Cortical Num1p Interacts with the Dynein Intermediate Chain Pac11p and Cytoplasmic Microtubules in Budding Yeast

Marian Farkasovsky and Hans Küntzel
Max-Planck Institute for Experimental Medicine, D-37075 Göttingen, Germany

Abstract. Num1p, a cortical 313-kD protein, controls cytoplasmic microtubule (cMT) functions and nuclear migration through the bud neck in anaphase cells. A green fluorescent protein (GFP)-Num1p fusion protein localizes at the bud tip and the distal mother pole of living cells, apparently forming cMT capture sites at late anaphase. In addition, galactose-induced GFP-Num1p is seen at the bud neck and in lateral regions of the mother cortex. The bud tip location of Num1p depends on Bni1p but does not require Kar9p, Dyn1p, or cMTs, whereas cMT contacts with polar Num1p dots are reduced in cells lacking Dyn1p. Num1p associates with the dynein intermediate chain Pac11p in the presence of Dyn1p, and with the α-tubulin Tub3p, as shown by coimmune precipitation of tagged proteins. Num1p also forms a complex with Bni1p and Kar9p, although Num1p is not required for Bni1p- and Kar9p-dependent nuclear migration to the bud neck in preanaphase cells.

Our data suggest that Num1p controls nuclear migration during late anaphase by forming dynein-interacting cortical cMT capture sites at both cellular poles. In addition, Num1p may transiently cooperate with an associated Bni1p–Kar9p complex at the bud tip of early anaphase cells.

Key words: microtubule • cytoskeleton • nuclear migration • dynein • yeast

Introduction

Two subsequent steps of nuclear migration are required for chromosome segregation in the budding yeast Saccharomyces cerevisiae: the proper positioning of the short preanaphase spindle at the mother bud neck, followed by the movement of the elongating spindle and nucleus through the neck during anaphase. Both nuclear migration steps depend on the functions of kinesin-related motor proteins and are controlled by physical interactions between the plus-ends of cytoplasmic microtubules (cMTs) and cortical proteins serving as cMT capture sites (Yeh et al., 1995; Carminati and Stearns, 1997; DeZwaan et al., 1997; Shaw et al., 1997, 1998; Kahana et al., 1998; Cottingham and Hoyt, 1997; Adames and Cooper, 2000; Heil-Chapdelaine et al., 2000; Hildebrandt and Hoyt, 2000).

The first step, nuclear migration to the bud neck and alignment of the short spindle along the mother–daughter axis in preanaphase cells, requires the interaction of the plus-ends of bud-oriented cMTs with the cortical protein Kar9p (Miller and Rose, 1998). The Kar9p localization at the bud tip cortex depends on its interaction with the formin Bni1p, the actin-interacting protein Bud6p/Aip3p, and F-actin (Lee et al., 1999; Miller et al., 1999; Theesfeld et al., 1999; Lee et al., 2000). Furthermore, the cMT capture by cortical Kar9p requires Bim1p/Yeb1p, a protein of the EB1 family localized at the plus-ends of microtubules (Schwartz et al., 1997; Tirnauer et al., 1999; Adames and Cooper, 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000), and the kinesin-related motor protein Kip3p (Cottingham and Hoyt, 1997; DeZwaan et al., 1997).

The movement of the elongating spindle through the bud neck during anaphase (the second step of nuclear migration) involves dynein (Dyn1p), a minus-end–directed microtubule motor, its actin-binding regulator dynactin (Act5p/Arp1p, Nip100p, Jnm1p), and the kinesin-related motor Kip2p (Saunders et al., 1995; Yeh et al., 1995; Carminati and Stearns, 1997; Shaw et al., 1997, 1998; Kahana et al., 1998; Miller et al., 1998; Adames and Cooper, 2000). Filamentous actin is not required for the dynein-dependent spindle movement through the neck (Heil-Chapdelaine et al., 2000).

Cortical proteins interacting with the dynein–dynactin complex at cMT capture sites have been postulated (Carminati and Stearns, 1997; Heald and Walczak, 1999; Karki and Holzbaur, 1999; Hildebrandt and Hoyt, 2000) but remain to be identified. A possible candidate is Num1p, a large protein (313 kD), which was localized by indirect immunofluores-
cence microscopy mainly to the mother cortex of budded cells (Kormanec et al., 1991; Farkasovsky and Küntzel, 1995). The cortical association and nuclear migration control functions of Num1p were shown to depend on a COOH-terminal pleckstrin homology (PH) domain (Farkasovsky and Küntzel, 1995). The deletion of NUM1 does not affect viability but causes the accumulation of large-budded cells with two DAPI-stained chromosomal regions in the mother compartment, especially after entering the stationary phase (Kormanec et al., 1991; Revardel and Aigle, 1993). num1Δ strains are synthetic lethal with certain cold-sensitive tub2 alleles and contain abnormally long and mislocated cMTs (Farkasovsky and Küntzel, 1995), as observed in dyn1Δ and arp1Δ mutants (Muhua et al., 1994; Carminati and Stearns, 1997).

A synthetic lethality screen with a strain deficient in the kinesin-related motor protein Cin8p has identified Num1p and Cin8p as a complex with the dynein heavy chain Dyn1p appears to be important to mediate Num1p–cMTs contacts.

