We reported recently that Bud2 also localizes to the presumptive bud sites that are distinct in different cell types (1). Although localization of Bud2 and Bud5 has not been examined in the same cells, the apparent colocalization of Bud2 and Bud5 at late G1 raised a question whether localization of Bud2 and Bud5 is interdependent. We found that Bud2-GFP localized at a random site in α *bud5Δ* cells and Bud5-GFP also localized at a random site in α *bud2Δ* cells at late G1 (H.-O. P., unpublished results). Bud5-GFP localized as a ring at the cell division site in α *bud2Δ* mutants as in wild-type cells (about 80% of unbudded cells), but then a new patch of Bud5 often appeared at a site away from the previous division site. However, both Bud2-GFP and Bud5-GFP still localized in a discrete patch rather than dispersed throughout the cytoplasm in the absence of the other protein, suggesting that each protein localized independently to the incipient bud site. Bud2-GFP and Bud5-GFP also localized similarly to those seen in wild-type cells during the rest of the cell division cycle in the absence of the other protein. Thus, it is likely that Bud2 and Bud5 interact independently with cell-type specific landmarks to be localized to a presumptive bud site. Isolation of *bud2* and *bud5* mutants that are specifically defective in bipolar budding pattern also supports this view (2; P. J. K. & H.-O. P, unpublished results). One intriguing point is that Bud2-GFP and Bud5-GFP fail to localize to the proper bud site in the absence of the other protein and cells fail to bud at the proper bud site, which is consistent with idea that localization of both Bud2 and Bud5 is important for coupling of spatial signals to the polarity establishment.

Methods and Material

Plasmids & Yeast strains

Yeast strains used in this study are listed in Supplemental Table 1.

Construction of strains expressing *BUD5-GFP*. To express Bud5 fused to GFP, a 720 bp fragment coding for GFP(S65T, V163A, S175G) (3) was inserted just before the stop codon of *BUD5* in pRS304-BUD5. The plasmid expressing Bud5-GFP was integrated into the *BUD5* locus of an α *bud5Δ* strain (IH2421) and an **a** *bud5Δ* strain (IH2423) (4), which bud in a random manner. The resulting strains, HPY307 (α *BUD5-GFP*) and HPY308 (**a** *BUD5-GFP*), budded in an axial manner, indicating that Bud5-GFP is fully functional (data not shown). Similarly, HPY342 (**a** *BUD5-GFP*) and HPY343 (α *BUD5-GFP*) were generated from YEF473A and YEF473B (gifts from J. Pringle), respectively. A diploid **a**/α strain expressing Bud5-GFP (HPY309) was constructed by mating HPY307 and HPY308. Cells of HPY309 exhibited the bipolar budding pattern (data not shown), indicating the Bud5-GFP is also functional in diploid **a**/α cells. Another diploid **a**/α strain (HPY358) expressing Bud5-GFP was generated by mating HPY342 and HPY343.

Construction of strains expressing *BUD5-b1-GFP*. Oligonucleotide site-directed mutagenesis was performed on pRS304-BUD5-GFP using QuickChange site-directed mutagenesis kit (Stratagene) to introduce *bud5-b1*$^{R17A, E18A}$ mutation. The resulting plasmid was integrated into the *BUD5* locus of IH2421 (α *bud5Δ*), generating HPY378 (α *bud5-b1*$^{R17A, E18A}$). A heterozygous diploid HPY388 was generated by mating HPY378 and IH2423 (**a** *bud5Δ*).

