Control of Mitotic Spindle Position by the *Saccharomyces cerevisiae* Formin Bni1p

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Abstract. Alignment of the mitotic spindle with the axis of cell division is an essential process in *Saccharomyces cerevisiae* that is mediated by interactions between cytoplasmic microtubules and the cell cortex. We found that a cortical protein, the yeast formin Bni1p, was required for spindle orientation. Two striking abnormalities were observed in *bni1Δ* cells. First, the initial movement of the spindle pole body (SPB) toward the emerging bud was defective. This phenotype is similar to that previously observed in cells lacking the kinesin Kip3p and, in fact, *BNI1* and *KIP3* were found to be in the same genetic pathway. Second, abnormal pulling interactions between microtubules and the cortex appeared to cause preanaphase spindles in *bni1Δ* cells to transit back and forth between the mother and the bud. We therefore propose that Bni1p may localize or alter the function of cortical microtubule-binding sites in the bud. Additionally, we present evidence that other bipolar bud site determinants together with cortical actin are also required for spindle orientation.

Key words: mitotic spindle apparatus • cell division • microtubule • actin • *Saccharomyces cerevisiae*

CELL division requires the correct positioning of the spindle within the cell in addition to assembly of the mitotic apparatus and segregation of the chromosomes (Hyman and Karsenti, 1996; Stearns, 1997). Significant progress has been made in characterizing the proteins involved in spindle construction and chromosome movement, but little is known about the proteins that mediate interactions between microtubules and the cell cortex to establish spindle position.

Various strategies to align the spindle with the plane of cell division are used in different organisms (Rhyu and Knoblich, 1995). In budding yeast, the axis of division is determined by the signaling molecules that control bud site selection (Freifelder, 1960; Chant and Pringle, 1995). The polarity of the dividing yeast cell is established first and then the spindle is aligned in response to this polarity to segregate the nuclei. This alignment is postulated to be mediated by interactions between cytoplasmic microtubules and asymmetrically localized cortical proteins (Snyder et al., 1991; Hyman and Stearns, 1992). Spindle positioning in plant cells is similar to yeast in that the spindle appears to be aligned by preestablished cortical cues (Staiger and Lloyd, 1991). By contrast, in animal cells spindle position determines the position of cytokinesis (Cao and Wang, 1996). This is important for development because correct spindle position is essential for generating asymmetric cell divisions. Furthermore, there are several examples in animal cells where spindle position appears to be developmentally regulated (Hyman and White, 1987; Hyman, 1989; Chenn and McConnell, 1995; Rhyu and Knoblich, 1995; Kraut et al., 1996). Despite the cell type differences in the relationship between spindle position, cell polarity, and cytokinesis, many of the molecular components involved in these processes are conserved, suggesting that some of the mechanisms linking microtubules to cortical polarity factors may also be conserved (Drubin and Nelson, 1996).

The establishment of spindle position in *Saccharomyces cerevisiae* occurs in distinct morphological steps (Yeh et al., 1995; DeZwaan et al., 1997; Carminati and Stearns, 1997; Shaw et al., 1997, 1998). First, the centrosome or spindle pole body (SPB)[1] migrates toward the incipient bud site. Around the time of SPB separation and the formation of a bipolar spindle, cytoplasmic microtubules penetrate into

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1. *Abbreviations used in this paper: CEN*, centromere-based; DAPI, 4′,6′-diamidino-2-phenylindole; DIC, differential interference contrast; GFP, green fluorescent protein; Lat-A, Latrunculin-A; SPB, spindle pole body.

the bud, and the spindle assumes a relatively stable position at the neck aligned with the mother–bud axis (Shaw et al., 1997). At anaphase, the spindle is inserted across the bud neck and then elongates, segregating the chromosomes between mother and daughter cells (Yeh et al., 1995). Genetic analysis has identified two partially redundant pathways for spindle orientation. One mediated by the kinesin Kip3p, is necessary for the initial movement of the SPB toward the bud. The other is mediated by cytoplasmic dynein which is necessary for the insertion of the spindle through the bud neck (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Stearns, 1997). It is not known if these motors act primarily by affecting the dynamics of cytoplasmic microtubules or whether they directly link cytoplasmic microtubules to the cell cortex.

One of the most interesting questions about the mechanism of spindle orientation is the identity and molecular function of the cortical proteins that interact with cytoplasmic microtubules. The existence of microtubule "capture sites," loosely analogous to those at the kinetochore, has been postulated, but little is known about the molecular composition of these cortical regions (Kaverina et al., 1998; Snyder et al., 1991). One factor that is clearly important for controlling spindle position in diverse organisms is the actin cytoskeleton. In *S. cerevisiae*, mutations in *ACT1*, the gene that encodes actin, result in defects in spindle position and nuclear segregation (Palmer et al., 1992; Drubin et al., 1993). In *Caenorhabditis elegans*, actin is required for the developmentally regulated rotation of the spindle during specific early embryonic cell divisions (Hyman and White, 1987; Hyman, 1989). Actin is also required for centrosome movement in human leukocytes (Euteneuer and Schliwa, 1985). The molecular role of actin in these different systems is not known. Cortical actin and associated proteins might bind microtubules or alternatively, they might be required to localize cortical microtubule-binding proteins. Recent genetic analysis in yeast has identified two novel cortical proteins, Num1p and Kar9p, that are required for spindle orientation. Num1p localizes to the mother cell cortex (Farkasovsky and Küntzel, 1995), whereas Kar9p localizes to a discrete region of the bud cortex and to the tip of the mating projection (shmoo). Although it has not been determined if these proteins bind microtubules, it is interesting that overexpressed Kar9p concentrates at sites of contact between cytoplasmic microtubules and the bud cortex (Miller and Rose, 1998).

Here we report a requirement for the conserved protein Bni1p in spindle orientation. Bni1p belongs to a family of proteins called formins, which are required for diverse cellular and developmental functions (Woychik et al., 1990; Castrillon and Wasserman, 1994; Emmons et al., 1995; Petersen et al., 1995; Zahner et al., 1996; Chang et al., 1997; Evangelista et al., 1997; Harris et al., 1997). Formins are characterized by conserved regions called formin homology (FH) domains. There is substantial evidence to suggest that Bni1p and other formins function as molecular scaffolds that link Rho-type GTPases with components of the actin cytoskeleton. The NH_2-terminal domain of Bni1p interacts with the activated forms of G proteins, Cdc42p and Rho1p, which regulate organization of the actin cytoskeleton (Kohno et al., 1996; Evangelista et al., 1997). The proline-rich FH1 domain of Bni1p binds profilin and this interaction may regulate actin polymerization at cortical sites (Evangelista et al., 1997; Imamura et al., 1997). The COOH-terminal domain of Bni1p interacts with another putative actin-binding protein, Aip3p/Bud6p (Amberg et al., 1997; Evangelista et al., 1997). In *S. cerevisiae*, a second formin, Bnr1p, has at least partial functional overlap with Bni1p (Imamura et al., 1997). Whether formins link polarity signals to other cytoskeletal systems, such as the microtubule cytoskeleton, has not been established.

Bni1p has been implicated in actin function during polarized morphogenesis and the establishment of cell polarity. Bni1p is localized in a cap-like structure (Jansen et al., 1996; Evangelista et al., 1997; Fujiwara et al., 1998), similar to that observed for Cdc42p and other polarity determinants (Ziman et al., 1993) at sites of polarized cell growth: the bud tip early in the cell cycle, the septal region late in the cell cycle, and to the shmoo tip. *bni1Δ* mutant cells contain normal cortical actin patches and cables, but are defective for the reorganization of these structures required for various aspects of cell polarization (Evangelista et al., 1997). *bni1Δ* cells are normal for bud emergence, but they are defective for polarized bud growth and appear to construct buds through isotropic growth, generating cells with a rounded shape. *bni1Δ* cells are also defective in shmoo formation, pseudohyphal growth, localization of *ASH1* mRNA to the bud, cytokinesis, and bipolar bud site selection (Jansen et al., 1996; Zahner et al., 1996; Evangelista et al., 1997; Mösch and Fink, 1997). The role of Bni1p in bipolar bud site selection is notable because Bni1p interacts with two other proteins, Spa2p and Bud6p, required for this process (Evangelista et al., 1997; Fujiwara et al., 1998). The molecular links between Bni1p and actin may also be relevant to bipolar bud site selection because many actin mutants also affect this process (Yang et al., 1997).

We have now found that Bni1p is required for specific steps in establishing the position of the mitotic spindle before anaphase. By fluorescence time-lapse microscopy, we observed that Bni1p was required for the initial movement of the SPB toward the incipient bud site. In addition, a strikingly abnormal movement of preanaphase spindles was observed in *bni1Δ* cells: short spindles were pulled back and forth between the mother cell and the bud. In wild-type cells, apparent pulling forces on the spindle were observed toward the bud, but not toward the mother cell. These findings suggest that in the absence of Bni1p, microtubule-binding sites at the cell cortex are either mislocalized or functionally altered, resulting in the loss of polarization of preanaphase spindle movement. Finally, analysis of mutations affecting the actin cytoskeleton suggest that actin structures involved in bipolar budding are also required for spindle orientation.

