Yeast Num1p Associates with the Mother Cell Cortex during S/G2 Phase and Affects Microtubular Functions

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Abstract. The NUM1 gene is involved in the control of nuclear migration in Saccharomyces cerevisiae. The content of NUM1 mRNA fluctuates during the cell cycle, reaching a maximum at S/G2 phase, and the translation product Num1p associates with the cortex of mother cells mainly during S, G2, and mitosis, as seen by indirect immunofluorescence. The nuclear spindle in NUM1-deficient cells, reaching a maximum at S/G2 phase, and the translocation product Numlp associates with the cortex of mother and bud compartment at G2, presumably mediated by AMTs.

At least four additional genes (DHC1/DYN1, JNM1, ACT3/ACT5 and NUM1) are involved in nuclear migration control, since strains lacking one of these nonessential genes accumulate cells with misoriented nuclei dividing in the mother compartment. DHC1/DYN1 encodes the heavy chain of the dynein motor complex which is probably involved in pulling the bud-proximal astral microtubules into the bud (Li et al., 1993; Eshel et al., 1993), whereas JNM1 appears to encode an accessory dynein protein involved in tethering astral microtubules to the bud cytoskeleton (McMillan and Tatchell, 1994). The actin/Arp1-related ACT3/ACT5 protein is a homolog of human α-actinin and a major component of a dynactin complex believed to support the dynein motor activity (Muhua et al., 1994; Clark and Meyer, 1994).

The NUM1 gene (Kormanec et al., 1991) encodes a 313-kD protein containing twelve near-identical tandem repeats of a peptide of 64 residues; the repeat unit itself is composed of two proline-punctuated homologous 32-amino acid peptides each containing two α-helical regions. The amino-terminal region exhibits a coiled-coil (heptad repeat) domain (Lupas et al., 1991) followed by a potential EF hand Ca2+-binding site, a cluster of potential phosphorylation sites and a pleckstrin homology domain. The latter domain appears to be involved in targeting Num1p to the mother cell cortex. Our data suggest that the periodically expressed NUM1 gene product controls nuclear migration by affecting astral microtubule functions.

The proper distribution of segregated chromosomes between mother and daughter cells requires the migration of the nucleus to the bud neck and the positioning of the pre-anaphase spindle along the mother/bud axis (Byers and Goetsch, 1975; Pringle and Hartwell, 1981). Nuclear migration and positioning critically depends on the functions of astral microtubules (AMTs) emanating from the cytosolic side of the two spindle pole bodies (SPBs) (Palmer et al., 1992), as manifested by the effects of microtubule inhibitors (Jacobs et al., 1988) and by the phenotypic properties of B-tubulin (tub2) mutants (Huffaker et al., 1988; Palmer et al., 1992). Furthermore, the nuclear migration defects of actin mutants suggest that actin may play a role in tethering astral microtubules to the cytoskeleton during the migration process (Palmer et al., 1992). The asymmetric segregation of the old and new SPB between mother and daughter cell, respectively (Vallen et al., 1992), suggests a specific functional orientation of SPBs toward the cortex of mother and bud compartment at G2, presumably mediated by AMTs.

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1. Abbreviations used in this paper: AMT, astral microtubule; GST, glutathione-S-transferase; MM, selective minimal medium; PH, pleckstrin homology; SPB, spindle pole body; YPD, rich medium.
More recently, a polymorphism of the repetitive region of NUM1 (allelic with RVS272) was reported in various strains, with repeat numbers varying between 1 and 24; furthermore, the percentage of binucleated large-budded mother cells in rvs272 or num1 populations was found to increase after entering stationary phase (Revardel and Aigle, 1993).

In this study we report the cell cycle—specific expression and cellular localization of the NUM1 gene product, the effects of NUM1 deletions on microtubule morphology and nuclear positioning, and genetic interactions with tubulin mutants. We further report evidence that the carboxy-terminal PH domain is required to target Numlp to the mother cortex. Our observations suggest that Numlp controls the proper alignment of the nuclear spindle along the mother/bud axis by affecting microtubule functions.

Materials and Methods

Reagents

β-Glucuronidase/Arylsulfatase, 4',6'-diamino-2-phenylindole (DAPI) and restriction endonucleases were purchased from Boehringer (Mannheim, FRG). Monoclonal anti-chicken brain α-tubulin, FITC-labeled goat anti-rabbit IgG and FITC-labeled rabbit anti-goat IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine-conjugated phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR). Glutathione-Sepharose and protein A-Sepharose were purchased from Pharmacia LKB (GmbH, Freiburg, FRG). Plasmids and E. coli strains were purchased from Stratagene GmbH (Heidelberg, FRG). Reagents for DNA purification, RNA, and protein purification were purchased from Sigma and from Qiagen GmbH (Hilden, FRG). Restriction enzymes, ligases, and nucleases were purchased from New England BioLabs (GmbH, Schwalbach, FRG). α-pheromone was a kind gift of W. Schmidt.

Plasmid Constructions

Fig. 1 shows the restriction map of the genomic KGD2-NUM1 region, indicating sites of deletions and subcloned regions of the NUM1 gene. The NUM1 flanking and coding region was subcloned in pUC19 as an up-stream 4.4-kb SalI-XbaI fragment (pK23), a central 4.3-kb XbaI fragment (pK28), and a downstream 8.4-kb HindIII fragment (pK27) (Kormane et al., 1991). pK28 was obtained by introducing a 1.6-kb Yep24/HindIII(repaired) fragment containing the URA3 gene into the XbaI-repaired site of pUC19.

