bni1 participate in apical growth. (A) Spa2, Pea2, Bud6, and Bni1 are required for the elongated bud morphology of the *cdc34* mutant grown at restrictive temperature. (B) Spa2 is required for the elongated cell shape of *clb2Δ* mutant cells. (C) Spa2 is required for the elongated bud morphology of the *cdc12* mutant at 37°C. Mid-log-phase cultures were transferred from room temperature to 37°C for 6 h and fixed at 37°C. The morphologies of these cells were visualized by Nomarski microscopy (A and B) or fluorescence microscopy with Calcofluor White staining (C).

during cytokinesis (75) (Fig. 4). In *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants, despite a generally polarized localization pattern, Sec4 staining is more diffuse than in the wild-type cells. In small and medium budded cells, it is often in patches throughout the bud (Fig. 4, arrowheads). Likewise, in large budded cells undergoing cytokinesis, it is also less concentrated at the neck (Fig. 4, arrows). The Sec4 staining pattern is least concentrated in the *bni1Δ* mutant, which exhibits the most-severe defects in both morphogenesis and bipolar bud site selection. Taken together, these results suggest that Spa2, Pea2, Bud6, and Bni1 function to help concentrate polarized growth and secretion not only at bud tips but also at mother-bud necks.

Consistent with the role of Spa2, Pea2, Bud6, and Bni1 at the neck region, *spa2Δ*, *spa2(1-2, 116-1466)*, *pea2Δ*, *bud6Δ*, and *bni1Δ* cells also exhibit abnormalities at the site of septation or cytokinesis. The mother-bud necks of these mutants are less constricted (wider) than those in wild-type cells; similar observations for *spa2*, *bud6*, and *bni1* mutants have been described (2, 36, 69, 78). We also observed cell wall protrusions on the mother cells under Nomarski microscopy (Fig. 1A, arrow heads); when stained with Calcofluor white and visualized by fluorescence microscopy, these structures correspond to bud scars. Together, these observations suggest that Spa2, Pea2, Bud6, and Bni1 are important for septation.

Ste20 plays a role in apical growth. Ste11 and Ste7 were previously identified as Spa2-interacting proteins by the yeast two-hybrid system (66). Ste11 and Ste7 are the MAPK kinase and MAPK kinase, respectively, of the Ste MAPK pathway and are required for mating and pseudohypha formation

(18, 44, 59, 70). Both of these processes also involve Spa2 (25, 48, 60). We therefore investigated whether Ste11, Ste7, and their upstream activator, Ste20, affect bud elongation of the temperature-sensitive *cdc34* mutant. As shown in Fig. 5A, the *cdc34 ste11Δ* and *cdc34 ste7Δ* double mutants, like the *cdc34* single mutant, form multiple elongated buds after 6 h at 37°C. Thus, STE11 and STE7 are not required for apical growth. Under the same conditions, *cdc34 ste20Δ* double mutants also form multiple buds, but most of these buds are not as elongated as those of the *cdc34* mutant (Fig. 5A), although some cells do make elongated buds (see Discussion). Moreover, *ste20Δ/ste20Δ* diploid cells are less elongated, with an average length/width ratio of 1.23, compared to 1.37 for the isogenic wild-type cells ($n = 125$ cells) (Fig. 5B and Table 2). The difference is even more pronounced in a *clb2Δ* background, in which the length/width ratios are 1.37 for *ste20Δ/ste20Δ clb2Δ/clb2Δ* cells, compared to 1.73 for *clb2Δ/clb2Δ* cells (Fig. 5B). Thus, like Spa2, Ste20 also plays a role in apical growth.

