

The *pot1*⁺ homologue in *Aspergillus nidulans* is required for ordering mitotic events

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Summary

Orderly progression through mitosis is essential to reduce segregation errors in the cell's genetic material. We have used a cytological screen to identify a mutant that progresses through mitosis aberrantly and have cloned the complementing gene, *nimU*, which encodes a protein related to Pot1 and other telomere end-binding proteins. We show that loss of *nimU* function leads to premature mitotic spindle elongation, premature mitotic exit, errors in chromosome segregation, and failure to delay mitotic exit under conditions that normally evoke the mitotic

spindle checkpoint response. Whereas premature mitotic exit is dependent upon anaphase promoting complex function, premature spindle elongation is not. We conclude that *nimU* is constitutively required for orderly mitotic progression under normal growth conditions and also required for the conditional mitotic spindle checkpoint response.

Key words: *Aspergillus nidulans*, Telomere end binding protein, Pot1, Mitotic exit, Spindle checkpoint

Introduction

The mitotic processes by which the nucleus divides occur in an orderly manner. Mitotic spindle assembly, elongation and disassembly are co-ordinated with chromatin re-organisation. In *Aspergillus nidulans*, as in other eukaryotes, mitotic progression is dependent upon Cdc2 protein kinase and the anaphase promoting complex/cyclosome, or APC/C (Osmani et al., 1994; Morris, 1976a; Peters et al., 1996; Lies et al., 1998). Cdc2 must first be activated through association with mitotic cyclin and later inactivated through cyclin destruction (Blanco et al., 2000; Wasch and Cross, 2002). The APC/C, which targets proteins for destruction, confers order on mitotic events by firstly acting upon proteins involved in sister chromatid cohesion, and then on mitotic cyclin (reviewed by Uhlmann, 2003; Zachariae and Nasmyth, 1999; Gardner and Burke, 2000). When the timing of mitotic events is perturbed, or when the mitotic spindle is damaged, the spindle assembly checkpoint prevents the activation of the APC/C. Thus the checkpoint can delay sister chromatid separation and mitotic exit. The *BUB* and *MAD* genes encode checkpoint components and were isolated from budding yeast by identifying mutants that failed to arrest chromatid separation in the presence of drug-induced spindle damage (Hoyt et al., 1991; Li and Murray, 1991). The checkpoint appears to be highly conserved. In *Aspergillus*, mutations in *BUB*-related genes confer sensitivity to spindle drugs (Efimov and Morris, 1998). Loss of the checkpoint is associated with abnormal chromosome numbers (aneuploidy) in human cancer cells (Li and Benezra, 1996; Cahill et al., 1998; Jin et al., 1998; Lengauer et al., 1998; Takahashi et al., 1999; Lee et al., 1999). Thus, mutations that perturb the timing of mitotic events and uncouple the checkpoint responses are likely candidates for inactivation in cancer.

A number of mutants that perturb progression through mitosis have been identified in *Aspergillus* (Morris, 1976b). For some of these, the corresponding genes have been isolated and shown to govern the intrinsic timing of mitotic events. The *bimA* (O'Donnell et al., 1991) and *bimE* (Osmani et al., 1988) genes encode homologues of APC/C components (Peters et al., 1996; Zachariae et al., 1996) and mutations in both genes lead to a delay in metaphase, presumably as a result of APC/C inactivation. Type 1 protein phosphatase, encoded by the *bimG* gene, is also required for progression through anaphase (Doonan and Morris, 1989). Mutation of the *bimB* gene leads to a transient mitotic delay and uncouples DNA replication from the completion of the previous mitosis (May et al., 1992). *bimB*-related genes in budding yeast (*ESP1*) and fission yeast (*cut1*⁺), both function as separases, which are required for sister chromatid separation in mitosis (Ciosk et al., 1998; Funabiki et al., 1996; McGrew et al., 1992). The *bimC* gene is the founding member of a class of motor proteins, called kinesins, that plays an essential role in spindle pole body separation in mitosis (Enos and Morris, 1990). A heat sensitive β tubulin mutation, *benA33*, that hyper-stabilises the mitotic spindle by blocking microtubule disassembly (Oakley and Morris, 1981), delays progression through anaphase.

The activities of two protein kinases are also required for entry into mitosis in *Aspergillus* (Osmani et al., 1991a; Ye et al., 1995) (reviewed by Osmani and Ye, 1996), and these must be inactivated for mitotic exit. The *nimX* gene, isolated by reverse genetics, encodes a Cdc2 homologue (Osmani et al., 1994), and the *nimA* gene encodes a second protein kinase required for chromosome condensation in mitosis (DeSouza et al., 2000). Whereas fluctuations in NIMX^{Cdc2} activity depend, in part, upon APC/C-mediated degradation of its activating subunit, NIME^{cyclinB} (Ye et al., 1997), NIMA is a direct target

for proteolysis (Pu and Osmani, 1995; Ye et al., 1995), and this also appears to be mediated by the APC/C (Ye et al., 1998). Both kinases contribute to each other's mitosis-promoting activity, since NIMA is hyper-phosphorylated and activated by NIMX^{Cdc2} (Ye et al., 1995), and NIMA is required for nuclear accumulation of NIME^{cyclinB}, the activating subunit of NIMX^{Cdc2} (Wu et al., 1998). Cdc2 kinase appears to play a role in APC/C regulation (reviewed by Pomeroy et al., 2003; Hershko, 1999), and a similar role has been proposed for NIMA (Ye et al., 1998). Thus the kinase activities that bring about mitosis are also involved in triggering mitotic exit.

The foregoing proteins are all required for mitotic progression, their mutation leading to arrest at G2/M or in mitosis. This contrasts with spindle checkpoint components, which act as inhibitors of mitotic progression, whose mutation leads to inappropriate progression through, and exit from, mitosis. Checkpoint components have been found to be essential in fly, worm and mouse (Basu et al., 1999; Kitagawa and Rose, 1999; Dobles et al., 2000; Kalitsis et al., 2000), indicating that inhibitors are absolutely required to ensure the fidelity of mitosis in metazoans. This contrasts with the lower eukaryotes in which the checkpoint genes are not essential and the intrinsic timing of mitosis has been proposed to be sufficient to ensure normal mitotic progression (Dobles et al., 2000). Here, we have identified a mutation, *nimU24*, which confers a lethal defect in mitotic progression. We report the cloning of *nimU*, and show that it encodes an essential protein structurally related to the Pot1 protein from fission yeast and humans and to other telomere end binding proteins (TEBPs) from protists. We show that *nimU* is required to prevent premature mitotic exit and chromosome segregation errors, as well as for the mitotic spindle checkpoint response to spindle damage.

Materials and Methods

Strains and media

Strains used were as follows: EM1 (*nimU24 pyrG89 chaA1*), EM2 (*nimU24 pyrG89 yA2 riboA1 choA1*), EM4 (*nimU24 pyrG89 yA2 riboA1 choA1*), GR5 (*pyrG89 pyroA4 wA3*), CP1 (*nimA5 pyrG89 yA2*), Dip32 (*pyrG89 yA2 // chaA1 acrA1 riboB2*), Dip37 (*nimU24 pyrG89 yA2 riboA1 // chaA1*), Dip38 (*nimU24 pyrG89 yA2 riboA1 // nimU24 chaA1*), GR5/Δ15 (*pyrG89 pyroA4 wA3 [pyr4⁺::nimU⁻]*), GR5/Δ42 (*pyrG89 pyroA4 wA3 [pyr4⁺::nimU⁻]*), CP1/1 (*nimA5 pyrG89 [pyr4⁺]*), CP1/Δ6 (*nimA5 pyrG89 [pyr4⁺::nimU⁻]*), SWJ224 (*nimU24 bimE7 methG1*), DBE4 (*bimE7 pyrG89*), 744Dn (*sldA744; pyrG89; yA2*), 937Dn (*sldB937; pyrG89; yA2*). Liquid culture experiments were performed in rich medium (0.5% w/v yeast extract, 2% w/v sucrose) and plate experiments on PDA rich medium (2% w/v potato dextrose agar, 2% w/v sucrose). Benomyl was used at 2.4 μg/ml.