Plasmid pFM18 contains the entire NUM1 flanking and coding region in the CENARS1HIS3 vector pRS313. Construction steps: a 4.3-kb pJK24-XbaI fragment was inserted into XbaI-linearized pJK23 to obtain pFM10; a 5.2-kb pFM10/PstI fragment was inserted into PstI-linearized pJK27 to obtain pFM24; a 4.1-kb pFM24/BstEII fragment was introduced into BstEII-linearized pJK28 to obtain pFM39; a 1.6-kb pFM39/EcoRI fragment was inserted into EcoRI-linearized pEMBL19 to obtain pFM13; a 5.1-kb pJK27/BstEII fragment was inserted into BstEII-linearized pFM13 to obtain pFM15; a 10-kb pm15/BamHI fragment (using a vector-derived BamHI site) was inserted into pRS313 to obtain pFM18.

Plasmid pFM70 (num1-Δ1::URA3) was obtained by inserting an end-repaired 1.6-kb Yep24/HindIII URA3 fragment between the flanking SpeI sites of pFM18. Alternatively, the same URA3 fragment was inserted between AsuIi and SpeI sites of pFM18, followed by the insertion of a 1-kb SacI fragment containing the NUM1 3' end into the single SacI site, to obtain pFM75 (num1-Δ2::URA3). The two num1-URA3 alleles were introduced into yeast as BstEII-HindIII fragments of pFM70 and pFM75, respectively.

Construction of pFM19 (num1-Δ3): a 4.3-kb pJK24/XbaI fragment was inserted into XbaI-linearized pBluescript II KS to obtain pFM44; a 1.2-kb BamHII-SalI fragment of pJK28 (using vector-derived sites) was placed between the respective sites of pFM44 to obtain pFM6; pFM7 was obtained by EcoRI digestion/religation of pFM6, deleting 11 out of 12 repeats; a 1.8-kb pFM7/XbaI fragment was inserted into the XbaI site of pJK23 to obtain pFM11; a 3.5-kb pFM11/BamHI fragment (using one vector-derived BamHI site) was inserted into the BamHI site of pUC19 to obtain pFM12; a 5.1-kb pJK27/BstEII fragment was inserted into the BstEII site of pFM12 to obtain pFM16; a 8.8-kb pFM16/BamHI fragment was introduced into the BamHI site of the centromeric plasmid pRS313 to obtain pFM19.

Construction of pFM26 (num1-Δ3,Δ4): a 1-kb SacI fragment was deleted from pJK27 to obtain pFM24; a 4.1-kb pFM24/BstEII fragment was inserted into the BstEII site of pFM24 to obtain pFM25; a 7.8-kb pFM25/BamHI fragment (using one vector-derived site) was inserted into pRS313 to obtain pFM26.

Construction of pFM34 (num1-Δ5): pJK24 was partially digested with AsuII and religated to obtain pFM37 (deletion of all 12 repeats); a 1.7-kb pFM37/XbaI fragment was inserted into the XbaI site of pJK23 to obtain pFM38; a 3.3-kb pFM38/BamHI fragment (using a vector-derived BamHI site) was inserted into the BamHI site of pUC19 to obtain pFM39; a 5.1-kb pJK27/BstEII fragment was inserted into the BstEII site of pFM39 to obtain pFM40; an 8.5-kb pFM40/BamHI fragment (using a vector-derived site) was inserted into pRS313 to obtain pFM42.

Construction of pFM35 (num1-Δ3,Δ6): a 0.8-kb SacI-HindIII fragment of pFM12 was inserted between the respective sites of pUC19 to obtain
pFM29, a 5' TAGCITAG-3' linker was placed between a Asel (partial digestion) and SpeI sites of pFM29 to obtain pFM30; a 1.4 kb BstEII (re-paired)/SacI fragment of pJK23 was introduced between the pUC19-derived repaired EcoRI site and the insert SacI site of pFM30 to obtain pFM31; a 6.4 kb BglII-SalI pFM16 fragment (flanked by the NUM1 downstream BglII site and a vector-derived end-repaired SalI site) was inserted between the single BglII site and the downstream end-repaired HindIII site of pFM31 to obtain pFM34; an 8-kb pFM34/BamHI fragment (using one vector-derived site) was inserted into the BamHI site of pRS133 to obtain pFM35.

Construction of pFM52 (num1Δ7): a 5.1 kb pFM15/BstEII fragment was inserted into a derivative of the two-hybrid vector pGBT9 (Clontech, Palo Alto, CA) containing a single BstEII site in the fusion site, to obtain pFM52; this plasmid encodes the DNA-binding domain of the GAL4 protein fused to the COOH-terminal half of Numlp.

Preparation of Antibodies

100 μg of affinity-purified GST-Numlp fusion protein was emulsified for Freund's complete adjuvant and injected subcutaneously in several places of the back of rabbits. After 14 and 45 d rabbits were boosted after the same procedure, except that incomplete adjuvant was used. Blood was collected 14 d after the last injection, and preimmune serum was collected three weeks before the initial injection. The IgG fractions were obtained by chromatography on protein A-Sepharose. Antibodies against GST were removed by incubation of IgG with GST immobilized on nitrocellulose strips, and anti-Numlp antibodies were affinity-purified on immobilized GST-Numlp fusion protein essentially as described (Smith and Fisher, 1984). To test the immunological specificity of antibodies, proteins of a crude yeast lysate were separated by SDS-PAGE using 5% polyacrylamide and blotted to nitrocellulose membranes for 2 h at 4°C and 8V/cm (Burnette, 1981). The blots were treated with TMBST (0.15 M NaCl, 50 mM Tris-HCl, pH 7.2, 0.1% Tween 20) containing 5% nonfat milk for 2 h at room temperature, and incubated for 1 h at room temperature with 1:200 diluted affinity-purified anti-Numlp antibodies in the same buffer. Immunodetection was accomplished by using biotinylated anti-rabbit antibodies and streptavidin-biotinylated horseradish peroxidase complex with the enhanced chemiluminescence Western blotting detection system (ECL, Amersham).

