Control of Mitotic Spindle Position by the \textit{Saccharomyces cerevisiae} Formin Bni1p

Laifong Lee,*† Saskia K. Klee,*‡ Marie Evangelista,§ Charles Boone,¶ and David Pellman*†

*Department of Pediatric Oncology, The Dana-Farber Cancer Institute and Department of Pediatric Hematology, The Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115; †Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544; §Institut für Biochemie, Universität Stuttgart, 70569 Stuttgart, Germany; and ¶Department of Biology, Queen’s University, Kingston, Ontario, Canada K7L 3N6

Abstract. Alignment of the mitotic spindle with the axis of cell division is an essential process in \textit{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 \textit{bni1} \textit{D} 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, \textit{BN1} and \textit{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 \textit{bni1} \textit{D} 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 • \textit{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 \textit{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) migrates toward the incipient bud site. Around the time of SPB separation and the formation of a bipolar spindle, cytoplasmic microtubules penetrate into

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 gene that encodes actin, result in defects in spindle po-
scaffolds that link Rho-type GTPases with components of
somes between mother and daughter cells (Yeh et al.,
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 cyto-
plasmic 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 cy-
toplasmic microtubules to the cell cortex.

One of the most interesting questions about the mecha-
nism of spindle orientation is the identity and molecular
function of the cortical proteins that interact with cyto-
plasmic microtubules. The existence of microtubule “cap-
ture sites,” loosely analogous to those at the kinetochore,
have been postulated, but little is known about the molecu-
lar composition of these cortical regions (Kaverina et al.,
1998; Snyder et al., 1991). One factor that is clearly impor-
tant 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 po-
nosition 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 cen-
tosome movement in human leukocytes (Euteneuer and
Schliwa, 1985). The molecular role of actin in these differ-
ent 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 microtu-
bules, it is interesting that overexpressed Kar9p concen-
trates 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 cel-
lar and developmental functions (Woychik et al., 1990;
Castrillon and Wasserman, 1994; Emmons et al., 1995; Pe-
tersen 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 homol-
gy (FH) domains. There is substantial evidence to sug-
gest that Bni1p and other formins function as molecular
scaffolds that link Rho-type GTPases with components of
the actin cytoskeleton. The NH2-terminal domain of Bni1p
interacts with the activated forms of G proteins, Cdc42p
and Rho1p, which regulate organization of the actin cy-
toskeleton (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 an-
other putative actin-binding protein, Aip3p/Bud6p (Am-
berg et al., 1997; Evangelista et al., 1997). In S. cerevisiae,
a second formin, Brn1p, has at least partial functional over-
lap 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 po-
larized morphogenesis and the establishment of cell polar-
ity. 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 determin-
ants (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 re-
quired 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 ap-
pear to construct buds through isotropic growth, gener-
ating cells with a rounded shape. bni1Δ cells are also
defective in shmoo formation, pseudohyphal growth, lo-
calization of ASH1 mRNA to the bud, cytokinesis, and bi-
polar 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 be-
cause 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 be-
fore 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, micro-
tubule-binding sites at the cell cortex are either mislo-
calized or functionally altered, resulting in the loss of
polarization of preanaphase spindle movement. Finally,
analysis of mutations affecting the actin cytoskeleton sug-
gest that actin structures involved in bipolar budding 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 nM. 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  | MATaasl::URA3 ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | D. Pellman |
| PY1994  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-52 | This study |
| PY1996  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-52 | [2 μ ASE1 ADE3 TRP1] This study |
| PY2400  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | [2 μ ADE3 TRP1] This study |
| PY2401  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | [2 μ ADE3 TRP1] This study |
| PY2403  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2409  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2513  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2515  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2516  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2517  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2581  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2582  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2583  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2595  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2596  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| PY2601  | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| Y66     | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| Y1353   | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |
| Y1445   | MATaasl::kanR ade2-100 ade3 leu2-3,112 lys2-801 trpl-1 ura3-1 | ade3-1 This study |