We further demonstrate that Num1p forms a complex with the dynein intermediate chain Pac11p, if Dyn1p is present, and with the α-tubulin Tub3p, suggesting a dynein-anchoring function of Num1p at cMT capture sites. In addition, we present evidence that Num1p coexists and associates with Bni1p and Kar9p at the bud tip of early anaphase cells, although Num1p is not required for Bni1p- and Kar9p-dependent nuclear migration to the bud neck in preanaphase cells.

Materials and Methods

Strains, Media, and Genetic Techniques

Genotypes of the yeast strains used in this study are listed in Table I. Media, genetic techniques, and the lithium acetate method of yeast transformation were as described (Ausubel et al., 1993). PCR-based gene deletions and modifications were carried out as described (Longtine et al., 1998), using DNA polymerase (Expand™ High Fidelity PCR System) was purchased from Roche Molecular Biochemicals. Plasmid pFM206 contains a KanMX4-GAL1p-yEGFP3 cassette and was constructed as follows: the GAL1 promoter was inserted as a 0.8-kb EcoRV/SpeI fragment of pFM110 (GAL1p in pBlueScript II KS) between the respective sites of pUG6 (Guldener et al., 1996). A 0.7-kb BamHI/SpeI PCR fragment containing yEGFP3 (Cormack et al., 1997) was inserted between the respective sites of pUG6-GAL1p to obtain pFM204. A linker encoding the decapeptide GPDGAPADGP was inserted between the SpeI and SacII...
media without (left) or with (right) 0.3 mg/ml polymyxin B.

Table II. Primers Used for PCR

| Name       | Sequence (5'-3')* |
|------------|------------------|
| NUM1-A1    | GCT GAT AGA CCA AGT GTG CAT C |
| NUM1-S1    | GCC AGG AAA GCG TCT CAA CCA ACA AGC GCG TAA CAA GGA TGG CCC TGA AGC TTC GTA GCC TCG AGG |
| NUM1-S2    | GCC AAA TGA TCG GTT TGG TAC TCT ATG TGG TAC TGA AGA CCC GCA TAG GCC AGT GGA TCT G |
| NUM1-N1    | GCT TTA GAA AGC ATA GAT TCG TCC TGA ATT TCT GGC CCC TTT ATT GCG CTG AAG CTT GCT AGC TGG CAG G |
| NUM1-N2    | GCT GTC TTT ATT TCT GTC TTT ATT TCT GGT GTG GGA CAT TGG ACC ATC AGC TGG AGC ACC |
| KAR9-A1    | GAT GGA TCA TGG ATA TGG AGC CCA GAT TGA CCA GGA TGG AGT GCT GAA GCT TAC GCT GCA GG |
| KAR9-S1    | GCA CTG CCA TGG ATA ATG AGC CCA GAT CTA TGA CCA GGA TGG AGT GCT GAA GCT TAC GCT GCA GG |
| KAR9-S2    | GGA GCC CTG CAA CGG GTT CTT CTT GTG GAA GGG ACC CCC CAA ACC GCA TAG GCC ACT AGT GGA TCT G |
| KAR9-N1    | GCA TTT CCT TCT AGT TGG AAT TGG AGC CTG AAT TCT AAT TCT TCA GTA TCG CAT CAG CAG CAG C |
| KAR9-N2    | GGA AGT CAT CCC CAA TGG CTA ATG TGC TGG GTC CAT CAT TAT CCA TGG GAC CAT CAG TGG CAG CAC C |
| BI1-A1     | GAC GGT GCG ATA GAC GGA |
| BI1-S1     | CGA ACT CAA AGG AAA GTC ATT CGA ATT CCA GTT GTA TAT TCC GTA TAT TCC GAA GAT TGG TAC GCT GCA GG |
| BI1-S2     | GCT GTG TCA CTA ATT TCG TCT TGA TCT TCG ACC GCA GTG TCC GTA ATC GCA CAC TAG TGG ATC TG |
| BI1-N1     | GCA CGG ACC CCT CTT ACA GAT AAG AGG AGA CTG AGC ACG GTG TGT TGT AGT CAG CTT GAG CAG CAG C |
| BI1-N2     | GAC TTT CCT TCT AGT TGG AAT TGG AGC CTG AAT TCT AAT TCT TCA GTA TCG CAT CAG CAG C |
| Dyn1-A1    | GAT GTG TCT ATC GTG TCT AAC |
| Dyn1-S1    | CGA AGA AGC CAT TAA GAG TAT ATC CCC TTC TTA TCA CGA AGG CTG AAG CTT GCT GAG CAG CAG C |
| Dyn1-S2    | CGA TCT CTC GAC TCT AGC TCT GCT TTA AGG ATA CTT CGA TAC TAA CAG ACC GAC CAA CTA GTG G |
| ARP1-A1    | GAA CAC AGT ACA CCA GTG TGA CG |
| ARP1-S1    | GCA AAT GCA GCC GCT AAG TGA CAG CTA TGC TTT GTA GAA TGA ACC GCT AGC TCG GAC GAC G |
| ARP1-S2    | GCG TCG TAT TCC GGC CAC TTA GGA TTA TGG ATG ACG ATA GGA TAT TGC ACC ACA ATT TAT TCT TCA GGG CAC GAT G |
| Kip2-A1    | CCA TGG GTT TAT TCT GCT GCT GC |
| Kip2-S1    | CCA AGC TTA AGG AGG CCA TGA AGC AGG TCT AGT GCC TCT GTG TGC AGT GCT GAA GCT TAC GCT GCA GG |
| Kip2-S2    | GCT TAT CCA GCA CAG GGT GCG GTG ATT GCT GTG TAT TAA TGA GTG GCC ATA GCC CAC TAG TGG ATC TG |
| Kip3-A1    | CCT TGT ATG ACT TTA GGA TGG |
| Kip3-S1    | CCA GCT GCC TAC TAT TGA CAC TAA CAT GAA GGT GCC TGA AAG GAG GCT GAA GCT TAC GCT GAC G |
| Kip3-S2    | GGA AAG ATG TTA TAT TGG CAT GAT TAC GTA TGC TTA GCC ATG GCC ACT AGT GGA TCT G |
| Tub3-A1    | CCA ACT GTG TAC AGA ACT GC |
| Tub3-N1    | CAA ACC CCT TAC ATA ATC CAT TAT AAA TAC TGC CAT GTA AGT ACA TAT GCT GAA GCT TAC GCT GAC G |
| Tub3-N2    | GGA AGG ATG TGA TTA TGC GAA GTT TAT GCA GAA GAT TCA ACA TAC TCA AGA CAA AGA TAT TCT TGC CAG G |
| EGFP-As-T1 | TTC GAA ATG TCC GTA ATT GCC GAA GGT GTA TCA ACT AGT |
| EGFP-As-T2 | CTT CCA GTG GGA CCC TGG AGT TGG TAC ATC TTA GCC ACA TGC |
| EGFP-Ba-T1 | GCC AGG TAC GAT TCA TGG GTA AGG TAA AGT CAT AAT TAC CAG |
| EGFP-Sp-T2 | GGT CCA CTA GGT GTG TGG TAC AAT TCA TCC ATA CCA TGG |