Bacterial Expression and Purification of a GST-Numlp Fusion Protein

The method of Smith and Johnson (1988) was used for induction and purification of fusion protein. The GST-Numlp fusion plasmid pFM71 was introduced into E. coli SURE competent cells (Stratagene, La Jolla, CA), and the resulting transformant was grown in M9 medium at 37°C to OD (600 nm) = 0.5 before adding isopropyl-β-thiogalactopyranosid to 1 mM. Cells were grown for another 2.5 h at 37°C and homogenized by sonication in PBS containing 1% Triton X-100. The fusion protein was purified by affinity chromatography on glutathione Sepharose.

Preparation of Antibodies

Table I. Yeast Strains Used in This Study

| Strain | Genotype | Source |
|--------|----------|--------|
| HK1b   | MATα ura3-52 his3Δ200 | a |
| DBY182 | MATα ura3-52 his3Δ200 trp1-1 leu2-3, 112 ade2 | b |
| DBY189 | MATα ura3-52 his3Δ200 trp1-1 leu2-3, 112 lys2-801 | b |
| FMY1   | MATα ura3-52 his3Δ200 trp1-1 leu2-3, 112 ade2 num1Δ1::URA3 | c |
| FMY2   | MATα ura3-52 his3Δ200 trp1-1 leu2-3, 112 lys2-801 num1Δ1::URA3 | c |
| RH270-2B | MATα ura3-52 leu2-3, 112 his4-539 lys2-801 bar1 | d |
| CC15   | MATα ura3-52 his3Δ200 ade2-101 cdc15-2 | e |
| DBY2499 | MATα ura3-52 his4-539 lys2-801 tub1-1 | f |
| DBY2500 | MATα ura3-52 ade2-101 tub1-1 | f |
| FMY60  | DBY2499 × DBY2500 | c |
| FMY61  | MATα/MATα ura3-52 his3Δ200 ade2-101/+ tub1-1/+ tub1-1 num1Δ1::URA3/+ | c |
| HKY80  | DBY2499 × FMY1 | c |
| HKY80-2B | MATα/MATα ura3-52 his3Δ200 trp1-1 leu2-3, 112 ade2-101/+ tub1-1/+ num1Δ1::URA3 | c |
| TH133  | MATα/MATα ura3-52 his4-539/+ ade2-101/+ tub2-403/404 | g |
| TH134  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-403/404 | g |
| TH135  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-402/403 | g |
| TH136  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-403/404 | g |
| TH137  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-404/405 | g |
| TH138  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-405/406 | g |
| FMY13  | MATα/MATα ura3-52 his4-539/+ ade2-101/+ tub2-404/405 num1Δ1::URA3/Δ1::URA3/Δ1::URA3 | c |
| FMY14  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-401/402 num1Δ1::URA3/Δ1::URA3 | c |
| FMY15  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-402/403 num1Δ1::URA3/Δ1::URA3 | c |
| FMY16  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-403/404 num1Δ1::URA3/Δ1::URA3 | c |
| FMY17  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-404/405 num1Δ1::URA3/Δ1::URA3 | c |
| FMY18  | MATα/MATα ura3-52 lys2-801/+ his4-539/+ ade2-101/+ tub2-405/406 num1Δ1::URA3/Δ1::URA3 | c |

a. Minder et al., 1988; b. Schütz et al., 1986; c. this study; d. obtained from H. Kierzmann; e. Zwierschke et al., 1994; f. Stearns and Botstein, 1988; g. Huffaker et al., 1988.
2B were treated with 0.025% glutaraldehyde (added directly to the growth medium) for 5 min, followed by the fixation by 3.7% formaldehyde in PBS. After 60 min incubation at room temperature the cells were washed 3x with PBS and suspended in 1 ml of solution A (1.2 M sorbitol, 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 10 mM 2-mercaptoethanol) containing 5 µl glucuronidase and 10 µl of a 30-µg/ml Zymolase solution. After 90 min incubation at 30°C cells were recovered by low speed centrifugation, washed twice with solution A, and incubated for 5 min at room temperature with 1% Triton X-100 in PBS. Cells were then washed with PBS containing 1% bovine serum albumin and allowed to settle for 3 min on poly-lysine-treated slides, which were subsequently air-dried and incubated for 30 min with a blocking solution (PBS containing 4% BSA and 1% nonfat dry milk). All subsequent incubations with antibodies were carried out in blocking solution at room temperature (or at 4°C if incubated overnight). Anti-Num1p and FITC-labeled antibody solutions were preabsorbed with nul1 cells (strain FMY2).

The first incubation was with a 1:50 diluted solution of affinity-purified and preabsorbed anti-Num1p for 3 x 1 h, followed by an overnight incubation. The second incubation was with 3 µg/ml FITC-labeled goat anti-rabbit IgG for 2 x 1 h. The third incubation was with 6 µg/ml FITC-labeled rabbit anti-goat IgG for 2 x 1 h, followed by incubation with 1.5 µg/ml FITC-labeled goat anti-rabbit IgG for 2 x 1 h.