Materials and Methods

Microbial Techniques

Media and genetic techniques were as described (Rose et al., 1990). The mating pheromone, α-factor, was added to log phase cultures at 6 μg/ml. Latrunculin-A (Lat-A) (from P. Crews, University of California, Santa Cruz, CA) was added to cultures at 400 mM. Benomyl sensitivity (a gift from E.I. du Pont de Nemours, Wilmington, DE) was tested in rich (YPD) medium. Geneticin (GIBCO BRL) was used at a concentration of 0.2 μg/

Table I. Strains Used

Strains	Genotype	Source
PY1746	*MAT***a** *ase1::URA3 ade2-100 ade3 leu2-3,112 lys2-801 trp1-1 ura3-1*	D. Pellman
PY1994	*MAT***a** *ase1::kanR ade2-100 ade3 leu2-3,112 lys2-801 trp1-1 ura3-52 {2μ ASE1 ADE3 TRP1}*	This study
PY1996	*MAT*α *ase1::kanR ade2-100 ade3 leu2-3,112 lys2-801 trp1-1 ura3-52 {2μ ASE1 ADE3 TRP1}*	This study
PY2400	*MAT***a** *kip3::HIS3 ade2-100 leu2-3,112 his3-11,15 trp1-1 ura3-1*	This study
PY2401	*MAT***a** *kip3::HIS3 bni1::kanR ura3-1 ade2-100 his3-11,15 trp1-1 leu2-3,112*	This study
PY2403	*MAT***a** *bni1::kanR ade2-100 leu2-3,112 his3-11,15 ura3-1 trp1-1 ade3*	This study
PY2409	*MAT***a** *kip2::URA3 ura3-1 leu2-3,112 his3-11,15 ade2-100 trp1-1*	This study
PY2513	*MAT***a** *bni1::kanR ade2-100 ade3 leu2-3,112 his3-11,15 trp1-1 ura3-1*	This study
PY2515	*MAT***a** *ase1::URA3 ade2-100 leu2-3,112 lys2 his3-11,15 ura3-1 trp1-1*	This study
PY2516	*MAT***a** *dyn1::URA3 ade3 leu2-3,112 ura3-1 trp1-1*	This study
PY2581	*MAT***a** *ase1::URA3 bni1::kanR ade2-100 leu2-3,112 lys2 his3-11,15 ura3-1 trp1-1*	This study
PY2582	*MAT*α *dyn1::URA3 ade2-100 leu2-3,112 his3-11,15 trp1-1 ura3-1*	This study
PY2583	*MAT*α *dyn1::URA3 bni1::kanR leu2-3,112 his3-11,15 trp1-1 ura3-1*	This study
PY2595	*MAT***a** *ade2-101 leu2-3,112 his3Δ200 ura3-52 can1 cry1 NUF2::GFP::URA3*	This study
PY2596	*MAT***a** *ade2-101 leu2-3,112 his3-Δ200 ura3-52 can1 cry1 NUF2::GFP::URA3 act1-116::HIS3*	This study
PY2608	*MAT***a** *bud6::TRP1 leu2Δ1 lys2-801 his3Δ200 trp1Δ63 ura3-52 NUF2::GFP::URA3*	This study
PY2611	*MAT***a** *leu2Δ1 lys2-801 his3Δ200 trp1Δ63 ura3-52 NUF2::GFP::URA3*	This study
AFS306	*MAT***a** *kip1::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1*	A. Murray (University of California, San Francisco, CA)
AFS462	*MAT***a** *kar3::TRP1 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1*	A. Murray
DBY3357	*MAT***a** *ade2-101 leu2-3,112 his3Δ200 ura3-52 can1 cry1*	D. Botstein (Stanford University, Stanford, CA)
KAY0227	*MAT***a** *sla1::URA3 his3Δ200 ura3-52 {HIS3 CEN SLA1}*	D. Drubin (University of California, Berkeley, CA)
KAY0228	*MAT***a** *sla1::URA3 his3Δ200 ura3-52 {HIS3 CEN sla1ΔSH3#3}*	D. Drubin
MAY2058	*MAT***a** *cin8::LEU2 ade2-101 leu2-3,112 lys2-801 his3Δ200 ura3-52*	M.A. Hoyt (The Johns Hopkins University, Baltimore, MD)
PY2061	*MAT*α *smy1::LEU2 leu2 ura3 his4 trp1*	S. Brown (University of Michigan Medical School, Ann Arbor, MI)
Y66	*MAT*α *far1-c ade2-100 leu2-3,112 lys1 his3-11,15 trp1-1 ura3-1*	C. Boone
Y1353	*MAT***a**/*MAT*α *bni1::HIS3/bni1::HIS3 leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63lys2-801/lys2-801*	C. Boone
Y1445	*MAT***a** *bni1::kanR bar1::LEU2 ade2-100 ade3 leu2-3,112 his3-11,15 trp1-1 ura3-1*	C. Boone

ml in YPD. Lists of the strains and plasmids used in this study are provided in Tables I and II. Except where indicated, all strains are congenic to W3031a.

Strain and Plasmid Construction

A complete deletion of the *BNI1* coding sequence was created by one-step gene replacement with the selectable marker kan^r, which confers Geneticin resistance to yeast (Wach et al., 1994). The *bni1Δ::kan^r* containing DNA sequence was generated by PCR using oligonucleotide primers: 5′ TATCTATCTTCTGTATTGAGGAGAAACATTTTACTCAAGCAGCTGAAGCTTCGTACGC and 5′GAGTAAAGATGAATGTAAAGTGTATCATAAGTGATCTATAGCATAGGCCACTAGTGGATCTG. Deletion of the *BNI1* locus was confirmed by Southern blot analysis of Geneticin-resistant transformants. This *BNI1* deletion strain, hereafter referred to as *bni1Δ*, was used for all subsequent analyses unless specified otherwise. The deletion of *ASE1* was also created by one-step gene replacement (Pellman et al., 1995).

Table II. Plasmids Used

Plasmid	Markers	Source
PB1046	*bni1-CTΔ1 LEU2*	This study
pFA-KanMX2	kan^r	P. Philippsen (University of Basel, Basel, Switzerland)
pJIL67	*NUF2::GFP URA3*	P. Silver (Dana-Farber Cancer Institute/ Harvard Medical School, Boston, MA)
pAFS125	*GFP::TUB1 URA3*	A. Murray
p182	*BNI1 URA3 CEN ARS*	C. Boone
p491	*bni1-CTΔ1 URA3 CEN ARS*	C. Boone
p1832	*BNI1 TRP1 CEN ARS*	C. Boone
p2029	*bni1-CTΔ1 TRP1 CEN ARS*	C. Boone
pRB1537	*act1-116::HIS3*	D. Botstein

The centromere-based *(CEN)* plasmid carrying *BNI1* (p182) has been described (Boone et al., 1992). This genomic sequence was also cloned into a *CEN ARS TRP1* plasmid (pRS314) (Sikorski and Hieter, 1989) to create p1832. The COOH-terminal deletion mutant, *bni1-CTΔ1*, lacks the coding sequence for amino acids 1,749–1,953 of Bni1p, and was created by the following strategy. p182 was digested with SstII which cuts *BNI1* at bp 5,249 and in the polylinker, and ligated to an oligonucleotide (5′GTAGGCGGCCGCCTACGC). This created a stop codon followed by a NotI site adjacent to the SstII site in *BNI1*. A *CEN* plasmid (p2029) derived from pRS314 and an integrating plasmid (PB1046) derived from pRS305 (Sikorski and Hieter, 1989) contain the *bni1-CTΔ1* sequence on a 6.3-kb BamHI/NotI restriction fragment. The strain containing PB1046 was used for genetic crosses, and the strain containing p2029 was used for morphological studies.

The *act1-116* and *sla1ΔSH3#3* strains and their isogenic wild-type controls are in the S288c genetic background. To construct PY2596, the *ACT1* locus was replaced by *act1-116::HIS3* from pRB1537 by homologous recombination in a strain containing *NUF2::GFP* (PB1000). This was done because previously constructed *act1-116* strains contain a linked *TUB2* mutation. Integration was confirmed by PCR analysis.

Genetic Analysis

The screen to identify mutations that were lethal in combination with an *ASE1*-null allele was performed using an *ADE3* sectoring assay as de-

scribed (Bender and Pringle, 1991). The starting strains *MAT*α and *MAT***a** *ase1::kan*^{r} *ade2 ade3 leu2 lys2 trp1 ura3 {2*μ *ASE1 ADE3 TRP1}* (PY1994 and PY1996) were mutagenized by transformation with a genomic library containing random insertions of a mini-Tn3lac*Z-LEU2* transposon (Burns et al., 1994). Candidate mutants were backcrossed to demonstrate that the mutation represented a single genetic locus and to confirm that the mutation cosegregated with the *LEU2* marker. The genomic site of transposon insertion was identified by direct sequencing of PCR products spanning the insertion site. These PCR products were generated by an arbitrary primed PCR strategy (Di Rienzo et al., 1996). The *bni1::Tn3::LEU2* allele has an insertion of the transposon at nucleotide 5,006 of the *BNI1*-coding sequence. This allele has an identical phenotype to the *bni1Δ::kan*^{r}-null allele described above. In addition to *BNI1*, genes that affect many aspects of microtubule function in *S. cerevisiae* were identified by this screen (to be described elsewhere).

For the *bni1Δ* × *ase1Δ* cross, 37 tetrads were dissected and 41 double mutants were recovered, all of which were temperature sensitive for growth. For the *bni1Δ* × *dyn1Δ* cross, 19 tetrads were dissected and 11 temperature-sensitive double mutants were recovered. For the *bni1Δ* × *kar3Δ* cross, 27 tetrads were dissected and 20 viable double mutants were recovered. *bni1Δ kar3Δ* double mutants formed smaller colonies than the *kar3Δ* single mutant at 23°C and were inviable at 30°C. For the *bni1Δ* × *cin8Δ* cross, 35 tetrads were dissected and 28 double mutants were recovered. For the crosses of *bni1Δ* with *kip1Δ* and *smy1Δ*, 23 and 17 tetrads were dissected respectively, and the double mutants were recovered at the expected frequency.

Quantitative mating assays (Sprague, 1991) were performed using a *MAT***a** *bni1::kan*^{r} *bar1::LEU2* strain (Y1445), carrying different alleles of *BNI1* on a *CEN* plasmid. The mating partner used for the mating assays was a *MAT*α *far1-c* strain (Y66) which was itself defective in mating and thus increased the sensitivity of the assay. The assays were performed in triplicate.

The strain background used for our initial genetic experiments and for our time-lapse analysis, W3031a, has a mixed random/bipolar budding pattern (Cvrckova et al., 1995). Therefore, in experiments where the movement of the SPB after telophase toward the bud was followed by fluorescence microscopy, the distance between the SPB and the bud was determined by overlaying images from each time point with a differential interference contrast (DIC) image obtained after bud emergence. The spindle orientation defect of *bni1Δ* that we observed is not specific to this strain background because we observed a similar spindle orientation defect in a YEF473-derived *bni1Δ* strain (Bi and Pringle, 1996) that has normal axial and bipolar bud site selection (data not shown).

