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

Christopher W. Pitt*, Eric Moreau, Patricia A. Lunness and John H. Doonan‡

John Innes Centre, Colney, Norwich, NR4 7UH, UK

*Present address: Telomere Biology Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

‡Author for correspondence (e-mail: john.doonan@bbsrc.ac.uk)

Accepted 27 August 2003

Journal of Cell Science 117, 199-209 Published by The Company of Biologists 2004
doi:10.1242/jcs.00844

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 I 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 (*ESPl*) 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...
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 (nimU5 pyrG89 yA2), Dip32 (pyrG89 yA2 // chaA1 acrA1 riboB2), Dip37 (nimU24 pyrG89 yA2 riboA1 // chaA1), Dip38 (nimU24 pyrG89 yA2 riboA1 // nimU24 chaA1), GR5/A15 (pyrG89 pyroA4 wA3 [pyr4⁻::nimU]), GR5/A42 (pyrG89 pyroA4 wA3 [pyr4⁺::nimU]), CP1/1 (nimU5 pyrG89 [pyr4⁺]), CP1/6 (nimU5 pyrG89 [pyr4⁺::nimU]), SW1224 (nimU24 bimE7 methG1), DBE4 (bimE7 pyrG89), 744Da (sldA74; pyrG89; yA2), 937Da (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 pBS with KpnI and BamHI and the 1.6 kb fragment gel isolated. The 5’ flank, together with the vector pBC, was isolated by digesting pBS 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 pAnimU. 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 nimAS 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 pAnimU, 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. [α32P]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: 6x SSC + 1 ml 50x 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 6x SSC + 0.1% SDS; twice with 2x SSC + 0.1% SDS; once with 0.1x 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 (axial 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 germings 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 germings, 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

pot1 homologue orders mitotic events

(End of Document)
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'-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. 3. Sequence comparison of NIMU and its higher eukaryotic homologues with the telomere end-binding protein (TEBP) α subunit 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/3dpsmm/) where amino acids were 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 TEBPa, TEBPb, telomere end binding protein α subunit; TEBPb, telomere end binding protein β subunit. The organisms are abbreviated as folllows: An, Aspergillus nidulans; At, Arabidopsis thaliana (accession no. NM_122714.1); 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_139391.1); Nc, Neurospora crassa (accession no. CAC28643.1); Ot, Oxytricha nova (Gray et al., 1991; Hicke et al., 1990); Ot, Oxytricha trifallax (Prescott et al., 1998; DuBois and Prescott, 1997); 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 TEBPα and β are indicated by black lines below the domain structures. Two strong regions of homology (black and black boxed regions) were identified between the NIMU/Pot1proteins and the TEBPα 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 TEBPβ subunits which overlap with the OB fold of TEBPβ. (B,C) Multiple sequence alignments of the two α subunit domains (black and black boxed 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 nimU24. 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 TEBPα 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. 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 TEBPα genes (both full-length and the first OB folds), as well as the O. nova TEBPβ 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 nimUΔ (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 nimUΔ 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 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 nimUΔ – strain CP1/Δ6) 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 nimUΔ, 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. 4. nimU is an essential gene. (A) Wild type, nimU24 and nimUΔ 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 nimUΔ spores produced grossly enlarged nuclei at both temperatures.
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. 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.

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− (bud1) and sldB− (bud3) 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 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

---

**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.
mitosis no more than once. The absence of \textit{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 \textit{nimU}+/\textit{nimU}24 diploid were due to chromosome missegregation (Table 1). Other characteristics of aneuploidy were also displayed by the \textit{nimU}+/\textit{nimU}24 and \textit{nimU}24/\textit{nimU}24 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 \textit{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.

\textit{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 \textit{nimU}24/\textit{nimU}24 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 \textit{nimU}24/\textit{nimU}24 strain grown at 25°C continually. This indicates that \textit{nimU}24 severely perturbs chromosome transmission at its restrictive temperature.