To confirm Num1p, actin, and chromosomal DNA, slides were washed after the final antibody treatment with PBS and incubated with a 1-µM solution of rhodamine-conjugated phallolidin in PBS for 2 h. In a last step, cells were treated with 0.02 µg/ml DAPI, which was added directly to the mounting solution (PBS containing 90% glycerol and 1 mg/ml p-phenylenediamine). Containing of microtubules and chromosomal DNA was performed by incubating slides (prepared as described above) with a 1:200 diluted solution of monoclonal anti-chicken brain α tubulin for 3 x 1 h. Slides were then washed with PBS and incubated with 1:4 diluted FITC-labeled rabbit anti-mouse IgG for 2 x 1 h. After washing, slides were mounted with a DAPI-containing mounting solution as described above. Cells were photographed on Kodak T-MAX 3200p using a Zeiss Axioskop equipped for epifluorescence microscopy.

**Results**

**Immunolocalization of Num1p**

Polyclonal antibodies were raised against a carboxy-terminal peptide (residues 2182-2519) of Num1p, which was expressed in *E. coli* as a glutathione-S-transferase (GST) fusion protein and purified by glutathione-Sepharose affinity chromatography. Western blot analysis of crude lysate proteins using anti-Num1p antibodies. (Lane A) Strain DBY1828 (relevant genotype NUM1); (lane B) strain FMY1 (num1-D1); (lane C) strain FMY1 containing plasmid pFM18 (num1-D1 NUM1); (lane D) strain FMY1 containing plasmid pFM19 (num1-D1 num1-D3). Size standards: rabbit myosin (200 kD), *E. coli* β-galactosidase (116 kD), and rabbit phosphorylase B (97.4 kD).

contain fluorescing material, supporting the specificity of the antibodies. DAPI staining of two budded num1-deficient cells (row C, second column) reveals the presence of two chromosomal spots in the mother compartment, a characteristic property of these mutants (Kormanec et al., 1991; Revardel and Aigle, 1993).

**Periodic Fluctuation of NUM1 mRNA**

Two observations prompted us to test a possible periodicity of NUM1 transcription and translation during the mitotic cell cycle: the NUM1 deletion affects the stage-specific process of nuclear migration (Kormanec et al., 1991), and Num1p specifically localizes to mother cells (see Fig. 3).

A pheromone-hypersensitive barl,NUM1 strain was synchronized by release from pheromone arrest, and aliquots were removed at 15-min intervals for cytological determinations (budding state, spindle morphology) and for the isolation of total RNA. A mixture of two radiolabeled DNA probes (a 740-bp EcoRI-BglII fragment of NUM1 and a 1.6-kb HindIII fragment containing the 8.5-kb NUM1 mRNA) together with the constitutively made 1-kb URA3 RNA by Northern hybridization. As an additional control we have also measured CDC6 and CLN1 mRNAs, which accumulate at late mitosis and G1 (START), respectively (Zwerschke et al., 1994; Kuntzel et al., 1994).

Fig. 4 demonstrates that the NUM1 mRNA content of pheromone-arrested cells (zero time) decreases to a minimum during the "return to the cycle" period at 15 min after release, and then reappears at a stage (45 min) where bud formation is initiated, while the nuclear spindle has not yet elongated. NUM1 mRNA drops to a low level during late mitosis and G1 phase (between 90 and 120 min) and reappears again at the onset of bud formation (135 min). Since bud initiation is a cytological marker associated with late S phase (Pringle and Hartwell, 1981), we conclude that NUM1 mRNA is induced at S phase and accumulates during S/G2. In contrast, CLN1 and CDC6 transcripts are not detected in pheromone-arrested cells.
and reappear already at 15 min after release. The second maximum of CDC6 mRNA is formed at the telophase stage (elongated spindle) at 90 min, followed by the second CLN1 mRNA maximum between the collapse of the mitotic spindle and bud initiation (around 120 min).

We consistently found that the level of NUM1 mRNA starts to decrease already ~15 min after its appearance during the second cell cycle (between 135 and 150 min), although this second wave of expression should be extended, rather than shortened, due to a progressive loss of synchrony. This apparent discrepancy can be explained, however, by a marked instability of NUM1 mRNA in cells grown under limiting nutrient supply (Kormanec, J., and H. Künstel, unpublished observations), and the second NUM1 transcription period in pheromone-synchronized cells is indeed extended if the culture is diluted into fresh medium at 120 min after release (data not shown).

We have also used an alternative method of cell cycle synchronization, the release of cdc15-2 cells from telophase arrest (Zwerschke et al., 1994), to confirm the NUM1 mRNA fluctuation pattern. As observed in pheromone-synchronized bar1 cells, the maxima of the three transcripts (CDC6, CLN1, and NUM1) followed in this order in intervals of 15–20 min, and the NUM1 mRNA maximum coincided with the onset of bud formation (data not shown).

**Periodic Fluctuation of Num1p**

Fig. 5 shows cells representative of populations removed from a pheromone-synchronized culture of the bar1, NUM1 strain RH-270-2B at indicated times (A–F, see Fig. 4). Cells were subsequently treated with anti-Num1p, rhodamine-conjugated phalloidin, and DAPI to visualize Num1p (first column from left), cortical actin dots (second column), and nuclear regions (third column), respectively. The fourth column shows cell images by phase contrast microscopy.