Microscopic Analysis of Cells

Nuclear morphology was observed by fluorescence microscopy in cells fixed and stained with 4′,6′-diamidino-2-phenylindole (DAPI) as described (Rose et al., 1990). To preserve green fluorescent protein (GFP) fluorescence for the analysis of spindle orientation, cells were fixed in culture medium with 3.7% formaldehyde for 30 min, washed, and then applied to slides. Indirect immunofluorescence was performed as described (Pellman et al., 1995). Experiments to measure nuclear or spindle position were performed at least twice.

Budding pattern was assayed after staining bud scars with calcofluor (Pringle et al., 1989). The strain used was a *bni1::HIS3/bni1::HIS3* YEF473 diploid (Y1353), containing alleles of *BNI1* on a *CEN* plasmid. Results from these strains were compared with results from an isogenic *BNI1/BNI1* diploid. The fact that *BNI1* carried on a *CEN* plasmid does not fully complement *bni1::HIS3/bni1::HIS3* suggests that the gene dosage of *BNI1* may be important for bipolar bud site selection. Mating projection assays using a W3031a *MAT*a *bni1::kan*^{r} *bar1::LEU2* strain (Y1445) containing the *bni1* alleles were performed in triplicate (Evangelista et al., 1997).

For live cell fluorescence microscopy, cells were applied to a microscope slide (DeZwaan et al., 1997) and observed at 23°C. The doubling time of cells in liquid culture at this temperature was 150 min, compared with the average doubling time of 176 min for cells observed with our time-lapse fluorescence protocol. A microscope (model ECLIPSE E600; Nikon) equipped with a 100×/1.4 NA Planapochromat objective, a 100-W mercury arc illuminator, a Z-axis focus motor (Biopoint Z-axis focus motor; Ludl Electronics), a fluorescence illumination shutter (Ludl Electronics), and an Endow GFP filter set (excitation 450–490 nm, dichroic 495, emission 500 nm LP; Chroma Optics) was used. Images were acquired with a cooled charge-coupled device camera (Hamamatsu 4742-95, Hamamatsu Photonics), containing a 1280 × 1024 Sony Interline chip (Hamamatsu Photonics). At each time point a series of fluorescence images in six focal planes spaced 0.5 μm apart were collected along with a DIC image. To minimize photo bleaching and photo damage of the cells, the light from the mercury lamp was attenuated to one-eighth of maximal intensity with neutral density filters and exposure times were limited to 200–400 ms. Images were analyzed using Openlabs Software (Improvision), and all statistical analysis was performed using StatView software (Abacus Concepts).

Results

BNI1 Has a Role in Microtubule Function

An allele of *BNI1* was unexpectedly isolated in a genetic screen to identify genes involved in microtubule function. The screen was for mutations that were lethal when combined with an *ase1Δ* allele (see Materials and Methods). *ASE1* encodes a nonmotor microtubule-associated protein that localizes to the spindle midzone and promotes spindle elongation (anaphase B) (Pellman et al., 1995; Juang et al., 1997). The genetic interaction between *BNI1* and *ASE1* suggested that *BNI1* might have a role in maintaining the integrity of the microtubule cytoskeleton.

A deletion allele of *BNI1* was created (see Materials and Methods). The *bni1Δ ase1Δ* double mutant strain displayed a marked temperature-sensitive growth defect (Fig. 1 a). Because this strain grew at a near normal rate at 23°C, we were able to test its sensitivity to the microtubule depolymerizing agent, benomyl. In contrast to the control strains, the *bni1Δ ase1Δ* strain had a strikingly increased sensitivity to benomyl (5 μg/ml). *bni1Δ* and *ase1Δ* mutations therefore have an additive detrimental effect on microtubule function.

bni1Δ Cells Have a Defect in Spindle Orientation

Because Bni1p is localized at the bud cortex and septum of mitotic cells and the shmoo tip in mating cells (Jansen et al., 1996; Evangelista et al., 1997; Fujiwara et al., 1998) (Evangelista, M., and C. Boone, unpublished results) we considered the possibility that *BNI1* affects microtubules through a role in spindle orientation and/or nuclear positioning. Here, defects in the location of the nucleus within the mother cell or defects in nuclear segregation that result in binucleate mother cells will be referred to as "nuclear position defects." Defects in the alignment of the spindle with the mother–bud axis will be described as "spindle orientation defects." The role of Bni1p in nuclear positioning was initially tested by examining *bni1Δ* and control cells stained with the DNA-binding dye, DAPI. Whereas mitosis in wild-type cells results in the faithful distribution of nuclei to mother and daughter cells (Fig. 1 b), mitosis in a significant fraction of *bni1Δ* cells was abnormal, resulting in two nuclei in the mother cell. The fraction of binucleate mother cells in *bni1Δ* strains increased at 37°C (Fig. 1 b).

We next tested whether the binucleate phenotype of *bni1Δ* cells was due to a defect in spindle orientation. *bni1Δ* and control strains were grown to early logarithmic stage and the angle between the spindle and the mother–bud axis was measured (Fig. 2). A chimeric gene expressing a fusion between Nuf2p, a spindle pole body protein, and GFP was introduced into both strains, allowing visualization of the poles by fluorescence microscopy (Kahana

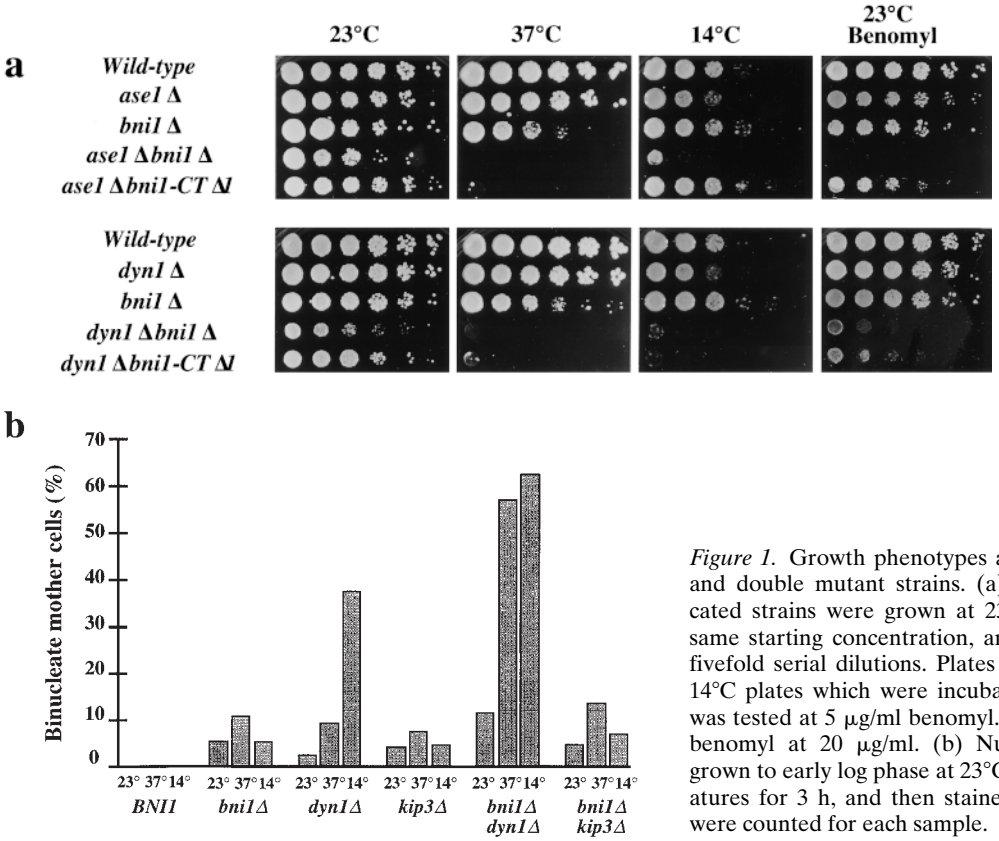

Figure 1. Growth phenotypes and nuclear segregation in *bni1Δ* and double mutant strains. (a) Growth phenotypes. The indicated strains were grown at 23°C to saturation, diluted to the same starting concentration, and then spotted onto medium in fivefold serial dilutions. Plates were incubated for 48 h, except 14°C plates which were incubated for 7 d. Benomyl sensitivity was tested at 5 μg/ml benomyl. Wild-type cells were sensitive to benomyl at 20 μg/ml. (b) Nuclear segregation. Strains were grown to early log phase at 23°C, shifted to the indicated temperatures for 3 h, and then stained with DAPI. At least 300 cells were counted for each sample.

et al., 1995; Kahana and Silver, 1996). In wild-type cells, more than 60% of cells had spindles oriented within 30 degrees of the mother–bud axis (Fig. 2 a). By contrast, spindle orientation in *bni1Δ* cells appeared to be random (Fig. 2 b). Furthermore, the distance between the neck-proximal SPB and the bud neck was significantly greater in *bni1Δ* than in *BNI1* cells: 1.0 and 0.7 μm, respectively (*t* test, $P < 0.0001$, $n = 180$). Thus, *bni1Δ* has a defect in spindle orientation.

Genetic Interactions Suggest that BNI1 and KIP3 Are in the Same Pathway

The entire set of *Saccharomyces cerevisiae* microtubule-based motor proteins has been defined. The genome contains a single gene encoding a dynein heavy chain, *DYN1*, and six genes encoding kinesin-related proteins, *KIP1, KIP2, KIP3, KAR3, CIN8,* and *SMY1*. Strains lacking any one of these genes are viable; however, combinations of different null alleles result in lethality. Only Dyn1p, Kip2p, Kip3p, and Kar3p are implicated in cytoplasmic microtubule function (Stearns, 1997; Winsor and Schiebel, 1997). Single mutant *kip3Δ* and *dyn1Δ* strains have the most striking effect on nuclear position. *kip3Δ* and *dyn1Δ* affect distinct steps in nuclear positioning: *kip3Δ* cells have a spindle orientation defect, whereas *dyn1Δ* cells have a defect in the insertion of the spindle across the bud neck. The fact that *kip3Δ dyn1Δ* double mutant strains are not viable suggests that there are two partially redundant pathways for spindle orientation (Cottingham and Hoyt, 1997; De Zwaan et al., 1997; Miller et al., 1998).