We also directly examined apical growth in the *ste20Δ* mutant using FITC-ConA labeling (Fig. 3). Whereas 48% of wild-

FIG. 3. Cell wall growth in wild type and mutants analyzed by FITC-ConA labeling experiments. (A) Wild-type, *spa2Δ*, and *ste20Δ* cells were labeled on their cell surface with FITC-ConA and then returned to the growth medium lacking ConA. For each strain, two cells exhibiting apical growth and one undergoing isotropic growth are shown. Arrows indicate the strong staining at the base of wild-type buds. Arrowheads indicate the weak but apparent staining at the bud tip of *spa2Δ* mutants. (B) Percentages of vegetative cells with the apical growth pattern in wild-type (WT) and mutant cells. One hundred cells were examined in each scoring, and each strain was scored at least three times. Error bars, standard errors.

FIG. 4. Indirect immunofluorescence staining of Sec4 in wild-type and polarity mutants. Green represents the anti-Sec4 staining pattern, and blue corresponds to nuclei stained by DAPI (4',6'-diamidino-2-phenylindole). Arrowheads and arrows indicate Sec4 staining at buds or bud tips and mother-bud necks, respectively.

type buds undergo apical growth, only 36% of the buds exhibit apical growth in the *ste20Δ* mutant (Fig. 3B). Thus, *ste20Δ* mutants may have a shorter apical growth phase compared with wild-type cells. Growth at the bud tip in *ste20Δ* mutants appears to be concentrated, similar to that in wild-type cells (Fig. 3A), but unlike *spa2Δ*, *bud6Δ*, and *bni1Δ* mutants.

***ste20Δ* is defective in choosing distal budding sites.** Since Ste20 is also involved in the control of apical growth, we examined whether Ste20 is required for bipolar bud site selection as is the case for Spa2, Pea2, Bud6, and Bni1. Haploid *bud3Δ* strains, which normally bud in a bipolar pattern, were used for this analysis; *bud3Δ* strains have been used successfully by Zahner et al. (78) to identify genes involved in bipolar bud site selection. The *ste11Δ* and *ste7Δ* mutations had little, if any, effect on the bipolar pattern (Fig. 6A). In contrast, the *ste20Δ* mutant displayed a unipolar budding pattern, with bud scars clustered adjacent to the birth scar, at the proximal pole (Fig. 6A). In these cells, individual bud sites can be observed adjacent to, overlapping, or within the birth scar and in the vicinity of other bud scars; however, these bud scars are not always immediately adjacent to the bud site of the previous cell cycle. This budding pattern is typical of the bipolar pattern at the proximal pole and is distinct from the axial budding pattern used by wild-type haploid cells. In the axial pattern, new buds form immediately adjacent to the preceding division site, producing a continuous chain of chitin rings (10, 22).

To ensure that the bud site selection defect of *ste20Δ* cells is independent of *bud3Δ*, we analyzed diploid mutants. *ste20Δ/ste20Δ* diploid cells are strongly biased toward choosing the proximal budding site and only rarely bud at the distal site (8% of the first buds, 0.7% of the second buds, and 0% of the third buds of *ste20Δ/ste20Δ* cells form at distal sites, compared to 97% of the first, 83% of the second, and 41% of the third buds

for wild-type cells [Fig. 7]). The budding pattern of heterozygous *ste20Δ/STE20* cells is similar to that of isogenic wild-type cells but is slightly biased towards the selection of proximal budding sites (data not shown). Thus, *ste11Δ* and *ste7Δ* mutants, which do not have a detectable defect in apical growth, display normal budding patterns, whereas *ste20Δ* mutants, which have defects in apical growth, are defective in distal bud site selection.

The phenotype of *ste20Δ* cells is very similar to that described for *bud8* mutants (78), suggesting that these two mutants function in the same pathway. Consistent with this hypothesis, *ste20Δ bud8Δ* double mutants display an identical budding pattern to those of single mutants, suggesting that they function in the same pathway for bud site selection (data not shown; see Discussion).