Cells removed at 60 min after pheromone release are mainly small-budded, and row A shows such a small-budded uninucleate cell with Num1p cortical dots located exclusively in the mother compartment, while phalloidin-stained actin is seen only in the bud and around the bud neck. 15 min later (75 min, row B) buds have increased in size, but are still devoid of anti-Num1p staining; actin is concentrated to the bud, but is also seen as cortical dots in the mother cell. Rows C and D (90 and 105 min, respectively) present mitotic cells with two chromosomes and elongated spindles (anti-tubulin fluorescence not shown here). Num1p remains associated mainly with the mother cell periphery, while the cortical actin dots are now evenly distributed between mother and daughter compartment. Row E (120 min) shows a large-budded cell with two reorganized nuclei and actin layers concentrated around the bud neck, indicating the stage of cytokinesis. Num1p has reached now a low level, while some weakly stained dots are visible both in mother and daughter compartment. Finally, row F (135 min) shows an apparently unbudded cell with a single nuclear region. The asymmetric distribution of cortical actin dots (second column) points to the initiation of a new bud, and the few Num1p dots (first column) may represent newly synthesized protein of an early S phase cell.

The cell types shown in Fig. 5 represent at least 60% (frequently 90%) of the various populations, indicating a reasonable degree of synchrony. The remaining cells were
also stained by anti-Numlp, but appeared to be in different stages of the cell cycle. Only 1% of cells were not stained.

The intensity of Numlp-specific immunofluorescence is not necessarily a function of Numlp content, due to a possible epitope masking effect. We have therefore measured the fluctuation of Numlp in pheromone-synchronized cells by Western blot analysis of total lysate protein, as shown in Fig. 6. The cytological determinations of this experiment (data not shown) indicated a somewhat different timing of events as compared with the data of Fig. 4: the maximum of telophase cells was reached ~15 min earlier (at 90 min) than in the previous experiment (at 105 min, see Figs. 4 and 5D). The intensity of the immunoreactive 310-kD band in Fig. 6 suggests a similar fluctuation pattern as revealed by the in situ fluorescence data (Fig. 5): the Numlp level is low in pheromone-arrested cells (zero time) and further decreases before the onset of bud formation (30 min after release). Numlp reappears during S phase (45 min), reaches a maximum at early mitosis (60 and 75 min), and drops to a minimum around the telophase stage at 105 min after release. A low amount of Numlp is seen at a stage shortly after cytokinesis (120 min), possibly produced by early S phase cells.

**Morphology of Microtubules in Num1-deficient Strains**

Fig. 7 shows some morphological features of microtubules in large-budded num1 cells (strain FMY2). Cells were grown to early stationary phase, a condition known to increase the number of binucleate mother cells (Revardel and Aigle, 1993), and treated with anti-tubulin and DAPI to visualize microtubules (left column) and nuclear regions (middle column), respectively. Row A shows a uninucleate cell with an abnormally elongated and misoriented bundle of AMTs (which should normally extend into the bud), and a short nuclear spindle which is not oriented along the mother/daughter axis. A misoriented spindle is also seen in cells B and C, showing two different stages of elongation. Again the two bundles of AMTs are unusually long, and one of them is seen to traverse the daughter compartment. The DAPI-stained cell C suggests an early stage of chromosome segregation within the mother compartment. Cell D contains a telophase nucleus (two separated chromosomal spots connected by an elongated nuclear spindle), which is correctly orientated along the mother/bud axis while remaining in the mother compartment. The cell contains short mother-oriented AMTs, whereas bud-oriented AMTs appear to be absent. Mitosis of a single

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**Figure 4.** Fluctuation of NUM1 mRNA in cells synchronized by release from pheromone arrest. Exponentially grown cells of the pheromone-hypersensitive strain RH-270-2B were treated for 1.5 h with α-pheromone. Arrested zygote cells were released at zero time by resuspension in preconditioned pheromone-free medium (Zwerschke et al., 1994), and aliquots were removed at 15-min intervals for cytological determinations, Northern hybridization analysis, and immunofluorescence microscopy. The upper part shows the percentage of small-budded cells (A–A), large-budded cells (O–O), and anaphase cells with an elongated nuclear spindle (●●●). The lower part shows the content of transcripts of NUM1, CLN1, CDC6, and URA3 in total RNA. The RNA input was controlled by measuring the O.D. at 260 nm, and by staining rRNA bands upon gel electrophoresis. Samples removed at indicated times (A–F, top) were used for immunofluorescence analysis (see Fig. 5).
tions, and were virtually absent from NUM1 cells. The data of Table II demonstrate that the percentage of cells with a misoriented spindle increases with the density of cell populations; a similar increase of cells with two chromosomal regions in the mother compartment is observed under the same conditions, confirming earlier findings (Revardel and Aigle, 1993). About 4% of cells contained a misoriented AMT bundle (as seen in Fig. 7 A), and this percentage was independent of the cellular density. Such misoriented AMTs were not observed in the isogenic wild-type strain DBY1829. It should also be noted that budding state and nuclear stain of cells showing extended mother-oriented AMTs (as in Fig. 7, B and C) correspond to the S/G2 period of maximal Numlp expression (see Figs. 4, 5, B and C, and 6), although the microtubule morphology was not analyzed in synchronized Numlp-deficient cells.

Genetic Interactions of num1 with Mutants Affecting Microtubules

We have replaced one copy of the NUM1 gene by the marker gene URA3 in the cold-sensitive tubl-1/tubl-1 strain FMY60 (see Table I), and the resulting strain FMY61 (relevant genotype tubl-1/tubl-1 num1-Δ1::URA3/) was sporulated and subjected to tetrad analysis, in order to test a possible interaction of the num1 null mutation with the α-tubulin mutation tubl-1. All twenty analyzed tetrads produced four viable spores with a 2:2 segregation of the num1-Δ1::URA3 marker, and all haploid progeny cells were cold-sensitive (growth arrest at 11°C), as expected. However, all tubl-1,num1 double mutant descendants were also temperature-sensitive (growth arrest at 37°C after performing about eight cell divisions) and grew at a somewhat slower rate at permissive temperature (30°C) than the temperature-resistant tubl-1,NUM1 descendants. At restrictive temperature, the double mutants accumulate large and osmotically fragile cells, often containing multiple and/or elongated buds, and most cells have lost their viability after 2 d incubation at 37°C.