Cloning and cDNA library construction

Sib selection was used to clone the *nimU* gene through DNA-mediated complementation of the *nimU24* phenotype. Since *nimU* had previously been mapped to chromosome VII (Morris, 1976b), banks of chromosome VII-specific cosmids [constructed in pWE15 (Evans and Wahl, 1987) and pLORIST (Gibson et al., 1987) and obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center)] were combined in a number of pools and tested for their ability to complement the temperature sensitive (*ts⁻*) growth defect in a *nimU24* strain (EM2). Clones present in complementing pools were

divided into sequentially smaller pools and tested until the complementing activity could be assigned to a single clone, pW04F01. Sub-clones derived from this cosmid showed that the complementing activity was contained in a 4.8 kb *BamHI/SacI* fragment: plasmid p5BS.

A two-step gene replacement (O'Connell et al., 1992) was performed to test whether the *nimU* gene, or a multi-copy extragenic suppressor, had been cloned. Briefly, the 4.8 kb *BamHI/SacI* fragment was cloned into the pRG3 integrative vector (carrying the *pyr4⁺* gene of *Neurospora crassa*, which complements the *A. nidulans pyrG89* mutation) and used to transform a *pyrG89 nimU24* strain (EM1) to uracil/uridine (UU) prototrophy. A *nimU24*-complemented transformant carrying a single copy of the plasmid was selected by Southern analysis. Spores from this strain were grown on plates containing the pyrimidine analogue 5'-fluoro-orotic acid (5-FOA) that severely inhibits growth of UU prototrophs, but not UU auxotrophs (Winston et al., 1984). This allowed selection of strains that had lost the *pyr4⁺*-containing vector through mitotic recombination. If the *nimU* gene had been cloned, then recombination between two tandemly repeated *nimU* sequences should produce UU auxotrophs that contain either the wild-type or the mutant copy of *nimU*. However, if a multi-copy suppressor had been cloned, then loss of the vector should remove the extra copy of the suppressor, and all such strains should be *ts⁻*. Fifty strains were isolated from 5-FOA medium, shown to be UU auxotrophs, and four of these were able to grow at the *nimU24*-restrictive temperature (i.e. they were *ts⁺*). A *ts⁺* isolate was crossed to a wild-type (*nimU⁺*) strain and the progeny were all found to be *ts⁺*, thereby confirming that the *ts⁻* allele had been eliminated from the genome. This confirmed that the *nimU* gene, and not a multicopy extragenic suppressor, had been cloned. The whole of the 4.8 kb *BamHI/SacI* fragment was sequenced and potential open reading frames mapped. To narrow down the precise *nimU* sequence the ability of restriction fragments, derived from p5BS, were tested for their ability to complement. This showed that the complementing activity was contained within a region encompassed by the *PstI* and *EcoRI* restriction sites. We identified three open reading frames in this region that contained consensus intron splice sites (Gurr et al., 1987) that suggested they might constitute three exons of the same gene. This was considered a strong candidate for the *nimU* gene.

We isolated *nimU* cDNA and used it to verify the intron/exon boundaries, by comparison with the genomic DNA sequence. A λ Unizap (Stratagene) cDNA library was constructed from total RNA isolated from a wild-type strain (GR5) grown overnight at *nimU24* permissive temperature, followed by a 3-hour incubation at the restrictive temperature. mRNA was isolated using the Promega PolyA Tract System and the library constructed according to the manufacturer's directions. Around 1.25 million plaques were screened using an 1824 bp *MunI* restriction fragment spanning the three predicted *nimU* exons. This led to the isolation of one cDNA (p6.1) that spanned the three exons. Sequencing on both strands showed the gene to consist of four exons. Co-transformation of *nimU24* strains (EM2 and EM4) with p6.1 and pSF20-1 [a selection plasmid that carries the *pyr4⁺* gene (Fidel et al., 1988)] showed that p6.1 could complement the *nimU24* phenotype. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ567920 (*nimU* genomic DNA) and AJ567922 (*nimU* cDNA).

Sequence analysis

Sequencing was carried out using an ABI automated sequencer (373 DNA Sequencing System) and sequencing reactions were performed using the Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems. Data was analysed using BESTFIT from the Wisconsin GCG package, Gene Construction Kit 2 (Textco), BLAST at NCBI, and DNASTar EditSeq and Megalign.

Construction of deletion plasmid

A DNA fragment flanking the 3' end of *nimU* was isolated by double-digestion of plasmid p5BS with *KpnI* and *BamHI* and the 1.6 kb fragment gel isolated. The 5' flank, together with the vector pBC, was isolated by digesting p5BS with *MunI* and *BamHI* and recovery of the 5.2 kb fragment. The *pyr4⁺* gene was isolated as a 2.1 kb fragment from plasmid pODC by double-digestion with *EcoRI* and *KpnI*. The three fragments were ligated to produce the 8.9 kb plasmid p Δ *nimU*. Note that the *MunI* and *EcoRI* cohesive ends are compatible, but both restriction sites are lost on ligation. The construct retains 57 bp of the 5' end of *nimU*, and has the potential to encode a 54 amino acid polypeptide having only the first 19 residues in common with *nimU*. Initial deletion analysis was performed in strain GR5. Subsequently, a deletion was generated in strain CP1, in order to attain cell cycle synchrony by utilising the G2 arrest of the *nimA5 ts⁻* allele.

Gene deletion

We carried out a gene deletion using heterokaryon rescue (Osmani et al., 1988). This technique relies upon the formation of multinucleated protoplasts, where transformed nuclei (carrying the deletion) are propagated alongside untransformed nuclei in a heterokaryon. This allows direct examination of the effect of gene deletion as uninucleate spores, derived from the heterokaryon, can be analysed on selective medium. GR5, a *pyrG89* strain (UU auxotroph), was transformed to uracil/uridine prototrophy with the 5.1 kb *EcoRI* fragment from plasmid p Δ *nimU*, thereby replacing approximately two-thirds of the *nimU* gene with the *pyr4⁺* gene of *N. crassa*. Transformants were selected on medium lacking uracil and uridine (–UU) and a total of 69 were isolated. On streaking out spores from six of these primary transformants we were unable to isolate *pyr4⁺* colonies on selective medium, suggesting that an essential gene had been deleted. Spores from these heterokaryons were then germinated on –UU medium. Only around 5% were able to form polarised cells, the remainder failed to germinate and therefore probably represented untransformed nuclei. The polarised cells were phenotypically indistinguishable from *nimU24* cells germinated at the restrictive temperature, forming highly branched hyphae, enlarged nuclei and they were unable to complete the asexual cycle. Site-specific integration of the deletion construct at the *nimU* locus was confirmed by PCR for all six of the putative deletants. This showed that the frequency of homologous recombination of this construct at the *nimU* locus was approximately 10%. This is similar to the frequency found for a γ tubulin deletion in *Aspergillus* (Oakley, 1990). For two of these transformants, the predicted gene replacement event was also demonstrated by Southern analysis of genomic DNA.

