

The Cortical Protein Num1p Is Essential for Dynein-dependent Interactions of Microtubules with the Cortex[Ⓢ]

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Abstract. In budding yeast, the mitotic spindle moves into the neck between the mother and bud via dynein-dependent sliding of cytoplasmic microtubules along the cortex of the bud. How dynein and microtubules interact with the cortex is unknown. We found that cells lacking Num1p failed to exhibit dynein-dependent microtubule sliding in the bud, resulting in defective mitotic spindle movement and nuclear segregation.

Num1p localized to the bud cortex, and that localization was independent of microtubules, dynein, or dynactin. These data are consistent with Num1p being an essential element of the cortical attachment mechanism for dynein-dependent sliding of microtubules in the bud.

Key words: microtubule attachment • dynein • mitosis • Num1p • yeast

Introduction

During some developmental processes, cells divide asymmetrically to produce daughter cells of different size or content (Stearns, 1997). In some cases of asymmetric cell division, the mitotic spindle is positioned in a specific orientation or location within the cell. In the budding yeast *Saccharomyces cerevisiae*, the position of cytokinesis is determined before mitosis by the selection of the bud site. The yeast cell then positions the mitotic spindle in the mother-bud neck so that each cell inherits one nucleus.

The position of the nucleus is controlled by cytoplasmic microtubules that connect the spindle pole body with the cell cortex (Sullivan and Huffaker, 1992; Shaw et al., 1997). Early in the cell cycle, a cortical attachment site for microtubules is established at the nascent bud site (Shaw et al., 1997; Adames and Cooper, 2000). If a growing microtubule contacts this site, the microtubule can be captured and then shrink, pulling the nucleus towards the emerging bud. In this process, the attachment of the microtubule to the cortex requires the cortical protein Kar9p, and the movement of the nucleus requires the kinesin Kip3p (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Miller and Rose, 1998; Miller et al., 1998).

Upon initiating anaphase, the elongating mitotic spindle moves into the neck (Yeh et al., 1995). The primary force for movement of the spindle into the neck comes from dynein-dependent sliding of cytoplasmic microtubules along the bud cortex, which pulls the spindle into the neck (Adames and Cooper, 2000). The cortical attachment molecule for dynein-dependent microtubule sliding is not known. Actin is not involved (Heil-Chapdelaine et al., 2000).

We have sought molecules involved in cortical attachment of microtubules for dynein-dependent movements. Mutants lacking cortical attachment molecules involved in dynein-dependent microtubule sliding should behave like dynein mutants in terms of cytoplasmic microtubule and spindle behavior. A cortical attachment molecule should be located at the cortex, and its localization should not require microtubules or dynein.

We considered Num1p as a candidate to mediate cortical attachment of microtubules in the bud because *num1Δ* mutants have a nuclear segregation phenotype and behave genetically like dynein in certain ways (Kormanec et al., 1991; Farkasovsky and Kuntzel, 1995; Geiser et al., 1997; Schwartz et al., 1997). Num1p has been described as localizing to the cortex, but primarily in the mother cell, which is the opposite direction of where the spindle needs to move (Farkasovsky and Kuntzel, 1995). Several models might explain this apparent paradox. First, Num1p may inhibit attachment sites in the mother, restricting active attachment sites to the bud. Alternatively, previously undetected Num1p may localize within the bud and work with

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dynein to capture and slide microtubules along the bud cortex, thus pulling the spindle into the bud neck.

We tested these models by examining the position and movement of the spindle and the cytoplasmic microtubules in wild type and *num1* Δ mutants. We also examined Num1p localization in live cells expressing Num1-GFP. Our data exclude the first model and support a role for Num1p at the cortex of the bud, working with dynein to pull the spindle into the neck via sliding of cytoplasmic microtubules along the cortex.

Materials and Methods

Reagents and Supplies

Yeast media was from Bio101. Frozen EZ transformation kit was from Zymo Research. Latrunculin A was from Dr. Philip Crews (Department of Chemistry, UCSC, NIH grant CA47135). GFP-TUB1 in plasmid pAFS92 was a gift from Aaron Straight and Andrew Murray (Department of Physiology, University of California, San Francisco, CA; Straight et al., 1997). All other reagents were from Sigma-Aldrich or Fisher.

NUM1 Deletion

The open reading frame for *NUM1* was deleted via homologous recombination using a PCR product composed of *HIS3* with 45 bp of sequence flanking the coding regions (Baudin et al., 1993). This PCR product was transformed into the diploid YJC1411. Genomic DNA from transformants was tested for the appropriate deletion by PCR with a forward primer upstream of the disruption and a reverse primer within the *HIS3* gene. Positive transformants were sporulated, and haploid mutants were isolated by tetrad dissection.