Double mutants were constructed to test if *BNI1* was in the *KIP3* pathway. Consistent with the similar spindle orientation defect observed in *bni1Δ* and *kip3Δ* strains, the *bni1Δ kip3Δ* strain was viable and grew indistinguishably from single mutant strains (Table III). The percentage of binucleate mother cells in the *bni1Δ kip3Δ* strain was also not increased relative to either single mutant strain (Fig. 1 b). By contrast, *bni1Δ* produced a striking synthetic interaction when combined with *dyn1Δ*. The *bni1Δ dyn1Δ* strain had a growth defect at 23°C and was inviable at 14° and 37°C (Fig. 1 a and Table III). Also, the *bni1Δ dyn1Δ* strain had a marked increase in binucleate mother cells relative to the single mutant strains (Fig. 1 b). Like the *bni1Δ ase1Δ* strain, the *bni1Δ dyn1Δ* strain had increased sensitivity to benomyl (Fig. 1 a).

The genetic interactions we observed between *bni1Δ* and the other motor gene deletions paralleled those observed with *kip3Δ* (Table III). Like *kip3Δ*, *bni1Δ* is lethal when combined with *kar3Δ*. *bni1Δ* did not show a significant synthetic interaction with either *kip1*, *cin8Δ*, or *smy1Δ*. This pattern of genetic interactions observed with *bni1Δ* suggests that *BNI1* and *KIP3* function in the same pathway.

Defective Movement of the SPB to the Incipient Bud in bni1Δ Cells

Because of the spindle orientation defect in *bni1Δ* and *kip3Δ* strains, we determined whether movement of the SPB to the incipient bud site was defective in *bni1Δ* cells

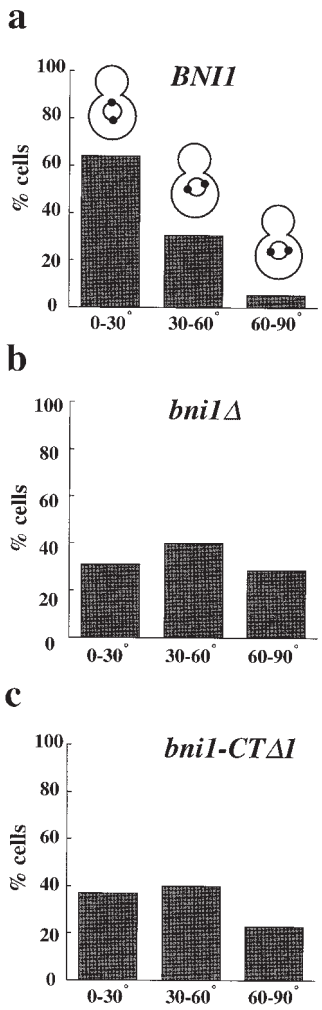

Figure 2. Spindle orientation in *BNI1* (a), *bni1Δ* (b), and *bni1-CTΔ1* (c) strains. Early log phase cultures were incubated at either 23° or 37°C for 3 h. The results shown are from the 37°C samples. The histograms show the percentages of cells with spindles oriented to the mother–bud axis at angles of 0°–30°, 30°–60°, and 60°–90°. 180 cells were examined for each sample. The mean spindle orientation angles are 23.7° (*BNI1*), 42.8° (*bni1Δ*), and 39.5° (*bni1-CTΔ1*) and the mean SPB-neck distances are 0.7, 1.0, and 1.1 μm, respectively. The mean spindle orientation angle of the *bni1Δ* and the *bni1-CTΔ1* strains were significantly different from that of the control (*t* test, $P < 0.0001$ for both). For samples maintained at 23°C, the mean spindle orientation angles were 25.2° (*BNI1*), 39.1° (*bni1Δ*), and 33.3° (*bni1-CTΔ1*). The mean angles of the *bni1Δ* and the *bni1-CTΔ1* strains at 23°C were also significantly different from the control strain (*t* test, $P < 0.0001$ and $P = 0.0005$, respectively).

Table III. Genetic Interactions between bni1Δ and Motor Mutations

	23°C	37°C	14°C	30°C
Growth phenotype of single mutants				
bni1Δ	+++	++	+++	ND
kip3Δ	+++	+++	+++	ND
dyn1Δ	+++	+++	+++	ND
kar3Δ	+	−	+	+
kip1Δ	+++	+++	+++	ND
kip2Δ	+++	+++	+++	ND
cin8Δ	+++	+/−	+++	ND
smy1Δ	+++	+++	+++	ND
Growth phenotype of double mutants				
bni1Δ kip3Δ	+++	++	+++	ND
bni1Δ dyn1Δ	++	−	−	ND
bni1Δ kar3Δ	+	−	+	−
bni1Δ kip1Δ	+++	++	+++	ND
bni1Δ kip2Δ	+++	++	+++	ND
bni1Δ cin8Δ	+++	−	+++	ND
bni1Δ smy1Δ	+++	++	+++	ND

Each strain was struck on a YPD plate and incubated at the appropriate temperatures. For 23°, 30°, and 37°C test, plates were scored after 48 h. For 14°C test, plates were scored after 7 d.

as previously observed in *kip3Δ* cells. Time-lapse fluorescence microscopy of live cells expressing either Nuf2p–GFP (Kahana et al., 1995; Kahana and Silver, 1996) or GFP–Tub1p (the major α tubulin gene in *S. cerevisiae*) was used to examine SPB movement early in the cell cycle in both *bni1Δ* and control cells (see Materials and Methods).

SPB movements in wild-type haploid cells were observed from the end of mitosis (telophase) through bud emergence and SPB separation (Fig. 3 a). At the end of mitosis (Fig. 3 a, 1.7 min, mother cell indicated by white arrow) the poles were extended 8–10 μm and located near the cell periphery. After spindle disassembly, the poles usually moved ~1–2 μm toward the center of the cell (Fig. 3 a and Fig. 4 a). In the wild-type strain, this initial movement of the SPB was followed by a concerted movement of the pole toward the site of the incipient bud. This movement of the SPB toward the bud occurred on average within 100 min ($n = 6$), and once the SPB had moved toward the bud its position became relatively stable. SPB separation usually occurred after the old SPB was positioned near the bud. Although the SPB underwent some oscillations as it moved toward the bud, large movements away from the bud were not observed in wild-type cells (Fig. 4 a).

In a significant fraction of posttelophase *bni1Δ* cells, prolonged movement of the SPB away from the bud was observed (Fig. 3 b and Fig. 4 b). In Fig. 3 b, the daughter cell (white arrow) shows this abnormal movement of the SPB (for example, 14.2–121.1 min). In the cells that exhibited the phenotype, the SPB spent almost as much time moving away from the bud as toward the bud. The more random direction of movement of the SPB with respect to the incipient bud in *bni1Δ* cells was similar to the abnormal SPB movements previously observed in *kip3Δ* cells (DeZwaan et al., 1997). This phenotype was detected in four out of 10 cells and was observed in both mother and daughter cells. The mother cell in Fig. 3 b is an example of a *bni1Δ* cell with relatively normal SPB movement. As in *kip3Δ* cells, the SPB eventually assumed a relatively stable position near the bud, suggesting that other factors may stabilize the interaction of the SPB with the bud neck.

Abnormal Spindle Rotation and Transiting of Short Mitotic Spindles in bni1Δ Cells

In addition to the defect in the movement of the unduplicated SPB toward the bud, we observed strikingly abnormal movements of short (1.5 μm) spindles that were unique to *bni1Δ* cells. In another series of experiments, spindle movements before anaphase were observed in *bni1Δ* and wild-type cells. In wild-type cells, short spindles aligned along the mother–bud axis as expected from our analysis of fixed cells and previous imaging of live cells (Yeh et al., 1995). These short spindles displayed some oscillations relative to the bud neck. However, short spindle movement in these cells was limited and only rarely were large rotations (90–180°) of the spindle observed. At anaphase, the spindles in wild-type cells were correctly inserted into the bud neck, and spindle elongation occurred through the bud neck (Fig. 3 a and Fig. 5 a).

By contrast, the spindles of *bni1Δ* cells underwent much more noticeable oscillations, and large rotations of the spindle were frequently observed. In the example shown

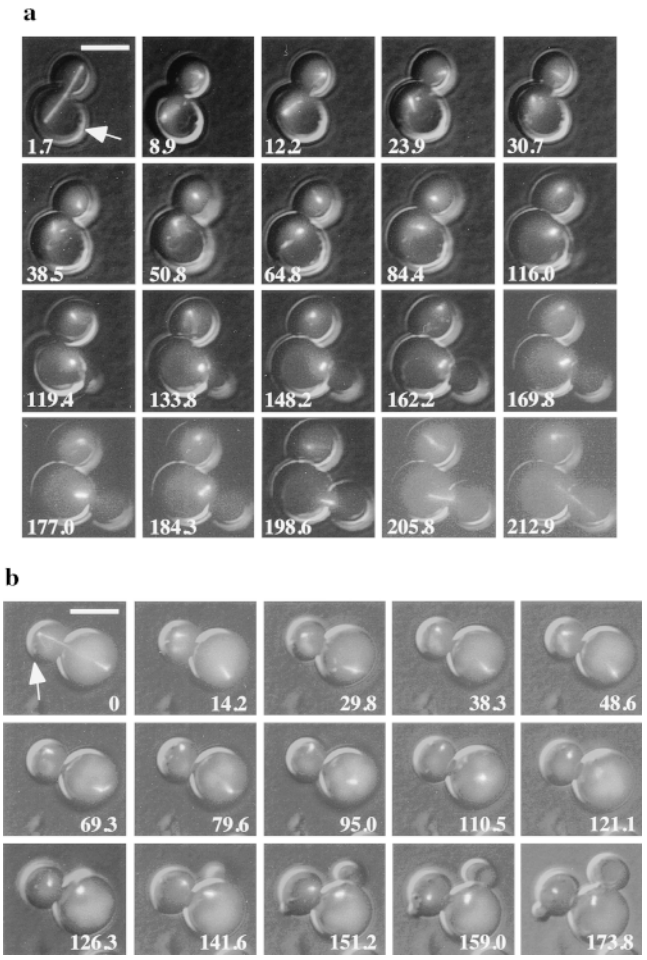

Figure 3. SPB movement toward the incipient bud in posttelophase wild-type and *bni1Δ* cells. Representative images from time-lapse series of *BNI1* (a) and *bni1Δ* (b) cells. Microtubules are labeled in vivo with GFP–Tub1. Cells were imaged from the time of maximal anaphase spindle elongation until SPB separation or longer. At each time indicated a DIC image and six fluorescent images separated by 0.5 μm in the z focal plane were acquired and overlaid. White arrow, the incipient bud site of the cell of interest. In the *BNI1* strain, spindle disassembly is at 8.9 min, bud emergence is at 116 min, SPB separation is at 162.6 min, spindle insertion is at 198.6 min, and spindle elongation is at 212.9 min. In the *bni1Δ* strain, spindle disassembly is at 0 min, bud emergence is at 141.6 min, and SPB separation is at 173.8 min. The complete data set for each cell is plotted in Fig. 4. Bar, 5 μm.