*Underlined sequences are portions of the corresponding genes.

sites of pFM204 to obtain pFM206. Insertion of the pFM206-derived PCR cassette at the NUM1 translational start leads to the galactose-inducible expression of a highly fluorescing yeast-enhanced GFP (yEGFP)-Num1p fusion protein. Plasmid pFM313 contains a NH2-terminal yEGFP-NUM1 fusion under the control of the NUM1 promoter in pRS416 (CEN6/ARS1/URA3). A 0.7-kb BstBI PCR fragment (EGFP-As-T1 and EGFP-As-T2 primers) containing yEGFP3 (Cormack et al., 1997) was inserted into the BstBI site of pJK23 located at the NUM1 translational start (Kormancew et al., 1991). Further construction steps were used as described for pFM18 (Farkasovsky and Küntzel, 1995).

**Immunological Methods**

Strains expressing epitope-tagged proteins were grown in yeast extract/peptone plus dextrose (YPD) at 30°C to A600 = 0.3. Cells were collected by centrifugation, washed twice with YPGal (1% yeast extract, 2% peptone, 2% galactose), resuspended in YPGal, and grown for 4 h at 30°C. Protein extract preparation, immunoprecipitation, and Western blot analysis was performed as previously described (Ansari et al., 1999), with the following modification: the pelletted protein A-conjugated sepharose beads containing the immunocomplex were washed twice with IP buffer containing 0.1% Triton X-100, 0.1% Tween 20, and resuspended in YPGal with 0.3% glucose. After 2–3 h incubation at 30°C (A600 = 0.6–0.8), living or fixed cells were immobilized with 2% agarose in PBS or minimal media. To preserve yEGFP fluorescence and to visualize microtubules or DNA, cells were fixed with formaldehyde added to a final concentration of 4% and incubated at room temperature for 90 min. Cells were then treated with monoclonal anti-α-tubulin, or with DAPI (Sigma-Aldrich), as described previously (Farkasovsky and Küntzel, 1995). For nocodazole treatment, a strain containing the yEGFP-

**Fluorescence Microscopy**

Overnight cultures of strains producing yEGFP-Num1p were diluted to A600 = 0.1 and grown in YPD to early log phase. Cells were collected by centrifugation, washed, and resuspended in YPGal with 0.3% glucose. After 2–3 h induction at 30°C (A600 = 0.6–0.8), living or fixed cells were immobilized with 2% agarose in PBS or minimal media. To preserve yEGFP fluorescence and to visualize microtubules or DNA, cells were fixed with formaldehyde added to a final concentration of 4% and incubated at room temperature for 90 min. Cells were then treated with monoclonal anti-α-tubulin, or with DAPI (Sigma-Aldrich), as described previously (Farkasovsky and Küntzel, 1995). For nocodazole treatment, a strain containing the yEGFP-

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**Figure 1.** Polymyxin B sensitivity of num1Δ and yEGFP3-NUM1 strains. Suspensions containing 10, 100, or 1,000 cells (from left to right) of haploid strains FMY691 (a, NUM1), FMY627 (b, yEGFP3-NUM1) and FMY693 (c, num1Δ) were dotted onto YPGal solid media without (left) or with (right) 0.3 mg/ml polymyxin B.
Cortical Num1p Interacts with Dynein and Microtubules