Nuclear Segregation and Microtubule Behavior

Nuclear segregation experiments were performed as described (Heil-Chapdelaine et al., 2000).

For analysis of microtubule behavior, cells were grown and viewed as described (Heil-Chapdelaine et al., 2000). Movies of cytoplasmic microtubules were made at 2 frames per second at a single focal plane using an ISIT video camera (Dage ISIT 68). Measurements were made using NIH Image 1.62. Additional movies for analysis of spindle movements were made by collecting 12 0.5- μ m slices at 1-min intervals and summing them into a two-dimensional projection.

Num1-GFP: Strain Construction, Microscopy, and Cytoskeletal Disruption

A Num1p-GFP fusion protein was made by integration at the *NUM1* locus, a PCR product containing GFP (S65G, S72A) and *HIS3*, flanked by 45 bases corresponding to the last 15 codons of *NUM1* and 45 bases immediately 3' of the *NUM1* stop codon. Positive transformants were confirmed by PCR and fluorescence microscopy. Num1-GFP functioned similarly to untagged Num1p as determined by assays of nuclear segregation and microtubule sliding.

Yeast strains are listed in Table I. To construct strains containing both Num1-GFP and various null mutations (*act5* Δ , *dhc1/dyn1* Δ , *jnm1* Δ , *pac10* Δ , *nip100* Δ , *bik1* Δ , *kip1* Δ , *kip2* Δ , *kip3* Δ , *kar3* Δ , *cin8* Δ , *smy1* Δ , *bni1* Δ , *spa2* Δ , *bud6* Δ , *kar9* Δ , *bem1* Δ , *bem4* Δ , *boi1* Δ , and *rho4* Δ), strain YJC2019 (Num1-GFP) was crossed to deletion strains from the deletion consortium (Research Genetics Inc.). Heterozygous diploids were sporulated in liquid sporulation media, and then vortexed with an equal volume of ether for 3 min. One drop of the aqueous phase was plated on synthetic media lacking histidine to select for yeast containing Num1-GFP. Colonies were patched on minus histidine plates and replica-plated onto G418 plates (200 μ g/ml) to test for the deletion.

For analysis of Num1-GFP localization, cells (YJC2019, YJC2037) were grown to early log phase in YPD, washed with nonfluorescent media, and single focal plane images were collected with a cooled CCD camera (DAGE-MTI-300T-RC). For analysis of Num1-GFP localization throughout the cell cycle, 12 0.5- μ m Z-slices were collected at time intervals with an ISIT camera and projected in two dimensions.

For analyzing Num1-GFP localization in the absence of microtubules, cells (YJC2151) were grown to early log phase ($OD_{600} \sim 0.3$) in YPD at

30°C, collected by centrifugation, resuspended in synthetic media lacking methionine to induce the GFP-tubulin and grown at 30°C for 1.5 h. Cells were then collected by centrifugation, and resuspended in nonfluorescent complete media (Waddle et al., 1996) containing 1% DMSO or 25 μ M nocodazole. Single focal plane images were collected every 30 min.

For analyzing localization of Num1-GFP in the absence of actin, cells (YJC 2151) were grown and induced as above, and resuspended in YPD plus 0.2 M hydroxyurea (HU)¹ for 1.5 h. Either 1% DMSO or 500 μ M latrunculin A was added. Single focal plane images were taken after 30 min on a cooled CCD camera. Localization of Num1-GFP in mutants was analyzed by collecting single focal plane images with a cooled CCD camera of HU-arrested cells expressing Num1-GFP.

Student's *t*-test assuming unequal variances was performed with Microsoft Excel. *P* values for two-tailed analyses are listed where appropriate.

Online Supplemental Material

Videos of microtubule behavior during movement of the spindle into the bud-neck: *num1* Δ mutants in both grayscale and pseudocolor (Video 1, black and white; and Video 2, color) and wild-type in both grayscale and pseudocolor (Video 3, black and white; Video 4, color); can be found at <http://www.jcb.org/cgi/content/full/151/6/1337/DC1>.

Results and Discussion

Mitotic Phenotype of *num1* Δ Mutants

We asked whether *num1* Δ mutants had a mitotic phenotype like that of dynein and dynactin mutants. Dynactin is an essential regulator of dynein (Muhua et al., 1994; Kahana et al., 1998). Previous studies of *num1* Δ mutants found an increased number of binucleate cells in logarithmically growing cells, enhanced by cold temperatures (Kormanec et al., 1991; Geiser et al., 1997; Schwartz et al., 1997). These results are similar to results for dynein and dynactin mutants. However, our search for dynein-like phenotypes revealed that a limited analysis of this sort can give false positives (Heil-Chapdelaine et al., 2000). A more thorough kinetic analysis of spindle and microtubule position and movement in movies of single cells, is necessary to identify a “dynein-like” mitotic phenotype with certainty (Heil-Chapdelaine et al., 2000).