Southern analysis

Blotting protocol was carried out using Hybond-N nylon membrane (Amersham Life Science), according to the manufacturer's instructions. [α^{32} P]dCTP was incorporated into DNA probes by oligolabelling, using a kit from Pharmacia Biotech (27-9250-01) and their standard protocol. Membranes were pre-hybridised with 50 ml of prehybridisation buffer: 6 $\times$ SSC + 1 ml 50 $\times$ Denhardt's solution [1% BSA; 1% Ficoll; 1% PVP] + 0.5% SDS + 2 mg salmon sperm DNA for 1 hour before adding the radio-labelled probe and 50 ml of hybridisation buffer (as prehybridisation buffer, except no salmon sperm DNA). Hybridisation was carried overnight at 65°C in a rotary incubator. Membranes were washed: once with 6 $\times$ SSC + 0.1% SDS; twice with 2 $\times$ SSC + 0.1% SDS; once with 0.1 $\times$ SSC + 0.1% SDS. Kodak scientific imaging film (X-OMAT AR) was exposed to the membrane.

Microscopy

Spores were germinated in liquid medium on coverslips then fixed in 4% w/v formaldehyde. Chromosome mitotic index (CMI) was

determined by staining chromatin with 4',6-diamidino-2-phenylindole (DAPI; Sigma, cat. no. D-9542). Spindle mitotic index was determined by indirect immunofluorescence using TAT-1 mouse anti- α tubulin as the primary antibody, and spindle pole bodies were visualised using mouse monoclonal anti- γ tubulin clone GTU-88 (Sigma, cat. no. T-6557). Alexa Fluor 568 goat anti-mouse antibody (A-21043) was used as the secondary antibody. Experiments were scored using a Nikon E600 fluorescence microscope.

Chromosome segregation experiments

Thirty inocula (each of 500 diploid conidiospores) of each strain were incubated at the restrictive temperature (42°C) for 6 hours then returned to the permissive temperature (25°C) to allow colony formation. Data presented in the text represent the mean and the standard error of three replicate experiments.

Results

nimU24 leads to defective mitoses without cell cycle arrest

Defective progression through mitosis often leads to aberrant nuclear structure (Enos and Morris, 1990; Doonan and Morris, 1989; Oakley and Morris, 1981). We therefore screened a collection of temperature sensitive conditionally lethal *Aspergillus* cell cycle mutants to identify strains that had aberrant nuclei at the restrictive temperature. *Aspergillus* conidiospores (asexual spores) are arrested at G1 phase of the cell cycle and on germination re-enter the cell cycle at this point. They then proceed through three nuclear division cycles before cytokinesis takes place, thus the germlings are syncytial. Surprisingly, we found that the *nimU24* mutant, previously reported to lead to arrest in the G2 phase of the cell cycle (Bergen et al., 1984), entered mitosis and developed enlarged nuclei with multiple spindle pole bodies (SPBs). These enlarged nuclei, which were present in all mutant germlings, appeared as distinct lobes of chromatin interconnected by strands of chromatin (Fig. 1A). In wild-type cells SPBs are duplicated once per nuclear division cycle and separated in mitosis through mitotic spindle formation. Therefore their number and separation provide a direct measure of mitotic history: prior to G2/M there is just one SPB per nucleus; at G2/M the SPBs have duplicated but remain paired; formation of the mitotic spindle during mitosis separates the SPBs; karyokinesis returns the SPB number to one per nucleus; failure to complete mitosis would increase the number of SPBs per nucleus above one; if the failed mitosis involved spindle formation then the SPBs should be separated but still associated with the same nucleus (Fig. 1B,C). Note that a single SPB is only associated with one nucleus. Our observations suggested that *nimU24* mutants did not arrest in G2 but continued through the cell cycle with defective mitoses, thereby failing to complete nuclear division. We measured the rate of entry into the first mitosis after germination by monitoring SPB separation using antibody against γ tubulin, a component of the SPB (Oakley et al., 1990). The data showed no significant difference in the rate of SPB separation between *nimU24* and wild-type cells (Fig. 1D), indicating that *nimU24* cells do not arrest in G2, but enter mitosis with similar dynamics to wild-type cells. Moreover, the nuclei in *nimU24* cells became progressively more enlarged and distorted with time, displaying multiple separated SPBs (Fig. 1A), whereas

wild-type cells had an average of just one SPB per nucleus (Fig. 1B), indicating continued cell cycle progression and failed mitoses in *nimU24* (Fig. 1A,C).

To confirm that the cell cycle continued in *nimU24* strains we monitored nuclear dynamics with the DNA dye 4',6'-

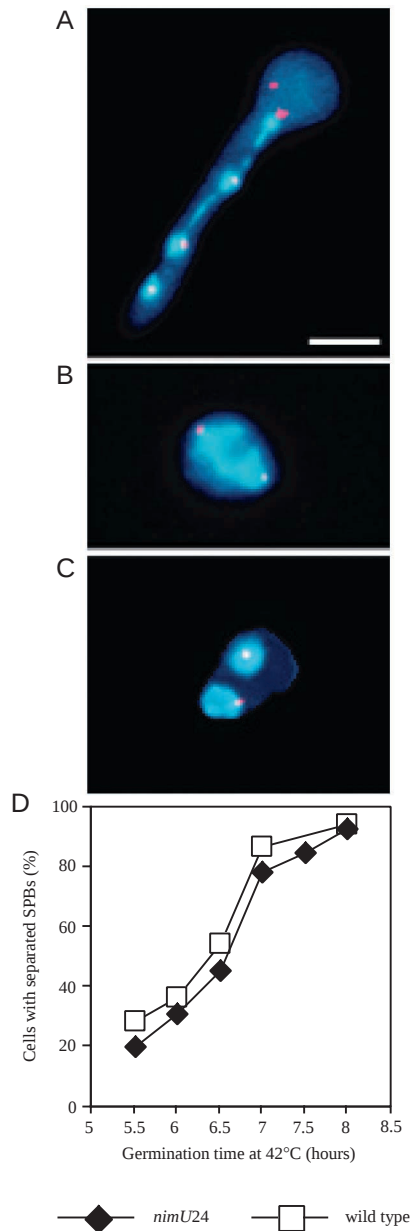


Fig. 1. *nimU24* strains fail to complete mitosis. Chromatin is shown in blue (DAPI staining) and spindle pole bodies in pink (indirect immunofluorescence with anti- γ tubulin antibody). (A) A *nimU24* cell (EM4) with multiple separated spindle pole bodies (SPBs) but foci of chromatin connected by strands of chromatin. In this image the chromatin appears condensed. (B) A uninucleate *nimU24* cell (EM4) with two separated SPBs. (C) A wild-type cell (GR5) containing two normal interphase nuclei, each with a single SPB. (D) SPB separation was scored in a *nimU24* strain (EM4) and a wild-type strain (GR5) through the time course indicated, at the restrictive temperature (42°C). Scale bar: 5 μ m.