Spa2 and Ste20 perform different functions in bipolar bud site selection. *ste20Δ* and *spa2Δ* have different phenotypes in apical growth and bud site selection. *ste20Δ* cells are defective only in distal budding, whereas *spa2Δ* mutants fail to bud precisely at both poles; we found that in *spa2Δ* mutants, even though the budding events may be categorized as either proximal or distal, they often are near, but not at, the pole of the cell (wild-type cells usually use the poles). This is consistent with a previous observation that the first two bud sites in *spa2* and *bud6* cells were generally not directly adjacent to each other (78). To further decipher the relationship between SPA2 and STE20 in the process of bipolar bud site selection, we analyzed the budding pattern of a *spa2Δ ste20Δ* double mutant in the *bud3Δ* background and compared it to isogenic *bud3Δ spa2Δ* and *bud3Δ ste20Δ* mutants. *spa2Δ ste20Δ* double mutants exhibit a combination of the bud site selection phenotypes observed in *spa2Δ* and *ste20Δ* single mutants. Similar to *ste20Δ* mutants, the double mutants use distal bud sites very

FIG. 5. Ste20 is important for apical growth. (A) Morphology of *cdc34*, *cdc34 ste20Δ*, *cdc34 ste11Δ*, and *cdc34 ste7Δ* cells after incubation at 37°C for 6 h. The formation of elongated buds in the *cdc34* mutant is hindered by *ste20Δ* but not by *ste11Δ* or *ste7Δ* mutations. Cells that failed to form elongated buds in the *cdc34 ste20Δ* culture are shown. (B) Length/width ratios of 125 individual cells from mid-log-phase wild-type (WT) *ste20Δ*, and *clb2Δ* cultures plotted in ascending order. *ste20Δ* cells are rounder than wild-type cells, and *clb2Δ ste20Δ* cells are not as elongated as *clb2Δ* cells.

infrequently (Fig. 6B). This is in contrast to *spa2Δ* mutants, which prefer distal sites for the first two budding events (68, 78). Thus, *spa2Δ* mutants are still capable of distinguishing the proximal pole from the distal pole, and *STE20* is required for both wild-type and *spa2Δ* cells to bud at distal sites. In the *spa2Δ ste20Δ* double mutant, most budding events occur within the proximal half of the cells, similar to the budding pattern of the *ste20Δ* mutant. However, the bud scars of the *ste20Δ* mutant are tightly clustered near the birth scar while the bud scars of the *spa2Δ ste20Δ* double mutant are more loosely distributed within the proximal hemisphere (Fig. 6B), indicating a role of Spa2 in confining budding within a small area at proximal poles. These data also indicate that Spa2 and Ste20 perform different functions in bipolar bud site selection.

Genes controlling cell cycle progression influence bipolar bud site selection. *ste20Δ* cells and polarity mutants *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* display defects in both apical growth and bipolar bud site selection. The strong correlation between these two processes prompted us to hypothesize that the apical growth phase is crucial for bipolar bud site selection. This idea was tested further using several yeast mutants. We first investigated whether extending bud tip growth can promote budding at distal poles. *grr1Δ* cells are elongated because apical growth is enhanced in these cells due to accumulation of high levels of G₁ cyclins (3, 4). Diploid *grr1Δ/grr1Δ* cells bud in a bipolar pattern. However, these cells display a stronger-than-

normal bias towards using distal bud sites; this bias is especially apparent in the later budding events (Fig. 7A). For example, in a vegetatively growing *grr1Δ* culture, 92% of the third buds form at distal poles, compared to only 51% of the third buds for wild-type cells (Fig. 7A). In addition, we also observed a decreased frequency of nonpolar (i.e., medial) budding in *grr1Δ* mutants for the first three budding events (Fig. 7A). Thus, *grr1Δ* mutants, which have increased apical growth at

FIG. 6. Evaluation of bud position for the first three budding events in wild-type (WT) and mutant cells (right panels). All strains used for this analysis are derivatives of *MATa bud3Δ* haploids. Bud scar staining is presented for each sample (left panels). Error bars, standard errors. (A) *ste20Δ* mutants bud almost exclusively at the proximal pole. (B) Ste20 is required for *spa2Δ* cells to bud at the distal pole; Spa2 is required for *ste20Δ* to bud precisely at the proximal pole.

