The bipolar budding pattern of \textit{a/ø} \textit{Saccharomyces cerevisiae} cells appears to depend on persistent spatial markers in the cell cortex at the two poles of the cell. Previous analysis of mutants with specific defects in bipolar budding identified \textit{BUD8} and \textit{BUD9} as potentially encoding components of the markers at the poles distal and proximal to the birth scar, respectively. Further genetic analysis reported here supports this hypothesis. Mutants deleted for \textit{BUD8} or \textit{BUD9} grow normally but bud exclusively from the proximal and distal poles, respectively, and the double-mutant phenotype suggests that the bipolar budding pathway has been totally disabled. Moreover, overexpression of these genes can cause either an increased bias for budding at the distal (\textit{BUD8}) or proximal (\textit{BUD9}) pole or a randomization of bud position, depending on the level of expression. The structures and localizations of Bud8p and Bud9p are also consistent with their postulated roles as cortical markers. Both proteins appear to be integral membrane proteins of the plasma membrane, and they have very similar overall structures, with long N-terminal domains that are both N- and O-glycosylated followed by a pair of putative transmembrane domains surrounding a short hydrophilic domain that is presumably cytoplasmic. The putative transmembrane and cytoplasmic domains of the two proteins are very similar in sequence. When Bud8p and Bud9p were localized by immunofluorescence and tagging with GFP, each protein was found predominantly in the expected location, with Bud8p at presumptive bud sites, bud tips, and the distal poles of daughter cells and Bud9p at the necks of large-budded cells and the proximal poles of daughter cells. Bud8p localized approximately normally in several mutants in which daughter cells are competent to form their first buds at the distal pole, but it was not detected in a \textit{bni1} mutant, in which such distal-pole budding is lost. Surprisingly, Bud8p localization to the presumptive bud site and bud tip also depends on actin but is independent of the septins.

INTRODUCTION
A central feature of morphogenesis in many types of cells is cell polarization, which involves the asymmetric organization of the cytoskeleton, secretory system, and plasma membrane components along an appropriate axis (Drubin and Nelson, 1996). In the budding yeast \textit{Saccharomyces cerevisiae}, such polarization allows asymmetric growth to form a bud, which becomes the daughter cell. An important feature of cell polarization is the selection of an appropriate axis. In \textit{S. cerevisiae}, axis selection is manifested in the selection of bud sites, which occurs in two different patterns depending on the mating type of the cells (Freifelder, 1960; Hicks \textit{et al.}, 1977; Chant and Pringle, 1995). In the axial pattern, as seen in \textit{MATa} or \textit{MATa} cells (such as normal haploids), the daughter cell’s first bud forms adjacent to the division site (as marked by the birth scar), and each subsequent bud forms adjacent to the immediately preceding bud site (as marked by the bud scar). This pattern appears to depend on a transient cortical marker that involves the Bud3p, Bud4p, and Axl2p/Bud10p/Sro4p proteins (Chant \textit{et al.}, 1995;
Table 1. *S. cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>YEF473</td>
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<td>Bi and Pringle, 1996</td>
</tr>
<tr>
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<td>Segregant from YEF473</td>
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<td>Robinson et al., 1988</td>
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<td>SEY6210D</td>
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<td>SEY6211</td>
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</tr>
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<tr>
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<td>W. Tanner</td>
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<td>W. Tanner</td>
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<td>Amberg et al., 1997</td>
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<td>YJZ427</td>
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<tr>
<td>YSI148</td>
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<td>M. Snyder (Gehrung and Snyder, 1990)</td>
</tr>
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<td>AB234</td>
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<td>A. Bender (Bender and Pringle, 1989)</td>
</tr>
</tbody>
</table>

ML130       | as YEF473A except bar1Δ | M. Longtime |
| LSY192     | as YEF473A except calc12-6 bar1Δ | This study* |
| LSY42      | as YEF473 except BUD9/pGAL::BUD9 | See text |
| LSY41      | as YEF473 except P_GAL::BUD9/P_GAL::BUD9 | See text |
| YHH141     | a his4 leu2 trp1 ura3-91 bud3::TRP1 | This study* |
| YHH145     | a his4 leu2 trp1 ura3-91 bud3::TRP1 | This study* |
| YHH1273    | a/a leu2/leu2 trp1/trp1 ura3-91/bud3::TRP1 | This study* |
| YHH1274    | a/a leu2/leu2 trp1/trp1 ura3-91/bud3::TRP1 | This study* |
| YHH1312    | a his4 leu2 trp1 ura3-91 | This study* |
| YHH1315    | a his4 leu2 trp1 ura3-91 | This study* |
| YHH1387    | as YEF473 except BUD8/bud8-Δ1 | See text |
| YHH1391    | as YEF473B except bud8-Δ1 | Segregant from YHH387 |
| YHH1393    | as YEF473A except bud8-Δ1 | Segregant from YHH387 |
| YHH1399    | a/a HIS3/his3-Δ200 his4/HIS4 leu2/leu2-Δ1 LYS2/lys2-801 trp1/trp1-Δ63 ura3/ura3-52 bud8-1/bud8-1 | YHH315 × YHH391 |
| YHH1415    | as YEF473 except bud8-Δ1/bud8-Δ1 | YHH391 × YHH394 |
| YHH1514    | as YEF473 except bud8-Δ1/bud8-Δ1 URA3::BUD8/URA3::BUD8 | See text |
| YHH1529    | as YEF473 except bud8-Δ1/bud8-Δ1 URA3::BUD8/URA3::BUD8 | See text |
| YHH1610    | as YEF473 except bud9/Δ1 | See text |
| YHH1613    | as YEF473A except bud9-Δ1 | Segregant from YHH610 |
| YHH1614    | as YEF473B except bud9-Δ1 | Segregant from YHH610 |
| YHH1615    | as YEF473 except bud9-Δ1/bud9-Δ1 | YHH613 × YHH614 |
| YHH1616    | as YEF473 except bud9-1/bud9-1 | YHH631 × YHH632 |
| YHH1625    | as YEF473 except bud8-Δ1/bud8-Δ1 bud9-Δ1/bud9-Δ1 | YHH631 × YHH632 |
| YHH1631    | as YEF473B except bud8-Δ1 bud9-Δ1 | YHH631 × YHH632 |
| YHH1632    | as YEF473A except bud8-Δ1 bud9-Δ1 | YHH631 × YHH632 |
| YHH1779    | a/a his4/his4 leu2/leu2 trp1/trp1 ura3/ura3 bud5::URA3 buds::URA3 | This study* |
| YHH1782    | a/a ade2/ade2 ade3/ade3 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 bud2::TRP1 bud2::TRP1 | This study* |

2498 Molecular Biology of the Cell
Bipolar Budding Markers in Yeast

Table 1. (Continued)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>YHH800</td>
<td>as YEF473 except bni1Δ::HIS3/bni1Δ::HIS3 [p39L12] [YEpGFP-BUD8]</td>
<td>This studya</td>
</tr>
<tr>
<td>YHH802</td>
<td>as YEF473 except bni1Δ::HIS3/bni1Δ::HIS3 [YEpGFP-BUD8]</td>
<td>This studya</td>
</tr>
</tbody>
</table>

a Plasmids are indicated in square brackets.
b The strain created by HO-induced diploidization of SEY6210.
c This allele has previously been referred to as Δ3::TRP1.
d A precise replacement of the BNI1 open reading frame by HIS3.
e Segregant from the cross of MB130 to a strain generated by seven backcrosses of a ∆c12-6 mutation (Adams and Pringle, 1984) into YEF473 A.
f The GFP sequences encode GFP with the S65T substitution.
g Segregants from crosses of YHH94 and YHH98 (Zahner et al., 1996), respectively, to YHH17 (a segregant from the same tetrad as YHH16 [Zahner et al., 1996]).
h Derived by mating other segregants from the crosses described in note g.
i Segregant from a cross of YHH141 to YHH141 (Zahner et al., 1996).
j Segregant from a cross of YHH145 to YHH137 (Zahner et al., 1996).
k A segregant from a cross of JC206 [similar to strain 205 (Chant et al., 1991)] to YEF473 A is mated to a segregant from a cross of JC207 (also similar to strain 205) to YEF473B, yielding YHH799.
l Derived by mating segregants from a cross of YHH632 to DDY206-3A (provided by D. DeMarini; it carries a complete deletion of the BUD3 open reading frame in the YEF473B background).
m Derived by mating HPY138 to HPY143a (both from H.-O. Park).
n Strains YJZ426 × YJZ427 were mated after transforming with plasmids p39L12 and p39C (Table 2), respectively. A subclone of the resulting diploid that had lost p39C was identified and transformed with YEpGFP-BUD8, yielding strain YHH800. A subclone of YHH800 that had lost p39L12 was named YHH802.

Halme et al., 1996; Roemer et al., 1996a; Sanders and Herskowitz, 1996); this marker is deposited at the mother-bud neck, and then distributed to the division site on both mother and daughter cells, during each cell cycle.