NUM1 fusion (FMY519) was grown to early log phase in YPD at 30°C. Nocodazole (Sigma-Aldrich) was added to 20 mg/ml and incubated for 1 h at 30°C. Cells were then incubated in YPGal with nocodazole (20 mg/ml) for 2 h at 30°C, fixed with formaldehyde, and stained for microtubules as above. Microscopy was carried out on an Axiophot microscope equipped with a 1.3 NA 100× Neofluar lens (Carl Zeiss, Inc.). Images were recorded using a SPOT camera (Visitron Systems GmbH) with a TWAIN driver. Adobe® Photoshop™ v5.0 and Canvas v3.5 were used to further optimize contrast.

Results

Cortical Num1p Dots at Mother/Daughter Poles May Serve as cMT Capture Sites during Late Anaphase

Num1p was previously localized in fixed cells by indirect immunofluorescence microscopy, using polyclonal antibodies against a COOH-terminal Num1p region (Farkasovsky and Küntzel, 1995). To study Num1p localization in living cells, we have introduced a PCR-generated cassette containing the kanMX4 gene, the GAL1 promoter, and a modified GFP gene (yEGFP) at the translational start of NUM1 (Cormack et al., 1997). The diploid strain FMY519 (expressing the galactose-inducible GFP-Num1p fusion protein) was grown in 2% galactose (YPGal), and 0.3% glucose was added to reduce the extent of overexpression (Carminati and Stearns, 1997). Functionality of overproduced GFP-Num1p was tested by using the antibiotic polymyxin B, which is known to break bacterial membranes and to activate phospholipases (Boguslawski, 1992). We found num1Δ strains to be hypersensitive against polymyxin B during a chemotyping screen of several orphan gene deletants in the framework of the EUROFAN program (Rieger et al., 1997).

Figure 2. (A and B) Subcellular distribution of GFP-Num1p in living diploid cells. (A) Strain FMY519 (relevant genotype GAL1p-yEGFP3-NUM1/NUM1) was grown in YPD to early exponential growth and induced in YPGal + 2–3 h. (B, upper row) CEN.PK2 cells containing pFM313 (NUM1p-yEGFP3-NUM1, CEN6/ ARSH4) were grown in YPD to early exponential phase. (B, lower row) CEN.PK2 cells containing the pRS416 derivatives pFM314 (a, GFP fused to Num1p residues 1–421), pFM315 (b, GFP fused to Num1p residues 1–1876) and the empty vector pRS416 (c). (C and D) Costaining of microtubules in FMY519 cells by indirect immunofluorescence using antitubulin antibodies. (D, upper row) Merged images of GFP-Num1p and microtubules; lower row, DAPI-stained nuclear regions.

Figure 3. Quantification of cell cycle–dependent localization of galactose-induced GFP-Num1p. 200–250 cells (FMY519) of different stages, as determined by nuclear and microtubule positions, were scored for prominent cortical dot positions at cellular poles and bud neck sites. Additional dots at the mother cortex are not depicted.

Figure 4. Quantification of cMT–cortex interactions in WT (FMY519) and dyn1Δ (FMY872) anaphase cells expressing galactose-induced GFP-Num1p. The percentage of cells with indicated cMT–cortex contacts was determined for early anaphase cells (short spindle entering the bud), mid anaphase cells (partially elongated spindle spanning the neck), and late anaphase cells (fully elongated spindle). (n) Number of cells analyzed. For details see legend to Fig. 2.
et al., 1999). The reason why the NUM1 deletion causes polymyxin B hypersensitivity is not known (see Discussion).

Fig. 1 indicates that the NUM1 wild-type strain FMY691 grows well on YPGal containing 0.3 mg polymyxin B (row a), whereas the num1Δ strain FMY693 does not grow under these conditions (row c). Strain FMY627 expressing a galactose-inducible yEGFP-NUM1 fusion gene grows like wild type (Fig. 1, row b), suggesting that the fusion protein is functional like Num1p in conferring polymyxin B resistance. Furthermore, a centromeric plasmid expressing yEGFP-NUM1 under the control of the NUM1 promoter (pFM313) rescues the nuclear migration defect of num1Δ strains (data not shown).

|     | A | B | C | D |
|-----|---|---|---|---|
| WT  | 99.5 | 0.5 | 0 | 0 |
| num1 | 69 | 3 | 21 | 7 |
| kar9 | 69 | 13 | 17 | 1 |
| bni1 | 89 | 6 | 4 | 1 |
| dyn1 | 81 | 11 | 8 | 0 |
| arp1 | 80 | 5 | 12 | 3 |
| kip2 | 85 | 6 | 3 | 0 |
| kip3 | 90 | 8 | 2 | 0 |
| num1 kar9 | 27 | 7 | 54 | 12 |
| num1 bni1 | 51 | 4 | 35 | 10 |
| num1 dyn1 | 82 | 6 | 10 | 2 |
| num1 arp1 | 81 | 3 | 11 | 5 |
| num1 kip2 | 75 | 7 | 15 | 3 |
| num1 kip3 | 25 | 9 | 49 | 17 |