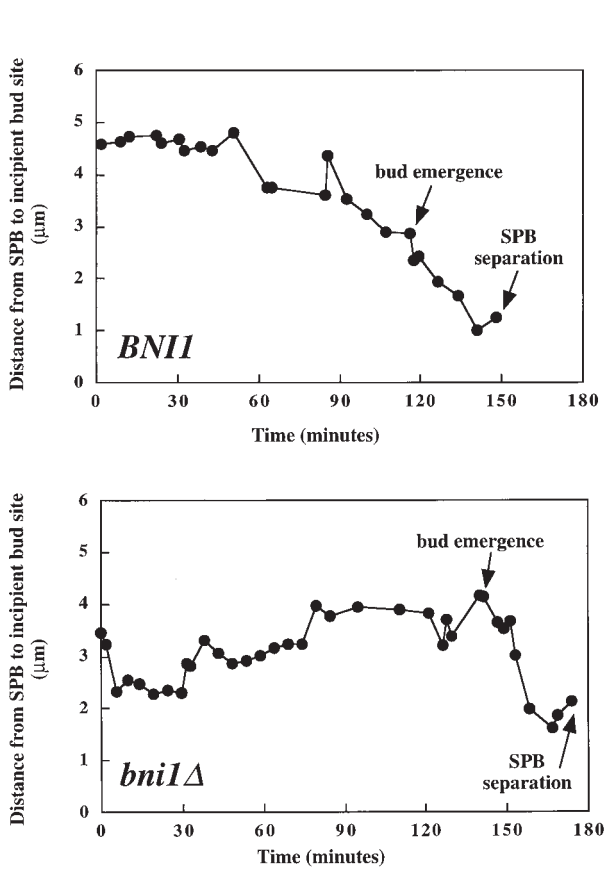

Figure 4. Spindle pole body migration in *BNI1* (a) and *bni1Δ* (b) cells from spindle disassembly to SPB duplication. The distance between the SPB and the incipient bud site is measured at each time point. At time points before bud emergence the position of the presumptive bud site was determined by comparison to later time points. Images for a subset of the time points acquired are shown in Fig. 3.

in Fig. 5 b the spindle underwent four 90–180° rotations in a 40-min time period before anaphase (crossing of lines with open and filled circles). Furthermore, in *bni1Δ* cells the short spindle frequently underwent what we refer to as transiting: movements of the entire short spindle into the bud or back into the mother cell. In the example shown in Fig. 5 b and Fig. 6, transiting of short spindles back and forth between the bud and mother cell was observed four times in the interval before anaphase (Fig. 6, 157.0, 167.5, 188.3, and 193.4 min). Like the defect in the initial SPB movement toward the bud observed in our other series of experiments, this phenotype was not completely penetrant (four out of 10 cells).

Abnormal Interactions between Microtubules and the Cortex in bni1Δ Cells

Recent live-cell fluorescence microscopy experiments suggest that spindle or SPB movement is driven by cytoplasmic microtubule contacts with the cell cortex (Carminati and Stearns, 1997; Shaw et al., 1997). Three types of contacts between cytoplasmic microtubules and the cell cortex have been correlated with changes in the position of the poles or spindles. Apparent pushing forces are generated when cytoplasmic microtubules remain in contact with the cortex while polymerizing. Apparent pulling forces are generated when they depolymerize while maintaining contact with the cortex. Finally, lateral movements of the SPB or spindles are seen as microtubules sweep across the cortex (Carminati and Stearns, 1997; Shaw et al., 1997).

To determine if the spindle movements in *bni1Δ* cells were due to abnormal interactions of cytoplasmic microtubules with cortical sites, image sets of wild-type and *bni1Δ* cells expressing GFP::Tub1p were acquired over short time intervals (30–60 s) using exposures that enabled us to clearly visualize cytoplasmic microtubules. In the wild-type cells with short spindles, the pattern of microtubule

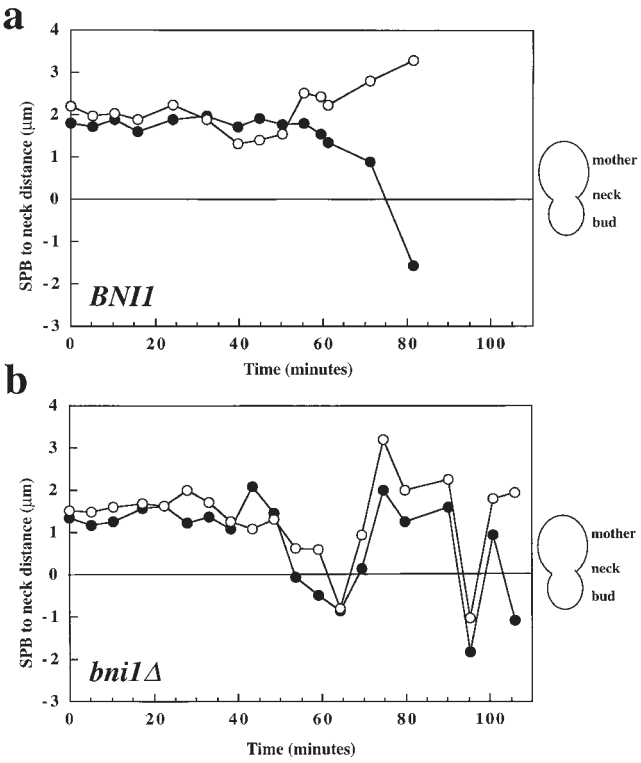

Figure 5. Preanaphase spindle movement in *BNI1* and *bni1Δ* cells. The distance between each SPB and the bud neck is measured at 5–10 min intervals. Cells were observed from SPB separation through spindle insertion. Images were acquired as in Fig. 3. Closed circles represent the SPB that is initially proximal to the bud and open circles represent the other SPB. The bud neck is at 0 μm, positive numbers are distances from the neck in the mother cell, and negative numbers are distances from the neck in the daughter cell. 0 min in Fig. 5 b corresponds to 97.9 min in Fig. 6.

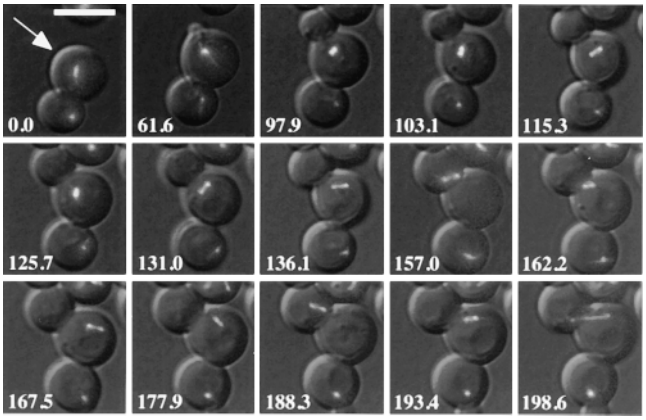

Figure 6. Defective preanaphase spindle movement in a *bni1Δ* cell: transiting of the short spindle between the mother and daughter cell. White arrow, the cell of interest. Images were collected as in Fig. 3. The images shown are a subset of the time points acquired and plotted in Fig. 5 b. Bud emergence and SPB separation occur at 61.6 and 97.9 min, respectively. Transiting of the preanaphase spindle into the bud is observed at 157.0 and 188.3 min, respectively, whereas transiting into the mother is observed at 167.5 and 193.4 min, respectively. Bar, 5 μm.

interactions with the cell cortex and the associated pattern of SPB and/or spindle movement were similar to that described above. Most notably, pulling events in our wild-type strain were observed in cells with short spindles when microtubules interacted with the bud cortex (five events observed over 140 min in five cells); however, such pulling events toward the mother cell were not observed (0 events over 140 min in five cells). Furthermore, in different experiments, we did not observe transiting of preanaphase spindles in seven wild-type cells, observed for an average time of 57 min per cell, over longer time intervals (data not shown). Although we did not observe spindle transits in our wild-type strain, low frequency spindle transits have been reported in some wild-type strains (Palmer et al., 1989).

We next examined microtubule interactions with the cell cortex in *bni1Δ* cells (Fig. 7). Unlike the control strain, pulling events between the cortex and the SPB in both the bud and mother cell were frequently observed (14 events toward bud and 10 events toward mother observed over 147 min in four out of 12 cells). An example of such interactions is shown in Fig. 7. In this cell, from time points 8.9–9.8 min, a microtubule end remained at a fixed position at the mother cell cortex whereas the microtubule shrank from 5.1 to 0 μm. A concomitant 5.6-μm movement of the spindle toward the mother cell cortex was observed. Again, from 15.8–16.6 min, a microtubule with one end at the mother cell cortex shrank from 4.2 to 1.7 μm, whereas the spindle moved 2.1 μm toward the cortex. Overall, we observed a strong correlation between pulling events and the abnormal transiting of short spindles between the mother and the bud in *bni1Δ* cells. Therefore, although microtubules in the *bni1Δ* cells still interact with the cortex, the inability to restrict pulling interactions toward the bud suggests that the microtubule cytoskeleton of the *bni1Δ* strain fails to sense the cell's polarity.