Strain and Plasmid Construction

A complete deletion of the BNI1 coding sequence was created by one-step gene replacement with the selectable marker kan<sup>T</sup>, which confers Geneticin resistance to yeast (Wach et al., 1994). The bni1<sup>T</sup>-kan<sup>T</sup> containing DNA sequence was generated by PCR using oligonucleotide primers: 5’ TATCTATCTCTGTTATGAGGAGAAACATT TACTCAGCC- and 5’ GAGTAAAGATGAATGTAAAGTGAAGCTTCGTACGC. Deletion of the BNI1 locus was confirmed by Southern blot analysis of Geneticin-resistant transformants. This BNI1 deletion strain, hereafter referred to as bni1<sup>Δ</sup>, 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).

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 (pR314) (Sikorski and Hieter, 1989) to create p1832. The COOH-terminal deletion mutant, bni1<sup>-CTΔ</sup>, lacks the coding sequence for amino acids 1,749–1,953 of Bni1p, and was created by the following strategy. p182 was digested with NotI site adjacent to the SstII site in pRS314 and ligated to an oligonucleotide coding sequence for amino acids 1,749–1,953 of Bni1p, and was created by the following strategy. p182 was digested with NotI site adjacent to the SstII site in pRS314 and ligated to an oligonucleotide (5’ GTAGCGCGCGCTACTAGGATT). This created a stop codon following by a NotI site adjacent to the SstII site in BNI1. A CEN plasmid (p2009) derived from pRS314 and an integrating plasmid (PY1046) derived from pRS305 (Sikorski and Hieter, 1989) contain the bni1<sup>-CTΔ</sup> sequence on a 6.3-kb BamHI/NotI restriction fragment. The strain containing p2009 was used for morphological studies.

The act1-H16 and sla1<sup>ΔSH3</sup> strains and their isogenic wild-type controls are in the S288c genetic background. To construct PY2596, the ACT1 locus was replaced by act1<sup>-H16::HIS3</sup> from pR1357 by homologous recombination in a strain containing NUF2:: GFP (PY1000). This was done because previously constructed act1<sup>-H16</sup> 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-
The starting strains MATa and MATα asel1::Kan ade2 ade3 leu2 lys2 trp1 ura3 (2 μA-SE1 ADE3 TRP1) (FY1994 and PY1996) were mutagenized by transformation with a genomic library containing random insertions of a mini-Tn3lacZ::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 transposon site. These PCR products were generated by an arbitrary primed PCR strategy (Di Rienzo et al., 1996). The bni1::Trp1::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-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 × asel1Δ cross, 37 tetrads were dissected and 41 double mutants were recovered, all of which were temperature sensitive for growth. For the bni1Δ × dyne1Δ 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 kipl1Δ and sny1Δ, 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 MATa bni1::Kan bar1::LEU2 strain (Y1445), carrying different alleles of BNI1 on a CEN plasmid. The mating partner used for the mating assays was a MATα kar1+ 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, W303α, 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).

**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 asel1Δ 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 asel1Δ 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 asel1Δ strain had a strikingly increased sensitivity to benomyl (5 μg/ml). bni1Δ and asel1Δ 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 Nut2p, a spindle pole body protein, and GFP was introduced into both strains, allowing visualization of the poles by fluorescence microscopy (Kahana...
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 \textit{bni1}D cells appeared to be random (Fig. 2 b). Furthermore, the distance between the neck-proximal SPB and the bud neck was significantly greater in \textit{bni1}D than in \textit{BNI1} cells: 1.0 and 0.7 μm, respectively (\textit{t} test, \textit{P}, 0.0001, \textit{n} = 180). Thus, \textit{bni1}D has a defect in spindle orientation.