Possible interactions of the num1 null mutation with cold-sensitive β-tubulin mutant alleles were tested by NUM1/URA3 gene replacement in the six homozygous tub2 diploid strains TH133 to TH138 containing the alleles tub2-104, tub2-401, tub2-402, tub2-403, tub2-404, and tub2-405, respectively. The resulting transformant strains FMY13 to FMY18 were allowed to sporulate, and 40 tetrad asci of each strain were dissected. The strains FMY13 and FMY14 (relevant genotypes tub2-104/tub2-104 num1-Δ1::URA3/+ and tub2-401/tub2-401 num1-Δ1::URA3/+, respectively)
produced four viable spores per tetrad with a 2:2 segregation of the numl-Δ1::URA3 marker. The double mutants tub2-104,numl and tub2-401,numl did not differ significantly from the corresponding tub2 single mutants in growth rate and cell morphology. Only two viable spores per tetrad were produced by strains FMY15, FMY16, FMY17, and FMY18 (relevant genotypes: tub2-202 alleles 402 to 405, numl-Δ1::URA3/+). All viable spores were uracil-requiring (NUM1), and the inviable spores (inferred genotype: tub2 alleles 402 to 405, numl-Δ1::URA3) either did not germinate, or arrested their growth after forming a microcolony of up to 100 cells.

Table II. Nuclear Morphology of numl-Δ1 Cells (FMY2) Grown to Increasing Densities

| Cellular density (OD at 600 nm) | Accumulation of cells |
|---------------------------------|-----------------------|
|                                 | With misoriented spindle | With two DNA regions in the mother compartment |
|                                 | %                      | %                          |
| 1.8                             | 13                     | 4                          |
| 4.0                             | 28                     | 20                         |
| 6.8                             | 37                     | 28                         |

We conclude from these observations that the numl null mutation shows synthetic lethality with the cold-sensitive β-tubulin mutant alleles tub2-402, tub2-403, tub2-404, and tub2-405, but not with the alleles tub2-104 and tub2-401.

**Functional Mapping of NUM1**

We have deleted various parts of the NUM1 gene (see Fig. 1) and introduced the gene variants as centromeric plasmids into NUM1-deficient strains, in order to test four functional parameters: (A) intracellular localization by indirect immunofluorescence microscopy, (B) suppression of the numl-specific nuclear migration defect, and (C) complementation of the temperature sensitivity of a tub1-1, numl-Δ1 double mutant.

Fig. 8 summarizes the protein maps of the resulting deletion variants together with their functional activities. All tested Num1p variants have retained residues 2182 to 2519, which were used to raise antibodies, and the intensity of indirect immunofluorescence has indicated that all variants are expressed at wild-type levels.

Fig. 9A shows the immunofluorescence pattern of numl cells expressing the truncated allele numl-Δ7 as a GAL4(bd) fusion protein (pFM52); this pattern (cortical dots in the mother cell) is virtually identical to that of NUM1 cells (Fig. 3A) or numl cells containing plasmids pFM18, pFM19, pFM26, pFM42, and pFM35 (data not shown). All Num1p variants encoded by these plasmids have in common their carboxy-terminal PH domain, and the only variant lacking this domain (encoded by pFM41) appears to be released preferentially into the cytoplasm of the mother compartment, as shown in Fig. 9B. These data strongly suggest a cortex-targeting function of the PH domain.

About 30% of large-budded FMY2 cells in cultures approaching stationary phase have two DAPI-stained chromosomal regions in the mother compartment, a morphology reflecting a nuclear migration defect (Kormanec et al., 1991; Revardel and Aigle, 1993). This defect is efficiently suppressed by plasmids pFM18, pFM19, and pFM26, since the content of binucleate mother cells is reduced to weight level (<0.1%). Plasmid pFM42 is partially active (15% binucleate mother cells), and the remaining three plasmids (pFM35, pFM52, and pFM41) are inactive in this assay (28–30% binucleate mother cells). The tubl-1,numl-Δ1 strain HKY80-2B contains >50% binucleate mother cells, if grown at 30°C to early stationary phase, indicating that the numl nuclear migration defect is more pronounced in the presence of the tub1-1 mutation. The relative suppressing activity of the tested plasmids (see Fig. 8B) was about the same in this strain.

Fig. 10 demonstrates the differential activity of these plas-
Discussion

Previous observations have indicated a role of the \textit{NUM1} gene product in the control of nuclear migration at the G2 phase of the cell cycle (Kormann et al., 1991). Here we demonstrate a temporal and spatial control of \textit{NUM1} gene expression: the \textit{NUM1} transcript transiently accumulates at the S/G2 phase of cells synchronized by two different methods, and the \textit{Num1} protein associates at the same period of the cell cycle to the cortex of mother cells, where it remains located mainly up to the stage of cytokinesis. Thus, \textit{NUM1} belongs to the class of periodically expressed genes encoding stage-specific cyclins, histones, transcriptional regulators, and proteins involved in DNA synthesis (for a recent review see Küntzel et al., 1994).

The \textit{NUM1} mRNA fluctuation pattern resembles that of histones (Osley, 1991) and of the mitotic cyclins \textit{CLB3} and \textit{CLB4} (Richardson et al., 1992). The latter two stage-specific activators of the \textit{CDC28} protein kinase are required for the assembly of a short nuclear spindle in proanaphase cells (Richardson et al., 1992; Schwob and Nasmyth, 1993).