Bni1p Is Not Required for Anaphase Spindle Elongation or Cell Cycle Progression

Because loss of *BNI1* affects cytoplasmic microtubule function, we next tested whether nuclear microtubule function was affected by measuring the kinetics of anaphase spindle elongation. Two major stages of anaphase have been observed in *S. cerevisiae* (Kahana et al., 1995; Yeh et al., 1995; Straight et al., 1997). There is an initial fast stage of elongation from an ~2-μm spindle to an ~4-μm spindle. The rate of elongation then slows as the spindle reaches maximal extension at 8–10 μm. Anaphase kinetics was measured in *bni1Δ* and wild-type cells (Fig. 8). Fast elongation was 0.55 μm/min ± 0.14 (n = 7) in the *bni1Δ* strain and 0.48 μm/min ± 0.10 (n = 5) in the wild-type strain. Slow elongation was 0.14 μm/min ± 0.04 (n = 7) in the *bni1Δ* strain and 0.12 μm/min ± 0.04 (n = 5) in the wild-type strain. Thus, loss of *BNI1* does not have a significant effect on anaphase spindle elongation.

Because the *bni1Δ* mutant could affect spindle orientation either through a direct effect on the cytoskeleton or indirectly by perturbing the cell cycle, we next tested whether *bni1Δ* strains were delayed in any specific stage of the cell cycle. *bni1Δ* strains grew slightly slower than control strains at 23°C. The distribution of cells throughout

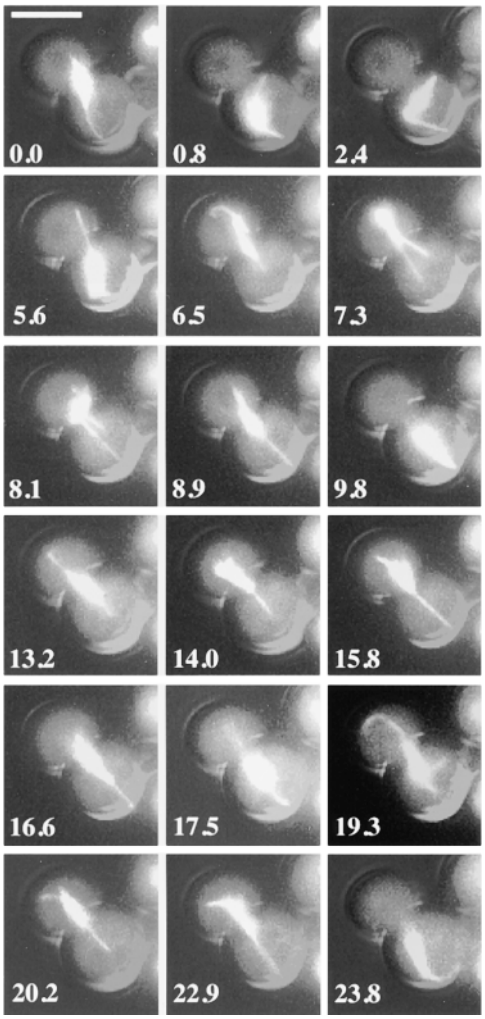

Figure 7. Relationship between preanaphase spindle transiting and cytoplasmic microtubules interactions with the cell cortex: time-lapse series of a preanaphase *bni1Δ* cell. At each time indicated a DIC image and eight to 10 fluorescent images separated by 0.3 μm in the z focal plane were acquired and overlaid. To enhance the visualization of cytoplasmic microtubules, images were collected using 2 × 2 binning and 400–700-ms exposures. The light from the mercury lamp was attenuated to one-eighth of the maximal intensity. This resulted in overexposure of the spindle microtubules. Spindle transits into the mother cell were observed at 0.8, 9.8, 17.5, and 23.8 min, and transits into the bud were observed at 7.3, 14.0, and 20.2 min. In all cases, spindle transit was correlated with shortening of the cytoplasmic microtubules which maintained contact with the cell cortex. Bar, 5 μm.

the cell cycle was first characterized by examining the distribution of cell morphologies in cultures of logarithmically growing cells (Table IV). The numbers of unbudded G1 cells, budded cells with undivided nuclei, and post-anaphase cells were nearly identical in *bni1Δ* and the wild-type strain. The ratio of cells in G1 and G2 cell cycle phases were also similar in both strains by FACS® analysis (data not shown). Finally, we analyzed our time-lapse experiments to measure the intervals between spindle disassembly and the initiation and completion of budding in *bni1Δ* and control cells. After completion of anaphase, the *bni1Δ* and control strains initiated budding on average at

Table IV. Cell Cycle Distribution of BNI1 and bni1Δ Cells

	Percent unbudded	Percent budded (preanaphase)	Percent anaphase
			
BNI1	46.8	43.1	10.1
bni1Δ	48.7	41.4	9.9

BNI1 and *bni1Δ* cells expressing GFP–Tub1p were grown in liquid culture at 23°C and observed by fluorescence microscopy. $n = 1{,}200$.

87 and 89 min, respectively. Budding was completed on average at 138 and 150 min, respectively, in these strains.

Therefore, although we cannot rule out a subtle effect on cell cycle progression in *bni1Δ* cells, our analysis shows that the cells do not exhibit a prominent delay at a specific stage of the cell cycle. This is consistent with the idea that loss of *BNI1* function affects spindle orientation through direct effects on the cytoskeleton rather than indirect effects on the cell cycle.

Bud6p and the Bud6p-binding Domain of Bni1p Are Required for Spindle Orientation and Bipolar Bud Site Selection

Bni1p associates with other proteins that are required for bipolar bud site selection (Amberg et al., 1997; Evangelista et al., 1997; Fujiwara et al., 1998). As an initial test to determine if these associations are required for spindle orientation, we characterized a *BNI1* allele *(bni1-CTΔ1)* lacking the COOH-terminal domain of Bni1p that interacts with the actin-associated protein Bud6p (Amberg et al., 1997; Evangelista et al., 1997). The *bni1-CTΔ1* strain is proficient both for shmoo formation and mating (Table V). By contrast, *bni1-CTΔ1* did not complement the bud site selection defect of a *bni1Δ/bni1Δ* diploid strain (Table V). Furthermore, *bni1-CTΔ1* cells had a significant defect in spindle orientation (Fig. 2 c). Like *bni1Δ* cells, the spindles in *bni1-CTΔ1* cells were also located farther from the bud neck and were highly variable in position (mean distance of 1.1 μm in *bni1-CTΔ1* versus 0.7 μm in *BNI1*, *t* test, $P < 0.0001$, $n = 180$) and, like the *bni1* null allele, the

Table V. Mating Projection Formation, Quantitative Mating Assay, and Bipolar Bud Site Selection

	Mating projection formation	Mating efficiency	Bipolar bud site selection
	%	%	%
bni1::kan^r {BNI1}	83.4 ± 5.5	0.17 ± 0.041	—
bni1::kan^r {bni1-CTΔ1}	77.2 ± 5.2	0.095 ± 0.013	—
bni1::kan^r {CEN URA3}	0	0.001 ± 0.0006	—
BNI1/BNI1	—	—	4 ± 1.8
bni1Δ/bni1Δ {BNI1}	—	—	31 ± 2.3
bni1Δ/bni1Δ {bni1-CTΔ1}	—	—	92 ± 3.5
bni1Δ/bni1Δ {CEN URA3}	—	—	93 ± 2.1

The mating projection assay measured the percentage of cells with mating projections after 3 h in α factor ($n = 300$). The quantitative mating assay (Sprague, 1991) measured the percentage of diploids formed after a 4-h mating with the *far1-c* tester strain. The result of the bipolar bud site selection assay (Pringle et al., 1989) shows the percentage of cells with a random budding pattern.

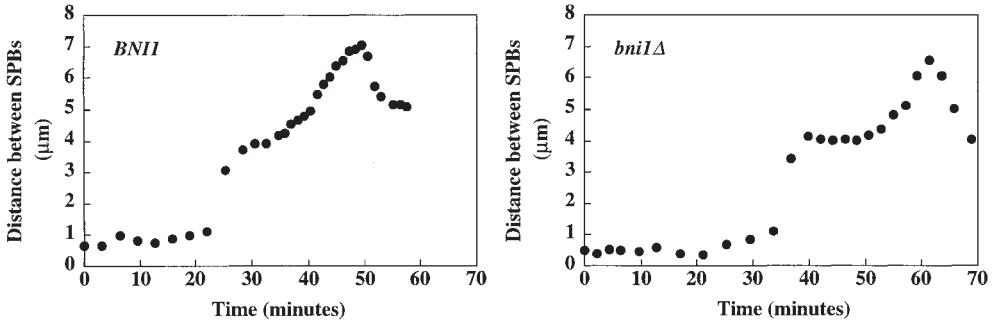

Figure 8. Anaphase kinetics in *BNI1* and *bni1Δ* cells. Time-lapse series of *BNI1* and *bni1Δ* cells collected from before anaphase through the completion of anaphase. Images were collected as in Fig. 3. The distance between the two SPBs was measured at each time point and plotted versus time.

bni1-CTΔ1 allele produced a striking temperature-sensitive growth defect when combined with *ase1Δ* or *dyn1Δ* (Fig. 1 a). *bni1-CTΔ1 ase1Δ* and *bni1-CTΔ1 dyn1Δ* strains were also more sensitive to benomyl, although the effect was less pronounced in the *bni1-CTΔ1 ase1Δ* strain (Fig. 1 a).

Spindle orientation was also affected in cells lacking Bud6p. At 23°C, the mean spindle orientation angle in a *bud6Δ* strain was 24.0°, compared with 17.8° in an isogenic wild-type (*t* test, $P < 0.0001$, $n = 290$). At 37°C, the mean angle in *bud6Δ* was 28.3° in contrast to 20.6° in the wild-type (*t* test, $P < 0.0001$, $n = 330$). These results demonstrate that Bud6p and the Bud6p interaction domain of Bni1p are required for spindle orientation and bipolar bud site selection, but suggest that Bud6p is less important for spindle orientation than Bni1p.