In dynein and dynactin mutants, cytoplasmic microtubules do not attach laterally (“plaster”) or slide along the cortex of the bud (Adames and Cooper, 2000). In contrast, microtubule plastering and sliding does occur in *kar9* Δ and *kip3* Δ mutants (data not shown). If Num1p functions at the bud cortex to promote dynein-dependent microtubule sliding, then loss of Num1p should cause loss of microtubule plastering and sliding. Alternatively, if Num1p acts to inhibit attachment in the mother, then sliding of cytoplasmic microtubules in the bud should be normal in *num1* Δ mutants. To discriminate between these two models, we examined cytoplasmic microtubule behavior during movement of the mitotic spindle into the neck in wild-type and *num1* Δ mutants expressing GFP-tubulin (YJC2027 and YJC2033). In 6 of 8 wild-type cells in which cytoplasmic microtubules were observed during movement of the spindle into the neck, microtubules did slide along the bud cortex (Fig. 1 A and B, and supplemental videos at <http://www.jcb.org/cgi/content/full/151/6/1337/DC1>). In 19 *num1* Δ cells, cytoplasmic microtubules were never seen to plaster or slide along the bud cortex during movement of the mitotic spindle into the neck (Fig. 1 A and B, and supplemental videos at <http://www.jcb.org/cgi/content/full/151/6/1337/DC1>). Instead, cy-

¹Abbreviations used in this paper: HU, hydroxyurea.

Table I. Yeast Strains

Strain	Genotype	Source
YJC0078	<i>MATa leu2 ura3-52 his4-539 cap2-1::URA3</i>	KT903 (Kelly Tatchell)
YJC0174	<i>MATα ura3-52 lys2-801amb ade2-101och trp1-Δ63 his3-Δ200 leu2-Δ1 cap1::TRP1</i>	Heil-Chapdelaine et al., 2000
YJC1160	<i>MATa ura3-52 lys2-801amb ade2-101och trp1-Δ63 his3-Δ200 leu2-Δ1 act5::HIS3</i>	Muhua et al., 1994
YJC1163	<i>MATa his3-200 leu2 ura3 ade2 jnm1::LEU2</i>	This study
YJC1193	<i>MATα leu2 ura3 his3-Δ200</i>	Karpova et al., 1998
YJC1210	<i>MATa dhc1-7::URA3 ACT5 ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1</i>	Muhua et al., 1994
YJC1411	<i>MATa leu2 ura3 his3-Δ200</i> <i>MATα leu2 ura3 his3-Δ200</i>	Karpova et al., 1998
YJC1618	<i>MATa leu2 ura3 his3-Δ200 nip100Δ::HIS3</i>	This study
YJC1871	<i>MATa leu2 ura3 his3-Δ200 num1Δ::HIS3</i> <i>MATα leu2 ura3 his3-Δ200 num1Δ::HIS3</i>	This study
YJC2019	<i>MATα Num1-GFP-HIS3 leu2 ura3 his3-Δ200</i>	This study
YJC2027	<i>MATa leu2 ura3 his3-Δ200 [pAFS92-GFP-TUB1::URA3]</i> <i>MATα leu2 ura3 his3-Δ200</i>	This study
YJC2033	<i>MATa leu2 ura3 his3-Δ200 num1Δ::HIS3 [pAFS92-GFP-TUB1::URA3]</i> <i>MATα leu2 ura3 his3-Δ200 num1Δ::HIS3</i>	This study
YJC2037	<i>MATa Num1-GFP-HIS3 leu2 ura3 his3-Δ200</i> <i>MATα Num1-GFP-HIS3 leu2 ura3 his3-Δ200</i>	This study
YJC2151	<i>MATa Num1-GFP-HIS3 leu2 ura3 his3-Δ200 [pAFS92-GFP-TUB1::URA3]</i> <i>MATα Num1-GFP-HIS3 leu2 ura3 his3-Δ200</i>	This study
YJC2363	<i>MATa Num1-GFP::HIS3 act5::Kan^R leu2 his3 ura3</i>	This study
YJC2365	<i>MATa Num1-GFP::HIS3 dhc1/dyn1::Kan^R leu2 his3 ura3</i>	This study
YJC2367	<i>MATa Num1-GFP::HIS3 jnm1::Kan^R leu2 his3 ura3</i>	This study
YJC2369	<i>MATa Num1-GFP::HIS3 pac10::Kan^R leu2 his3 ura3</i>	This study
YJC2371	<i>MATa Num1-GFP::HIS3 nip100::Kan^R leu2 his3 ura3</i>	This study
YJC2373	<i>MATa Num1-GFP::HIS3 bik1::Kan^R leu2 his3 ura3</i>	This study
YJC2375	<i>MATa Num1-GFP::HIS3 kip1::Kan^R leu2 his3 ura3</i>	This study
YJC2377	<i>MATa Num1-GFP::HIS3 kip2::Kan^R leu2 his3 ura3</i>	This study
YJC2379	<i>MATa Num1-GFP::HIS3 kip3::Kan^R leu2 his3 ura3</i>	This study
YJC2381	<i>MATa Num1-GFP::HIS3 kar3::Kan^R leu2 his3 ura3</i>	This study
YJC2383	<i>MATa Num1-GFP::HIS3 cin8::Kan^R leu2 his3 ura3</i>	This study
YJC2385	<i>MATa Num1-GFP::HIS3 smy1::Kan^R leu2 his3 ura3</i>	This study
YJC2387	<i>MATa Num1-GFP::HIS3 bni1::Kan^R leu2 his3 ura3</i>	This study
YJC2389	<i>MATa Num1-GFP::HIS3 spa2::Kan^R leu2 his3 ura3</i>	This study
YJC2391	<i>MATa Num1-GFP::HIS3 bud6::Kan^R leu2 his3 ura3</i>	This study
YJC2393	<i>MATa Num1-GFP::HIS3 kar9::Kan^R leu2 his3 ura3</i>	This study
YJC2395	<i>MATa Num1-GFP::HIS3 bem1::Kan^R leu2 his3 ura3</i>	This study
YJC2397	<i>MATa Num1-GFP::HIS3 bem4::Kan^R leu2 his3 ura3</i>	This study
YJC2401	<i>MATa Num1-GFP::HIS3 boi1::Kan^R leu2 his3 ura3</i>	This study
YJC2403	<i>MATa Num1-GFP::HIS3 rho4::Kan^R leu2 his3 ura3</i>	This study