FIG. 7. (A) Deletion of *GRR1* enhances distal budding. (B) Deletion of *CLB2* partially restores distal bud site selection of *ste20* mutants. Bud positions were scored for the first three budding events in diploid wild-type (*WT*) *grr1Δ*/*grr1Δ*, *ste20Δ/ste20Δ*, *clb2Δ/clb2Δ*, and *ste20Δ/ste20Δ clb2Δ/clb2Δ* cells. Error bars, standard errors.

bud tips, preferentially bud at distal poles and display less random budding.

We next analyzed several strains that differ in their timing of the apical-to-isotropic switch: the fidelity of bipolar bud site selection was examined for wild-type, *clb2Δ*, and *swe1Δ* vegetative cells with three or more bud scars. Clb2-Cdc28 triggers the switch from apical growth to isotropic growth and prevents entry into cytokinesis (42, 72). Deletion of *CLB2* results in an extended apical growth phase (42) and a more elongated cell shape (42, 58, 72). *SWE1* is homologous to the *Schizosaccharomyces pombe* *wee1* gene and encodes a kinase that negatively regulates the activity of the Clb1, 2-Cdc28 kinase (8). Using FITC-ConA labeling experiments, we found that *swe1Δ* cells exhibit less apical growth (Fig. 3B). In addition, the shape of *swe1Δ* cells is less elongated, with a length/width ratio of 1.25, than that of wild-type cells, with a ratio of 1.42 (Table 2). Decreased apical growth and shorter cell length of the *swe1Δ* mutant indicate that this mutant switches to isotropic growth earlier.

In our analysis, 6% of wild-type cells displayed a random

budding pattern (for definition, see Materials and Methods). In contrast, only 1% of *clb2Δ* mutant cells, which exhibit more apical growth, budded randomly, whereas *swe1Δ* mutants, with reduced apical growth, showed a random bud scar distribution in 18% of the cells (Fig. 8). Inspection of individual cells in the *swe1Δ* and wild-type populations reveals that most of the random-budding cells are rounder than their bipolar counterparts in the same culture. Together, these observations demonstrate that cells with enhanced apical growth use bipolar bud sites with higher fidelity, whereas cells with less apical growth display a reduced fidelity of bipolar bud site selection.

The bud site selection defect of *spa2Δ* and *ste20Δ* cells can be suppressed by deletion of *CLB2*. We reasoned that if insufficient or defective apical growth is responsible for the random budding phenotype of the *spa2Δ* mutant, this phenotype might be corrected by extending the period of apical growth in *spa2Δ* cells. To test this idea, we examined and compared the budding patterns of diploid *spa2Δ* mutants and *spa2Δ clb2Δ* double mutants, which exhibit more apical growth (70%) than *spa2Δ* single mutants (45%), as judged from the FITC-ConA labeling

FIG. 8. The fidelity of bipolar bud site selection is affected by the timing of the switch from apical to isotropic growth. (A) Bud scar staining of wild-type (*WT*) *swe1Δ*, *spa2Δ*, and *clb2Δ spa2Δ* cells. Although most of the *swe1Δ* cells exhibit a bipolar budding pattern, random-budding cells from the *swe1Δ* culture are shown here. Defects in bipolar bud site selection of the *spa2Δ* mutant are partially suppressed by *clb2Δ*. (B) Percentage of random-budding cells in the mid-log-phase wild-type (*WT*) *swe1Δ*, *clb2Δ*, *spa2Δ*, and *clb2Δ spa2Δ* cultures. Error bars, standard errors.

experiment (Fig. 3B). Approximately 84% of diploid *spa2Δ* cells that bud three or more times exhibit random budding, whereas this percentage decreases significantly to approximately 28% in the *spa2Δ clb2Δ* double mutant (Table 2). However, in some *spa2Δ clb2Δ* double mutant cells that display bipolar budding, the bud scars are not tightly concentrated at the poles as in wild-type cells (Fig. 8A), which presumably reflects the role of Spa2 in concentrating polarized growth. Nevertheless, deletion of *CLB2*, which extends the duration of polarized growth, can partially suppress the bud site selection defect of *spa2Δ* cells.