Genetic Interactions Suggest that \textit{BNI1} and \textit{KIP3} Are in the Same Pathway

The entire set of \textit{Saccharomyces cerevisiae} microtubule-based motor proteins has been defined. The genome contains a single gene encoding a dynein heavy chain, \textit{DYN1}, and six genes encoding kinesin-related proteins, \textit{KIP1}, \textit{KIP2}, \textit{KIP3}, \textit{KAR3}, \textit{CIN8}, and \textit{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 \textit{kip3}D and \textit{dyn1}D strains have the most striking effect on nuclear position, \textit{kip3}D and \textit{dyn1}D affect distinct steps in nuclear positioning: \textit{kip3}D cells have a spindle orientation defect, whereas \textit{dyn1}D cells have a defect in the insertion of the spindle across the bud neck. The fact that \textit{kip3}D and \textit{dyn1}D 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 \textit{BNI1} was in the \textit{KIP3} pathway. Consistent with the similar spindle orientation defect observed in \textit{bni1}D and \textit{kip3}D strains, the \textit{bni1}D \textit{kip3}D strain was viable and grew indistinguishably from single mutant strains (Table III). The percentage of binucleate mother cells in the \textit{bni1}D \textit{kip3}D strain was also not increased relative to either single mutant strain (Fig. 1 b). By contrast, \textit{bni1}D produced a striking synthetic interaction when combined with \textit{dyn1}D. The \textit{bni1}D \textit{dyn1}D strain had a growth defect at 23°C and was inviable at 14°C and 37°C (Fig. 1 a and Table III). Also, the \textit{bni1}D \textit{dyn1}D strain had a marked increase in binucleate mother cells relative to the single mutant strains (Fig. 1 b). Like the \textit{bni1}D \textit{ase1}D strain, the \textit{bni1}D \textit{dyn1}D strain had increased sensitivity to benomyl (Fig. 1 a).

The genetic interactions we observed between \textit{bni1}D and the other motor gene deletions paralleled those observed with \textit{kip3}D (Table III). Like \textit{kip3}D, \textit{bni1}D is lethal when combined with \textit{kar3}D. \textit{bni1}D did not show a significant synthetic interaction with either \textit{kip1}, \textit{cin8}D, or \textit{smy1}D. This pattern of genetic interactions observed with \textit{bni1}D suggests that \textit{BNI1} and \textit{KIP3} function in the same pathway.

Defective Movement of the SPB to the Incipient Bud in \textit{bni1}D Cells

Because of the spindle orientation defect in \textit{bni1}D and \textit{kip3}D strains, we determined whether movement of the SPB to the incipient bud site was defective in \textit{bni1}D cells.
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
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 \( bni1\Delta \) 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 \( bni1\Delta \) 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 \( bni1\Delta \) cells were due to abnormal interactions of cytoplasmic microtubules with cortical sites, image sets of wild-type and \( bni1\Delta \) 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
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 bni1\(\Delta\) 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 \(\mu m\). A concomitant 5.6-\(\mu m\) 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 \(\mu m\), whereas the spindle moved 2.1 \(\mu m\) 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 bni1\(\Delta\) cells. Therefore, although microtubules in the bni1\(\Delta\) cells still interact with the cortex, the inability to restrict pulling interactions toward the bud suggests that the microtubule cytoskeleton of the bni1\(\Delta\) strain fails to sense the cell’s polarity.

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

Because loss of BNII 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-\(\mu m\) spindle to an ~4-\(\mu m\) spindle. The rate of elongation then slows as the spindle reaches maximal extension at 8–10 \(\mu m\). Anaphase kinetics was measured in bni1\(\Delta\) and wild-type cells (Fig. 8). Fast elongation was 0.55 \(\mu m/min \pm 0.14 (n = 7)\) in the bni1\(\Delta\) strain and 0.48 \(\mu m/min \pm 0.10 (n = 5)\) in the wild-type strain. Slow elongation was 0.14 \(\mu m/min \pm 0.04 (n = 7)\) in the bni1\(\Delta\) strain and 0.12 \(\mu m/min \pm 0.04 (n = 5)\) in the wild-type strain. Thus, loss of BNII does not have a significant effect on anaphase spindle elongation.