toplastic microtubules rotated freely about the spindle pole body, flailing about the cytoplasm of the bud. Occasionally, cytoplasmic microtubules were observed to encounter the cortex at their distal ends, become anchored for a short time, and sometimes shrink, similar to what was observed for dynein and dynactin mutants (Adames and Cooper, 2000). Loss of microtubule sliding in the bud supports the model in which Num1p functions in the bud to assist in dynein-mediated microtubule sliding. The result is inconsistent with the model in which Num1p inhibits microtubule attachment in the mother.

If Num1p functions in the bud with dynein, then loss of Num1p should result in the elongating mitotic spindle residing near the neck and ultimately moving into the neck inefficiently, as seen in dynein mutants (Yeh et al., 1995; Adames and Cooper, 2000). Alternatively, if Num1p functions in the mother to inhibit microtubule attachment and pulling toward the mother cortex, then the mitotic spindle should occupy a position near the mother cortex. To distinguish between these two models, we examined spindle position and movement in movies of wild-type, dynein, and *num1Δ* mutants expressing GFP-tubulin. The dis-

tance between the mitotic spindle and the neck at the onset of anaphase was similar in *wt*, *dhc1Δ*, and *num1Δ* cells (Fig. 1 D). None of the differences were significant. Thus, the data are inconsistent with Num1p inhibiting microtubule attachment in the mother cell.

In this experiment, we also assessed the efficiency of spindle movement, by measuring the length to which the spindle elongated before it entered the neck and the time required for the spindle to enter the neck. In *num1Δ* cells, spindles elongated to twice the length of spindles in wild-type cells before they entered the neck, similar to *dhc1Δ* cells (Fig. 1 C). *num1Δ* cells also required more time for the spindle to enter the neck after initiating elongation, similar to *dhc1Δ* cells (Fig. 1 C). Together, these results support a model in which Num1p functions in the bud with dynein.

Loss of microtubule sliding in dynein and dynactin mutants results in poor nuclear segregation, which is most readily revealed in synchronized cell assays (Heil-Chapdelaine et al., 2000). We examined nuclear segregation in synchronized cultures of *num1Δ* mutants comparing them with dynein and dynactin mutants. In wild-type cells (YJC1193), only 1% of mitoses occurred in the mother