diamidino-2-phenylindole (DAPI). This facilitates direct observation of chromosome condensation at mitosis in *Aspergillus*, allowing the distinction between mitotic and interphase nuclei to be made. We characterised the *nimU24* mutant by measuring the percentage of mitotic cells (the chromosome mitotic index – CMI) at the permissive (32°C) and restrictive (42°C) temperatures. At 42°C the average CMI through a 14-hour time course was 5% for a wild-type strain but only 2% for a *nimU24* strain (Fig. 2A). This CMI remained approximately constant over the course of the experiment, confirming that cells did not arrest. However, the *nimU24* nuclei became progressively enlarged and misshapen as a function of time (Fig. 2B). As we have shown that the rate of entry into mitosis is normal in the mutant, the lowered mitotic index suggests that *nimU24* cells spend less time in mitosis than wild type. The enlarged nuclei indicate continuing rounds of chromosome replication without nuclear division.

nimU is structurally related to fission yeast and mammalian genes

The *nimU* gene was cloned and a cDNA isolated by DNA mediated complementation of the *nimU24* mutant (see

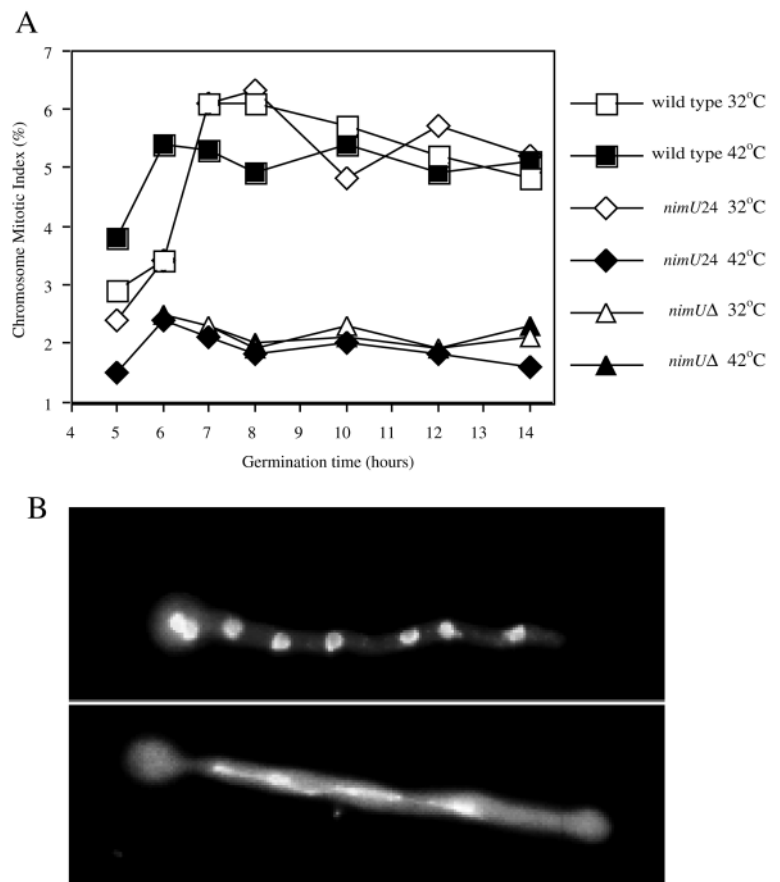


Fig. 2. Mitotic failure in the absence of *nimU* function. (A) Chromosome mitotic index was scored in wild-type (GR5), *nimU24* (EM4), and *nimUΔ* (GR5/ Δ 15) cells at *nimU24* permissive and restrictive temperatures. (B) After 10 hours at the restrictive temperature a typical wild-type germling has undergone three mitoses to produce eight nuclei (top panel). Under the same conditions a *nimU24* germling has an enlarged, misshapen nucleus (lower panel).