Construction of mutant strains expressing *BUD5-GFP*. The *bud3* and *bud4* deletion mutants carrying *BUD5-GFP* were isolated by meiotic tetrad analysis from crosses HPY307 x JC1028 (5) and HPY308 x SY298 (6), respectively. The *axl2* deletion mutant carrying *BUD5-GFP* was generated by polymerase chain reaction (PCR) as described in Roemer et al. (7). The *axl1* mutant carrying *BUD5-GFP* was generated by one-step gene disruption using pHP871 (pUC19-axl1::HIS3). The haploid **a** (HPY345) and α *bud9* deletion (HPY346) mutants carrying *BUD5-*

GFP were generated by integration of pRS304-BUD5-GFP at the *BUD5* locus in YHH613 and YHH614 (gifts from J. Pringle), respectively, after deletion of the endogenous *BUD5* gene. HPY359 (**a**/α *bud9Δ/bud9Δ BUD5-GFP/BUD5-GFP*) was generated by mating HPY345 and HPY346. The α (HPY367) and **a** (HPY368) haploid strains carrying *bud8Δ* and *BUD5-GFP* were isolated by meiotic tetrad analysis from crosses HPY343 x YHH394 (gift from J. Pringle) and HPY342 x YHH391 (gift from J. Pringle), respectively. HPY373 (**a**/α *bud8Δ /bud8Δ BUD5-GFP/BUD5-GFP*) was generated by mating HPY368 and HPY367. The genotype of the strains was confirmed by genomic PCR.

Construction of a strain expressing *BUD5-HA* and plasmids carrying AXL2-Protein C and GST-AXL2^{C}. To express HA epitope-tagged Bud5, a 111 bp DNA fragment encoding three copies of the HA epitope was inserted just before the stop codon of *BUD5* in pRS304-BUD5. The resulting plasmid was integrated into the *BUD5* locus in a protease-deficient strain, HPY16, resulting in HPY421. A diploid strain (HPY429) expressing *BUD5-HA* was generated from HPY421 using YCp-HO (8). To express Protein C-epitope-tagged Axl2, a double-stranded oligonucleotide encoding 12 amino acids (EDQVDPRLIDGK) of the Protein C epitope (9) was inserted just before the stop codon of *AXL2* in YEp-AXL2 (7). Expression of Bud5-HA and Axl2-Protein C restored the wild-type budding pattern to *bud5Δ* and *axl2Δ* strains (data not shown), respectively, indicating that the tagged proteins are fully functional. To express the cytoplasmic domain of Axl2 as a GST fusion protein (GST-Axl2^{C}) in yeast, a 1.33 Kb BamHI - SalI fragment encoding amino acid residues 529 - 823 of Axl2 was inserted into pRD56 (10).

Immunoprecipitation and Glutathione-Sepharose Column Chromatography

Yeast cell extracts were prepared from protease-deficient strains, HPY421 and HPY429 (see Supplemental Table 1), carrying YEp-AXL2-Protein C using lysis buffer (50 mM HEPES, 50 mM KCl, 1mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 1% Triton X-100) and glass beads as

previously described (10). Subsequently, Axl2-Protein C was immunoprecipitated using an anti-Protein C affinity matrix (Roche Molecular Biochemicals) in the presence of 1mM $CaCl_2$ and the association of Bud5 with Axl2 was determined by immunoblotting with antibodies against HA epitope (HA11 from BAbCo).