In contrast, the bipolar pattern, as seen in MATa/MATα cells (such as normal diploids), appears to depend on persistent markers that are deposited at both the birth-scar-distal and birth-scar-proximal poles of the daughter cell, as well as at the division site on the mother cell (Chant and Pringle, 1995). These markers can direct bud formation to the marked site either in the next cell cycle or in a later one. A screen for mutants with normal axial bud-site selection but defective bipolar bud-site selection led to the identification of the BUD8 and BUD9 genes, which have mutant phenotypes suggesting that they might encode components of the markers at the distal and proximal poles of the daughter cell, respectively (Zahner et al., 1996). Specifically, the bud8 mutants bud almost exclusively around the proximal pole, whereas the bud9 mutants bud almost exclusively around the distal pole.

We report here the cloning of BUD8 and BUD9 and the initial molecular analyses of their products. These analyses suggest that Bud8p and Bud9p do indeed mark the opposite poles of the daughter cell. Both proteins contain large extracellular domains, which may anchor them in their specific locations by interacting with the cell wall, and short cytoplasmic domains. The cytoplasmic domains are very similar to each other in sequence and may provide the recognition sites for the Rsr1p/Bud2p/Bud5p GTPase signaling module, which appears to transmit the positional information from the axial and bipolar cortical markers to the proteins responsible for cell polarization (Pringle et al., 1995; Roemer et al., 1996b; Chant, 1999).

MATERIALS AND METHODS

Strains, Plasmids, Growth Conditions, and Genetic and Recombinant DNA Methods

Yeast strains used in this study are listed in Table 1; the construction of strains containing deletions and/or tagged genes is described below. Plasmids used in this study are listed in Table 2 or described where appropriate below. Cells were grown on YM-P or YPD rich liquid medium, solid YPD medium, synthetic complete (SC) medium lacking appropriate nutrients, or minimal medium plus casamino acids (Lillie and Pringle, 1980; Guthrie and Fink, 1991; Salmon et al., 1998), as indicated; 2% glucose was used as carbon source except where noted. Cells were grown at 23°C except where noted. Cells expressing Bud8p or Bud9p tagged with the green fluorescent protein (GFP) were grown in the dark to minimize photobleaching.

For the experiments using a mating pheromone to produce synchronous populations of unbudded, G1-phase cells, α factor (Sigma Chemical Co., St. Louis, MO) was added (final concentration, 25 ng/ml) to cultures growing exponentially (10⁷ cells/ml) in SC-Leu medium. After 90 min, the cultures were diluted threefold with fresh SC-Leu medium that had been prewarmed to 37°C and contained 25 ng/ml α factor, and incubation was continued for 30 min at 37°C, at which point >90% of the cells were unbudded. Cells were collected by centrifugation at 2000 rpm for 5 min, resuspended in half the original volume of SC-Leu medium (without α factor) at 37°C, and incubated further at 37°C.

For the experiments using Latrunculin A (Lat A; Molecular Probes, Eugene, OR) to depolymerize F-actin, a preculture in 2°C synchronus populations of unbudded, G1-phase cells, α factor (Sigma Chemical Co., St. Louis, MO) was added (final concentration, 25 ng/ml) to cultures growing exponentially (10⁷ cells/ml) in SC-Leu medium. After 90 min, the cultures were diluted threefold with fresh SC-Leu medium that had been prewarmed to 37°C and contained 25 ng/ml α factor, and incubation was continued for 30 min at 37°C, at which point >90% of the cells were unbudded. Cells were collected by centrifugation at 2000 rpm for 5 min, resuspended in half the original volume of SC-Leu medium (without α factor) at 37°C, and incubated further at 37°C.
Leu medium (like SC-Leu, but with all ingredients at twice their normal concentrations) containing 0.2% glucose as carbon source was inoculated to normal concentrations containing 0.2% glucose as carbon source and 32P-labeled DNA fragments were used to probe a integrated DNA Technologies (Coralville, IA). For physical map- chemicals, Indianapolis, IN). Oligonucleotide primers were from merase or the Expand High Fidelity System (Roche Molecular Bio- enzymes were purchased from New England Biolabs (Beverly, MA). The polymerase chain reaction (PCR) used either Vent DNA poly- et al., 1995) were used except where noted. Except where noted, et al. (1989). Cloning and Sequencing of

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS315</td>
<td>CEN6 ARSH4 LEU2 (low copy)</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
<tr>
<td>pRS316</td>
<td>CEN6 ARSH4 URA3 (low copy)</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
<tr>
<td>pRS423</td>
<td>LEU2 (high copy)</td>
<td>Christianson et al., 1992</td>
</tr>
<tr>
<td>pRS426</td>
<td>URA3 (high copy)</td>
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<td>YCplac111</td>
<td>CEN4 ARS1 LEU2 (low copy)</td>
<td>Gietz and Sugino, 1988</td>
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<td>YEpplac181</td>
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<td>Gietz and Sugino, 1988</td>
</tr>
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<td>YEpplac195</td>
<td>URA3 (high copy)</td>
<td>Gietz and Sugino, 1988</td>
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<td>YIplac211</td>
<td>URA3 (integrating)</td>
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</tr>
<tr>
<td>YCplF2</td>
<td>CEN4 ARS1 LEU2 P&lt;sub&gt;Gal&lt;/sub&gt;</td>
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<td>YEp352-SUC2</td>
<td>URA3 SUC2 (high copy)</td>
<td>Lussier et al., 1996, 1997a</td>
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<td>CB&lt;sub&gt;6&lt;/sub&gt;12-Kre1p-HA</td>
<td>URA3 P&lt;sub&gt;Adh1&lt;/sub&gt;-HA-KRE1 (high copy)</td>
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<td>BUD8 in YEpplac181</td>
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<tr>
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<td>H. Fares</td>
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<tr>
<td>p39C</td>
<td>BNI1 LEU2 (low copy)</td>
<td>H. Fares</td>
</tr>
</tbody>
</table>

<sup>a</sup> The GFP sequences encode GFP with the S65T substitution.

<sup>b</sup> The GFP sequences encode GFP with the F64L, S65T substitutions.

Cloning and Sequencing of BUD8 and BUD9

BUD8 and BUD9 were cloned by transforming strains YHH274 and YHH273 (Table 1) with an S. cerevisiae genomic DNA library in plasmid YCP50-LEU2 (kindly provided by F. Spencer and P. Hieter, Johns Hopkins University, Baltimore, MD). Transformants were stained with Calcofluor to visualize bud scars (see below) and examined as described previously (Zahner et al., 1996) for complementation of the budding-pattern defects. Of 1700 transformants examined for strain YHH274, one showed a plasmid-dependent restoration of normal bipolar budding. Of 4032 transformants examined for strain YHH273, two showed a plasmid-dependent restoration of normal bipolar budding. Restriction mapping indicated that the two plasmids had similar or identical inserts.

For further analysis of BUD8, an ~3.0-kb BamHI-XbaI fragment that proved to contain the entire BUD8 open reading frame (ORF) plus 547 bp of upstream sequence and 608 bp of downstream sequence (Figure 1A) was subcloned into BamHI/XbaI-digested pBluescript KS<sup>±</sup> (Stratagene, La Jolla, CA) to generate plasmid pK5-BUD8 and into BamHI/XbaI-digested pRS316 to generate plasmid YCPBUD8. In addition, an ~2.5-kb HindIII-Scal fragment from YCPBUD8 (containing the BUD8 ORF plus 290 bp of upstream sequence and 391 bp of downstream sequence) was subcloned into HindIII/Smal-digested YEplac181 to generate plasmid YEPBUD8. To construct a plasmid expressing BUD8 under control of the GAL1/10 promoter (P<sub>Gal</sub>), site-directed mutagenesis was used to introduce a BamHI site immediately upstream of the BUD8 start codon and a HindIII site 141 bp downstream of the BUD8 stop codon. To this end, the HindIII-Scal fragment from pKS-BUD8 (see above) was subcloned into HindIII/Smal-digested YEplac181 to generate plasmid pALT-BUD8. Mutagenesis was then performed as recommended by Promega, using primers HH1 and HH2 (Table 3), to generate plasmid pALT-BUD8(B/H).
The BamHI-HindIII fragment was then subcloned into BamHI/HindIII-digested YCplac111 to generate plasmid YCpGal-BUD8.

For further analysis of BUD9, an ~2.7-kb NsiI fragment that proved to contain the entire BUD9 ORF plus 864 bp of upstream sequence and 192 bp of downstream sequence (Figure 1B) was subcloned into NsiI-digested YCplac111 to generate plasmid YCp-BUD9 and into NsiI-digested YEpplac195 to generate plasmid YEp-BUD9.