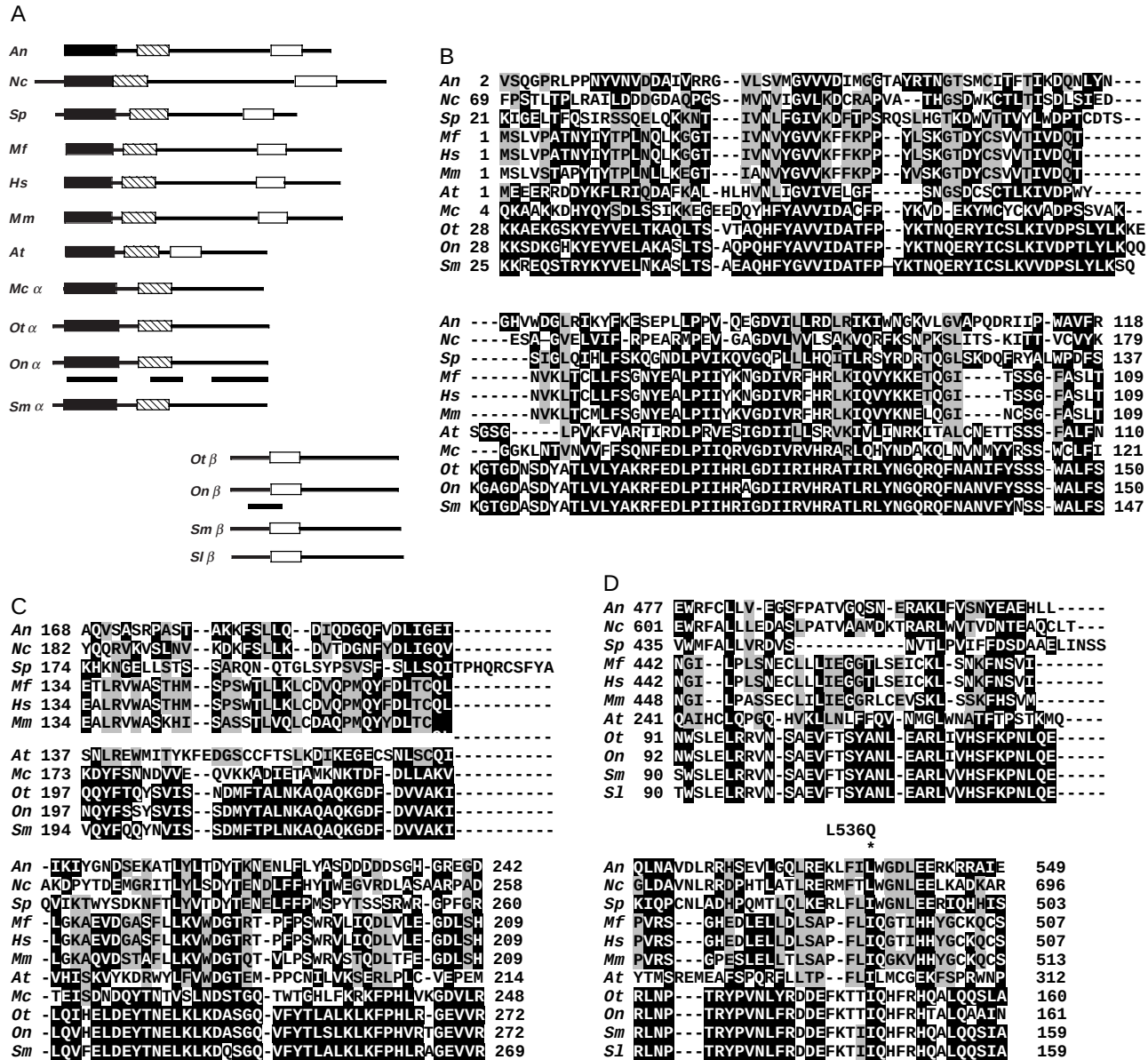


Fig. 3. Sequence comparison of NIMU and its higher eukaryotic homologues with the telomere end-binding protein (TEBP) subunits α and β of ciliated protozoa. Multiple sequences alignments were obtained using the 3D-PSSM program [(Kelley et al., 2000) online at <http://www.sbg.bio.ic.ac.uk/servers/3dpssm/>] where amino acids are placed into the following conserved groups based on secondary structure predictions: LIVM, PSTAG, QNEHDRK, CW, FY, followed by minor manual adjustment. The mammalian and plant sequences were manually aligned to the TEBP β . TEBP α , telomere end binding protein α subunit; TEBP β , telomere end binding protein β subunit. The organisms are abbreviated as follows: An, *Aspergillus nidulans*; At, *Arabidopsis thaliana* (accession no. NM_120714.1); Hs, *Homo sapiens* (Baumann and Cech, 2001); Mc, *Moneuplotes crassus* (Wang et al., 1992); Mf, *Macaca fascicularis* (accession no. AB066545.1); Mm, *Mus musculus* (accession no. NM_133931.1); Nc, *Neurospora crassa* (accession no. CAC28643.1); On, *Oxytricha nova* (Gray et al., 1991; Hicke et al., 1990); Ot, *Oxytricha trifallax* (Prescott et al., 1998; DuBois and Prescott, 1997); Sl, *Stylonychia lemnae* (accession no. AAF87600); Sm, *Stylonychia mytilis* (Fang and Cech, 1991); Sp, *Schizosaccharomyces pombe* (Baumann and Cech, 2001). (A) Domain structure of the three most highly conserved regions of homology between the NIMU/Pot1 proteins and the TEBP α and β subunits of protozoa. The positions of oligonucleotide/oligosaccharide-binding (OB) folds of *O. nova* TEBP α and β are indicated by black lines below the domain structures. Two strong regions of homology (black and cross hatched boxes) were identified between the NIMU/Pot1 proteins and the TEBP α subunits corresponding to regions overlapping the first two OB folds. A third region of homology (white box) was found between the NIMU/Pot1 proteins and the TEBP β subunits which overlap with the OB fold of TEBP β . (B,C) Multiple sequence alignments of the two α subunit domains (black and cross hatched boxes in A). Starting and ending amino acid numbers are shown. Residues conserved in at least four sequences (including both ciliate and non-ciliate sequences) are shown as white letters shaded in black. Residues conserved in at least four of the non-ciliate sequences and representing at least two kingdoms are shown as black letters shaded in grey. (D) Multiple sequence alignment of the β subunit domain (white box in A). Shading as in C. An asterisk indicates the L536Q mutation in *nimU*24. Sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ567920 (*nimU* genomic DNA) and AJ567922 (*nimU* cDNA).

Materials and Methods). The cDNA predicts a 614 amino acid protein (NIMU) with a central basic region and a C-terminal leucine zipper/leucine rich region. Sequencing of the *nimU24* allele revealed a single T to A missense mutation that alters the leucine at position 536 to glutamine (L536Q). Homology searches using the primary amino acid sequence showed it to be most closely related to the fission yeast and human Pot1 (protection of telomeres) proteins and also to TEBP α of ciliated protozoa (Fig. 3A-C), as well as other predicted fungal, mammalian and plant proteins (the *A. nidulans* NIMU protein displayed 25% identity and 41% similarity with the *S. pombe* Pot1 protein, and these identical/similar regions were distributed throughout the lengths of the two proteins). Secondary structure predictions further confirmed these relationships (Fig. 3B,C) and also indicated a weaker relationship between a region of the C termini of the *Aspergillus* and fission yeast proteins with a region of the TEBP β of *Oxytricha nova* (Fig. 3D). Interestingly, the mutated residue in the *nimU24* mutant (L536Q) falls within this TEBP β domain, and is conserved as either a leucine or isoleucine in the alignment shown (Fig. 3D). Notably, the three strongest regions of homology between the non-ciliate sequences correspond to the regions of homology with the TEBP α and β subunits. All three of these regions are within, or overlap, oligonucleotide/oligosaccharide-binding (OB) folds of the α and β proteins (Fig. 3A) that interact with telomeric ssDNA. We used the *S. pombe* *pot1*⁺ and *O. nova* TEBP α genes (both full-length and the first OB folds), as well as the *O. nova* TEBP β and *nimU* itself, to search the *A. nidulans* genome but were unable to identify any gene other than *nimU*. Taken together, these findings suggest that *nimU* represents the sole *pot1*⁺ homologue in *A. nidulans* and that the Pot1/NIMU proteins represent a fusion of the protozoan TEBP α and β subunits.

nimU is an essential gene

The *nimU24* mutation is recessive to its wild-type allele in heterozygous diploids and probably represents a loss-of-function mutation. To test this assumption we carried out a gene deletion using heterokaryon rescue (Osmani et al., 1988) (as described in Materials and Methods). Germinated *nimU Δ* (*nimU* deleted) spores were phenotypically indistinguishable from *nimU24* cells germinated at the restrictive temperature, forming highly branched hyphae and enlarged nuclei, and were unable to complete the asexual cycle (Fig. 4). The chromosome mitotic index for the *nimU Δ* strain was similar to that of a *nimU24* strain grown at the restrictive temperature (Fig. 2A,B). These results show that the *nimU24* phenotype is indistinguishable from the *nimU* deletion, suggesting that *nimU24* results in a loss of *nimU* function when cells are grown at the restrictive temperature.

Loss of *nimU* function leads to premature exit from mitosis

To test whether loss of *nimU* allows early mitotic exit we synchronised cells at the first G2/M after germination using a temperature sensitive mutation in the *nimA* gene, which encodes a protein kinase required for entry into mitosis (Osmani et al., 1991b). The *nimA5* allele allows synchronous

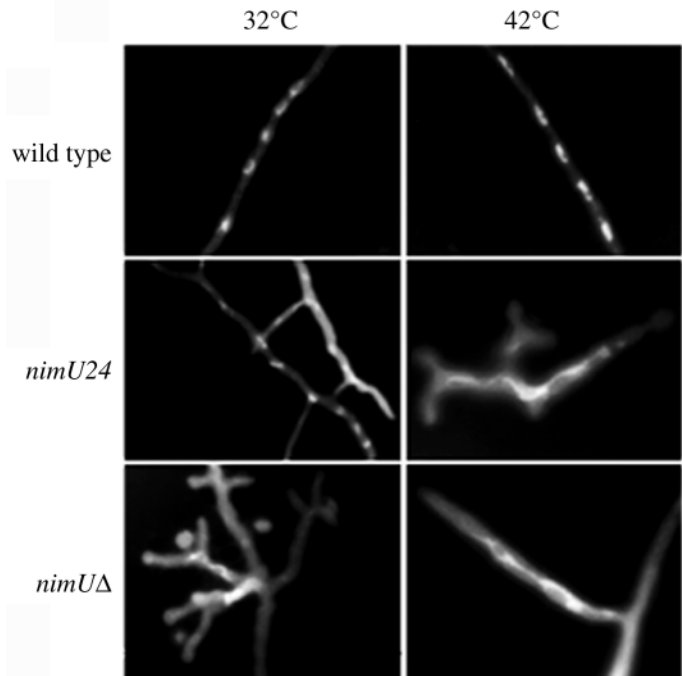


Fig. 4. *nimU* is an essential gene. (A) Wild type, *nimU24* and *nimU Δ* spores were grown for 14 hours at permissive and restrictive temperatures for *nimU24*, then fixed and stained with DAPI. The wild-type strain grew normally at both temperatures, producing multinucleate hyphae. *nimU24* spores produced multinucleate hyphae at the permissive temperature, but only grossly enlarged nuclei at the restrictive temperature. The *nimU Δ* spores produced grossly enlarged nuclei at both temperatures.