Because the bni1\(\Delta\) mutant could affect spindle orientation either through a direct effect on the cytoskeleton or indirectly by perturbing the cell cycle, we next tested whether bni1\(\Delta\) strains were delayed in any specific stage of the cell cycle. bni1\(\Delta\) strains grew slightly slower than control strains at 23°C. The distribution of cells throughout

---

**Figure 6.** Defective preanaphase spindle movement in a bni1\(\Delta\) 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 \(\mu m\).

**Figure 5.** Preanaphase spindle movement in BNII and bni1\(\Delta\) 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 \(\mu m\), 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.
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 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 IV. Cell Cycle Distribution of BNI1 and bni1Δ Cells |
|-----------------------------------------------|
| Percent un budded | 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.

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 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 test, 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 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. 2c). 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. MatingProjection Formation, Quantitative Mating Assay, and Bipolar Bud Site Selection |
|-----------------------------------------------|
| Mating projection formation | Mating efficiency | Bipolar bud site selection |
|-----------------------------|-------------------|--------------------------|
| % | % | % | % |
| bni1::kan’ [BNI1] | 83.4 ± 5.5 | 0.17 ± 0.041 | — |
| bni1::kan’ [bni1-CTΔ1] | 77.2 ± 5.2 | 0.095 ± 0.013 | — |
| bni1::kan’ [CEN URA3] | 0 | 0.001 ± 0.0006 | — |
| BNI1/bni1Δ | 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.
bni1-CTΔ1 allele produced a striking temperature-sensitive growth defect when combined with aselΔ or dyn1Δ (Fig. 1 a). bni1-CTΔ1 aselΔ and bni1-CTΔ1 dyn1Δ strains were also more sensitive to benomyl, although the effect was less pronounced in the bni1-CTΔ1 aselΔ 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 slalΔΔSH3#3 strain with abnormal cortical actin structure did not have a defect in bipolar bud site selection (Yang et al., 1997). Strains with this slal allele accumulated abnormal cortical actin aggregates (Fig. 9 f) and like slalΔ strains, were temperature-sensitive for growth. Like slalΔ, these strains were also hypersensitive 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 slalΔΔSH3#3 strain. As shown in Fig. 9, c and d, spindle orientation in slalΔΔSH3#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 (slalΔΔSH3#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).
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 *bni1*Δ 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 *bni1*Δ 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 *bni1*Δ cells the orientation of preanaphase spindles was random with respect to the mother–bud axis.

Our real-time analysis demonstrated two striking phenotypes in *bni1*Δ 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 *bni1*Δ 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 *kip3*Δ cells (DeZwaan et al., 1997), and parallels the common spindle orientation defect observed in fixed populations of *bni1*Δ and *kip3*Δ 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-

**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.
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 cells (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 budoΔ 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 (slalΔSH3#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-CTΔ1p 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.

The authors wish to thank K. Salisbury and S. Randall of Improvement (Coventry, UK) for assistance with the imaging system and software, K. Ayscough (University of California, Berkeley, CA), K. Bloom (University of North Carolina, Chapel Hill, NC), S. Brown, D. Drubin, M.A. Hoyt, J. Kahana (Dana-Farber Cancer Institute/Harvard Medical School, Boston, MA), R. Miller, A. Murray, P. Silver, and A. Straight (Harvard Medical School) for plasmids and strains. A. Datta (Ludwig Institute for Cancer Research, La Jolla, CA) for help with cloning by PCR, N. Lee (Massachusetts Institute of Technology, Lexington, MA) for mathematical advice, K. Bloom, D. Drubin, D. Lew (Duke University, Durham, NC), R. Miller (Princeton University, Princeton, NJ), and M. Rose (Princeton University, Princeton, NJ) for helpful discussion, M. Christman (University of Virginia, Charlottesville, VA), R. Li (Harvard Medical School), M. McLaughlin (Brigham and Women’s Hospital, Harvard Medical School), D. Roof (University of Pennsylvania, Philadelphia, PA), S. Schuyler (Harvard Medical School), and J. Tirnauer (Dana-Farber Cancer Institute) for comments on the manuscript, and members of the Pellman lab for stimulating discussions.