To sequence BUD9, pKS-BUD9 (see above) was subjected to Bal31 nuclease treatment to generate a deletion series (Ausubel et al., 1995). Both strands of the BUD9 region were then sequenced from the BamHI site to just beyond the Scal site (Figure 1A) using the M13 primers and the Sequenase reagent kit (USB, Cleveland, OH) according to the manufacturer’s instructions. To sequence BUD9, both strands of the ~2.7-kb NsiI fragment in YCpBUD9 were sequenced by the University of North Carolina-Chapel Hill DNA Sequencing Facility with the use of sequentially generated primers. Comparison of our sequences to those from the genome project (all sequences can be accessed through the Saccharomyces Genome Database; see accession numbers given in RESULTS) revealed some discrepancies, some of which lead to changes in the predicted amino acid sequences (Figure 4A). These discrepancies may represent polymorphisms in the DNA sequences or errors in the various sequences.

Deletion and Tagging of BUD8 and BUD9

To delete BUD8, the ~1.2-kb Sma1-NdeI fragment containing TRP1 from plasmid pJJ280 (equivalent to pJJ246 [Jones and Prakash, 1990]) was ligated into Hpal/NdeI-digested pKS-BUD8 to generate plasmid pKS-TRP. This deletes BUD8-region sequences from 247 bp upstream of the BUD8 start codon (no other gene overlaps this region) to 118 bp upstream of the BUD8 stop codon (Figure 1A). The ~2.2-kb Sma1-SaclII fragment (both sites from the vector) from pKS-TRP was ligated into HindIII-digested YEF473, Trp+ transformants were isolated, and these transformants were analyzed by Southern blotting after digestion of genomic DNA with BamHI and BglII, using the BamHI-XbaI fragment (Figure 1A) as probe. Strain YHH3587 displayed the bands expected for a BUD8/bud8-Δ1 heterozygote, and tetrad analysis showed the expected correlated segregation of Trp+ : Trp- with the appropriate bands as detected by Southern blotting (our unpublished results).

The BUD9 coding region was precisely deleted by the PCR method of Baudin et al. (1993), using HIS3-containg plasmid pRS303 (Sikorski and Hieter, 1989) as template and primers ΔBUD9-F and ΔBUD9-R (Table 3). The PCR product was transformed into strain YEF473, selecting for His+. PCR using primers ΔBUD9-F and BUD9-R indicated that transformant YHH610 had one copy of BUD9 replaced by HIS3, and tetrad analysis showed a 2:2 segregation of His+ : His- (our unpublished results).

To create plasmids carrying GFP cassettes that could be used to tag BUD8 and BUD9, the GFP ORF was amplified by PCR using primers GFP-F and GFP-R (Table 3) and plasmid pS65T-C1 (Clontech, Palo Alto, CA) as template. The PCR product was cut with EcoRI (sites indicated in the primers) and cloned into EcoRI-cut pBluescript KS(-) to create plasmid pKS-GFP, which encodes full-length GFP carrying the S65T substitution (Heim et al., 1995). A series of three steps then generated plasmid pKS-GFP+. First, the MscI-MfeI fragment (both sites in the GFP ORF) of pKS-GFP65ST was replaced by the corresponding fragment from a plasmid (kindly provided by C. Albright and H. McDonald, Vanderbilt University, Nashville, TN) that encodes GFP with both the F64L and S65T substitutions (Cormack et al., 1996), thus creating plasmid pKS-GFP64LF, S65T. Second, the Ndel-MfeI fragment (both sites in the GFP ORF) of pKS-GFP65ST was replaced by the corresponding fragment from plasmid pAFS135 (Straight et al., 1998), which encodes GFP with the V163A substitution, thus creating plasmid pKS-GFP65ST, V163A. Third, the PmlI-KpnI fragment (former site downstream of GFP codon 65, latter site in the vector polylinker) of pKS-GFP64LF, S65T, V163A was replaced by the corresponding fragment from pKS-GFP65ST, V163A, thus creating plasmid pKS-GFP+, which encodes GFP carrying the F64L, S65T, and V163A substitutions.

To tag Bud8p, the pALTER system, plasmid pALT-BUD8 (see above), and primer HH3 were used to introduce a NorI site immediately after the BUD8 start codon. NorI fragments carrying sequences encoding three copies of the hemagglutinin epitope (HA; Stinchcomb et al., 1993; Straight et al., 1998), the pGFP65ST cassette that could be used to create plasmids carrying GFP carrying the F64L, S65T, and V163A substitutions. For each tagged BUD8 allele, three different pairs of transformants were mated to check the budding pattern of a/a cells. The resulting diploids (including strains YHH514 and YHH529) displayed approximately normal bipolar budding (Figure 2, O and P; Figure 3, A–C), indicating that the HA-tagged and GFP-tagged Bud8p provided approximately normal Bud8p function. To construct additional HA-BUD8 plasmids, the HindIII-SacI fragment (latter site from the vector polylinker) carrying the tagged allele was subcloned from pALTER into HindIII/SacI-digested pRS315 to generate plasmid YcPH-A-BUD8. The same sites were then used to subclone the HA-BUD8 fragment from YcPH-A-BUD8 into pRS425 and pRS426, thus generating plasmids YEpHA-BUD8-5 and YEpHA-BUD8-6, respectively. Control plasmid YEpBUD8-6 was constructed by using the HindIII and SacI sites (latter site from the vector) to subclone the fragment carrying untagged BUD8 from YCPBUD8 into HindIII/SacI-digested pRS426. To construct additional GFP-BUD8 plasmids, the HindIII-KpnI fragment (latter site from the vector polylinker) carrying the tagged allele was subcloned from pALTER into HindIII/KpnI-digested YE-
plac181, generating plasmid YEpGFP-BUD8. This plasmid also provided an approximately normal Bud8p function (Figure 3D). The NotI-BamHI fragment (former site in the N-terminal portion of the GFP coding region, latter site introduced with primer GFP-R [Table 3]) was then replaced by the corresponding fragment from pKS-GFP, generating plasmid YEpGFP-BUD9.

To generate plasmids expressing tagged versions of Bud9p, the following strategy was used. First, using plasmid YcpgBUD9 as a template PCR reactions were conducted. One used forward primer LS33 (corresponding to vector sequences outside the polylinker and near the upstream end of the BUD9-containing insert) and reverse primer LS34 (corresponding to sequences overlapping the BUD9 start codon and incorporating a NotI site just downstream of this start codon) (Table 3). The other used forward primer LS35 (corresponding to sequences overlapping the BUD9 start codon and incorporating a NotI site just downstream of this start codon) and reverse primer LS36 (corresponding to sequences within the BUD9 ORF). The products from these two reactions were digested with NotI and ligated together, and the resulting linear product was used as template in a second round of PCR using primers LS33 and LS36. The resulting product was digested with KpnI (one site from the original vector polylinker, just outside the junction with the BUD9 upstream sequences, and the other site in the 5' part of the BUD9 ORF) and ligated into KpnI-digested YcpgBUD9. After sequencing the entire KpnI segment to confirm the presence of the NotI site and the absence of mutations introduced by the PCR, NotI fragments containing triple-HA and GFP coding sequences (see above) were cloned into the NotI site, thus generating plasmids YcpgHA-BUD9 and YcpgGFP-BUD9. In addition, SplI fragments from YcpgHA-BUD9 and YcpgGFP-BUD9 (one site near the upstream end of the BUD9 upstream sequences; the other site from the vector polylinker) were subcloned into SplI-digested YEpplac195 to generate plasmids YEpHA-BUD9 and YEpGFP-BUD9, respectively. Diploid strains carrying YcpgHA-BUD9 or YcpgGFP-BUD9 as their sole source of Bud9p showed a partial restoration of Bud9p-dependent proximal-pole-budding (Figure 3, E and G; cf. Figure 3M), and use of the proximal pole approaches wild-type levels (Figure 3A) when YEpHA-BUD9 or YEpGFP-BUD9 provided the sole source of Bud9p (Figure 3, F and H). These data suggest that the tagged versions of Bud8p and Bud9p are partially, but not completely, functional.

To construct a strain expressing GFP-Bud9p at the BUD9 chromosomal locus under Pgal control, we used the PCR method (Longtine et al., 1996b). A fragment generated using plasmid pFA6a-His3MX6-Pgal1-GFP as template and primers LS10 and LS11 (Table 3) was transferred into strain YEF473, selecting for stable His+ transformants, which should have Pgal sequences fused in frame to BUD9 sequences. Strain LSY42 was one such transformant. A PCR check using forward primer ML135 (corresponding to sequences within the BUD9 ORF) and reverse primer LS8 (corresponding to sequences downstream of the BUD9 start codon) confirmed that BUD9 had been modified as expected, and tetrad analysis showed that LSY42 was heterozygous for the modified and wild-type BUD9 alleles. Two Pgal-GFP-BUD9 segregants from LSY42 were mated to produce strain LSY41. When grown on 2% raffinose, strain LSY41 displayed approximately normal levels of budding at the proximal pole (Figure 3I), consistent with the other evidence (see above) that GFP-tagged Bud9p is at least partially functional.

### Analysis of Growth Rates and Mating Efficiencies

Growth rates in liquid medium were analyzed by growing cells to exponential phase, recording the OD660, diluting the culture twofold with prewarmed medium, and determining the time required to grow back to the original OD660. Growth on plates was analyzed by comparing colony sizes. Tests of sensitivity to Calcofluor and caf-
Mating efficiencies were analyzed by growing an a and a strains to exponential phase in YM-P medium, collecting the cells on filters as described previously (Reid and Hartwell, 1977), incubating the filters on YPD plates for 3 h, removing the cells by vortexing in YM-P medium, sonicking for 2 s, and counting the numbers of zygotes.