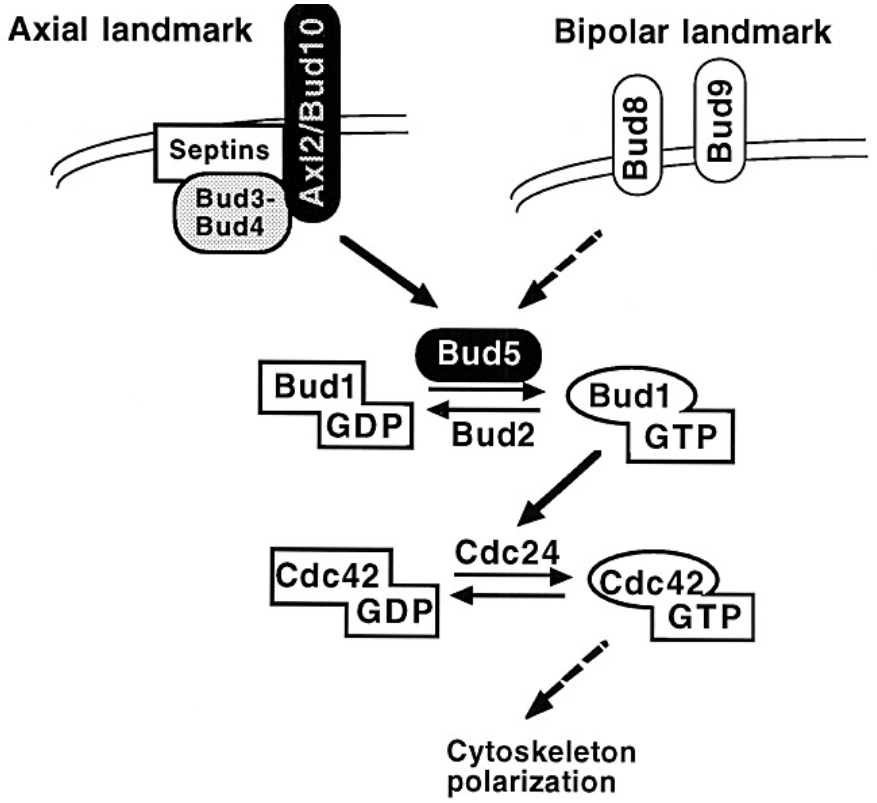
GST-$AXL2^C$ was purified from yeast strains, HPY421 and HPY429, carrying pRD56-$AXL2^C$ as previously described (10). To determine whether Bud5 interacts with the cytoplasmic domain of Axl2, eluents of the glutathione-Sepharose column (Pharmacia) were analyzed by immunoblotting with antibodies against HA epitope.

Microscopy

To view Bud5-GFP, cells were grown to early log phase and visualized using a Nikon Eclipse microscope fitted with a 100X immersion objective (N.A.= 1.30) as previously described (1). Images were collected using a Micromax digital camera (Princeton Instruments) and analyzed with IPLab software (Signal Analytics Corporation, Vienna, VA). Bud scars and birth scars were visualized by staining with Calcoflour as previously described (11).

Supplemental Figure 1. A model for spatial control of cell polarity during yeast budding.

Bud5 is likely to be localized to the presumptive bud site in haploid **a** and α cells through the interaction with Axl2, a component of the axial landmark. Bud5 locally activates Bud1 to the GTP-bound state, which then associates with proteins necessary for bud site assembly. Bud5 may interact with Bud8 or Bud9, putative landmarks of diploid **a**/α cells, thus direct polarity establishment for bipolar budding pattern (see text).



Supplemental Table 1. Yeast Strains Used in this Study

Strain	Relevant Genotype	Source/Reference
IH2390	α *HMRα HMLα his4 trp1 ura3*	12
IH2421*	α *bud5Δ::URA3*	4
IH2423*	**a** *bud5Δ::URA3*	4
HPY307*	α *bud5Δ::URA3 BUD5-GFP*	This study
HPY308*	**a** *bud5Δ::URA3 BUD5-GFP*	This study
HPY309*	**a**/α *bud5Δ::URA3 BUD5-GFP/bud5Δ::URA3 BUD5-GFP*	This study
HPY378*	α *bud5Δ::URA3 bud5-b1$^{R17A, E18A}$-GFP*	This study
HPY388*	**a**/α *bud5Δ::URA3 bud5-b1$^{R17A, E18A}$-GFP/bud5Δ::URA3*	This study
JC1028*	**a** *bud3Δ::URA3*	5
SY298*	α *bud4Δ::TRP1*	6
HPY352*	α *bud3Δ::URA3 bud5Δ::URA3 BUD5-GFP*	This study
HPY353*	**a** *bud4Δ::TRP1 bud5Δ::URA3 BUD5-GFP*	This study
YEF473A	**a** *trp1 leu2 ura3 his3 lys2*	J. Pringle
YEF473B#	α	J. Pringle
YHH613#	**a** *bud9Δ::HIS3*	J. Pringle
YHH614#	α *bud9Δ::HIS3*	J. Pringle
YHH394#	**a** *bud8Δ::TRP1*	J. Pringle
YHH391#	α *bud8Δ::TRP1*	J. Pringle
HPY342#	**a** *bud5Δ::URA3 BUD5-GFP*	This study
HPY343#	α *bud5Δ::URA3 BUD5-GFP*	This study
HPY345#	**a** *bud9Δ::HIS3 bud5Δ::URA3 BUD5-GFP*	This study
HPY346#	α *bud9Δ::HIS3 bud5Δ::URA3 BUD5-GFP*	This study
HPY357#	**a** *axl2Δ::HIS3 bud5Δ::URA3 BUD5-GFP*	This study
HPY358#	**a**/α *bud5Δ::URA3 BUD5-GFP/bud5Δ::URA3 BUD5-GFP*	This study