### Discussion

We show that loss of \textit{nimU} function leads to a variety of mitotic defects but, surprisingly, does not lead to cell cycle arrest. Instead, cells lacking \textit{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 \textit{nimU} leads to increased chromosomal instability, segregation errors and a loss of viability. As the \textit{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 \textbeta{} subunit in \textit{Aspergillus}. Our findings suggest there may be no separate \textbeta{} subunit in other eukaryotes, rather, the \textalpha{} and \textbeta{} subunits are incorporated into a single protein. In the protist \textit{Oxytricha nova} the TEBP \textalpha{} subunit can bind telomeric ssDNA alone (although differently than in the context of an \textalpha{}\textbeta{} complex) whereas the \textbeta{} subunit binds only very weakly (Gray et al., 1991). However, the \textalpha{} and \textbeta{} 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 \textalpha{} and \textbeta{} domains into proximity with the DNA and each other. Our finding that a mutation within the proposed \textbeta{} domain confers a conditional null phenotype on NIMU supports this hypothesis.

\textbf{\textit{nimU}24 does not block cell cycle progression}

\textit{nimU}24 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 \textit{Aspergillus} temperature sensitive conditionally lethal ‘\textit{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 \textit{nimU}24 does not arrest the cell cycle: (i) we have followed the rate of entry into the first mitosis in \textit{nimU}24 and wild-type cells by monitoring the duplication and separation of SPBs and found the rates to be similar; (ii) the nuclei of \textit{nimU}24 and \textit{nimU}A 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 \textit{nimU}24 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 \textit{nim} mutants, particularly those that gave ambiguous results in the earlier work (Bergen et al., 1984).

\textbf{\textit{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 germllings 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.

We thank R. Morris and S. James for provision of strains and T. Toda and J. Cooper for critical reading of the manuscript. E.M. was funded by the John Innes Foundation. C.W.P. was funded by the Biotechnology and Biological Sciences Research Council and the Cancer Research Campaign.

References

Baumann, P. and Cech, T. R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 292, 1171-1175.

Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E. and Goldberg, M. L. (1999). Mutations in the essential spindle checkpoint gene bub1 cause chromosome mis-segregation and fail to block apoptosis in Drosophila. J. Cell Biol. 146, 13-28.

Berg, L. G., Upshall, A. and Morris, N. R. (1984). S-phase, G2, and nuclear division mutants of Aspergillus nidulans. J. Bacteriol. 159, 114-119.

Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K. V., Markowitz, S. D., Kinzler, K. W. and Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. Nature 392, 300-303.

Blanco, M. A., Sanchez-Diaz, A., de Prada, J. M. and Moreno, S. (2000). APC(ste9/swr1) promotes degradation of mitotic cyclins in G(1) and is inhibited by cdc2 phosphorylation. EMBO J. 19, 3945-3955.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell 93, 1067-1076.

De Souza, P. C., Osmani, A. H., Wu, L.-P., Spotts, J. L. and Osmani, S. A. (2000). Miotic histone H3 phosphorylation by the NIMA kinase in Aspergillus nidulans. Cell 102, 293-302.

Dobles, M., Liberal, V., Scott, M. L., Benezra, R. and Sorger, P. K. (2000). Chromosome mis-segregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 101, 635-645.

Doonan, J. H. and Morris, N. R. (1989). The bimG gene of Aspergillus nidulans, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase-1. Mol. Cell. Biol. 17, 987-996.

DuBois, M. L. and Prescott, D. M. (1997). Volatility of internal eliminated segments in germ line segments of hypotrichous ciliates. Mol. Cell. Biol. 17, 326-337.

Efimov, V. P. and Morris, N. R. (1998). A screen for dynein synthetic lethals in Aspergillus nidulans identifies spindle assembly checkpoint genes and other genes involved in mitosis. Genetics 149, 101-116.

Enos, A. P. and Morris, N. R. (1990). Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in A. nidulans. Cell 60, 1019-1027.

Evans, G. A. and Wahl, G. M. (1987). Cosmid vectors for genomic walking and rapid restriction mapping. Methods Enzymol. 152, 604-610.

Fang, G. W. and Cech, T. R. (1991). Molecular cloning of telomere-binding protein genes from Stylophoronychia mytilus. Nucleic Acids Res. 19, 5515-5518.

Fang, G. and Cech, T. R. (1993). Oxytricha telomere-binding protein: DNA-dependent dimerization of the alpha and beta subunits. Proc. Natl. Acad. Sci. USA 90, 6056-6060.

Fidel, S., Doonan, J. H. and Morris, N. R. (1988). Aspergillus nidulans contains a single actin gene which has unique intron locations and encodes a gamma-actin. Gene 70, 283-293.