Figure 5. Nuclear migration defects in null mutant cells. Haploid MATa cells were grown at 30°C to OD_{600} = 1.2 in YPD, fixed, and stained with DAPI, and 250–300 large-budded cells were scored for the position of nuclear regions. Strains: FMY691 (WT), FMY693 (num1Δ), FMY705 (kar9Δ), FMY711 (bni1Δ), FMY859 (dyn1Δ), FMY856 (arp1Δ), FMY848 (kip2Δ), FMY852 (kip3Δ), FMY799 (num1Δ kar9Δ), FMY821 (num1Δ bni1Δ), FMY789 (num1Δ dyn1Δ), FMY793 (num1Δ arp1Δ), FMY850 (num1Δ kip2Δ), and FMY854 (num1Δ kip3Δ). D includes cells with three or more nuclei in the mother compartment. >80% of the num1Δ kar9Δ, num1Δ bni1Δ, and num1Δ kip3Δ cells listed under D are multinucleate and multibudded.

|     | A | B |
|-----|---|---|
| WT  | 0 | 0 |
| num1 | 82 | 8 |
| dyn1 | 80 | 4 |
| kar9 | 14 | 25 |

Figure 6. (A) cMT orientation in MATa mutant cells containing two DAPI-stained chromosomal regions in the mother compartment. Strains FMY691 (WT), FMY705 (kar9Δ), FMY859 (dyn1Δ), and FMY693 (num1Δ) were grown to late exponential phase at 30°C and fixed for indirect immunofluorescence using antitubulin antibodies. 120–150 binucleate cells per mutant strain were scored for cMT orientation. (B) Nuclear position in preanaphase MATa wild-type and mutant cells. Strains: FMY691 (WT), FMY693 (num1Δ), FMY705 (kar9Δ), FMY859 (dyn1Δ), FMY799 (num1Δ kar9Δ), FMY789 (num1Δ dyn1Δ). Strains were grown to early exponential phase at 30°C, and 200–250 DAPI-stained preanaphase cells (single nucleus in the mother) were scored for each strain.
Fig. 2 A visualizes the cellular distribution of galactose-induced GFP-Num1p in living diploid cells. In addition to the previously noted dot-like Num1p distribution at the mother cortex, we observe prominent dots at the bud tip, the opposite pole of the mother compartment, and the bud neck, which were not visible in fixed NUM1 cells treated with anti-Num1p antibodies (Farkasovsky and Küntzel, 1995). A similar distribution of GFP-Num1p was observed in galactose-grown haploid cells (data not shown). Furthermore, a yEGFP3-NUM1 fusion gene was placed under the control of the NUM1 promoter and introduced into the diploid wt strain CEN.PK2 as a centromeric plasmid (pFM313).

Fig. 2 B (upper row) shows the localization of GFP-Num1p as distinct dots at both poles of large-budded pFM313–containing cells. The exposure time of Fig. 2 B (GFP-Num1p controlled by the NUM1 promoter) was increased fourfold compared with Fig. 2, A, C, and D (GFP-Num1p controlled by the GAL1 promoter). Therefore, the intensities of the GFP signals do not reflect the level of expression. The lower row of Fig. 2 B shows large-budded cells expressing GFP-fusions to NH2-terminal Num1p residues 1–421 (a) and 1–1876 (b) under the control of the NUM1 promoter (pFM314 and pFM315, respectively). As previously noted, Num1p variants lacking the COOH-terminal PH domain are found in the cytoplasm and do not localize to the cell cortex (Farkasovsky and Küntzel, 1995).

Fig. 3 summarizes the appearance of prominent galactose-induced GFP-Num1p dots in cells of various cell cycle stages (less prominent dots at the mother cortex are not depicted). Two opposite Num1p dots are seen in 96% of G1 cells; one of the two dots is close to the incipient bud site and appears to persist at the mother side of the neck through S, G2, and M phase. The tip of the growing bud is marked by a Num1p dot at S/G2 and persists through mitosis.

The interactions of cMTs with galactose-induced cortical Num1p dots at the poles of anaphase cells (Fig. 2, C and D) are summarized in Fig. 4. A contact of cMTs to both polar Num1p dots is not observed in early anaphase cells, but is seen in 9% of late anaphase cells. cMTs appear to contact
Num1p at the bud tip but not at the opposite mother pole in 23% of early anaphase, 65% of mid anaphase, and 28% of late anaphase cells (Fig. 4, column B). cMTs contacting lateral regions of the bud and mother cortex, instead of touching the cellular poles, are seen in 72% of early anaphase, 26% of mid anaphase, and 8% of late anaphase cells (Fig. 4, column C).

We have also studied cMT contacts with polar Num1p dots in a diploid dyn1Δ/dyn1Δ strain expressing galactose-induced GFP-Num1p (FMY872). As summarized in Fig. 4, the percentage of late anaphase cells showing cMT contacts to both Num1p dots (A) is significantly reduced in the absence of Dyn1p (36% in dyn1Δ compared with 64% in wild type), whereas the percentage of cells with no cMT–Num1p contact (C) increases from 8% (wild type) to 42% (dyn1Δ). These observations suggest that the dynein heavy chain Dyn1p is involved in cMT capture at polar Num1p sites.