We also investigated whether deletion of *CLB2* can suppress the inability of *ste20* cells to choose distal sites, which we speculate is due, in part, to insufficient apical growth in these cells. Deletion of *CLB2* extended the apical growth phase of *ste20* mutants (36% for *ste20Δ/ste20Δ* and 51% for *ste20Δ/ste20Δ clb2Δ/clb2Δ* double mutants [Fig. 3B]). *ste20Δ/ste20Δ clb2Δ/clb2Δ* double mutants display a bipolar budding pattern and an increased usage of distal budding sites (27.7% of the first buds, 20.5% of the second buds, and 11.7% of the third buds are placed distally [Fig. 7B]) compared to *ste20Δ/ste20Δ* cells (7.7% of the first buds, 0.7% of the second buds, and 0% of the third buds are placed distally). We do not expect restoration of distal budding in *ste20Δ/ste20Δ clb2Δ/clb2Δ* double mutants to a wild-type level since deletion of *CLB2* is expected to initiate repolarization earlier and is likely to cause a longer repolarization phase, which we think might also promote budding at the proximal pole (see Discussion). Thus, although proximal bud sites of *ste20Δ/ste20Δ clb2Δ/clb2Δ* double mutants are still more preferred than distal sites compared to wild-type cells, increasing apical growth can partially restore distal site usage in *ste20Δ* mutants.

DISCUSSION

In this study, we demonstrated that *SPA2*, *PEA2*, *BUD6*, *BNI1*, and *STE20* are required for the proper execution of apical growth and that *STE20*, like *SPA2*, *PEA2*, *BUD6*, and *BNI1*, is required for bipolar bud site selection. The intimate relationship between apical growth and bipolar bud site selection was further demonstrated by analyzing cell cycle mutants that exhibit altered durations of polarized growth phases. We propose that apical growth at bud tips is important for establishing the bud site tags at distal poles, and that repolarization of growth materials at mother-bud necks prior to cytokinesis is a crucial process for marking proximal poles (Fig. 9A). This model is similar to but more detailed than one of the possibilities suggested by Chant and Pringle (10). Since apical growth is also an important determinant of cell shape, this model suggests a means by which the establishment of a cell division plane can be coupled to cell shape, as is evident during pseudohyphal differentiation in budding yeast.

The role of Spa2, Pea2, Bud6, and Bni1 in polarized growth and bipolar bud site selection. We demonstrated that Spa2, Pea2, Bud6, and Bni1 are all required for elongation of cells and buds in wild-type and *cdc34* strains and that Spa2 is also required for cell and bud elongation in *clb2Δ* and *cdc12* cells. Thus, Spa2, Pea2, Bud6, and Bni1 control cell shape by participating in apical growth. Since the length of the apical growth phase is similar in wild type and *spa2Δ* cells (Fig. 3B), we presume that the quality of apical growth is affected in the mutants (see below).