The Role of Actin in Spindle Orientation and Bipolar Bud Site Selection

Because Bni1p and Bud6p are both thought to be regulators of actin function, we next characterized the nature of actin's role in spindle orientation and nuclear positioning. Previous studies demonstrated that actin mutations interfere with normal nuclear position (Palmer et al., 1992; Drubin et al., 1993). To determine if actin is required for spindle orientation, we treated logarithmically growing wild-type cells with Lat-A for a brief period (5 min), fixed the cells, and then measured spindle orientation (Fig. 9 b). Staining with rhodamine-phalloidin revealed a complete loss of polymerized actin after treatment with Lat-A (data not shown). By contrast with mock-treated cultures (Fig. 9 a), the 5-min treatment with Lat-A–randomized spindle orientation. Thus, actin is required for spindle orientation in normal growing cells. The rapid effect on spindle orientation suggests that the role of actin in controlling spindle orientation may be direct.

Genetic experiments have provided evidence for a specialized actin structure that mediates bipolar bud site selection (Yang et al., 1997). Many, but not all, mutations that affect the actin cytoskeleton affect bipolar bud site selection. A *sla1ΔSH3#3* strain with abnormal cortical actin structure did not have a defect in bipolar bud site selection (Yang et al., 1997). Strains with this *sla1* allele accumulated abnormal cortical actin aggregates (Fig. 9 f) and like *sla1Δ* strains, were temperature-sensitive for growth. Like *sla1Δ*, these strains were also supersensitive to osmotic stress at 23°C (data not shown). On the other hand, two "pseudo-wild-type" actin alleles, *act1-116* and *act1-117*, exhibited normal growth and cortical actin structures but had a defect in bipolar bud site selection (Yang et al., 1997). The reciprocal phenotypes of these mutants suggested that a specific actin structure might mediate bipolar bud site selection. This postulated structure (or substructure) might also contain bipolar bud site determinants since these proteins are known to interact with actin.

The work by Yang et al. (1997) together with our analysis of *bni1Δ* and *bni1-CTΔ1* prompted us to test the hypothesis that similar actin structure(s) required for bipolar budding are required for spindle orientation. We first examined spindle orientation in the *sla1ΔSH3#3* strain. As shown in Fig. 9, c and d, spindle orientation in *sla1ΔSH3#3* cells was indistinguishable from that of the isogenic wild-type strain. This demonstrates that alterations in the actin cytoskeleton per se do not affect spindle orientation and supports the idea that a specific actin structure may be required for both spindle orientation and bipolar bud site selection.

We next examined spindle orientation in the *act1-116* strain. For our analysis we chose *act1-116* of the two pseudo-wild-type actin alleles previously studied because it has the most noticeable effect on bipolar bud site selection (Yang et al., 1997). The mean angle for spindle orientation in the *act1-116* strain was significantly greater than that for the isogenic wild-type strain (25.8° and 19.1° respectively, *t* test, $P = 0.0002$, $n = 360$). The percentage of cells with obviously misoriented spindles was also greater in the *act1-116* strain (10.4% with spindle orientation between 60° and 90° compared with 3.4% for the wild-type strain). Although the spindle orientation defect in *act1-116* was small, it was consistent with the magnitude of the defect in bipolar bud site selection pattern (35% of *act1-116/act1-116* diploid cells had a random budding pattern; data not shown). The fact that a mutant strain with no detectable cortical actin defect (*act1-116*) disrupts spindle orientation and bipolar bud site selection, whereas another mutant strain (*sla1ΔSH3#3*) with abnormal cortical actin does not, supports the idea that spindle orientation and bipolar bud site selection may be mediated by a similar actin-containing structure.

Discussion

The Role of Bni1p in Spindle Orientation

The formins are important for cytokinesis and aspects of development in many organisms (Frazier and Field, 1997).

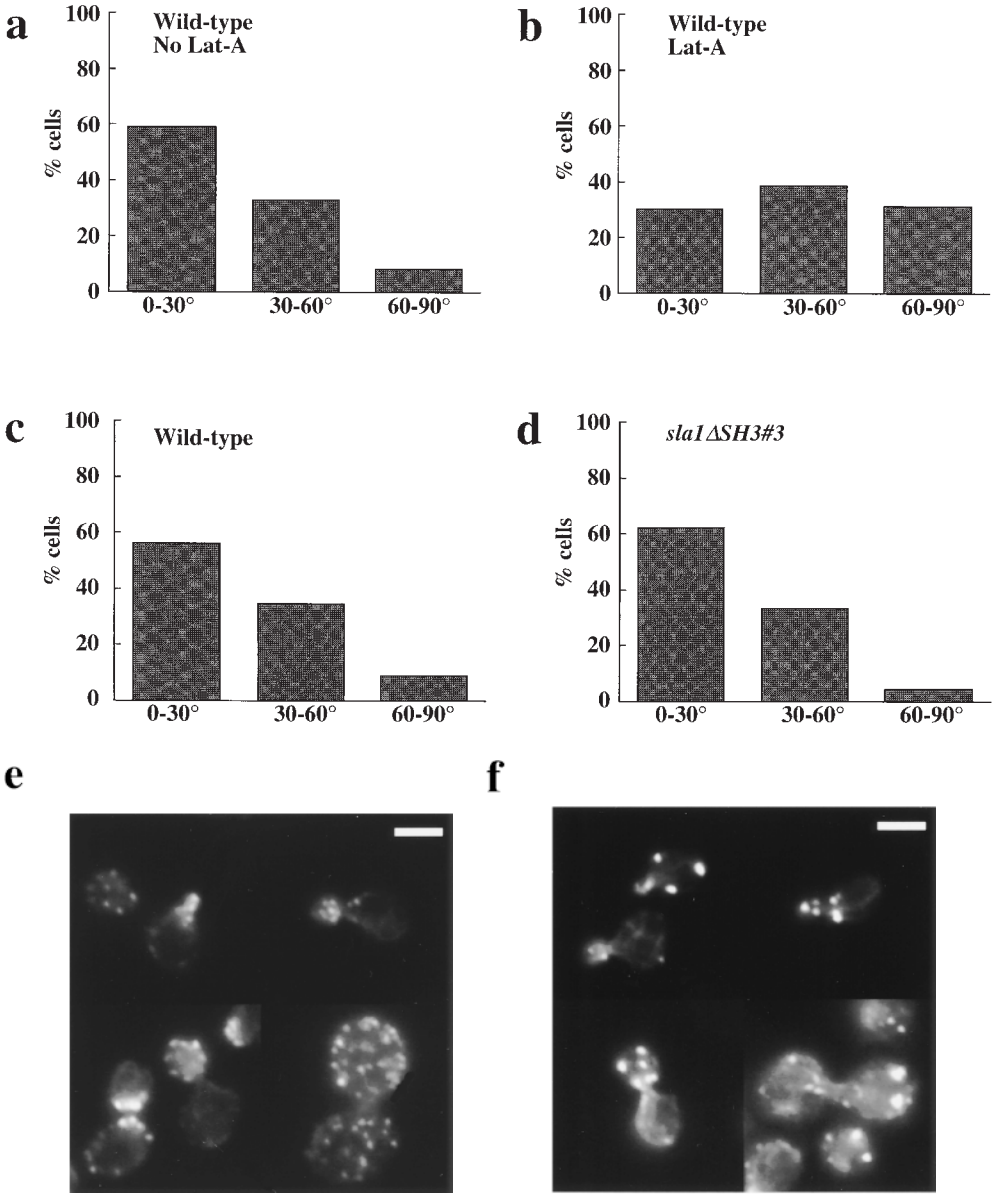

Figure 9. The role of actin in spindle orientation. Early log phase wild-type cells expressing Nuf2p–GFP were treated with DMSO (a) or Lat-A (b) for 5 min. Cells were fixed and spindle orientation was measured as in Fig. 2. Spindle orientation angles are shown on the x axis. A sample from each culture was stained with rhodamine-phalloidin to visualize actin. Spindle orientation angles were measured in *sla1ΔSH3#3* (d) and isogenic wild-type strains (c). Spindles were visualized by indirect immunofluorescence using the anti-tubulin mAb, YOL1/34. *sla1ΔSH3#3* (f) and isogenic wild-type strains (e) were stained with rhodamine-phalloidin to visualize actin structures. Bar, 3 μm.

On the molecular level, formins are thought to be regulators of the actin cytoskeleton, but their exact role in actin function is not defined. Here we report that the yeast formin, Bni1p, is required for orientation of preanaphase mitotic spindles in *S. cerevisiae*. Our analysis of the spindle positioning defect in *bni1Δ* cells suggests that Bni1p is required to assemble or transport cortical microtubule-binding sites into the bud.

The idea that Bni1p has a role in spindle orientation was prompted by genetic interactions we observed between *bni1Δ* and mutations in genes encoding components of the microtubule cytoskeleton. The localization of Bni1p to the bud cortex early in mitosis and to the septum late in mitosis (Jansen et al., 1996; Evangelista et al., 1997; Fujiwara et al., 1998) (Evangelista, M., and C. Boone, unpublished observation) suggested that Bni1p would most likely regulate cytoplasmic microtubules. Indeed, we observed that in *bni1Δ* cells the orientation of preanaphase spindles was random with respect to the mother–bud axis.

Our real-time analysis demonstrated two striking phenotypes in *bni1Δ* cells. First, Bni1p was required for the initial directed movement of the SPB toward the incipient bud site. Unlike wild-type cells, where the SPB makes a concerted (but not absolutely direct) movement toward the incipient bud site, the SPB in *bni1Δ* cells spent almost as much time moving away from the incipient bud site as toward it. Consistent with the idea that a partially redundant pathway is able to compensate for the loss of *BNI1* (in some cells better than others), this defect was observed in four out of 10 cells. This phenotype is very similar to that previously observed in *kip3Δ* cells (DeZwaan et al., 1997), and parallels the common spindle orientation defect observed in fixed populations of *bni1Δ* and *kip3Δ* cells.