**Num1p Acts in a Dynein–Dynactin–Kip2p-dependent Anaphase Pathway but Is Not Required for Kar9p-dependent Preanaphase Nuclear Positioning**

The three double mutants num1Δ kar9Δ, num1Δ bni1Δ, and num1Δ kip3Δ exhibit additive defects in nuclear migration through the bud neck, as summarized in Fig. 5: the percentage of large-budded cells with more than one DAPI spot in the mother compartment (“binucleate mother cells”) is increased in comparison to the single mutants. In contrast, the num1Δ combinations with dyn1Δ, arp1Δ, and kip2Δ are not significantly affected. Furthermore, the double mutants num1Δ kar9Δ and num1Δ kip3Δ grow slowly at 30°C and arrest at 15°C, whereas all other single and double mutants, including num1Δ bni1Δ, grow like wild type at both temperatures.

Fig. 6 A quantifies the distribution of binucleate mother cells with differing cMT morphologies in num1Δ, dyn1Δ, and kar9Δ strains, as revealed by indirect immunofluorescence of cells treated with antitubulin antibodies and DAPI. Approximately 80% of num1Δ and dyn1Δ cells of this nuclear phenotype contain long cMTs traversing the bud neck, as previously noted (Farkasovsky and Küntzel, 1995; Carminati and Stearns, 1997; Miller et al., 1998), whereas cMTs do not enter the bud in the majority of kar9Δ binucleate mother cells (Miller and Rose, 1998). It is interesting to note that bni1Δ mutants are much more related to num1Δ or dyn1Δ mutants than to kar9Δ or bim1Δ mutants according the cMT phenotype of binucleate mother cells: cMTs extend into the bud in 79–90% of binucleate bni1Δ cells (Fujiiwara et al., 1999; Miller et al., 1999).

The data of Fig. 6 B demonstrate that nuclear migration to the bud neck in preanaphase cells does not require Num1p or Dyn1p, but partially depends on Kar9p. The double mutant num1Δ dyn1Δ (FMY789) corresponds phenotypically to the two single mutants (nuclear position close to the bud neck in 96% of preanaphase cells), whereas the num1Δ kar9Δ strain FMY799 exhibits a similar migration defect (only 62% of preanaphase cells with a nuclear position at the bud neck) as the kar9Δ single mutant.

The properties and genetic interactions of num1Δ mutants, as summarized in Figs. 5 and 6 and Table III, imply that Num1p acts in a Dyn1p–Arp1p–Kip2p pathway, but is not involved in a partially redundant Kar9p–Bim1p–Kip3p pathway.

**Num1p Forms a Complex with the Dynein Intermediate Chain Pac11p and with the α-Tubulin Tub3p**

The observed cMT capture by polar Num1p dots, the partial Dyn1p-dependency of these cMT–cortex interactions and the function of Num1p within a dynein pathway led us to test physical interactions of Num1p with the α-tubulin Tub3p and the dynein intermediate chain Pac11p by coimmunoprecipitation of epitope-tagged proteins. The nuclear migration defect of pac11Δ mutants is similar to that of num1Δ and dyn1Δ strains, suggesting that the three proteins act in the same functional class (Geiser et al., 1997). We introduced the epitopes 3xMyc or 3xHA at the translational starts of NUM1, PAC11, and TUB3 by inserting PCR-derived kanMX4-GAL1p-3xMyc or kanMX4-
The functionality of the epitope-tagged proteins was demonstrated by an unimpaired nuclear migration phenotype of galactose-grown haploid strains expressing c-Myc (Myc)– or hemagglutinin (HA)-tagged proteins. In addition, haploid num1::kanMX4-GAL1p-3xMyc-NUM1 strains showed the polymyxin B sensitivity of wild-type strains (Fig. 1), if grown in YPGal (data not shown). Western blot analysis of crude lysates of galactose-grown strains showed the expression of a 320-kD Myc-Num1p and a 60-kD HA-Pac11p protein (data not shown). Crude lysates of cells grown in galactose medium were treated with purified anti-Myc mAbs, and the immunocomplex was absorbed to protein A–conjugated Sepharose beads. The immunoprecipitate was then probed by Western blotting using anti-HA or anti-Myc antibodies.

Fig. 7 A demonstrates that HA-Pac11p coprecipitates with Myc-Num1p in a wild-type background (lane 3, strain FMY905) but not in the absence of Dyn1p (lane 4, strain FMY912). Furthermore, the 60-kD HA-Pac11p band is absent from anti-Myc precipitates of cells expressing only HA-Pac11p (lane 2, strain FMY899), supporting the specificity of the coprecipitation. Lanes 5 to 8 of Fig. 7 A confirm the presence or absence of Myc-Num1p in the anti-Myc precipitates. Similarly, the results shown in Fig. 7 B indicate that HA-Tub3p (50 kD) coprecipitates with Myc-Num1p (Fig. 7, lane 3), and Myc-Num1p coprecipitates with HA-Tub3p (Fig. 7, lane 9). The specificity of these coprecipitations is documented in Fig. 7, lanes 2 and 8.