HPY359#	**a**/α *bud5Δ::URA3 BUD5-GFP/bud5Δ::URA3 BUD5-GFP bud9Δ::HIS3/bud9Δ::HIS3*	This study
HPY367#	α *bud8Δ::TRP1 bud5Δ::URA3 BUD5-GFP*	This study
HPY368#	**a** *bud8Δ::TRP1 bud5Δ::URA3 BUD5-GFP*	This study
HPY373#	**a**/α *bud5Δ::URA3 BUD5-GFP/bud5Δ::URA3 BUD5-GFP bud8Δ::TRP1/bud8Δ::TRP1*	This study
HPY458#	**a** *axl1::HIS3 bud5Δ::URA3 BUD5-GFP*	This study
HPY16	**a** *ura3 his3 leu2 trp1 prb1 pep4 prc1*	10
HPY421+	**a** *BUD5-HA*	This study
HPY429+	**a**/α *BUD5-HA/BUD5-HA*	This study

* Isogenic to IH2390 except where noted.

Isogenic to YEF473A except where noted.

+ Isogenic to HPY16 except where noted.

Legends to Supplemental Table 2. Summary of Bud5-GFP localization

The Bud5-GFP localization pattern is summarized for unbudded cells and cells with large-sized buds (included cells whose bud size is about 3/4 of the mother cell or bigger). Each number represents percent of cells showing each localization pattern where n is the total number of cells examined. Localization of Bud5-GFP in haploid and diploid wild-type cells was analyzed in two different strain backgrounds. The results were very similar in haploid cells and minor differences were observed in diploid cells of different strain backgrounds (data not shown).

a. Mixed pattern of localization.

b. Bud5-GFP signal in these cells did not appear clearly as a ring compared to those seen at the division site in wild type cells.

c. It was difficult to distinguish in this case whether a new patch overlapped with the ring inherited from the previous division.

d. It was difficult to distinguish whether a single ring or a double ring was present. Signals at the neck of diploid cells were not as discrete as those in haploid cells.

e. These numbers in () indicate the percentage of cells with Bud5-GFP signal only at the poles of mother cells or bud tip, but no signal at the neck.

Supplemental Table 2.

Relevant Genotype					other[a]	n
a WT	80	<1	5	14		200
α *bud3Δ*	12[b]	10[b]	29	47	2	246
a *bud4Δ*	24[b]	9[b]	41	26		150
a *axl2Δ*	26[b]	38[b]	30	4	2	435
a/α WT	25	70	<1	5		252
a/α *bud8Δ/bud8Δ*	67[c]	<1	13	16	4	257
a/α *bud9Δ/bud9Δ*	18	49	9	16	8	181

Relevant Genotype										other[a]	n
a WT	80	15	4							1	150
α *bud3Δ*	21[d]		6 (12)[e]	3 (1)[e]	2			(9)[e]	42	4	279
a *bud4Δ*	25	32	15 (2)[e]	9	4				11	2	100
a *axl2Δ*	1[d]		1 (18)[e]	7 (2)[e]	4 (44)[e]	13		(2)[e]	4	4	174
a/α WT	10[d]		36 (7)[e]	13	27			3	1	3	200
a/α *bud8Δ/bud8Δ*	26[d]			5	<1		19	12 (11)[e]	24	3	196
a/α *bud9Δ/bud9Δ*	15[d]		46 (3)[e]					14 (13)[e]	6	3	257

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