**Protein Analyses**

**Protein Extraction.** Yeast cells were grown at 30°C in SC medium lacking particular nutrients as needed for selection of various plasmids. Total cell lysates were prepared from late-exponential-phase cultures (OD$_{600}$ of ~3.0) essentially as described by Ljungdahl et al. (1992). Briefly, cells were harvested by centrifugation at 23°C,
washed once with lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 300 mM sorbitol, plus one protease inhibitor cocktail tablet [Roche Molecular Biochemicals] per 25 ml [i.e., twice the normal dosage]), and resuspended in lysis buffer to an OD₆₀₀ of ~0.110. The cells were then broken by six cycles of vortexing for 30 s with glass beads interspersed with 30-s periods of cooling in an ice bath. The crude lysate was centrifuged at 1600 rpm (200 × g) for 5 min to remove nonlysed cells, and the supernatant was collected as the total cell lysate and stored at 80°C.

Electrophoresis and Immunoblotting. Samples were diluted fivefold with 5×-concentrated sample buffer (Laemmli, 1970), heated at 100°C for 5 min, and subjected to SDS-PAGE using 8% gels (Laemmli, 1970). For immunoblotting, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electrophoresis overnight at 30 V in a Bio-Rad (Hercules, CA) Mini-Protean II apparatus. The membranes were blocked for 1 h at 23°C in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk powder and then incubated for 1 h at 23°C in the same buffer containing anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals; used at a 1:2000 dilution), an anti-invertase polyclonal antiserum (Lussier et al., 1996; used at a 1:1000 dilution), or affinity-purified anti-Bud8p antibodies (see below; used at a 1:100 dilution). The membranes were then washed in TBST buffer and incubated for 1 h at 23°C in TBST buffer containing 5% nonfat dry milk powder and a 1:2000 dilution of horseradish peroxidase-conjugated sheep anti-mouse-IgG or donkey anti-rabbit-IgG secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ), as appropriate. The blots were then washed further with TBST, and proteins were visualized using the enhanced chemiluminescence Western-blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Figure 3. Quantitative evaluation of bud position in wild-type and mutant strains, strains overexpressing Bud8p or Bud9p, and strains expressing tagged versions of Bud8p or Bud9p. Cells of the indicated strains were grown and stained as described in the legend to Figure 2, A–P, except that strain LS1741 was grown with 2% raffinose instead of 2% glucose as carbon source. For each experiment, the positions of all bud scars were determined for 100 cells with one bud scar, 100 cells with two bud scars, 100 cells with three bud scars, and 100 cells with four bud scars. (Thus, for each experiment, the “1 bud scar” bars represent 100 total bud scars, the “2 bud scars” bars represent 200 total bud scars, etc.) The positions of bud scars were scored as distal pole (the third of the cell most distal to the birth scar), equatorial (the middle third of the cell), or proximal pole (the third of the cell centered on the birth scar). In each panel, the average values from three independent experiments are shown. (A) Wild-type strain YEF473; (B) bud8Δ/bud8Δ URA3:HA-BUD8/URA3:HA-BUD8 strain YHH514; (C) bud8Δ/bud8Δ URA3:GFP-BUD8/URA3:GFP-BUD8 strain YHH529; (D) bud8Δ/bud8Δ strain YHH415 carrying the high-copy GFP-BUD8 plasmid YEpGFP-BUD8; (E–H) bud9Δ/bud9Δ strain YHH415 carrying plasmid YCpHA-BUD9 (E), YEpHA-BUD9 (F), YCpGFP-BUD9 (G), or YEpGFP-BUD9 (H); (I) P_GAL-GFP-BUD9/P_GAL-GFP-BUD9 strain LS1741; (J) bud8Δ/bud8Δ strain YHH415; (K) bud8Δ/bud8Δ bud9Δ/bud9Δ strain YHH625; (L) strain YEF473 carrying the high-copy BUD8 plasmid YEpBUD8; (M) bud8Δ/bud9Δ strain YHH615; (N) bud8Δ/bud8Δ bud9Δ/bud9Δ bud3Δ::HIS3/bud3Δ::HIS3 strain YHH772; (O) strain YEF473 carrying the high-copy BUD8 plasmid YEpBUD8.

YEpBUD8: (M) bud8Δ/bud9Δ strain YHH615; (N) bud8Δ/bud8Δ bud9Δ/bud9Δ bud3Δ::HIS3/bud3Δ::HIS3 strain YHH772; (O) strain YEF473 carrying the high-copy BUD9 plasmid YEpBUD9.
Analysis of Membrane Association. To 50 μl of total cell lysate were added 20 μl of lysis buffer (as control), 0.5 M NaCl, 8 M urea, 20% Triton X-100, or 10.1% SDS. These samples were incubated for 20 min on ice (or at 23°C for the Triton and SDS samples) and then centrifuged for 1 h at 100,000 × g to separate the insoluble membrane materials in the pellet from the soluble materials in the supernatant. These fractions were then analyzed by SDS-PAGE and immunoblotting. Alternatively, to test for possible plasma membrane association (Gould et al., 1988), an aliquot of total cell extract was centrifuged for 5 min at 10,000 × g at 4°C, and the supernatant and pellet fractions were analyzed separately by SDS-PAGE and immunoblotting.

Analysis of Protein Glycosylation. Possible O-linked glycosylation was investigated by analyzing proteins from appropriate mutant strains (see RESULTS). Possible N-linked glycosylation was investigated by analyzing proteins from appropriate mutant strains (see RESULTS) and/or by digestion of proteins in total cell lysates either with a recombinant endo-β-N-acetylgalactosaminidase H/maltose-binding-protein fusion protein (EndoH; New England Biolabs) or with a mixture of endoglycosidase F and peptide-N-glycosidase F (EndoF/PNaseF; Oxford GlycoSciences, Bedford, MA). EndoH was used essentially as described by the manufacturer (see also Roemer et al., 1994). For EndoF/PNaseF digestion, several microliters of total cell lysate were mixed with water and a denaturation buffer cocktail to give a total volume of 12.5 μl at final concentrations of 20 mM sodium phosphate, pH 7.5, 55 mM EDTA, 0.075% SDS, 0.5% β-mercaptoethanol, 2 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. This mixture was heated to 100°C for 10 min and then cooled to 23°C. To counteract the inhibition of PNaseF by SDS, 5.5 μl of an octylglycoside preparation (1.82% octylglycoside in 20 mM sodium phosphate, pH 7.5, 55 mM EDTA, plus protease inhibitors as described above) was added, followed by 2.0 μl of the EndoF/PNaseF preparation (in 20 mM potassium phosphate, pH 7.2, 50 mM EDTA), giving a final enzyme concentration of 340 deglycosylation units/ml. After incubation overnight at 37°C, samples were analyzed by SDS-PAGE and immunoblotting as described above. In these experiments, controls were mock-digested by being subjected to identical treatment without the deglycosylation enzymes.

Generation of Antibodies to Bud8p
To generate fusions of E. coli maltose-binding protein (MBP) and TrpE to Bud8p, the BUD8-containing BamH-HindIII fragment from pALT-BUD8/B/H (see above) was subcloned into the corresponding sites of pMAL-c2 (New England Biolabs) and pATH3 (Koerner et al., 1991) to generate plasmids pMAL-BUD8 and PATH-BUD8. Sequencing across the junctions confirmed that in-frame fusions had been constructed. When E. coli DH5α cells containing these plasmids were induced by standard procedures (Ausabel et al., 1995), only extensively degraded proteins were observed by SDS-PAGE (our unpublished results). Thus, shorter fusion genes (containing 877 bp of BUD8 coding sequence; Figure 1A) were generated by digesting pMAL-BUD8 and PATH-BUD8 with SalI and HindIII, blunting the ends with Klenow-fragment polymerase, and religating. Induction of these genes in E. coli DH5α cells and analysis by SDS-PAGE revealed primarily undegraded fusion proteins of the expected sizes of 72 kDa (MBP-Bud8pSal) and 70 kDa (TrpE-Bud8pSal) (our unpublished results). MBP-Bud8pSal was purified on an amylose-agarose column (New England Biolabs) as described previously (Ausubel et al., 1995) and used to raise polyclonal rabbit antisera by standard procedures (Cocalico Biologicals, Reamstown, PA). Antisera were tested by immunoblotting against TrpE-Bud8pSal. Antibodies from a serum obtained after four booster injections were affinity purified first against TrpE-Bud8pSal and then against MBP-Bud8pSal, using nitrocellulose strips containing the fusion proteins (Pringle et al., 1991). The purified antibodies were concentrated using a Centricon-3 filter (Amicon, Danvers, MA), and bovine serum albumin (BSA) was added to 0.1%.