This work was supported by the National Institutes of Health (GM55772) and a Kimmel Scholar Award to D. Pellman, grants from the Natural Sciences and Engineering Research Council of Canada and the National Cancer Institute of Canada to C. Boone, and a Natural Sciences and Engineering Research Council of Canada postgraduate fellowship to M. Evangelista.

Received for publication 7 October 1998 and in revised form 6 January 1999.

References

Amberg, D.C., J.E. Zahner, J.W. Muholland, J.R. Pringle, and D. Botstein. 1997. Aip3p/Bud3p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites, Mol. Biol. Cell, 8:729–753.

Ayscough, K.R., J. Stryker, N. Pokala, M. Sanders, P. Crews, and D.G. Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor Latrunculin-A, J. Cell Biol, 137:399–416.

Bender, A., and J.R. Pringle. 1991. Use of a screen for synthetic lethal and mul-
Hyman, A.A., and T. Stearns. 1992. Spindle positioning and cell polarity. *Cell Biol. 11:1295–1305.

Boeke, D.J., and R.F. Proctor. 1991. Evidence for a role of the *HMR region in the control of cell shape in *Saccharomyces cerevisiae. *Mol. Cell Biol. 11:1439–1452.

Castrillon, D.H., and S.A. Wasserman. 1994. A cortical site determining centrosome position. *J. Cell Biol. 128:629–641.

The Journal of Cell Biology, Volume 144, 1999 960

BROWN, A.A., and T. Stearns. 1992. Spindle positioning and cell polarity. *Cell Biol. 11:1295–1305.

Boeke, D.J., and R.F. Proctor. 1991. Evidence for a role of the *HMR region in the control of cell shape in *Saccharomyces cerevisiae. *Mol. Cell Biol. 11:1439–1452.

Castrillon, D.H., and S.A. Wasserman. 1994. A cortical site determining centrosome position. *J. Cell Biol. 128:629–641.
Stearns, T. 1997. Motoring to the finish: kinesin and dynein work together to orient the yeast mitotic spindle. *J. Cell Biol.* 138:957–960.

Straight, A.F., W.F. Marshall, J.W. Sedat, and A.W. Murray. 1997. Mitosis in living budding yeast: anaphase A but no metaphase plate. *Science.* 277:574–578.

Valt, N., and I. Herskowitz. 1996. Pca2 protein of yeast is localized to sites of polarized growth and is required for efficient mating and bipolar budding. *J. Cell Biol.* 135:725–739.

Wach, A., A. Brachat, R. Pöhlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast.* 10:1793–1808.

Winsor, B., and E. Schiebel. 1997. Review: an overview of the *Saccharomyces cerevisiae* microtubule and microfilament cytoskeleton. *Yeast.* 13:399–434.

Woychik, R.P., R.L. Maas, R. Zeller, T.F. Vogt, and P. Leder. 1990. “Formins”: proteins deduced from the alternative transcripts of the *limb deformity* gene. *Nature.* 346:850–853.

Yang, S., K.R. Aycough, and D.G. Drubin. 1997. A role for the actin cytoskeleton of *Saccharomyces cerevisiae* in bipolar bud-site selection. *J. Cell Biol.* 136:111–123.

Zahner, J.E., H.A. Harkins, and J.R. Pringle. 1996. Genetic analysis of the bipolar pattern of bud site selection in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 16:1857–1870.

Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. *Mol. Biol. Cell.* 4:1307–1316.