entry into mitosis after accumulation at the G2/M arrest point and release to the permissive temperature (Oakley and Morris, 1983). A gene deletion was generated in a *nimA5* strain (CP1) by heterokaryon rescue and a control strain was also constructed by transforming strain CP1 with an empty vector (pRG3) carrying the *pyr4*⁺ gene. After a 7-hour incubation at the restrictive temperature, cells were released from the arrest in the presence (*nimA5 nimU*⁺ – strain CP1/1) and absence (*nimA5 nimU Δ* – strain CP1/ Δ 6) of *nimU* function and the chromosome mitotic index, spindle mitotic index (SMI) and rate of entry into mitosis were monitored. SMI was measured by monitoring for the presence or absence of the mitotic spindle using an antibody against α tubulin. Rate of entry into mitosis was measured by monitoring SPB separation. Prior to determination of mitotic indices, the presence of paired spindle pole bodies was determined at 0 minutes release for both strains. The percentage of observable paired SPBs was approximately the same for both strains (*nimA5 nimU*⁺, 81%; *nimA5 nimU Δ* , 85%). This indicated that both strains had accumulated at the *nimA5* G2/M block point prior to release, i.e. *nimA5* did not affect progression through G2 when *nimU* function was lost. The mitotic indices (Fig. 5A,B) showed the rates of entry into – and exit from – mitosis to be independent of *nimU* function, but the timing of mitotic exit was early in the absence of *nimU*. The SPB separation assay also showed entry into mitosis to be independent of *nimU* function (Fig. 5C). This supports the data presented in Fig. 1D, since SPB

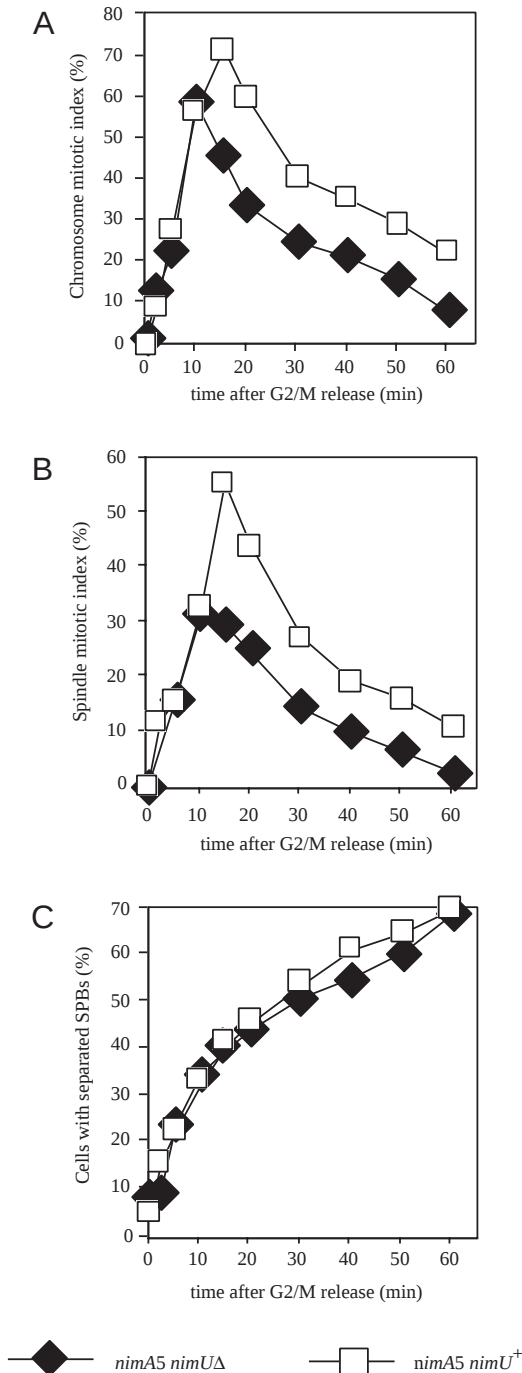


Fig. 5. Early mitotic exit when *nimU* function is lost. Mitotic progression in the presence and absence of *nimU* function was monitored by following G2/M synchronised cells (strains CP1/1 and CP1/Δ6) through mitosis. Synchrony was achieved by utilising the temperature sensitivity of the *nimA5* allele. Samples were taken through the time course indicated and the chromosome mitotic index (A), the spindle mitotic index (B), and the rate of entry into mitosis (C), were determined.

separation is correlated with mitotic spindle formation, and is not merely due to SPB diffusion in the nuclear envelope. Therefore, loss of *nimU* function does not delay mitotic entry but does lead to premature mitotic exit.

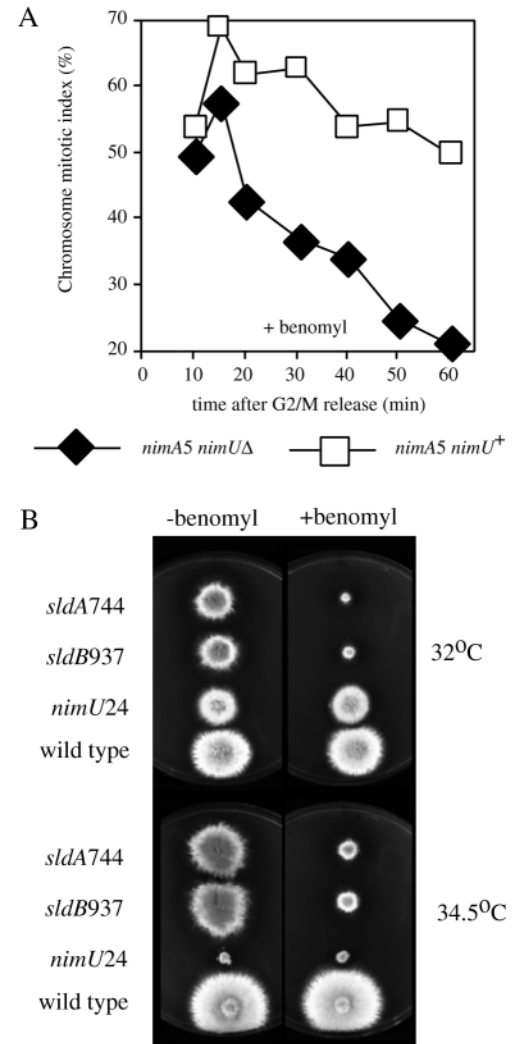


Fig. 6. The spindle assembly checkpoint cannot delay mitotic exit in the absence of *nimU*. (A) The G2/M block/release experiment was repeated in the presence of benomyl and the chromosome mitotic index determined. (B) Conidiospores were spot-inoculated onto rich medium with or without benomyl and germinated at semi-permissive (32°C) or restrictive (34.5°C) temperatures for the *nimU24* mutant for several days. *sldA744* and *sldB937* are loss of function mutations in the spindle checkpoint components *BUB1* and *BUB3*, respectively.