Spa2, Pea2, Bud6, and Bni1 may serve to confine growth to a restricted region rather than to establish polarized growth directly. Unlike mutations in polarity establishment genes, such as *cdc24* and *cdc42* (15, 55), *spa2Δ*, *pea2Δ*, *bud6Δ*, and

bni1Δ cells are still able to undergo polarized growth to form buds or mating projections. These mutants also exhibit polarized distribution of the actin cytoskeleton and a secretion marker, Sec4. However, unlike wild-type cells, these mutants form buds that are quite round and fail to concentrate Sec4 at sites of polarized growth. Furthermore, the mating-projection tip of the *spa2Δ* mutant is not pointed and contains actin patches that are less concentrated (25) (data not shown). Given that Spa2, Pea2, Bud6, and Bni1 are able to interact with one another and colocalize at the tips of buds and mating projections (2, 19, 24, 25, 66, 68, 74), we suggest that these polarisome proteins confine growth by forming a multiprotein complex that helps concentrate the actin cytoskeleton and/or exocytic vesicles at growth sites.

Prior to cytokinesis, a similar complex containing Spa2, Pea2, Bud6, and Bni1 may form at the mother-bud neck to help concentrate growth materials to this region. This idea is consistent with the following observations. First, Spa2, Pea2, Bud6, and Bni1 localize to the mother-bud neck just before and during septation (2, 34, 68, 69, 74). Second, Sec4 is less concentrated in this region in *spa2Δ*, *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants (Fig. 4). Third, we have observed structural abnormalities at neck regions and in bud scars in these mutants (Fig. 1), consistent with previous observations that *spa2Δ*, *bud6Δ*, and *bni1Δ* cells have cytokinesis defects (2, 36, 69, 78). It is thus likely that Spa2, Pea2, Bud6, and Bni1 concentrate the actin cytoskeleton and secretory vesicles at growth sites during both apical growth and septation.

How might apical growth and septation defects in the *spa2Δ* mutant account for its random budding pattern? Two observations indicate that the two poles are probably still marked in *spa2* mutants despite the random budding phenotype. First, the first budding event in *spa2* mutants is generally positioned at the distal pole, suggesting that a functional bud site selection signal exists there. Second, in the *ste20Δ* background, which buds proximally, deletion of *SPA2* does not simply lead to random budding, as would be expected for mutants completely lacking bud site tags. Instead, *spa2Δ ste20Δ* double mutants bud within the proximal hemisphere, although their bud scars are not tightly clustered (Fig. 6B); this indicates that the proximal poles are marked in these cells. Thus, in *spa2Δ* mutants, the bud site tags are probably still directed to both poles of the daughter cell. However, as discussed earlier, *spa2Δ* mutants fail to concentrate growth at both poles, and hence bipolar tags are deposited in a less-compacted fashion (Fig. 9B). As a result, diploid *spa2Δ* cells fail to choose bud sites with precision and therefore display a "random-like" budding pattern. The random budding pattern observed in diploid *pea2Δ*, *bud6Δ*, and *bni1Δ* mutants may be due to the same reason. A large number of other mutants affecting the actin cytoskeleton and secretory pathway disrupt bipolar bud site selection similar to *spa2Δ* (7, 16, 20, 29, 67, 77). We speculate that these mutants also affect apical growth as well as repolarization to the mother-bud neck and that defects in these processes lead to their diploid-specific random budding phenotypes.

Role of Ste20 in apical growth and bipolar bud site selection. We also demonstrated that Ste20 is involved in both apical growth and bipolar bud site selection. Disruption of *STE20* results in cell elongation defects in wild-type, *cdc34*, and *clb2Δ* strains (Fig. 5 and Table 2) and shortens the apical growth phase (Fig. 3B). Ste20 is also required for proper bipolar bud site selection: diploid *ste20Δ* mutants rarely bud at distal poles (Fig. 7B). We suggest that the distal poles of *ste20Δ* mutants are not properly marked due, at least in part, to insufficient apical growth (Fig. 9B). In addition, the Ste20 kinase may affect bud site selection by mechanisms indepen-

FIG. 9. (A) A model to explain how components involved in cell morphogenesis (shaded) contribute to the establishment of bipolar bud site tags in yeast. (B) Budding patterns of wild-type, *spa2Δ*, *ste20Δ*, *grr1Δ*, *clb2Δ*, and *swe1Δ* cells interpreted by the model proposed above. Apical growth and repolarization are indicated by arrows. The relative length of each growth period corresponds to the length of the arrow. Growth direction is represented by the direction of the arrowhead. Parallel arrows indicate confined growth patterns, while dispersed arrows indicate diffuse growth patterns. The distribution of bipolar bud site tags is indicated by gray-shaded areas. See Discussion for more details.

dent of its role in apical growth, for example, by directly phosphorylating the distal tag (perhaps Bud8; see below).