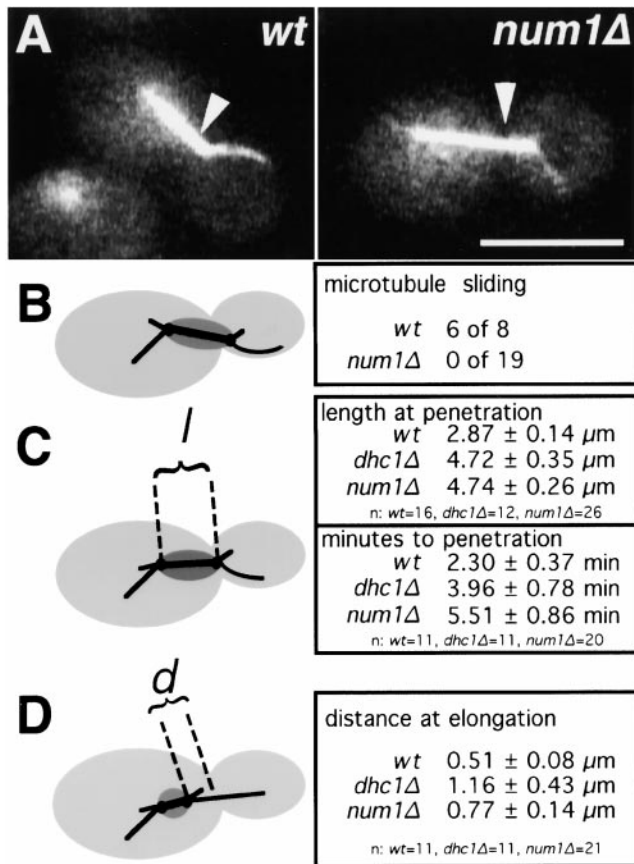


Figure 1. Microtubule and spindle behavior in *num1Δ* mutants. **A**, Frames from videos of GFP-labeled microtubules during movement of the spindle into the bud neck (see <http://www.jcb.org/cgi/content/full/151/6/1337/DC1>). The arrow marks the bud-neck. The cytoplasmic microtubule is “plastered” along the bud cortex in the *wt* cell, but not in the *num1Δ* cell. Bar, 5 μm. The accompanying videos show sliding in a *wt* cell and lack of sliding in a *num1Δ* cell. **B**, Frequency of cytoplasmic microtubules sliding along the cortex in *wt* and *num1Δ* cells. **C**, The length of the spindle 1 min before it penetrated the neck. Spindles in *num1Δ* cells were longer than spindles in *wt* cells, similar to spindles in *dhc1Δ* cells (*num1Δ* vs. *wt*, $P = 2.6 \times 10^{-7}$; *num1Δ* vs. *dhc1Δ*, $P = 0.97$). Also, the time from initiation of spindle elongation to penetration of the neck was determined. Spindles took longer to enter the neck in *num1Δ* cells than in *wt* cells ($P = 2 \times 10^{-3}$), but no longer than in *dhc1Δ* cells ($P = 0.19$). **D**, The distance of the spindle from the neck at initiation of spindle elongation. None of the differences are statistically significant (*num1Δ* vs. *wt*, $P = 0.13$; *dhc1Δ* vs. *wt*, $P = 0.17$; *num1Δ* vs. *dhc1Δ*, $P = 0.41$).

cell. Many mitoses occurred in the mother in cells lacking dynein (YJC1210) or the dynactin components Arp1p (YJC1160), Jnm1p (YJC1163), or Nip100p (YJC1618), but not capping protein (YJC0078 and YJC0174). Therefore, the yeast orthologues of p150^{Glued} (Nip100p), p50^{dynamitin} (Jnm1p), and Arp1 (Arp1p/Act5p) are essential for dynein function in spindle positioning while capping protein is not. In *num1Δ* cells, mitosis occurred within the mother in 25% of mitoses (Fig. 2). Therefore, *num1Δ* mutants also resembled dynein mutants in this assay for nuclear segregation.

Num1p Localization

The model for Num1p working as an essential element of dynein-dependent sliding of cytoplasmic microtubules along the bud cortex predicts that Num1p should localize

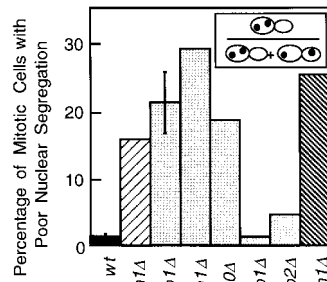


Figure 2. Nuclear segregation in *num1Δ*, dynein, and dynactin mutants. The frequency of cells with both nuclei in the mother as a percentage of total mitotic events is plotted. Error bars represent SEM (*wt*, $n = 7$; *arp1Δ*, $n = 5$).

to the bud. To test this model, we tagged Num1p with GFP to determine the location of Num1p in live cells throughout the cell cycle. We tagged Num1p by integration of GFP at the *NUM1* locus. Thus, Num1–GFP was under the control of its endogenous promoter. Num1–GFP was fully functional as assessed by rescue of nuclear segregation and microtubule sliding phenotypes (data not shown).