Funahiki, H., Kumada, K. and Yanagida, M. (1996). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J. 15, 6617-6628.

Gardner, R. D. and Burke, D. J. (2000). The spindle checkpoint: two transitions, two pathways. Trends Cell Biol. 10, 154-158.

Gibson, T. J., Coulson, A. R., Sulsan, J. E. and Little, P. F. R. (1987). Lorist2, a cosmid with transcriptional terminators insulating vector genes in Eukaryotic microorganisms. In Eukaryotic vectors (ed. J. R. Kinghorn), pp. 93-139. Oxford, UK: IRL Press at Oxford University Press.

Harris, S. D. and Hamer, J. E. (1995). sepB: an Aspergillus nidulans gene involved in chromosome segregation and the initiation of cytokinesis. EMBO J. 14, 5244-5257.

Hershko, A. (1999). Mechanisms and regulation of the degradation of cyclin B. Proc. R. Soc. London B Biol. Sci. 354, 1571-1575.

Hicke, B. J., Celderar, D. W., MacDonald, G. H., Price, C. M. and Cech, T. R. (1990). Two versions of the gene encoding the 41-kilodalton subunit of the telomere binding protein of Oxytricha nova. Proc. Natl. Acad. Sci. USA 87, 1481-1485.

Horvath, M. F., Schweiker, V. L., Bevilacqua, J. M., Ruggles, J. A. and Schultz, S. C. (1998). Crystal structure of the Oxytricha nova telomere end binding protein complexed with single strand DNA. Cell 95, 963-974.

Hoyt, M. A., Totis, L. and Roberts, B. T. (1991). Ces cervesia genes required for cell cycle arrest in response to loss of microtubule function. Cell 66, 507-517.

James, S. W., Mirabito, P. M., Scacheri, P. C. and Morris, N. R. (1995). The Aspergillus nidulans bim1 (blocked-in-mitosis) gene encodes multiple
cell cycle functions involved in mitotic checkpoint control and mitosis. *J. Cell Sci.* 108, 3485-3499.

Jin, D.-Y., Spencer, F. and Jeang, K.-T. (1998). Human T cell leukemia virus type 1 oncprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell* 93, 81-91.

Kafer, E. and Upshall, A. (1973). The phenotypes of the eight disomics and trisomics of *Aspergillus nidulans*. *J. Hered.* 64, 35-38.

Kalitsis, P., Earle, E., Fowler, K. J. and Choo, K. H. (2000). Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev.* 14, 2277-2282.

Kelly, L. A., MacCallum, R. M. and Sternberg, M. J. E. (2000). Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* 299, 499-520.

Kitagawa, R. and Rose, A. M. (1983). A mutation in *Aspergillus nidulans* is a component of the spindle pole body that is essential for microtubule attachment. *Cell* 34, 837-845.

Lee, H., Trainer, A. H., Friedman, L. S., Thistlethwaite, F. C., Evans, M. J., Ponder, A. J. and Venkitaraman, A. R. (1999). Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, *Brca2*. *Mol. Cell* 4, 1-10.

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1997). Identification of *BIME* as a subunit of the anaphase-promoting complex/cyclosome, is required for a G2 checkpoint blocking entry into mitosis in the absence of NIMA function. *J. Cell Sci.* 111, 1453-1465.

May, G. S., McGoldrick, C. A., Holt, C. L. and Denison, S. H. (1992). The *bimB* mutation of *Aspergillus nidulans* uncouples DNA replication from the completion of mitosis. *J. Biol. Chem.* 267, 15737-15743.

McGrew, J. T., Goetsch, L., Byers, B. and Baum, P. (1992). Requirement for *ESP1* in the nuclear division of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 3, 1443-1454.

Morris, N. R. (1976a). A temperature-sensitive mutant of *Aspergillus nidulans* reversibly blocked in nuclear division. *Exp. Cell Res.* 98, 204-210.

Morris, N. R. (1976b). Mitotic mutants of *Aspergillus nidulans*. *Genet. Res.* 26, 237-254.

Oakley, B. R. and Morris, N. R. (1980). Nuclear movement is β-tubulin-dependent in *Aspergillus nidulans*. *Cell* 19, 255-262.