When tested by immunoblotting (see above), the purified antibodies recognized two polypeptides, with apparent molecular weights of −120 and −74 kDa, in extracts of cells carrying a high-copy HA-BUD8 plasmid (our unpublished results). Because the −120-kDa species was not detected in extracts of cells carrying a control plasmid, it appears to represent Bud8p, consistent with the results obtained with the use of HA-specific antibodies (Figures 5–7, below). In immunofluorescence experiments, the purified antibodies yielded a signal in bud8Δ cells that were overexpressing Bud8p from the GAL promoter (Figure 8C). Because this signal was not detected in wild-type cells or in bud8Δ cells containing the control plasmid YciP1F2 (our unpublished results), it appears to be specific for Bud8p.

Staining and Microscopy
For characterization of budding patterns, cells were generally fixed with 3.7% formaldehyde and stained with 0.1–1 mg/ml Calcofluor as described by Pringle et al. (1989). For some counts and for simultaneous visualization of a GFP signal together with birth and bud scars, unfixed cells were stained with 1 μg/ml Calcofluor as described by Zahner et al. (1996). Cells were examined and photographed on a Nikon Microphot SA microscope using an Apo 60X/1.40 NA oil-immersion objective and Kodak T-Max 400 film.

Living cells expressing GFP-Bud8p or GFP-Bud9p were observed either by conventional fluorescence microscopy (as just described) or by time-lapse digital-imaging microscopy. The time-lapse experiments were performed essentially as described by Salmon et al. (1998). Cells were grown overnight in minimal medium plus casamino acids and observed in the same medium containing 25% gelatin. Observations were made using a Nikon Microphot FXA microscope equipped with a Hamamatsu charge-coupled device camera and an Apo 60X/1.4 NA oil-immersion objective. Images were collected at 1-min intervals using 3-s exposures and analyzed using Metamorph software (Universal Imaging, West Chester, PA).

Cells to be used for immunofluorescence were fixed by adding formaldehyde directly to the growth medium to a final concentration of 3.7% and swirling gently for 1.5 h at 23°C. Localization of Cdc11p and of tubulin was performed essentially as described previously (Pringle et al., 1991) using a rabbit polyclonal anti-Cdc11p (Ford and Pringle, 1991) and the YOL1/34 rat monoclonal anti-tubulin (Kilmartin and Adams, 1984; obtained from Accurate Chemical and Scientific, Westbury, NY). The secondary antibodies were Cy2-conjugated goat anti-rabbit-IgG and rhodamine-conjugated goat anti-rat-IgG (both from Jackson Immunoresearch, West Grove, PA). For immunolocalization of Bud8p, the fixed cells were washed once in phosphate-buffered saline (PBS) and once in solution A (40 mM potassium phosphate, pH 6.5, 0.5 mM MgCl2, 1.2 M sorbitol [Pringle et al., 1991]), followed by treatment with 1% β-mercaptoethanol and 0.5 mg/ml lyticate (catalog no. 152270; ICN Biomedicals, Costa Mesa, CA) in solution A for 40 min at 37°C to remove cell walls. The cells were then washed twice with solution A and once with PBS, resuspended in PBS containing 1% BSA (PBS/BSA), and applied to polylysine-coated slides as described previously (Pringle et al., 1991). Affinity-purified anti-Bud8p antibody diluted 1:10 in PBS/BSA was then applied, and the slide was incubated for 1 h at 23°C. After washing with PBS/BSA, the cells were incubated for 1 h at 23°C in BODIPY-FL-conjugated goat anti-rabbit-IgG antibody (Molecular Probes) diluted 1:200 in PBS/BSA. The cells were then washed further with PBS/BSA and mounted as described previously (Pringle et al., 1991).

RESULTS
Cloning of BUD8 and BUD9
To clone BUD8 and BUD9, we transformed bud8 and bud9 mutant diploid strains with a yeast genomic-DNA library in
a low-copy vector and examined individual transformants by fluorescence microscopy for restoration of the bipolar budding pattern (see MATERIALS AND METHODS). Subcloning localized the bud9-complementing activity to an ~30-kb BamHI-XbaI fragment (Figures 1A and 2, C and D). This fragment hybridized to a λ* clone carrying DNA from chromosome arm XII between ILV5 and CDC3, very close to the map location of bud8-1 (Zahner et al., 1996). Sequencing of the BamHI-XbaI fragment (Figure 1A; accession no. L37016) revealed one complete and one partial ORF. Subsequent release of chromosome XII sequence by the genome project (accession no. U19102) identified the complete ORF as YLR335W and the adjacent incomplete ORF as YLR334C (TAL1). A diploid strain homozygous for a deletion of YLR335W (Figure 1A; see MATERIALS AND METHODS) budded only from the proximal pole (Figure 2E), like the original bud8-1 mutant (Figure 2C), and the BamHI-XbaI fragment on a low-copy plasmid restored bipolar budding (our unpublished results). In addition, the deletion failed to complement bud9-1 (Figure 2F). The similar phenotypes, noncomplementation, and coincidence in map position establish that YLR335W is BUD8.

Subcloning localized the bud9-complementing activity to an ~2.7-kb NsiI fragment (Figures 1B and 2, G and H). This fragment hybridized to λ* clones carrying DNA from chromosome arm V between KSS1 and RME1, very close to the map position of bud9-1 (Zahner et al., 1996). The NsiI fragment was sequenced (accession no. AF302239) and found to contain a single ORF. Comparison of our sequence to that from the genome project (accession no. Z72826) identified this ORF as YGR041W. A diploid strain homozygous for a deletion of YGR041W (Figure 1B; see MATERIALS AND METHODS) budded only from the distal pole (Figure 2I), like the original bud9-1 mutant (Figure 2G), and the NsiI fragment on a low-copy plasmid restored bipolar budding (our unpublished results). In addition, the deletion failed to complement bud9-1 (Figure 2J). The similar phenotypes, noncomplementation, and coincidence in map position establish that YGR041W is BUD9.

**Deletion and Overexpression Phenotypes of BUD8 and BUD9**

As noted above, diploid strains homozygous for the bud8-1Δ and bud9-1Δ deletions resembled the original bud8-1 and bud9-1 mutants in budding pattern. However, quantitative analysis showed that the deletion phenotypes were somewhat more extreme, at least in the case of BUD8. Although bud8-1 cells produce a few buds at their distal poles (Zahner et al., 1996), bud8-1Δ cells budded almost exclusively at their distal poles through their first four cell cycles (Figure 3F). Similarly, bud9-1Δ cells budded almost exclusively from their distal poles (Figure 3M). Like bud8-1 and bud9-1 (Zahner et al., 1996), the bud8-1Δ and bud9-1Δ mutations had no detectable effects on axial budding (Figure 2, K and L). However, it should also be noted that the bud scars at the proximal pole for budding or randomize bud position (by producing a delocalized signal). Indeed, both effects could be observed. When BUD8 was expressed from its own promoter on a high-copy plasmid, the cells showed a more persistent bias for the use of the distal pole (Figure 3L), whereas the presence of a high-copy BUD9 plasmid resulted in increased use of the proximal pole during the first few cell cycles (Figure 3O). (Strikingly, although cells whose first four buds were all at the proximal pole were very rare in wild-type strains [Chant and Pringle, 1995], 9% of the cells were of this type in the population shown in Figure 3O.) In contrast, when BUD8 was expressed (presumably to high levels) from the GAL promoter, bud-site positions were largely random (Figure 2, Q and R).

**Analysis of Bud8p and Bud9p Sequences**

The predicted sequences of Bud8p and Bud9p revealed that the two proteins are very similar in overall structure despite a modest difference in length (Figure 4). Each protein has a long, hydrophilic N-terminal domain followed by two short hydrophobic domains surrounding a short hydrophilic domain. The program TM-pred (Hofmann and Stoffel, 1993) predicts that each of the hydrophobic domains is membrane-spanning and that the N-terminal domain of each protein is in the extracytoplasmic space despite the absence of N-terminal signal sequences. This prediction is consistent with the presence of multiple potential sites for N-linked glycosylation within each N-terminal domain and with their high serine + threonine content (suggestive of possible O-linked glycosylation [Orlean, 1997]). Although there is little...
similarity in sequence between the N-terminal portions of the proteins, there is strong similarity (53% sequence identity) between their C-terminal 116 amino acids (Figure 4A). Interestingly, the regions with sequence similarity include the predicted transmembrane domains (including the two conserved prolines in the upstream domains) and the 30 amino acids just N-terminal to them, as well as the predicted cytoplasmic loops. Surprisingly, Bud9p does not share the cluster of positively charged amino acids found just C-terminal to the first predicted transmembrane domain in Bud8p, which might have been thought to be critical in determining the orientation of insertion of this domain into the endoplasmic reticulum membrane (Hartmann et al., 1989; von Heijne, 1996; Säaf et al., 1999). To date, no homologs of Bud8p or Bud9p from other organisms have appeared in the databases.