Cells expressing Num1–GFP (YJC2037) had bright spots at the mother cortex. Small buds showed no fluorescence, but medium-sized buds had punctate fluorescence at the cortex (Fig. 3 A). In large buds, the cortical spots were brighter and clustered at the tip of the bud (Fig. 3 A).

To investigate the assembly and dynamics of Num1p cortical patches, we made time-lapse movies of Num1–GFP cells. At each time point, several focal planes were collected, providing images through the entire thickness of the cell. These images were summed into a two-dimensional projection. The Num1–GFP spots in the mother cortex were relatively immobile, based on images at 5-min intervals (Fig. 3 B). Cortical spots appeared in the bud de novo and accumulated as a crescent at the bud tip (Fig. 3 B). Bud spots also did not move. The accumulation of Num1p at the bud tip occurs at the time that Num1p is synthesized (Farkasovsky and Kuntzel, 1995), suggesting that newly synthesized Num1p may go to the bud.

Num1p remained at the tip of the bud until after cell separation (Fig. 4 A, open arrow), in contrast to certain cell polarity and secretory proteins such as Sec3p and Cdc24p, which leave the tip of the bud and accumulate at the neck (Finger and Novick, 1998; Toenjes et al., 1999). After cell separation, the crescent of Num1p at the bud tip faded and became less tightly aggregated, suggesting that the crescent is a collection of smaller aggregates or that smaller aggregates are formed by breakdown of a larger complex (data not shown).

Num1p and Filamentous Actin

Work by ourselves and others has shown that movement of the spindle into the neck of large-budded cells occurs independently of actin (Theesfeld et al., 1999; Heil-Chapdelaine et al., 2000). If Num1p functions with dynein to slide microtubules along the cortex of larger buds, then maintenance of Num1p in the bud should also be independent of actin. To test this prediction, we arrested cells with large buds and short spindles by treatment with HU. Dynein-based movement of the spindle is independent of actin in cells released from such an arrest (Theesfeld et al., 1999; Heil-Chapdelaine et al., 2000). In arrested cells (YJC2151), Num1–GFP was concentrated at the tip of the bud (Fig. 4 B). Arrested cells were treated with latrunculin A to depolymerize actin filaments, and Num1–GFP remained in the bud tip (Fig. 4

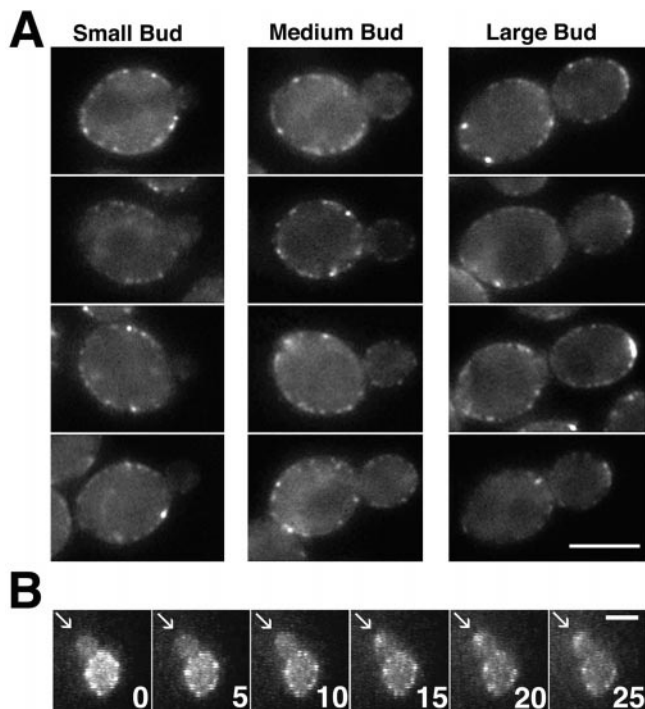


Figure 3. Num1p localization. A, Representative cells expressing Num1-GFP are shown. Bar, 5 μ m. B, Num1-GFP localization in a single cell over time. 16 focal planes were collected every minute in diploid cells expressing Num1-GFP and projected to two dimensions. Shown are frames at 5 min intervals. At $t = 0$ the mother has bright cortical spots and the bud has no staining. The spots in the mother do not move. At 15 min, a small amount of fluorescence staining appears at the bud tip. The bud tip staining enlarges over time (arrow). Bar, 5 μ m.

B). Therefore, maintenance of Num1-GFP in the bud is independent of filamentous actin, which is consistent with Num1p being an essential element for actin-independent sliding of microtubules by dynein.