Ste20 and Spa2 contribute differently in the processes of apical growth, cytokinesis, and bipolar bud site selection. Ste20 does not seem to be required for restricting growth to a small region as does Spa2; the shape of buds in *cdc34 ste20Δ* double mutants is more pointed, unlike that of the rounder *cdc34 spa2Δ* buds, and *ste20Δ* mutants can still confine cell wall expansion to a small region during apical growth (Fig. 3A). Furthermore, the machinery that facilitates apical growth may still be functional, because some of the *cdc34 ste20Δ* buds are able to elongate. Finally, since *ste20Δ* mutants exhibit less apical growth (Fig. 3B), Ste20 may be required for timely activation or for maintenance of the activity of the apical growth machinery rather than being a component of the machinery itself. Ste20 has been shown to localize to bud tips like

Spa2, but unlike Spa2, it has not been found at sites of cytokinesis (40, 54). In addition, we do not observe septation defects in *ste20Δ* cells. Thus, Ste20 is probably not critical for septation and therefore, as predicted by our model, does not affect budding at the proximal pole (Fig. 9B).

The role of Ste20 in apical growth and bipolar bud site selection is unique among its associated signaling components, including its downstream kinases, Ste11 (70) and Ste7 (18), and another p21-activated kinase, Cla4 (14). We have found that Ste11 and Ste7 are not required for apical growth and these two kinases and their scaffolding protein Ste5 (11, 56) are not important for bipolar bud site selection (Fig. 6; data not shown). Consistent with our finding, it was reported that Ste20, but not Cla4 or Ste7, is required for cell elongation and polarization of the actin cytoskeleton in the *cdc28-4* mutant arrested at 37°C (17). These observations suggest that either

Ste20 has additional targets that control these functions independently of Ste11 and Ste7 or that a redundant pathway substitutes for Ste11 and Ste7 functions. We also found that deletion of *CLA4* does not result in the same morphological abnormality and budding pattern alteration in diploid cells as the *ste20Δ* mutation (data not shown). Hence, Ste20 and Cla4 have nonredundant vegetative functions.

How might Ste20 exert its vegetative function in apical growth? During apical growth, Ste20 may transmit signals from the polarity establishment protein Cdc42 (reviewed in references 12 and 15), a Rho-type GTPase that interacts with Ste20 (79), to components that execute or maintain apical growth. Proper localization of Ste20 in both vegetative and mating cells requires its ability to bind Cdc42 (40, 54). Thus, the functions of Ste20 during apical growth may be controlled by Cdc42, similar to that observed during the mating response and filamentous differentiation. Given the localization of the Ste20 kinase to bud tips, it may phosphorylate and activate key components at bud tips to initiate and/or maintain apical growth that leads to proper establishment of distal bud site tags.

The identical budding pattern of diploid *ste20Δ*, *bud8Δ*, and *ste20Δ bud8Δ* double mutants indicates that Ste20 and Bud8 function in the same pathway to promote budding at the distal pole. Bud8 is thought to be the distal pole marker or involved in proper positioning of the marker (78). Thus, Ste20 might play a role in phosphorylating the potential distal tag in addition to its role in apical growth. Alternatively, Bud8 may function in apical growth like Ste20. Perhaps the Ste20 kinase phosphorylates and regulates Bud8 function, or perhaps Bud8 regulates the kinase activity of Ste20.