Oakley, B. R. and Morris, N. R. (1981). A β-tubulin mutation in *Aspergillus nidulans* that blocks microtubule function without blocking assembly. *Cell*, 24, 837-845.

Oakley, B. R. and Morris, N. R. (1983). A mutation in *Aspergillus nidulans* that blocks the transition from interphase to prophase. *J. Cell Biol.* 96, 1155-1158.

Oakley, B. R., Oakley, C. E., Yoon, Y., and Jung, M. K. (1990). γ-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* 61, 1289-1301.

O'Connell, M. J., Osmani, A. H., Morris, N. R. and Osmani, S. A. (1992). An extra copy of *nimA* elevates pre-MPF levels and partially suppresses mutation of *nimRtdc2* in *Aspergillus nidulans*. *EMBO J.* 11, 2139-2149.

O'Donnell, K. L., Osmani, A. H., Osmani, S. A. and Morris, N. R. (1991). *bimA* encodes a member of the tetratricopeptide repeat family of proteins and is required for the completion of mitosis in *Aspergillus nidulans*. *J. Cell Sci.* 99, 711-719.

Osmani, A. H., McGuire, S. L. and Osmani, S. A. (1991a). Parallel activation of the NIMA and P34cdc2 cell cycle-regulated protein kinases is required to initiate mitosis in *Aspergillus nidulans*. *Cell* 67, 283-291.

Osmani, A. H., O’Donnell, K., Pu, R. T. and Osmani, S. A. (1991b). Activation of the nimA protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the bimE checkpoint. *EMBO J.* 10, 2669-2679.

Osmani, A. H., van Peij, N., Mischke, M., O’Connell, M. J. and Osmani, S. A. (1994). A single p34cdc2 protein kinase (encoded by *nimXtdc2*) is required at G1 and G2 in *Aspergillus nidulans*. *J. Cell Sci.* 107, 1519-1528.

Osmani, S. A., Engle, D. B., Doonan, J. H. and Morris, N. R. (1988). Spindle formation and chromatid condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell* 52, 241-251.

Osmani, S. A. and Ye, X. S. (1996). Cell cycle regulation in *Aspergillus* by two protein kinases. *Biochim. Biophys. Acta* 317, 633-641.

Pang, T. L., Wang, C. Y., Hsu, C. L., Chen, M. Y. and Lin, J. J. (2003). Exposure of single-stranded telomeric DNA causes G2/M cell cycle arrest in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 278, 9318-9321.

Peters, J. M., King, R. W., Höög, C. and Kirschner, M. W. (1996). Identification of BIME as a subunit of the anaphase-promoting complex. *Science* 274, 1199-1204.

Pomerening, J. R., Sontag, E. D. and Ferrell, J. E. (2003). Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2. *Nat. Cell Biol.* 5, 346-351.

Prescott, J. D., DuBois, M. L. and Prescott, D. M. (1998). Evolution of the scrambled germline gene encoding alpha-telomere binding protein in three hypotrichous ciliates. *Chromosoma* 107, 293-303.

Pu, R. T. and Osmani, S. A. (1995). Mitotic destruction of the cell cycle regulated NIMA protein kinase of *Aspergillus nidulans* is required for mitotic exit. *EMBO J.* 14, 995-1003.

Takahashi, T., Haruki, N., Nomoto, S., Masuda, A., Saij, S., Osada, H. and Takahashi, T. (1999). Identification of frequent impairment of the mitotic checkpoint and molecular analysis of the mitotic checkpoint genes, *hsMad2* and *p55CDC*, in human lung cancers. *Oncogene* 18, 4295-4300.

Uhlmann, F. (2003). Chromosome cohesion and separation: from men and molecules. *Curr. Biol.* 13, R104-R114.

Wang, W., Skopp, R., Schofield, M. and Price, C. (1992). Euplotes cassus has genes encoding telomere-binding proteins and telomere-binding protein homologs. *Nucleic Acids Res.* 20, 6621-6629.

Wun, I.-H., Li, Y. and Benezra, R. (1996). Identification of *pot1* homologue orders mitotic events in hypotrichous ciliates. *Chromosoma* 107, 293-303.

Wang, W., Skopp, R., Schofield, M. and Price, C. (1992). Euplotes cassus has genes encoding telomere-binding proteins and telomere-binding protein homologs. *Nucleic Acids Res.* 20, 6621-6629.