The \textit{Num1} gene maps close to the dihydrolipoyl trans-succinylase gene \textit{KGD2} (Repetto and Tzagoloff, 1990), and both genes share a 561-bp promoter region controlling a divergent transcription of opposite DNA strands (see Fig. 1). However, both genes appear to be differentially regulated: \textit{KGD2} transcription is glucose-repressible (Repetto and Tzagoloff, 1990) and constant throughout the cell cycle, whereas the periodic \textit{NUM1} transcription is enhanced in cells grown in glucose media (our unpublished data).

Indirect immunofluorescence microscopy of \textit{NUM1} cells treated with anti-\textit{Num1p} has visualized the \textit{Num1p} antigen as cortical dots located at the periphery of mother cells, while the bud region is not stained by antibody. This asymmetrical distribution pattern is opposed to that of cortical actin patches which concentrate around the bud neck and within small buds during the S/G2 stage of the cell cycle (for review see Welch et al., 1994). The cellular content of \textit{Num1p} cortical dots decreases at the cytokinesis stage of the cell cycle and increases again when small buds are visible. Western blot analysis of pheromone-synchronized cells has also shown a pronounced fluctuation of \textit{Num1p} during the cell cycle.

Our functional mapping data suggest that the carboxy-terminal PH domain of \textit{Num1p} serves as a "cortex-targeting" signal: all tested \textit{Num1} variants containing the PH domain associate with the mother cell cortex, even after deleting functionally important regions, and only the removal of the PH domain leads to a cytosolic localization of \textit{Num1p}. PH domains are found in cytoskeletal proteins like spectrin, pleckstrin, and dynamin, but also in many proteins involved in signal transduction, such as GTPase-activating proteins, protein kinases, and phospholipases (Musacchio et al., 1993; Gibson et al., 1994). Interestingly, a \textit{Num1} region including the PH domain (residues 2301 to 2742) shows significant sequence similarity with a corresponding region of the \textit{Aspergillus nidulans ApsA} protein, which is required for nuclear positioning and entry of nuclei into primary buds (sterigmata) during the development of conidiophores (Fischer and Timberlake, 1995). The similarity between \textit{Num1p} and ApsA is most pronounced within the PH domain, whereas other common

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
plasmids & cortex & cytoplasm \\
\hline
pFM18 & + & - \\
pFM19 (num1-\Delta 3) & + & - \\
pFM26 (num1-\Delta 3,\Delta 4) & + & - \\
pFM42 (num1-\Delta 5) & + & - \\
pFM35 (num1-\Delta 3,\Delta 6) & - & - \\
pFM52 (num1-\Delta 7) & - & - \\
pFM41 (num1-\Delta 3,\Delta 8) & - & - \\
\hline
\end{tabular}
\caption{Functional mapping of \textit{Num1p}. Features of the \textit{Num1p} map (upper line) are explained in the legend to Fig. 1. Plasmids indicated at left were introduced into the \textit{NUM1}-deficient strain FM2, and the resulting transformants were analyzed by (A) indirect immunofluorescence microscopy, and (B) by determining the percentage of large-budded cells with two DAPI-stained regions in the mother, after growth to early stationary phase. Strains containing actively suppressing plasmids (+) produce less than 1% binucleate mother cells, and strains with inactive plasmids (−) produce about the same percentage (28-30%) as plasmids in the \textit{tub1-1,num1-\Delta 1} strain HKY80-2B in order to test a complementation of temperature sensitivity (+, growth at 37°; −, arrest at 37°).}
\end{table}
features of both proteins (an NH$_2$-terminal coiled-coil domain and central tandem repeats) are not conserved in their primary structure.

A common function of PH domains could be the recognition of phosphorylated serine/threonine residues in specific peptides (Gibson et al., 1994) and/or the interaction with the membrane lipid phosphatidyl-inositol-4,5-bisphosphate (Harlan et al., 1994). The latter function would imply a direct association of Numlp with lipids of the mother cell membrane; alternatively, Numlp could be anchored via its PH domain to proteins associated with the plasma membrane of mother cells.

Several observations suggest that Numlp affects microtubular functions. (a) NUM1-deficient strains accumulate large-budded cells with a misoriented nuclear spindle and elongated AMTs, a phenotype characteristic of conditional-lethal β-tubulin and actin mutants (Huffaker et al., 1988; Palmer et al., 1992), as well as of null mutants lacking either the dynein heavy chain Dhc1p/Dynlp (Li et al., 1993; Eshel et al., 1993), the centrin homolog Act3p/Act5p (Muhua et al., 1994; Clark and Meyer, 1994), or the dynein-associated protein Jnm1p (McMillan and Tatchell, 1994). (b) The AMTs of numl cells are sometimes misoriented and do not extend into the bud (see Fig. 7 A). This is in contrast to jnm1 mutant cells, where bud-oriented AMTs always appear to extend to the mother/bud neck (McMillan and Tatchell, 1994). (c) Misoriented nuclei tend to perform mitosis and nuclear division in the mother compartment of large-budded numl cells (see Fig. 7) leading to the accumulation of “binucleate mother cells” (Kormanec et al., 1991; Revardel and Aigle, 1993). Again, this behavior is seen in many other mutants affecting microtubular functions, and it confirms that the mitotic functions of the nuclear spindle are not affected by mutations impairing AMTs (Sullivan and Huffaker, 1992). (d) The deletion of the nonessential NUM1 gene has synthetic effects in the cold-sensitive α-tubulin mutant tubl-l (Stearns and Botstein, 1988) and in certain cold-sensitive β-tubulin (tub2) mutant alleles (Huffaker et al., 1988). The numl−Δ null mutation confers conditional lethality at 37°C to tubl-l strains, and is lethal at all temperatures in combination with the alleles tub2-402, tub2-403, tub2-404, and tub2-405. The four tub2 mutations interacting with numl−Δ are clustered within a COOH-terminal β-tubulin domain (residues 375-424), while the noninteracting allele tub2-401 maps more upstream at residues 233-245 (Huffaker et al., 1988). The four numl-interacting tub2 alleles have quite different effects on the content of nuclear and astral microtubules at restrictive temperature: two mutant alleles (tub2-403 and tub2-405) contain greatly diminished levels of both kinds of microtubules, one allele (tub2-402) contains predominantly astral microtubules, and allele tub2-404 contains prominent nuclear and astral microtubule arrays; the two noninteracting alleles contain predominantly nuclear microtubules (tub2-104) or apparently lack both kinds of microtubules (tub2-401) (Huffaker et al., 1988). It is difficult to deduce from these data a common property distinguishing numl-interacting from noninteracting tub2 alleles; perhaps the ability to produce AMTs is critical for lethal interaction with numl−Δ.