The spindle assembly checkpoint cannot delay mitotic exit in the absence of *nimU*

To test whether activation of the spindle assembly checkpoint could prevent early mitotic exit in the absence of *nimU*, we repeated the *nimA5* block/release experiment in the presence of the microtubule destabilising drug benomyl (Bergen et al., 1984). This inhibits mitotic spindle formation in *Aspergillus* (Oakley and Morris, 1980) and activates the spindle checkpoint, which maintains chromosomes in their condensed state. Incubation with the drug led to a substantial delay in mitotic exit of *nimA5 nimU+* cells after release from the G2/M arrest (Fig. 6A), whereas *nimA5 nimUΔ* cells failed to exhibit such a delay, and exited mitosis at a similar rate to that observed without drug treatment. Therefore, the mitotic delay seen for wild-type cells when the spindle checkpoint response

is evoked is bypassed or disabled when *nimU* function is absent. This result led us to test the benomyl sensitivity of the *nimU24* strain at a semi-permissive temperature (Fig. 6B). Notably, *nimU24* strains did not show sensitivity to benomyl at semi-permissive temperature in contrast to *sldA⁻* (*bub1*) and *sldB⁻* (*bub3*) spindle checkpoint mutants (Fig. 6B), suggesting that *nimU* is not part of the spindle checkpoint per se, although it is possible that its checkpoint function is not defective at this temperature.

Premature mitotic exit, but not premature spindle elongation, is APC/C dependent

One possible explanation of the early mitotic exit is that cells lacking *nimU* function are unable to maintain chromatin in a condensed state, thereby triggering an early exit from mitosis. However, *nimU24 bimE7* cells have previously been found to arrest in mitosis with condensed chromatin (James et al., 1995). *bimE* encodes a subunit of the APC/C and its loss of function leads to metaphase arrest. This indicates that the early exit seen in this work is dependent upon APC/C function. We were able to recapitulate this result, showing that *nimU24 bimE7* cells accumulated in mitosis with condensed chromatin at the same rate and extent as single *bimE7* mutants (Fig. 7A) confirming that loss of *nimU* function does not lead to a defect in maintaining condensed chromatin.

In the same experiment cells were scored for metaphase spindle length and this showed that the average metaphase spindle in *nimU24 bimE7* double mutants was significantly longer than that for the single *bimE7* mutant (Fig. 7B). Furthermore, as mitotic spindle length was longer in the double mutants at time points prior to the mitotic peak (Fig. 7A) we conclude that aberrant spindle elongation is a consequence of loss of *nimU* function on entry into mitosis, rather than reflecting leak-through from the *bimE7* metaphase arrest. This suggests that *nimU* acts as a negative regulator of mitotic spindle elongation. Additionally, in agreement with previous results (James et al., 1995), we found that approximately 12% of *nimU24 bimE7* were able to segregate chromatin into two masses, unlike the single *bimE7* mutant in which all mitotic cells displayed a single mass of condensed chromatin (data not shown). In those *nimU24 bimE7* cells that did produce two chromatin masses the chromatin remained condensed, indicating that premature mitotic exit in *nimU24* cells is APC/C dependent.

Chromosome segregation errors accumulate in the absence of *nimU*

We reasoned that a mutation leading to premature mitotic exit would also induce errors in chromosome segregation. We investigated whether such errors could be detected under conditions that lead to transient or partial loss of *nimU* function. To do this we performed a chromosome missegregation assay (Harris and Hamer, 1995). *nimU⁺/nimU⁺* (Dip32), *nimU⁺/nimU24* (Dip37) and *nimU24/nimU24* (Dip38) diploids were made, each carrying the recessive *yA2* and *pyrG89* mutations on opposite arms of one copy of chromosome I. When these mutations are homozygous or hemizygous, *yA2* leads to the formation of yellow spores and *pyrG89* to uracil/uridine auxotrophy. The green spored

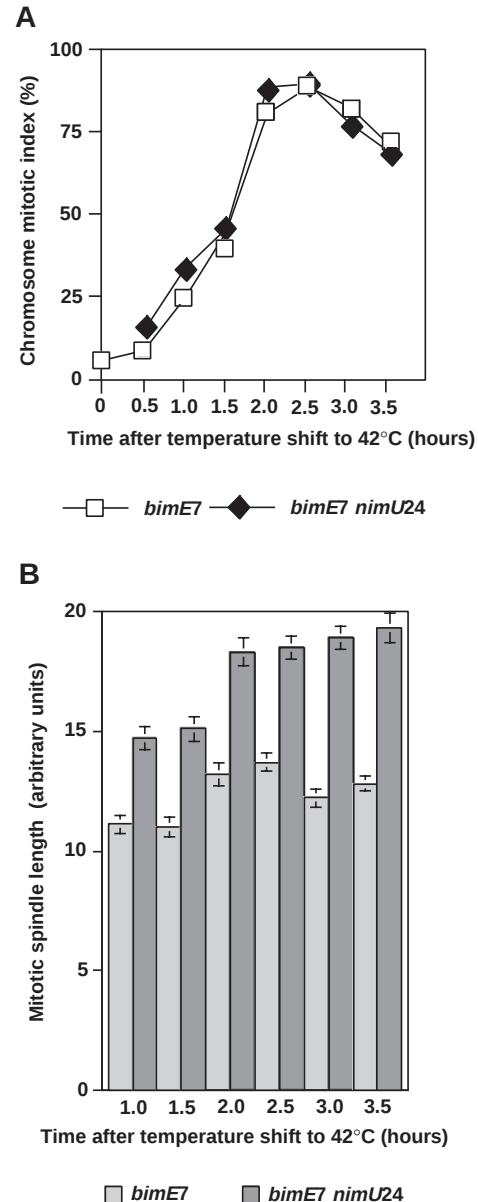


Fig. 7. Mitotic arrest but premature spindle elongation in *nimU24 bimE7* cells. (A) *bimE7* and *nimU24 bimE7* cells were germinated overnight at the permissive temperature and then shifted to the restrictive temperature and fixed at 0.5 hourly intervals. The chromosome mitotic index was determined after staining with DAPI. (B) The same samples were also stained for mitotic spindles (anti- α tubulin immunofluorescence) and 100 mitotic spindles were measured for each time point using IP Lab software. Values plotted are arbitrary units and represent the mean and standard error for the 100 measurements.

diploids give rise to yellow spored colony sectors through recombination and/or chromosome missegregation, which can be resolved by determining whether yellow sectors are prototrophic (recombination) or auxotrophic (chromosome missegregation) for uracil/uridine. To transiently inactivate *nimU*, spores were germinated for 6 hours at the restrictive temperature (42°C), followed by incubation at 25°C. Under these conditions germinating spores would have attempted

Table 1. Elevated levels of chromosome missegregation in the absence of *nimU* function

Genotype	Yellow sectors	Recombination	Missegregation
<i>nimU</i> ⁺ / <i>nimU</i> ⁺	6.0	5.0 (±2.4)	1.0 (±1.4)
<i>nimU</i> ⁺ / <i>nimU24</i>	4.0	4.0 (±1.4)	0.0
<i>nimU24/nimU24</i>	52.3	4.3 (±2.4)	48.0 (±3.7)

Germination of *nimU24* homozygous diploids at the restrictive temperature for 6 hours produced aneuploid colonies with yellow sectors. Under the same conditions diploids wild type for *nimU* function (*nimU*⁺/*nimU*⁺ and *nimU*⁺/*nimU24*) produced normal colonies with yellow sectors. Yellow sectors were tested for recombination and chromosome missegregation (see Results).

mitosis no more than once. The absence of *nimU* function led to higher numbers of yellow sectors relative to wild type (Table 1), and whereas wild-type yellow sectors were attributable to recombination only, most of the yellow sectors formed by the *nimU24/nimU24* diploid were due to chromosome missegregation (Table 1). Other characteristics of aneuploidy were also displayed by the *nimU24/nimU24* diploids including colony sectoring, irregular colony edge, and expression of other recessive alleles (data not shown). These were not seen for the same strain at 25°C, nor for the wild-type diploid at either temperature. No yellow sectors were obtained for any of the strains when grown only at 25°C. Thus, transient inactivation of *nimU* leads to approximately 48 times the level of chromosome missegregation seen for wild type. Moreover, this high level of missegregation is attributable to just one passage through defective mitosis, underscoring the severity of the mitotic defect.