The polarity of the cell is established before bud emergence. Bni1p and other proteins are localized to the incipient bud before bud emergence (Chant, 1996; Ayscough et al., 1997). Initial studies of fixed populations of cells detected interactions between cytoplasmic microtubules and the in-

cipient bud site in unbudded cells (Snyder et al., 1991). These observations suggested that the microtubule cytoskeleton must be able to sense and respond to polarity cues even before the bud emerges. This study and previous fluorescence time-lapse microscopy studies of wild-type cells demonstrate that the SPB does move toward the bud site before bud emergence (DeZwaan et al., 1997). The finding that Bni1p was required for this initial movement of the SPB suggests that Bni1p is either directly involved in the physical interactions between cytoplasmic microtubules and the cortex, or that it regulates these interactions.

It is important to note that, although there was a profound delay in movement of the SPB in *bni1Δ* cells toward the incipient bud, the SPB eventually assumed a position close to the bud before SPB separation. Therefore it is possible that other proteins can also mediate the cortical interaction, but that they assemble at cortical sites later than Bni1p. Alternatively, there may be a Bni1p-independent pathway for spindle orientation. Because of the genetic evidence of functional overlap between *BNI1* and *DYN1*, cytoplasmic dynein is one candidate to mediate an alternate mechanism for spindle orientation.

A second striking phenotype that was unique to *bni1Δ* cells was the abnormal rotations of the preanaphase spindle and transiting of these spindles between the mother and the bud. The transiting motions of the spindle were correlated with shortening of the cytoplasmic microtubules that maintain contact with the cell cortex. We interpret these movements to be the result of pulling forces between the cortex and the spindle, because the shortening microtubule consistently maintained contact with the cell cortex. These spindle movements did not appear to result from pushing forces, because we did not observe consistent contact between the cortex and cytoplasmic microtubules from the distal SPB.

What was most remarkable about the *bni1Δ* cells was that these apparent pulling movements were observed with approximately equal frequency toward the mother cell body as toward the bud. Apparent pulling interactions between cytoplasmic microtubules and the bud cortex were observed in wild-type cells during spindle orientation. However, apparent pulling movements of the spindle into the mother cell were not observed in our wild-type samples.

The simplest explanation for the high frequency of transiting of preanaphase spindles in *bni1Δ* cells is that Bni1p is required to concentrate microtubule-binding sites to the bud cortex while spindle orientation occurs. In the absence of Bni1p, these microtubule-binding sites would no longer be asymmetrically localized and would be distributed evenly between the mother and the bud. Bni1p could participate in the assembly or transport of microtubule-binding sites into the bud. Other models are also possible. For examples, cortical attachment sites might be evenly distributed throughout the cell, but Bni1p might specifically alter those in the bud, therefore biasing pulling movements toward the bud. Interestingly, we have found that the second formin in yeast, Bnr1p, is not required for spindle orientation (Lee, L., and D. Pellman, unpublished results). This is consistent with Bnr1p's specific localization to the septal regions and not the bud cortex (Kamei et al., 1998).

The Genetic Pathways Controlling Spindle Orientation

Two major pathways for spindle positioning have been identified. As discussed above, the kinesin Kip3p is required for the initial migration of the SPB toward the incipient bud site before SPB duplication (DeZwaan et al., 1997). Cytoplasmic dynein is required for insertion of the preanaphase spindle into the bud neck, but not for the initial alignment of the spindle with the axis of division (Yeh et al., 1995; DeZwaan et al., 1997). These pathways are at least partially overlapping, because cells lacking both *KIP3* and *DYN1* are inviable (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Miller et al., 1998). The pattern of genetic interactions that we observed between *bni1Δ* and null alleles of different motor genes was remarkably similar to the pattern of genetic interactions previously observed with *kip3Δ*. These findings together with the fact that *bni1Δ kip3Δ* double mutant cells were viable and did not have an exacerbated defect in nuclear segregation relative to the single mutant cells, place *KIP3* and *BNI1* in the same genetic pathway.

There are several molecular models that can explain the relationship between *BNI1* and *KIP3*. One possibility is that Kip3p is a minus end–directed motor, and that the pulling of the spindle toward the bud is mediated by Kip3p tethered to Bni1p. Arguing against this idea is the fact that Kip3p is not detected at the cell cortex (DeZwaan et al., 1997; Miller et al., 1998). Also, Kip3p and Bni1p do not coimmunoprecipitate under a variety of conditions, and the localization of Kip3p is not altered in a *bni1Δ* mutant (Ellingson, E., L. Lee, and D. Pellman, unpublished results). However, it remains possible that there is a physical interaction that is transient or of low affinity.

Another explanation for the similar phenotypes and genetic interactions observed with *bni1Δ* and *kip3Δ* strains is that Kip3p may promote microtubule dynamics that are required for cortical microtubule capture. Dynamic microtubules could be required to increase the number of encounters between the ends of the cytoplasmic microtubules and the cortex, thereby increasing the likelihood that the microtubules will find a binding site. Finally, Kip3p might mediate microtubule depolymerization after the cytoplasmic microtubule is tethered to the bud cortex through interactions with another protein. Microtubule depolymerization in this manner would generate a pulling force on the spindle. Consistent with the idea that Kip3p has an important role in controlling microtubule dynamics, *kip3Δ* cells have longer cytoplasmic microtubules and are resistant to microtubule-depolymerizing agents (Cottingham and Hoyt, 1997; DeZwaan et al., 1997). Interestingly, we have noted that *bni1Δ* cells also have longer cytoplasmic microtubules than control cells, but we have not characterized microtubule dynamics in *bni1Δ* cells in detail (Lee, L., and D. Pellman, unpublished observation).

Bipolar Bud Site Determinants, Actin, and Cortical Microtubule Capture

Several lines of evidence suggest that other bipolar budding determinants in addition to Bni1p may be involved in spindle orientation. First, several genes required for bipolar budding appear to define a functional group based on similar mutant phenotypes, similar genetic interactions,

and similar localization of the encoded proteins (Snyder, 1989; Valtz and Herskowitz, 1996; Zahner et al., 1996; Amberg et al., 1997; Winsor and Schiebel, 1997). Furthermore, there is evidence for physical interactions among at least four of these proteins. Bni1p and Spa2p interact by two-hybrid analysis and in vitro, and the localization of Bni1p is dependent upon Spa2p function (Fujiwara et al., 1998). There is also evidence for physical interactions between Spa2p, Pea2p, and Bud6p (Sheu et al., 1998). Second, we found that *bud6Δ* cells exhibit a defect in spindle orientation. Third, cells expressing Bni1-CTΔ1p, which lack the Bud6p-interacting domain of Bni1p, are defective in bipolar budding and spindle orientation but not Bni1p mating functions. Although Bud6p is necessary for normal spindle positioning, our results demonstrate that it is not as important as Bni1p. This suggests that the biochemical mechanism for cortical microtubule capture may be complex, perhaps involving several partially redundant factors.

The actin cytoskeleton is also required for both spindle orientation and bipolar budding (Palmer et al., 1992; Yang et al., 1997). We have extended previous studies by demonstrating that spindle orientation in normal logarithmically growing cells becomes random within 5 min of depolymerizing cellular actin with Lat-A. The rapid effect of Lat-A on spindle orientation argues that the role of actin may be direct.

Our findings are also consistent with the idea that actin structures postulated to be required for bipolar budding (Yang et al., 1997), are also required for spindle orientation. The supposition that a specific actin structure is required for both bipolar budding and spindle orientation is based on several lines of evidence. First, a mutation that results in abnormal cortical actin (*sla1ΔSH3#3*) does not affect bipolar budding or spindle orientation, demonstrating that alterations in the actin cytoskeleton per se do not perturb these two processes. Second, an *act1-116* allele that does not alter cortical actin or cell growth is defective for both bipolar budding and spindle orientation. Third, both *bni1Δ* and *bni1-CTΔ1* cells have normal appearing actin structures (Lee, L., and D. Pellman, unpublished data) but are defective for spindle orientation and bipolar bud site selection. Together, these findings are consistent with a model where Bni1p is a component of an actin complex that mediates bipolar bud site selection and spindle orientation.

Although our results suggest that Bni1p is necessary to concentrate microtubule/cortical interaction sites into the bud, the molecular nature of this postulated microtubule-binding site remains to be defined. As discussed above, one possibility is that the interaction is mediated by transient association of a microtubule motor such as Kip3p with the cortex. Another possibility is that cytoplasmic microtubules are captured by microtubule-binding proteins located at cortical sites. One candidate cortical protein that might have microtubule-binding activity is Kar9p (Miller and Rose, 1998). The *kar9Δ* phenotype partially overlaps with that of *bni1Δ*. Furthermore, Kar9p is mislocalized in *bni1Δ* cells, although not obviously to cortical sites in the mother cell (see accompanying paper by Miller et al., 1999). Another candidate for a cortical interaction site is coronin (Heil-Chapdelaine et al., 1998; Goode et al., 1999). Coronin colocalizes with actin patches in vivo and, interestingly, binds to both microtubules and actin in vitro. Since most of the proteins required for spindle orientation are not essential, it seems likely that there may be several cortical proteins that interact with microtubules.

Whatever the nature of the cortical microtubule-binding sites, it is likely that their activity is regulated during the cell cycle. One plausible mechanism to achieve this would be through Rho-type GTPases. Because Bni1p links Rho-type GTPases to the actin cytoskeleton (Evangelista et al., 1997; Imamura et al., 1997; Fujiwara et al., 1998), it is interesting to note that genetic evidence suggests a role for Rho2p and the GDP/GTP exchange factor Rom2p in microtubule function (Manning et al., 1997). Whether the interaction between Bni1p and the activated forms of GTPases is required to maintain spindle orientation remains to be determined.

The results described here define an important role for an *S. cerevisiae* formin, Bni1p, in regulating spindle position. Whether formins in other organisms have similar functions is unknown. However, we note that mutations in the genes encoding the *Drosophila* formins, *cappuccino* and *diaphanous*, result in abnormal microtubule structures (Emmons et al., 1995; Giansanti et al., 1998). *cappuccino* mutant oocytes contain long and thick microtubule bundles around the cell cortex. During male meiosis in *diaphanous* mutants the structure of both the actin contractile ring and the central spindle is disrupted. These findings raise the possibility that formins have a conserved role in microtubule function.

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