Num1p Localization Does Not Depend on Microtubules, Dynein, Dynactin, Lis1, or Molecules in the Kip3p/Kar9p Nucleus Positioning Mechanism

If Num1p serves as a cortical attachment molecule for dynein, dynactin, and/or microtubules, its localization at the bud cortex should not depend on them. We treated cells expressing GFP-tubulin and Num1-GFP (YJC2151) with nocodazole to depolymerize the microtubules and followed growing buds. Loss of GFP-labeled microtubules documented that microtubules were depolymerized throughout the experiment. Cells with small buds did not contain fluorescent spots in the bud. As the buds grew, spots of fluorescence appeared in the bud, and eventually a crescent was formed (Fig. 4 A). The size and intensity of the crescent was normal. Therefore, neither assembly nor maintenance of Num1-GFP in the bud depends on microtubules. We also localized Num1-GFP in dynein (*dhc1 Δ* , YJC2365), and dynactin mutants (*act5 Δ* , YJC2363; *jnm1 Δ* , YJC2367; and *nip100 Δ* , YJC2371). The localization of Num1-GFP was indistinguishable from *wt* cells (YJC2019, data not shown). Therefore, Num1p localization at the bud cortex does not require dynein or dynactin.

Lis1 is a protein implicated in dynein function during neuronal development in *Drosophila* and humans, and in nu-

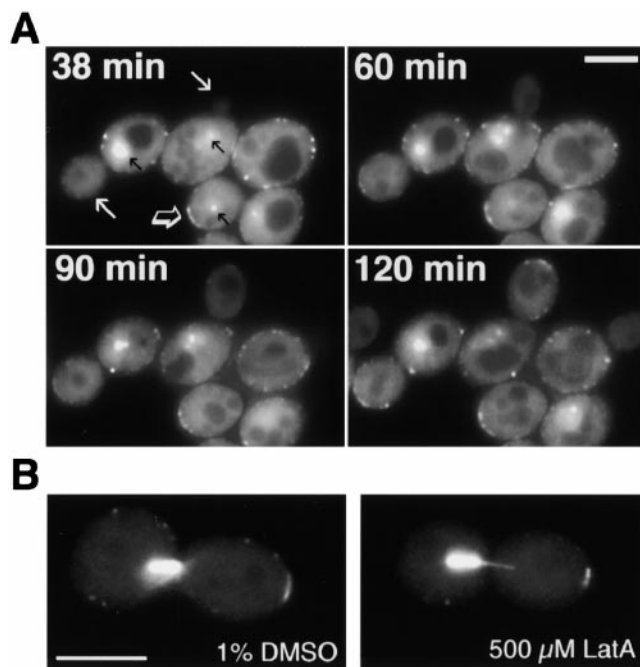


Figure 4. Localization of Num1p without microtubules and without filamentous actin. A, Localization of Num1-GFP does not require microtubules. Log phase diploid cells expressing GFP-tubulin and Num1-GFP were treated with 25 μ M nocodazole at $t = 0$ min to depolymerize microtubules, and images were collected at 30-min intervals. The black arrows indicate residual GFP-tubulin fluorescence at spindle pole bodies. The white arrows indicate growing buds. The larger of the two growing buds has dim cortical fluorescence that increases over time. The smaller of the two growing buds begins to show cortical fluorescence at 90 min after nocodazole addition, and a bright cortical crescent appears at 120 min. The open arrow shows a crescent of spots at the tip of a large budded cell (mother cell is above and to the right of the bud). Cell separation occurs between 38 and 60 min, manifested by a shift in the position of the bud relative to the mother. Fluorescence remains at what was the tip of the bud throughout the time course. Bar, 5 μ m. These results are identical to those for cells not treated with nocodazole (data not shown). B, Maintenance of Num1-GFP to the bud tip does not require filamentous actin. Cells expressing Num1-GFP and GFP-tubulin were treated with 200 mM HU to arrest them as large budded cells with short mitotic spindles. The cells were treated with 1% DMSO as a control or 500 μ M latrunculin A to depolymerize actin. Both DMSO-treated and latrunculin A-treated cells show bright fluorescence at the tip of the bud after 10 min of treatment. Similar results were obtained from 5 to 30 min after treatment. Bar, 5 μ m.

clear migration in fungi (Morris, 2000). To test the epistasis of Lis1p in Num1p localization, we localized Num1-GFP in mutants lacking the yeast homologue of Lis1, Pac1p (*pac1 Δ*) (Geiser et al., 1997). The localization of Num1-GFP in the *pac1 Δ* mutant was indistinguishable from *wt* cells (data not shown). Therefore, Num1p localization does not require Lis1/Pac1p. These data are consistent with Num1p localization not requiring dynein function.