**Membrane Association and Glycosylation of Bud8p and Bud9p**

To test the membrane association and topology predicted from the Bud8p and Bud9p sequences, we carried out fractionation experiments and tests of glycosylation using strains expressing functional HA-epitope-tagged versions of the proteins. Although expression of HA-Bud8p from a low-copy plasmid (YcpHA-BUD8) resulted in only a faint signal upon immunoblotting with the anti-HA antibody (our unpublished results), a somewhat fuzzy band was visualized clearly when a high-copy plasmid was used (Figure 5A, lane 2). This band was not seen in control extracts (Figure 5A, lane 1) and corresponded to a polypeptide of apparent molecular weight 69 kDa, much larger than the 64 kDa predicted from the Bud8p and HA sequences. On fractionation in lysis buffer or in the presence of Na2CO3, NaCl, urea, or Triton X-100, this polypeptide remained in the pellet fraction (Figure 5A, lanes 3-12), but it was extracted into the supernatant upon treatment with SDS (Figure 5A, lanes 13-14). Essentially identical results were obtained with HA-Bud9p, except that in this case the signal was observed clearly even when a low-copy plasmid was used (Figure 5B). HA-Bud9p had an apparent molecular weight of 110 kDa, much larger than the 64 kDa predicted from the Bud9p and HA sequences. Because treatment with NaCl or urea should release most peripheral membrane proteins and...
treatment with Na₂CO₃ should release soluble proteins from membrane vesicles (Fujiki et al., 1982; Goud et al., 1988; Ljungdahl et al., 1992; Roemer et al., 1994, 1996a), the data suggest that Bud8p and Bud9p are indeed integral membrane proteins. When an extract of cells expressing HA-Bud8p was centrifuged at 10,000 g (see MATERIALS AND METHODS), most Bud8p was found in the pellet (our unpublished results), suggesting that most Bud8p was in the plasma membrane (Goud et al., 1988).

It seemed likely that the high apparent molecular weights of Bud8p and Bud9p reflected glycosylation of the proteins. To test for possible N-linked glycosylation, we treated cell extracts with enzymes that remove N-linked glycosyl side chains. For both HA-Bud8p and HA-Bud9p, treatment either with an Endo F/PNGase mixture (Figure 6, A and E) or with Endo H (our unpublished results) led to a substantial decrease in apparent molecular weight, although both proteins still migrated considerably more slowly than expected from the sizes of their polypeptide chains. Because all of the potential sites for N-linked glycosylation are in the N-terminal domains of the proteins (Figure 4A), this evidence for N-linked glycosylation strongly supports the hypothesis that the N-terminal domains are in the extracytoplasmic space.

To characterize the N-linked glycosylation further, HA-Bud8p and HA-Bud9p were expressed in a strain lacking Mnn9p, which is required for the elaboration of N-glycan outer chains (Yip et al., 1994; Orlean, 1997; Shahinian et al., 1998). As controls, an HA-tagged Kre1p (which is heavily O-glycosylated but not N-glycosylated [Boone et al., 1990; Roemer and Bussey, 1995]) and Suc2p (which is both O- and N-glycosylated, with highly elaborated N-glycosyl outer chains [Esmon et al., 1987; Reddy et al., 1988]) were ex-
pressed in the same mnn9 mutant strain. As expected, HAKre1p from the mnn9 and wild-type strains showed no difference in electrophoretic mobility (Figure 6C), whereas Suc2p migrated much more rapidly when expressed in the mnn9 strain (Figure 6D). Surprisingly, HA-Bud8p from the mnn9 and wild-type strains showed no detectable difference in mobility (Figure 6B), whereas HA-Bud9p from the mnn9 strain showed only a small increase in mobility (Figure 6F). HA-Bud8p also showed no increase in mobility when expressed in strain HAB881, which is deficient in Och1p, the α-1,6-mannosyltransferase required for the initiation of outer chain formation (Nakayama et al., 1992; Orlean, 1997; Shahnian et al., 1998) (our unpublished results). Thus, it appears that most or all of the N-linked glycosyl chains on Bud8p and Bud9p consist of core units only, without elaborated outer chains.

To test for possible O-linked glycosylation, HA-Bud8p and HA-Bud9p were expressed in strains lacking one or more of the protein O-mannosyltransferase (Pmt) enzymes, which transfer the initial mannosyl residue from dolichyl-P-mannose to serine or threonine residues in the polypeptide (Tanner and Lehle, 1987; Gentzsch and Tanner, 1996; 1997; Orlean, 1997). When expressed in pmt1, pmt2, pmt3, pmt4, pmt5, or pmt6 single-mutant strains, HA-Bud8p and HA-Bud9p showed slightly increased (Figure 7, A and D) or essentially the same (our unpublished results) mobilities relative to the proteins from wild-type cells. Expression of the proteins in strains carrying certain combinations of pmt mutations led to larger increases in mobility (Figure 7, A, B, and D), although for HA-Bud9p, even the largest increase in mobility observed (Figure 7D, pmt2/4) was rather modest. As expected, enzymatic removal of N-glycosyl chains from HA-Bud8p extracted from a pmt mutant strain led to a further increase in mobility (Figure 7C). Interestingly, however, the protein still migrated significantly more slowly than predicted from the size of its polypeptide chain (see DISCUSSION).

Localization of Bud8p and Bud9p in Wild-Type Cells

Previous analyses of bipolar budding had suggested that the distal-pole marker would arrive at the presumptive bud site before bud emergence and remain at the tip of the bud as the bud grew, thus eventually marking the distal pole of the daughter cell (Chant and Pringle, 1995; Amberg et al., 1997). These analyses also suggested that the proximal-pole marker would arrive at the mother-bud neck shortly before cell division and remain in place during division, thus marking the proximal pole of the daughter cell. To ask whether Bud8p and Bud9p behaved as predicted for the distal-pole and proximal-pole markers, we localized these proteins with the use of an antibody specific for Bud8p and GFP-tagged fusion proteins that were at least partially functional (see MATERIALS AND METHODS).

In a strain carrying a chromosomal copy of GFP-BUD8, only ~10% of the cells showed any detectable GFP signal. Among such cells, GFP-Bud8p was observed in patches at presumptive bud sites on unbudded cells (Figure 8A, cells 1–3) and at the tips of buds of various sizes (Figure 8A, cells 4–6). Most of the unbudded cells had a single patch of GFP-Bud8p (Figure 8A, cell 1), but some had patches of GFP-Bud8p at both poles (Figure 8A, cells 2 and 3). When GFP-BUD8 was expressed from a high-copy plasmid, the GFP signal was observed in a higher proportion (20–25%) of the cells and was typically somewhat brighter, but the patterns observed were very similar (Figure 8B; see also Figure 10A). In this case, however, most unbudded cells with a detectable signal had patches of GFP-Bud8p at both poles. When immunofluorescence was performed on wild-type cells using the affinity-purified anti-Bud8p antibodies, no signal was observed. However, when cells expressing BUD8 under control of the GAL promoter were induced for several hours and then examined, patterns of Bud8p staining were observed that were essentially the same as those observed with GFP-tagged Bud8p (Figure 8C). Taken together, the results support the hypothesis that Bud8p is a component of the distal-pole marker, although the observation of unbudded cells with Bud8p at both poles is surprising.
Staining with Calcofluor revealed that all of the unbudded cells with GFP-Bud8p at both poles were daughter cells that had never budded (our unpublished results), and time-lapse analysis (Figure 9) revealed how this pattern of Bud8p localization was generated. For example, in panels 2 and 3, cell a had a large bud with a rather diffuse patch of GFP-Bud8p at its distal tip and no detectable GFP-Bud8p signal at its neck. Signal then appeared at the neck (panels 4–7), and when the mother and daughter cells separated (panel 8), most or all of this signal partitioned to the daughter cell. Cell b showed the same phenomena (panel 19 ff.). The time-lapse analysis also revealed several other features of interest. First, although the patches of GFP-Bud8p at incipient bud sites and on newly formed buds were quite tight (cell b, panel 3 ff.; cell c, panel 2 ff.; cell d, panel 8 ff.; cell e, panel 16 ff.), they became rather diffuse later in bud growth (cells b and c, panel 12 ff.). This explains why newborn daughter cells (e.g., cell a’s daughter, panel 8; cell b’s daughter, panel 23; also, presumably, cells d and e, panel 2) had diffuse patches of GFP-Bud8p at their distal poles and tight patches at their proximal poles. Second, the diffuse patches of GFP-Bud8p at the distal poles of newborn daughters became dramatically tighter during the several minutes just before bud emergence at that pole (cell d, panels 6–11; cell e, panels 12–18; this rapid tightening is particularly vivid in the accompanying movie).