The most prominent structural feature of Numlp is a 12-fold near-perfect repetition of a peptide of 64 residues (Kormanec et al., 1991). However, our deletion experiments suggest that the amplification of this peptide is not critical for function, since a single repeat unit is sufficient to suppress the nuclear migration defect in TUB1/numl cells as well as the temperature sensitivity of tubl-1/numl mutants. A similar conclusion can be drawn from the observed polymorphism of the repetitive region in different yeast wild-type strains (Revardel and Aigle, 1993).

The deletion of the remaining single repeat unit in allele numl−Δ5 reduces the ability to suppress the nuclear migration defect in TUB1/numl cells, but surprisingly does not affect the rescue of conditional lethality of the tubl-1/numl double mutation. The opposite effect is observed if the last 30 residues of Numlp are removed, which include a cluster of potential phosphorylation sites of cAMP-dependent protein kinase and protein kinase C (Kormanec et al., 1991): this deletion abolishes the ability to rescue the temperature sensitivity of tubl-1/numl double mutants, but does not affect the suppression of the nuclear migration defect in TUB1/numl cells. Both activities are lost by deleting a potential EF-hand Ca$^{2+}$-binding site (Δ6) or a carboxy-terminal region including the PH domain (Δ8).

Figure 9. Immunolocalization of truncated Numlp derivatives encoded by alleles numl-Δ7 (A, strain FM2-S2) and numl-Δ3,Δ8 (B, strain FM2-41). (Left column) Indirect immunofluorescence of cells treated with anti-Numlp antibodies. (Right column) Phase contrast microscopy.
Figure 10. Complementation of tub1-1::num1-Δ1 temperature sensitivity (strain HKY80-2B) by plasmids containing intact and deleted NUM1 variants. Strains and relevant genotypes: 18, tub1-1::num1-Δ1 pFM18 (NUM1); 19, tub1-1::num1-Δ1 pFM19 (num1-Δ3); 26, tub1-1::num1-Δ1 pFM26 (num1-Δ3,Δ4); 42, tub1-1::num1-Δ1 pFM42 (num1-Δ5); 35, tub1-1::num1-Δ1 pFM35 (num1-Δ3,Δ6); 52, tub1-1::num1-Δ1 pFM52 (num1-Δ7); 41, tub1-1::num1-Δ1 pFM41 (num1-Δ3,Δ8). Cells were incubated for 2 d on selective medium at 30°C and 37°C.

These observations point to differential roles of Numlp in controlling AMT functions and/or nuclear migration. However, the specific association of Numlp with the mother cell cortex is difficult to reconcile with effects on mother-distal AMTs, which are sometimes not properly oriented towards the bud; furthermore, both mother-distal and mother-proximal AMTs are often abnormally elongated in numl cells. A similar problem concerns the role of Jnm1p, a protein located in the bud or near the bud-oriented SPB. This protein affects not only bud-oriented, but also mother-oriented AMTs, which both appear to be abnormally elongated in JNM1-deficient strains (McMillan and Tatchell, 1994).

The first evidence for a functional orientation of SPBs (via their AMTs) toward the cortex of mother and bud compartment was provided by the observation that the old SPB remains in the mother cell, whereas the new SPB (formed by SPB duplication) segregates into the daughter cell (Vallen et al., 1992).

A more recent model of nuclear migration in budding yeast has postulated the positioning of the pre-anaphase nucleus by transiently tethering AMTs to cortical sites at the mother compartment and at the bud or bud/mother neck region (Schroer, 1994). Candidates for tethering bud-oriented AMTs are components of the dynactin/dynein complex encoded by DHC1/DYN1, ACT3/ACT5, and JNM1 (Schroer, 1994; Macmillan and Tatchell, 1994), whereas Numlp is a possible candidate for tethering AMTs to the mother cortex during its transient expression period at S/G2 phase. The failure to attach either mother- or bud-oriented AMTs to their respective cortical sites (e.g., by deleting NUM1, DHC1/DYN1, ACT3/ACT5, or JNM1) could possibly lead to the release from both attachment sites, followed by the abnormal elongation of both kinds of AMTs.

Alternatively, Numlp may control AMT functions more indirectly, e.g., by acting in a signaling pathway involved in the structural modification of microtubules or microtubule-associated proteins. Indeed, some functionally important elements of Numlp (a potential EF hand Ca2+-binding site, a PH domain frequently found in signaling proteins, and clusters of potential phosphorylation sites for cAMP-dependent protein kinase and/or protein kinase C) point to a role of this mother-specific protein in signal transduction.

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