Aspergillus can tolerate low levels of chromosome imbalance (aneuploidy) but this leads to abnormal colony growth (Kafer and Upshall, 1973; Harris and Hamer, 1995). In a separate experiment, diploid conidiospores were spread onto solid medium and incubated at 33.5°C (semi-permissive temperature) for 2 days and then returned to the permissive temperature (25°C). Only 41% of *nimU24/nimU24* diploids were able to survive this treatment (relative to the same strain grown only at 25°C), and 71% of surviving colonies displayed one or more characteristics of aneuploidy, indicative of high levels of chromosome missegregation. These conditions had no effect on survival or colony growth of a wild-type diploid control strain, or the *nimU24/nimU24* strain grown at 25°C continually. This indicates that *nimU24* severely perturbs chromosome transmission at its restrictive temperature.

Discussion

We show that loss of *nimU* function leads to a variety of mitotic defects but, surprisingly, does not lead to cell cycle arrest. Instead, cells lacking *nimU* appear to progress through the cell cycle normally until metaphase, and thereafter mitotic events become uncoordinated. We find that spindle elongation occurs prematurely in the absence of APC/C function. Mitotic exit is also premature but early exit is dependent on APC/C and does not respond to the spindle assembly checkpoint. Transient and partial inactivation of *nimU* leads to increased chromosomal instability, segregation errors and a loss of viability. As the *nimU* protein is structurally related to TEBPs, these data suggest that TEBPs mediate a pathway that links telomere

structure to mitotic control, or alternatively, that TEBPs play mitotic roles in addition to their telomeric function(s).

Until now, no homologue of the protist TEBPβ subunit has been reported in other organisms. Consistent with this, we were unable to identify a β subunit in *Aspergillus*. Our findings suggest there may be no separate β subunit in other eukaryotes, rather, the α and β subunits are incorporated into a single protein. In the protist *Oxytricha nova* the TEBP α subunit can bind telomeric ssDNA alone (although differently than in the context of an αβ complex) whereas the β subunit binds only very weakly (Gray et al., 1991). However, the α and β subunits form a complex on ssDNA, and this interaction is DNA dependent (Fang and Cech, 1993; Horvath et al., 1998). Our data suggest that Pot1 associates with telomeric ssDNA through an intramolecular interaction that brings both the α and β domains into proximity with the DNA and each other. Our finding that a mutation within the proposed β domain confers a conditional null phenotype on NIMU supports this hypothesis.

nimU24 does not block cell cycle progression

nimU24 was originally classified as a G2-phase arresting cell cycle mutant based on the results of reciprocal shift experiments (Bergen et al., 1984). Those experiments aimed to determine at which points in interphase *Aspergillus* temperature sensitive conditionally lethal ‘*nim*’ (never in mitosis) mutants arrested. Briefly, in a temperature upshift experiment mutants were held in S phase at the permissive temperature using the DNA replication inhibitor hydroxyurea (HU). Uninucleate cells were released from HU arrest at restrictive temperature and the number of cells that became binucleate was scored. The reciprocal downshift experiment was performed by releasing cells from the restrictive temperature block point to the permissive temperature in the presence of HU. The rationale was that a G1 arresting mutant would become binucleate on upshift but not on downshift. The reverse would be true of a G2 arresting mutant. An S phase arresting mutant would not become binucleate in either experiment. However, this logic is only valid if there is a discrete cell cycle arrest point. Here, a number of lines of evidence show that *nimU24* does not arrest the cell cycle: (i) we have followed the rate of entry into the first mitosis in *nimU24* and wild-type cells by monitoring the duplication and separation of SPBs and found the rates to be similar; (ii) the nuclei of *nimU24* and *nimUΔ* cells increase in size and accumulate multiple SPBs; (iii) the chromosome mitotic index never reaches zero in these cells; (iv) Osmani et al. (Osmani et al., 1991a) found that *nimU24* cells, when released from the restrictive temperature into benomyl containing medium, showed a reduced level of accumulation in mitosis. This was interpreted as a failure to release efficiently from the G2 arrest, but in the light of our own experiments, is consistent with no arrest. It would therefore be of interest to determine the kinetics of SPB separation in other *nim* mutants, particularly those that gave ambiguous results in the earlier work (Bergen et al., 1984).

nimU and cell cycle checkpoints

Our approach to isolating mutants defective in mitotic

progression led to the identification of *nimU* as a gene encoding a component essential for mitotic integrity. The subsequent finding that *nimU* is a telomere end binding protein homologue was surprising and requires us to consider the response of cells lacking *nimU* function to the potential presence of uncapped telomeres.

The majority of DNA in a eukaryotic cell is organised as linear molecules, the chromosomes, which have free ends. DNA damage can also create free ends and these normally trigger the ATM kinase-dependent DNA damage checkpoint to prevent mitotic entry, but under normal circumstances the free ends in telomeres do not. In budding yeast, exposed telomeric DNA caused by mutation of the telomeric ssDNA binding protein, CDC13, leads to cell cycle arrest at the G2/M transition (Pang et al., 2003) but this can be suppressed by over-expression of other telomeric binding proteins. Since absence of CDC13 triggers G2/M delay in yeast it seems surprising that absence of *nimU* in *Aspergillus* fails to trigger a similar delay (note, however, that the presence of a G2 delay when *pot1*⁺ function is lost in other organisms has not been reported). As such, our findings suggest that either *nimU* is required for the DNA damage checkpoint in *Aspergillus* or that lesions (for instance, uncapped telomeric DNA) created through the absence of *nimU* are not normally detected by the DNA damage checkpoint. Our preliminary findings show that *nimU24* mutants display wild-type sensitivity to the DNA damaging agent methyl methanesulphonate (data not shown) and this argues against a checkpoint role for *nimU*. It would therefore be of interest to determine whether loss of Pot1p function in fission yeast and humans also fails to trigger this checkpoint.

Whether the mitotic catastrophe observed in the absence of *nimU* is a consequence of failing to detect uncapped telomeres or due to a separable function for *nimU* is an interesting question for future work. Fission yeast cells deleted for *pot1*⁺ show chromosome segregation defects (Baumann and Cech, 2001) similar to the situation in *Aspergillus* germlings lacking *nimU*. These investigations showed that *pot1*⁻ cells underwent rapid telomere loss and chromosome end-to-end fusions, thus the chromosome segregation defects could be attributed to the formation of dicentric chromosomes. The integrity of the mitotic spindle checkpoint, however, was not assessed. It seems plausible then that the mitotic defects seen in *Aspergillus* when *nimU* function is lost may be directly attributable to telomere uncapping. Our findings raise the possibility of a hitherto unsuspected role for telomere structures in the spindle checkpoint response. Improper execution of this role may contribute to the genomic instability seen in *pot1*⁻ cells.

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