**Num1p Associates with Bni1p–Kar9p at the Bud Tip of Early Anaphase Cells**

Num1p may transiently coexist or even cooperate with a Bni1p–Kar9p complex at the bud tip, although the two cor-

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**Table III. Properties of Nuclear Migration Mutants**

| Mutant | cMTs extend into bud in most cells* | Cell stage affected | Reference |
|--------|-----------------------------------|---------------------|-----------|
| kar9Δ  | No                                | Preanaphase         | Miller et al., 1999 |
| bim1Δ  | No                                | Preanaphase         | Adames and Cooper, 2000 |
| kip3Δ  | No                                | Preanaphase         | Miller et al., 1998 |
| bni1Δ  | Yes                               | Preanaphase         | Miller et al., 1999; Fujiwara et al., 1999 |
| num1Δ  | Yes                               | Anaphase            | Farkasovsky and Küntzel, 1995; Geiser et al., 1997; this study |
| dyn1Δ  | Yes                               | Anaphase            | Miller et al., 1998; Carminati and Stearns, 1997 |
| kip2Δ  | Yes                               | Anaphase            | Miller et al., 1998 |

*Binucleate mother cells (telophase in mother).
tical cMT capture sites appear to operate at different stages of the cell cycle (see Discussion). To test this possibility, we have studied the influence of Bni1p, Kar9p, and Dyn1p on the bud tip localization of GFP-Num1p. The two Bni1p-deficient diploid cells (FMY795) shown in Fig. 8 A contain a prominent GFP-Num1p dot at a lateral region of the bud cortex, in addition to several dots at the mother cortex, whereas a Num1p dot at the bud tip is clearly missing in virtually all observed budded cells. In contrast, a prominent GFP-Num1p dot is seen at the bud tip of cells lacking either Kar9p (Fig. 8 B) or Dyn1p (Fig. 8 C).

We have also addressed the question whether cMTs influence the Num1p deposition at the bud tip by treating cells with nocodazole before galactose induction of GFP-Num1p. 51% of nocodazole-treated large-budding cells lacked microtubules but contained the bud tip dot of GFP-Num1p, as shown in Fig. 8 E, whereas the bud tip dot of GFP-Num1p was observed in 28% of mock-treated (DMSO) large-budded cells (Fig. 8 D), suggesting that cMTs are not required for targeting Num1p to the bud tip cortex.

Fig. 9 depicts cells of a diploid strain (FMY861) coexpressing galactose-inducible yEGFP3-NUM1 and 3xHA-KAR9 fusion genes. Cells were grown in YPGal to early exponential phase and treated with anti-HA mAbs after fixation. DAPI was added to the mounting medium to stain chromosomal regions. Fig. 9, row A, shows a preanaphase cell containing a HA-Kar9p patch at the bud tip (lanes b and d), which apparently represents a cortical cMT/Kar9p capture site visualizing the plus-end of cMTs by microtubule-bound Kar9p (Miller et al., 1999). The same cell contains a GFP-Num1p patch at the bud tip, which is only partially resolved from the HA-Kar9p patch, as seen in the merged image of Fig. 9, lane d. Fig. 9, row B, shows a cell at early anaphase stage with a GFP-Num1p dot (lane a) and a cMT/HA-Kar9p capture site (lane b) at the bud tip. The merged image of Fig. 9, lane d, clearly resolves the two apparently adjacent cortical GFP-Num1p and HA-Kar9p dots and further demonstrates that the Kar9p-decorated cMTs are in contact with cortical HA-Kar9p but not with GFP-Num1p. HA-Kar9p and GFP-Num1p contact or near proximity was observed in 29% of early anaphase and 75% of mid anaphase cells with Kar9p localization at the bud cortex (dot or line). Anti–HA-stained HA-Kar9p has completely disappeared from the bud at the late anaphase/telophase stage (>90%), as seen in Fig. 9, row C, lane b, whereas GFP-Num1p persists at the bud tip (Fig. 9, lanes a and b).

The close neighborhood of Num1p and Kar9p dots at the bud tip of early anaphase cells may indicate a transient physical association of Num1p with Kar9p-binding Bni1p at the bud tip cortex. Indeed, we observe a coimmunoprecipitation of Myc-Num1p with HA-Bni1p and HA-Kar9p, as shown in Fig. 10, lanes 4 and 5, respectively. Anti-Myc precipitates of cells expressing only HA-Bni1p (FMY783) or HA-Kar9p (FMY810) do not contain the 219-kD HA-Bni1p band (Fig. 10, lane 2) or the 74 kD HA-Kar9p band (Fig. 10, lane 3), supporting the specificity of the coprecipitation. The presence of Myc-Num1p in the anti-Myc precipitates was confirmed by anti-Myc probing (Fig. 10, lanes 6, 9, and 10).