Genetic and morphological evidence points to two sequential yet partially overlapping and redundant mechanisms for moving the mitotic spindle into the bud neck: a Kip3p-dependent mechanism and a dynein-dependent mechanism (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Miller and Rose, 1998; Miller et al., 1998). Deletion of both mechanisms severely impairs nuclear segregation

and cell viability. If Num1p is part of the dynein-dependent mechanism, then loss of both Num1p and Kar9p should reduce viability and localization of Num1p should be independent of Kar9p or Kip3p. Tetrad analysis revealed that *num1Δ* is synthetic lethal with *kar9Δ* (of 26 double mutants 20 were dead and 6 grew extremely poorly), but not with *dyn1Δ* (all of 25 double mutants grew well). This result supports a role for Num1p in the dynein-dependent sliding of microtubules, but not the Kar9p, Kip3p-dependent mechanism of nuclear positioning. To examine Num1p localization in *kip3Δ* and *kar9Δ* cells, these strains expressing Num1-GFP (YJC2393 and YJC2379) were arrested as large budded cells with HU. Both strains contained cortical spots in the mother, and crescent-like aggregates of Num1p at the tip of the bud. Therefore, neither Kip3p or Kar9p are required for Num1p localization.

We also tested Num1p localization in a number of mutants involved in microtubule function or cell polarity: *pac10Δ*, *bik1Δ*, *kip1Δ*, *kip2Δ*, *kar3Δ*, *cin8Δ*, *smy1Δ*, *bni1Δ*, *spa2Δ*, *bud6Δ*, *bem1Δ*, *bem4Δ*, *boi1Δ*, and *rho4Δ* (YJC2369, YJC2373, YJC2375, YJC2377, YJC2381, YJC2383, YJC2385, YJC2387, YJC2389, YJC2391, YJC2395, YJC2397, YJC2401, and YJC2403). Num1p accumulation in the bud was normal in all these mutants.

Perhaps no protein is necessary for localization of Num1p to the cortex. Num1p contains a COOH-terminal pleckstrin homology domain that is essential and sufficient for localization to the mother cortex and may therefore bind directly to membrane lipids (Farkasovsky and Kuntzel, 1995). Pleckstrin homology domains are found on a variety of membrane proteins and many bind polyphosphoinositides (Rebecchi and Scarlata, 1998). An asymmetric distribution of lipids between the mother and bud might account for newly synthesized Num1p being directed to the bud cortex. Alternatively, lipids could be symmetrically distributed and a protein-based mechanism may transport Num1p into the bud.

The function of Num1p in the mother is uncertain. Dynein-dependent sliding of microtubules does occur in the mother, but not until late in mitosis (Adames and Cooper, 2000). Therefore, dynein in the mother appears to be inhibited early in mitosis, but this inhibition is not due to the absence of Num1p. Num1p in the mother may function late in mitosis with dynein to move microtubules.

The dynein-dependence of microtubule sliding in yeast suggests that dynein should localize to the cortex. The fact suggests that *num1Δ* mutants lack microtubule sliding that localization of dynein to the cortex may be lost in *num1Δ* mutants. Unfortunately, despite extensive work by ourselves and others, no conclusive evidence exists for localization of dynein complex to the cortex (Yeh et al., 1995; Shaw et al., 1997; our unpublished data). Should a method be developed to visualize dynein at the cortex it will be interesting to test if loss of Num1p affects that localization.

Num1p is the first protein identified in yeast that localizes exclusively to the cortex and is essential for dynein-dependent sliding of microtubules along the cortex. How dynein interacts with membranes or the cortex in other organisms is unclear. p62, of the dynactin complex, may link dynein to the actin cytoskeleton (Garces et al., 1999). Also, dynein intermediate chains may link dynein to or-

ganelles through interactions with p150^{Glued} (Steffen et al., 1997). However, except for evidence that dynein can bind directly to phospholipids in vitro (Lacey and Haimo, 1994), no molecule has been found that has an analogous function to Num1p.

BLAST analysis of the Num1p sequence does not reveal any obvious homologues in other organisms. However, biochemical evidence and secondary structure analysis suggest that Nip100p and Jnm1p are part of the yeast dynactin complex, even though BLAST analysis shows only weak similarity of Nip100p to p150^{Glued} and of Jnm1p to dynamin. Therefore, identifying what proteins may complex with Num1p could allow the identification of analogous complexes in other organisms.

In conclusion, we have shown that Num1p is in the bud when dynein slides microtubules along the cortex, and this localization is independent of dynein or microtubules. We also showed that loss of Num1p completely mimics the complete loss of dynein or dynactin function. Taken together, these data show that Num1p is an essential element of the cortical attachment mechanism for dynein-dependent sliding of microtubules along the cortex.

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