Regulation of cell morphogenesis and budding pattern by the cell cycle machinery. We found that the cell cycle machinery, which regulates bud morphogenesis (41), also influences bipolar bud site selection. In diploid *grr1Δ* mutants, which stabilize G₁ cyclins (3), apical growth is enhanced and buds are preferentially formed at distal poles (Fig. 7A). Deletion of *SWE1* shortens the apical growth phase and results in a rounder cell shape, and these cells also show a decreased fidelity in bipolar bud site selection (Fig. 8B). In contrast, deletion of *CLB2*, which results in a longer apical growth phase, leads to the formation of elongated cells (42, 58, 72) and improves the fidelity of bipolar bud site selection (Fig. 8B). The analyses of *clb2Δ*, *swe1Δ*, and *grr1Δ* cells further indicate a strong link between the control of cell morphogenesis and bipolar bud site selection.

Quantification of individual budding events shows that the *clb2Δ* mutant does not simply increase its distal site usage as does the diploid *grr1Δ/grr1Δ* mutant (Fig. 7); rather, it reduces the number of randomly budding cells (Fig. 8B). Given that Clb2 also has a negative role in repolarization of actin to septation sites after nuclear division (42), this situation can be explained by our model: the extended duration of both the apical growth phase and the repolarization phase would allow both distal and proximal tags to be reinforced in *clb2Δ* cells (Fig. 9B). The fact that deletion of *CLB2* can partially suppress the bud site selection defect of *spa2Δ* and restore distal bud site usage of *ste20Δ* mutants further supports this idea. The enhancement of bud site tags in both poles may be part of the reason why deleting *CLB2* in the *ste20Δ* mutant does not restore distal budding to a wild-type level. In contrast to *clb2Δ* cells, *swe1Δ* cells may enter the repolarization phase later and therefore have a shorter duration for this process and weaker proximal tags. Together with the shorter apical growth phase, *swe1Δ* cells probably have weaker bipolar bud site tags at both poles and hence decreased fidelity of bipolar bud site selection (Fig. 9B). Therefore, the length of apical growth and repolar-

ization affects not only cell shape but also the fidelity of bipolar budding.

Role of apical growth in morphogenesis and bud site selection in filamentous differentiated cells. Cells undergoing filamentous differentiation, such as pseudohyphal and haploid invasive growth, alter both their shape and budding pattern. Filamentous growing cells are extremely elongated and bud more frequently at distal poles (26, 59, 60). How are these two features simultaneously achieved? A possible mechanism is by prolonging or enhancing apical growth, which would facilitate bud elongation and strengthen distal bud site tags. Consistent with this idea, components involved in apical growth play important roles in filamentous differentiation. Spa2, Pea2, and Bni1 are required for formation of elongated filamentous cells, and Spa2 is essential for bud site selection during this process (48, 60). Ste20 is also essential for signaling during filamentous differentiation (45, 59). Contrary to our observation in vegetatively growing cells, it has been reported that Ste20 is not required for the budding pattern of filamentous cells (44, 59). This discrepancy might be explained by a longer apical growth phase that helps restore the usage of distal bud sites in filamentous differentiating *ste20Δ* cells. Thus, the defect of filamentous differentiation in *spa2*, *pea2*, and *bni1* mutants and perhaps part of the filamentous growth defect in *ste20Δ* mutants are likely due to defects in apical growth.

Cell cycle regulators involved in apical growth are also crucial for filamentous differentiation. Cln1 and Cln2 are required for filamentous differentiation (4, 53); these G₁ cyclins are partially stabilized in pseudohyphal cells (4). In addition, the *clb2Δ* mutant is more sensitive to pseudohyphal induction (37). The involvement of Cln1, Cln2, and Clb2 in filamentous growth suggests that the cell cycle machinery may help promote this differentiation process by prolonging the duration of apical growth. Therefore, filamentous differentiation of yeast cells demonstrates that cell elongation and budding pattern alteration can be coordinated by promoting apical growth.

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