Discussion

The data presented in this study suggest that Num1p controls the dynein-dependent migration of the elongating nuclear spindle through the bud neck by forming a cortical cMT capture site at the bud tip; a second Num1p/cMT capture site at the opposite mother pole appears to be involved in late anaphase/telophase cells. We further suggest that cortical capture and pulling of cMTs at the Num1p sites is mediated by the dynein intermediate chain Pac11p. Our conclusions and suggestions are based on the following observations: (a) Genetic and phenotypic analyses of num1A strains indicate that Num1p is not required for Bni1p–Kar9p–Bim1p–Kip3p-dependent nuclear migration to the bud neck in preanaphase cells but acts within a dynein–dynactin–Kip2p pathway, controlling nuclear migration through the bud neck and spindle elongation in anaphase cells; (b) GFP-Num1p localizes at the bud tip and the opposite pole of the mother cortex in living cells, and cMTs appear to intersect the Num1p dot at the bud tip during mid anaphase and to touch both polar Num1p dots at late anaphase/telophase; and (c) Num1p forms a complex with the dynein intermediate chain Pac11p and with the α-tubulin Tub3p, as shown by coimmunoprecipitation of Myc- or HA-tagged proteins. Pac11p has previously been shown to coprecipitate with the dynein heavy chain Dyn1p (Kahana et al., 1998) and to act together with Num1p (= Pac12p) in a cytoplasmic dynein pathway (Geiser et al., 1997). Furthermore, our data indicate that the Num1p–Pac11p interaction as well as the Num1p–cMT contacts depends on the presence of Dyn1p, suggesting that Num1p serves to attach the Dyn1p motor domain and the plus-end of cMTs to the cellular cortex.
Another component of the yeast dynein pathway is the 54-kD Pac1 protein (Geiser et al., 1997), a homologue of the Aspergillus nidulans NudF protein (Xiang et al., 1995) and the human lissencephaly protein Lis1 (Reiner et al., 1993). Mammalian Lis1p is required for microtubule organization and the regulation of cytoplasmic dynein during neuronal proliferation, migration, and morphogenesis (Faulkner et al., 2000; Smith et al., 2000). The Drosophila homologue DLis1 acts as a cortical anchor for the dynein–dynactin complex during nuclear positioning in oocytes (Swan et al., 1999). Although Pac1p has not yet been localized in yeast cells, it may participate in cortical cMT capture by interacting with Num1p and/or Dyn1p.

Num1p contains a COOH-terminal PH domain that is essential for cortical association and nuclear migration functions (Farkasovsky and Küntzel, 1995). PH domains of several proteins are known to interact with the lipid phosphatidylinositol 4,5-bisphosphate (PIP2) (Lemmon et al., 1996), and Num1p may bind to the inner face of the plasma membrane via its PH domain. We have shown that the Num1p PH domain is sufficient to target GST (glutathione S-transferase) to the cortex of yeast cells; furthermore, Num1p physically interacts with PIP2-specific phospholipase C, as revealed by two-hybrid and coimmunoprecipitation experiments (Martin, 1998; our unpublished data).

The postulated membrane interaction of Num1p may possibly be involved in protecting yeast cells against the membrane-breaking antibiotic polymixin B (Boguslawski, 1992), resulting in the observed hypersensitivity of num1Δ strains.

Two observations suggest that Num1p interacts with the formin Bni1p, another cortical protein forming a cMT capture site at the bud tip (Heil-Chapdelaine et al., 2000; Lee et al., 1999; Korinek et al., 2000; Lee et al., 2000): Myc-Num1p coprecipitates with HA-Bni1p, and the GFP-Num1p dot at the bud cortex is shifted to a lateral region in the absence of Bni1p. Costaining experiments have revealed that the Bni1p-binding protein Kar9p localizes at the bud tip of early and mid anaphase cells at a region adjacent to, but not superimposing with, the Num1p dot. Furthermore, bud-oriented cMTs appear to intersect a cortical Kar9p capture site, without being in contact with the adjacent Num1p dot at the bud tip (Miller and Rose, 1998; Lee et al., 2000; this study, Fig. 9 B).

Thus, the two types of cortical cMT capture sites (Bni1p–Kar9p and Num1p–Pac1p) seem to be in close proximity or even in physical contact at least during early and middle anaphase, although they control two temporally separated steps of nuclear migration. Our observation that the GFP-Num1p localization depends on Bni1p but not on Kar9p (Fig. 8) suggests an indirect Num1p–Kar9p interaction via Bni1p; indeed, a two-hybrid assay failed to reveal physical interactions between Num1p and Kar9p peptides (data not shown).

A cell cycle–specific transient interaction between the two cortical complexes Bni1p–Kar9p and Num1p–dynein may help to control the transition from Kar9p- to dynein-dependent nuclear migration: Kar9p disappears from the bud cortex at late anaphase/telephase (Miller and Rose, 1998), whereas Num1p remains at the bud tip and the opposite pole of the mother cell. This final stage of mitosis may require both Num1p sites to draw the two spindle pole bodies (SPBs) close to the cellular poles, and both Num1p/cMT capture sites may bind the minus-end-directed dynein motor via its subunit Pac1p. It is interesting to note that the dynein-activating dynactin subunit Nip100p localizes to the SPBs throughout the cell cycle, but is never seen at the cell cortex; furthermore, the dynactin subunits Nip100p, Arp1p, and Jnm1p form a 15.5S complex that does not include the dynein subunits Dyn1p and Pac1p (Kahana et al., 1998). These observations would predict that the SPB-bound dynactin complex can transiently interact with the cortically associated dynein complex only after pulling the SPBs close to the cellular poles. The cortex–SPB contact may then trigger signals for spindle collapse, nuclear reorganization, and cytokinesis.

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