In strains carrying a low-copy or high-copy GFP-BUD9 plasmid, little or no signal was detected. However, when cells expressing GFP-BUD9 under control of the GAL promoter were induced for 4 h and then examined, a GFP signal could be detected in ~30% of the cells. In cells with medium-sized or large buds, GFP-Bud9p was typically observed at the mother-bud necks and appeared to be asymmetrically localized to the bud side of the neck (Figure 8D, cells 1–5). These concentrations of Bud9p appeared to remain in place during division, because unbudded cells typically showed a single patch of GFP-Bud9p at one pole of the cell (Figure 8E, cells 1–5), and staining with Calcofluor revealed that such cells were all daughter cells and that the GFP-Bud9p patches were at their proximal poles (Figure 8F). These results support the hypothesis that Bud9p is a component of the proximal-pole marker. Unexpectedly, however, GFP-Bud9p was also observed at the bud tips of some budded cells (Figure 8D, cells 5 and 6), including some cells with small buds (our unpublished results), as well as at both poles of some unbudded daughter cells (Figure 8E, cell 6). The proportion of cells with bud-tip localization of GFP-Bud9p increased with increasing times of expression in galactose medium.

**Localization of Bud8p in Mutant and LatA-treated Cells**

To explore some of the functional relationships among proteins involved in bipolar budding, we examined the localization of GFP-Bud8p in strains carrying mutations in sev-
eral other relevant genes. Because Bud9p does not appear to be involved in budding at the distal pole, it seemed unlikely that bud9 mutations would affect the localization of Bud8p. Indeed, the distribution of GFP-Bud8p in a bud9 deletion strain (Figure 10B) resembled that in wild-type cells (Figures 8, A–C, and 10A). In addition, although Rsr1p, Bud2p, and Bud5p are essential for bipolar (and axial) budding, these proteins are thought to function downstream of the spatial markers (see INTRODUCTION). Consistent with this model, the distribution of GFP-Bud8p in rsr1 (strain AB324), bud2 (strain YHH782), and bud5 (strain YHH759) mutant cells was essentially the same as in wild-type cells (our unpublished results).

It was of particular interest to examine Bud8p localization in bud6, spa2, and bni1 mutants. In diploid bud6 or spa2 mutant cells, the first buds appear to form more-or-less normally at the distal pole (Zahner et al., 1996; Amberg et al., 1997), although the positioning of subsequent bud sites is nearly random. (There are some subtle effects on the positions of the first buds that have been considered elsewhere [Zahner et al., 1996; Amberg et al., 1997; Sheu et al., 2000].) These observations suggested that the localization of Bud8p would be approximately normal in these mutants. In contrast, diploid bni1 mutants appear to bud in random locations in the first as well as in subsequent cell cycles (Zahner et al., 1996), suggesting that Bud8p localization might be lost in a bni1 mutant. Expression of GFP-Bud8p in appropriate mutant strains confirmed both of these expectations (Figure 10, C–F).

Bud6p and Spa2p are involved in the organization of the actin cytoskeleton (Amberg et al., 1997; Fujiwara et al., 1998; Sheu et al., 1998; Jaquenoud and Peter, 2000). Mutations in several other genes that affect the actin cytoskeleton also produce bipolar-budding phenotypes similar to those of bud6 and spa2 mutants (Yang et al., 1997). Taken together, these data suggested strongly that the delivery of the distal-pole marker was actin independent, a conclusion that seemed plausible given that a variety of proteins is known to arrive at the presumptive bud site in an actin-independent manner (Ayscough et al., 1997). Although Bni1p is also involved in the organization of the actin cytoskeleton (Evangelista et al., 1997; Fujiwara et al., 1998; Bi et al., 2000; Jaquenoud and Peter, 2000), it also interacts genetically with the septins (Fares and Pringle, unpublished data). Thus, a plausible interpretation of the loss of distal-pole budding (and of Bud8p localization) in bni1 mutants was that the delivery of Bud8p to the presumptive bud site was septin dependent. Remarkably, however, Bud8p localization appears to be actin dependent and septin independent.

Figure 9. Time-lapse observations of GFP-Bud8p localization. Cells of bud8Δ1/bud8Δ1 strain YHH415 carrying plasmid YEpGFP-BUD8 were grown and imaged as described in MATERIALS AND METHODS. Images were collected at 1-min intervals for 159 min. The figure shows every fourth image for 84 min (panels 1–22) followed by the images collected at 116, 124, and 128 min (panels 23–25, respectively). Some cells are marked for reference in the text. The entire image series can be accessed as a QuickTime movie.
To test for a possible role of actin, we used the inhibitor LatA, which produces a very rapid and essentially complete loss of filamentous actin (Ayscough et al., 1997). Stationary-phase cells were mostly unbudded and devoid of incipient bud sites (as judged by septin staining), and they showed no detectable patches of GFP-Bud8p signal (Figure 11A, t = 0'). On resumption of growth in the absence of LatA, patches of GFP-Bud8p appeared at presumptive bud sites within 2 h (Figure 11A, t = 120', DMSO). In contrast, in the presence of LatA, although incipient bud sites could be recognized by 120 min on the basis of their septin staining (as expected from the previous work of Ayscough et al., 1997), no patches of GFP-Bud8p could be detected (Figure 11A, t = 120', LatA). Thus, at least under these conditions, the delivery of Bud8p to the presumptive bud site appears to be actin dependent.

In contrast, when the possible septin dependence of Bud8p localization was evaluated with the use of the cdc12-6 septin mutation (which causes a rapid and seemingly complete loss of septin organization upon shift to restrictive temperature: Adams and Pringle, 1984), it was clear not only that existing bud-tip patches of GFP-Bud8p could be maintained but that new patches of GFP-Bud8p could form at incipient bud sites (Figure 11B; note especially the new buds forming at 120 and 180 min). Thus, localization of Bud8p to the bud site and bud tip, and thus eventually to the distal pole of the daughter cell, appears to be septin independent. In contrast, GFP-Bud8p localization to the mother-bud neck was not observed in the septin mutant, although it was seen clearly in wild-type cells grown under the same conditions (Figure 11B, 60'). Thus, Bud8p localization to the neck, like that of most other proteins that localize to that site (Longtine et al., 1996, 1998a, 2000; Longtine and Pringle, 1999), appears to be septin dependent.

DISCUSSION

Bud8p and Bud9p as the Apparent Markers of Bipolar Budding Sites

Despite some complications (discussed below), the results presented here suggest strongly that Bud8p and Bud9p are
essential components of the spatial markers for bipolar budding at the distal and proximal poles, respectively (Figure 12). Deletion of BUD8 or BUD9 has no detectable effect on the axial budding pattern, but diploid bud8 deletion strains show an essentially complete loss of ability to bud at the distal pole, whereas diploid bud9 deletion strains show an essentially complete loss of ability to bud at the proximal pole. Moreover, diploid bud8 bud9 double-deletion strains bud in essentially random locations (when their ability to use axial budding cues is also disabled by deleting BUD3), and strains overexpressing BUD8 or BUD9 show either an enhanced use of the corresponding pole or a randomization of budding pattern (apparently depending on the level of expression). It should also be noted that Mösch and Fink (1997) identified transposon-insertion mutations in BUD8 in a screen for mutants defective in pseudohyphal growth, which depends on budding at the distal poles of daughter cells (Gimeno et al., 1992; Kron et al., 1994).

The apparent use of axial budding cues by diploid bud8 bud9Δ bud9Δ cells was surprising, because axial budding appears to depend on Axl1p, whose expression is repressed in a/a cells (Fujita et al., 1994; Chant, 1999). Evidently, either AXL1 is not completely repressed in a/a cells or axial budding does not depend absolutely on Axl1p, so that when the bipolar budding markers are absent, the axial marker (whose known components are expressed in a/a cells: Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996a; Sanders and Herskowitz, 1996) can be recognized with limited efficiency. These observations focus attention on the very interesting questions of how haploid cells normally use axial sites with such high fidelity (Madden and Snyder, 1992; Chant and Pringle, 1995), despite the apparent presence of all components needed for bipolar budding (Chant and Herskowitz, 1991; Madden and Snyder, 1992; Chant and Pringle,

Figure 11. Dependence of Bud8p localization to the bud tip on actin but not on the septins. All strains contained plasmid YEpGFP-BUD8. (A) Strain YHH415 (bud8Δ/bud8Δ) was treated with LatA in DMSO or DMSO alone as described in MATERIALS AND METHODS. Samples taken at the beginning of the incubations with LatA or DMSO (t = 0’) and at intervals thereafter were examined for GFP fluorescence or fixed and processed for immunofluorescence using anti-tubulin antibodies (to demonstrate successful permeabilization of the stationary-phase cells for immunofluorescence) or anti-Cdc11p antibodies (to score for the presence of incipient bud sites). At t = 0’, most cells were unbudded and showed no localized septin staining; a few budded cells were present and showed septin staining at the neck, as expected. (B) Strains ML130 (wild type) and LSY192 (cdc12-6) were synchronized with α factor as described in MATERIALS AND METHODS. Samples taken at intervals after the release from α factor arrest at 37°C were examined for GFP fluorescence. Other samples taken at the same times were fixed and examined for septin localization by immunofluorescence using anti-Cdc11p antibodies; these observations verified that the septins localized normally in strain ML130 but were undetectable throughout the period of the experiment in strain LSY192, as expected.

Figure 12. Proposed localization, membrane topology, and function of Bud8p and Bud9p. The cytoplasmic domain of Bud8p or Bud9p appears to be recognized (directly or indirectly) by Bud2p (circle) and Bud5p (square), the regulatory factors that control the Ras1p GTPase (triangle), which in turn communicates positional information to the polarity-establishment proteins. See text for further discussion.
brane proteins, the bulk of whose mass (polypeptide plus cate that both Bud8p and Bud9p are integral plasma-mem-
their glycosylation patterns, and their localizations all indi-
yses of their sequences, their behaviors during fractionation,
limited by interactions with the cell wall (Chant and Pringle,
might involve transmembrane proteins whose mobility is
persistence of the markers led to the prediction that they
suggested that it depends on spatially and temporally per-
imal poles. Detailed analysis of the bipolar pattern had
support the hypothesis that they mark the distal and prox-
Bud8p and Bud9p. Although we had little success in visualizing Bud8p or
localization without overexpression, our observa-
tions are mostly consistent with the postulated roles of these
proteins. As expected (Chant and Pringle, 1995; Amberg et
bud8 was found at presumptive bud sites, the
tips of growing buds, and the distal poles of daughter cells,
whereas Bud9p was observed at the bud side of the neck in
large-budded cells and at the proximal poles of daughter
in addition, as expected (Zahner et al., 1996; Amberg et
seq 1997; see RESULTS), Bud8p localization appeared ap-
proximately normal in bud6 and spa2 mutants but was un-
detectable in a bni1 mutant. Surprisingly, however, in wild-
type cells, we sometimes also observed Bud8p at the neck or
the proximal pole of the daughter cell or Bud9p at the bud
tip or the distal pole of the daughter cell. Although this point
will require further investigation, we suspect that these ob-
servations are artifacts resulting from overexpression of the
proteins: the mechanisms that direct these structurally sim-
ilar proteins to their normal locations (see below) seem likely
to be overwhelmed when the proteins are overproduced,
causing each protein to be delivered to the location normal
for the other as well to its own normal location. Consistent
with this interpretation, the frequency of the putatively ab-
errant localization appeared to be higher when the levels of
overexpression were greater (see RESULTS).

Although the extracytoplasmic domains of Bud8p and
Bud9p have only very limited similarity in sequence, the
cytoplasmic domains are very similar. This similarity may
allow the cytoplasmic components of the bud-site-selection
pathway to recognize essentially the same signal at the two
poles of the cell. Although it is not known how this recog-
nition occurs or whether the same components are involved
at the two poles, the identification of special alleles of BUD2
and BUD5 that disable bipolar but not axial budding (Zah-
ner et al., 1996) suggests that Bud2p and Bud5p (which are
regulatory elements of the Rsr1p module) may be directly
involved (Figure 12). These observations do not appear to
help solve (and indeed might be said to deepen) the mystery
of why daughter cells show such a strong bias for forming
their first few buds at the distal pole (Chant and Pringle,
1995; Zahner et al., 1996; Figure 3A).

Other Unsolved Problems

Although the working model of Figure 12 seems likely to be
correct at least in outline, it leaves many questions unas-
swered in addition to those already noted above. Some of
these questions relate to the structures of Bud8p and Bud9p
themselves. For example, it is not clear that the N- and
O-linked glycosylation can fully account for the differences
between the expected and observed electrophoretic mobili-
ties of these proteins. It is possible that the deglycosylated
polypeptides migrate atypically during SDS-PAGE. Alterna-
tively, there may be residual O-linked glycosylation on
Bud8p and/or Bud9p expressed in strains with the combi-
nations of pmt mutations used in this study (Gentzsch and
Tanner, 1996, 1997; Orlean, 1997; Sanders et al., 1999), the
proteins may have N-glycosyl moieties that are resistant to
cleavage by the enzymes used (Van Rinsum et al., 1991;
Orlean, 1997), or both. Nonetheless, it also seems possible
that Bud8p and Bud9p are also modified in some other
way(s).

It is also unclear whether the glycosylation serves any
specific role(s) in the function of the proteins. One plausible
possibility is that the extended conformations expected to
result from O-glycosylation (Jentoft, 1990) cause Bud8p and
Bud9p to project through the periplasmic space and into
intimate interaction with the cell wall; as noted above, such
interaction could help to explain how the bipolar markers
can apparently remain in place through multiple cell cycles.
The interactions could be either with polysaccharide com-
ponents of the wall or with one or more of the cell-wall
proteins that are themselves linked to the polysaccharides
(Orlean, 1997; Kapteyn et al., 1999). In this regard, it is
intriguing that not only the polysaccharide chitin but also
some of the cell-wall proteins (Bony et al., 1998; Ram et al.,
1998) have restricted spatial distributions.

However, these arguments also raise a difficult question: if
Bud8p and Bud9p are really anchored in the cell wall, how
can we explain the apparent movements of Bud8p (its ap-
parent dispersion late in the cell cycle and coalescence just
before bud emergence), as seen in Figure 9 and the accom-
panying movie? Although we cannot yet answer this ques-
tion, it should be noted that the apparent dispersion of
Bud8p may actually be an artifact (of the overexpression
used, of gradual inactivation of the GFP, of cleavage fol-
lowed by diffusion of the GFP moiety, or of some combina-
tion of these factors), and the apparent coalescence may
actually be the delivery of a new bolus of Bud8p to the
presumptive bud site (which is expected in any case, given
that daughter cells do not always form their first buds at
their distal poles).

Another important question is whether the distal-pole and
proximal-pole markers consist of Bud8p and Bud9p alone or
of these proteins in combination with others. Although Zah-
ner et al. (1996) identified two bud8 mutants and three bud9
mutants, this screen was almost certainly not saturated,
particularly as mutations affecting the hypothetical Bud8p and Bud9p partners might not have such strong effects on the budding patterns. In this regard, the inability of Triton X-100 to solubilize Bud8p or Bud9p may be relevant. Although this inability could just reflect variations in the efficiency with which integral-membrane proteins are extracted by this detergent (Deshaias and Schekman, 1990; Roemer and Bussey, 1995; Jiang et al., 1996; Lin et al., 1998; Lodder et al., 1999), it might also reflect association of Bud8p and Bud9p with other proteins in complexes that are not disrupted by Triton X-100.

Another very interesting question is how the structurally similar Bud8p and Bud9p proteins become localized to the opposite poles of the cell. Clearly, one possibility is that each polypeptide contains targeting signals that direct it (by unknown mechanisms) to the appropriate site. However, another attractive possibility is that localization depends primarily on time of expression during the cell cycle. Ideally before bud emergence, many proteins are delivered to the presumptive bud site and form a “cap” there and subsequently at the tip of the emerging bud (Lew and Reed, 1995; Pruyn and Bretscher, 2000a,b); the delivery is actin-dependent in some cases and actin-independent in others (Ayssough et al., 1997). Thus, if vesicles containing Bud8p are available for delivery to the plasma membrane only at that time, Bud8p could arrive at the presumptive bud site as part of this general traffic. Similarly, late in the cell cycle, many of the “cap proteins” (plus some others) become relocalized to the neck region (Lew and Reed, 1995; Pruyn and Bretscher, 2000a,b); thus, if vesicles containing Bud9p are available for delivery to the plasma membrane only at that time, Bud9p could arrive at the neck region as part of this general traffic (although this hypothesis would not in itself explain the asymmetric distribution of Bud9p at the neck). Consistent with this model, it has been reported that both BUD8 and BUD9 mRNAs peak periodically during the cell cycle (Cho et al., 1998; Spellman et al., 1998). This model also suggests that overexpression (or mistimed expression) of either protein would result in its appearance at the site appropriate for the other, as we have apparently observed.

The experiments with LatA appear to establish that the Bud8p that will mark the distal pole of the daughter cell arrives at the presumptive bud site of the mother cell in an actin-dependent manner. These observations presumably mean that the effect of bni1 mutations on distal-pole budding and the localization of Bud8p can be interpreted in terms of Bni1p’s known role in the organization of the actin cytoskeleton and without invoking a connection to the septins (see RESULTS). However, the actin dependence of Bud8p localization also creates a real mystery: how can so many other mutations that perturb the actin cytoskeleton have little or no effect on distal-pole budding and Bud8p localization (see RESULTS)?

Possible Relevance to Other Organisms

Determining an appropriate axis is a central problem for all cells that must polarize or divide asymmetrically. It remains unclear whether the mechanisms used by S. cerevisiae cells to select bud sites have close parallels in other types of cells. In particular, no homologs of Bud8p or Bud9p have been identified as yet in other organisms (including, rather surprisingly, Candida albicans). Nonetheless, we suggest that the mechanism of generating a spatially and temporally persistent marker by anchoring a transmembrane protein in extracellular material has such obvious utility that it will also be found in other types of cells (or at least among other cells with cell walls). Such mechanisms could have arisen independently during evolution or even be homologous to that in S. cerevisiae, because the sequence constraints on the marker proteins seem likely to be weak (so that residual sequence homology could be difficult or impossible to detect). It will be interesting to seek such parallels as more is learned about the mechanisms for axis selection in other types of cells.

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