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Mitotic destruction of the cell cycle regulated NIMA protein kinase of *Aspergillus nidulans* is required for mitotic exit

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NIMA is a cell cycle regulated protein kinase required, in addition to p34^{cdc2}/cyclin B, for initiation of mitosis in *Aspergillus nidulans*. Like cyclin B, NIMA accumulates when cells are arrested in G_2 and is degraded as cells traverse mitosis. However, it is stable in cells arrested in mitosis. NIMA, and related kinases, have an N-terminal kinase domain and a C-terminal extension. Deletion of the C-terminus does not completely inactivate NIMA kinase activity but does prevent functional complementation of a temperature sensitive mutation of *nimA*, showing it to be essential for function. Partial C-terminal deletion of NIMA generates a highly toxic kinase although the kinase domain alone is not toxic. Transient induction experiments demonstrate that the partially truncated NIMA is far more stable than the full length NIMA protein which likely accounts for its toxicity. Unlike full length NIMA, the truncated NIMA is not degraded during mitosis and this affects normal mitotic progression. Cells arrested in mitosis with non-degradable NIMA are able to destroy cyclin B, demonstrating that the arrest is not due to stabilization of p34^{cdc2}/cyclin B activity. The data establish that NIMA degradation during mitosis is required for correct mitotic progression in *A.nidulans*.

Key words: *Aspergillus nidulans*/cyclin B/mitosis/NIMA protein kinase/protein degradation

Introduction

Initiation of mitosis in *Aspergillus nidulans* requires the cooperative action of two cell cycle regulated protein kinases, p34^{cdc2}/cyclin B and NIMA, which both have maximal kinase activity during mitosis (Osmani *et al.*, 1991a,b). In addition, both kinases are inactivated during mitotic progression and both are stabilized if normal mitotic progression is artificially prevented (Ye *et al.*, 1995). Thus there exists a mechanism which is able to monitor mitotic progression and then act on this information to inactivate these two mitotic protein kinases at a specific point(s) during mitotic progression. It is not currently known if the mechanisms of inactivation of p34^{cdc2} and NIMA are related in any way. Nor is the functional significance of NIMA inactivation to mitotic exit understood. The current work addresses these two issues.

Mitotic p34^{cdc2} H1 kinase consists of a complex between p34^{cdc2} and cyclin B called MPF (Draetta, 1990; Nurse, 1990; Norbury and Nurse, 1992). The p34^{cdc2} catalytic component is phosphorylated at threonine 167 and this phosphorylation is essential for its kinase activity (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1992, 1993; Fesquet *et al.*, 1993; Poon *et al.*, 1993). Inactivation of p34^{cdc2}/cyclin B H1 kinase activity during mitosis is thought to occur by ubiquitin mediated proteolysis of the cyclin B component of the kinase complex (Evans *et al.*, 1983; Draetta and Beach, 1988; Booher *et al.*, 1989; Glotzer *et al.*, 1991), but threonine 167 dephosphorylation could also play a role in the inactivation of the kinase (Lorca *et al.*, 1992).

Exactly how the ubiquitin dependent proteolysis of cyclin B is regulated is not understood. The process is known to involve identification of a conserved motif in the N-terminal region of cyclin B, called the cyclin destruction box, by the ubiquitin conjugating machinery (Glotzer *et al.*, 1991). Once polyubiquitinated during mitosis, cyclin B is degraded by the ubiquitin proteolysis pathway. It has therefore been possible to generate truncated versions of cyclin B which are resistant to mitotic destruction but which are able to activate p34^{cdc2} as a mitotic H1 kinase. Such stable cyclin B proteins are toxic to cells as they allow mitotic entry but then cause arrest of normal mitotic progression (Murray *et al.*, 1989; Luca *et al.*, 1991; Gallant and Nigg, 1992). Such data have been interpreted to indicate that cyclin B destruction is required for correct exit from mitosis. Data have also been presented suggesting that other proteins, in addition to cyclin B, may also need to be degraded by the ubiquitin pathway in order for cells to complete mitosis (Holloway *et al.*, 1993).

By investigating the functional role of the C-terminal extension of NIMA we have generated a version of NIMA that is no longer regulated through the cell cycle. This C-terminal truncated form of NIMA is extremely toxic and prevents normal mitotic progression because, unlike full length NIMA, it is very stable. The relationship between mitotic cyclin B destruction and NIMA destruction has been studied and is discussed.

Results

The C-terminus of NIMA is required for function but not for kinase activity

NIMA, and related protein kinases, have an N-terminal catalytic domain and a C-terminal domain of unknown function. Full length NIMA has protein kinase activity when expressed in *Escherichia coli* (Lu *et al.*, 1993, 1994). To determine the importance of the C-terminus of NIMA to its kinase activity we synthesized both full length NIMA and the kinase domain of NIMA in *E.coli* using the IPTG-inducible pET expression system.

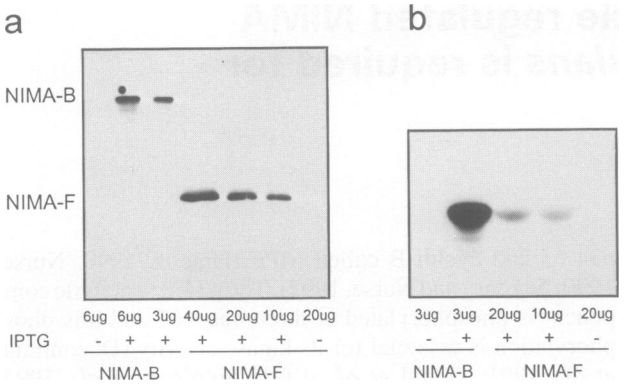

Fig. 1. Western blot (**a**) and kinase activity (**b**) of full length NIMA (NIMA-B) and the kinase domain of NIMA (NIMA-F) expressed in *E.coli*. For Western blot the indicated amounts of soluble protein extract prepared from *E.coli* strains induced with IPTG to express either full length NIMA or the kinase domain of NIMA were separated by SDS−PAGE and analyzed by Western blot with anti-T7 antiserum. For kinase assays the indicated amounts of the soluble *E.coli* protein were immunoprecipitated using T7 antiserum and assayed for β-casein kinase activity. Kinase reactions were stopped and ^{32}P-labelled β-casein was resolved by SDS−PAGE and exposed to X-ray film.

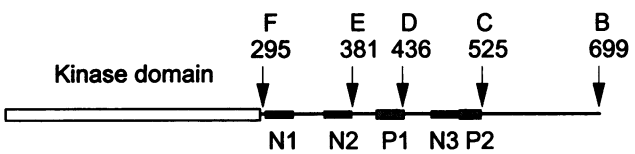

Fig. 2. Nomenclature and position of the C-terminal NIMA truncation series. The kinase catalytic domain of NIMA is located in the N-terminus. The relative positions of three putative nuclear localization signals (N1, N2 and N3) and two PEST sequences (P1 and P2) are indicated together with the positions of the truncation sites.

Although by Coomassie Blue staining no NIMA size band was detectable after IPTG induction (data not shown) both full length and truncated NIMA could be detected by Western blot of soluble protein extracts prepared from *E.coli* induced to synthesize NIMA (Figure 1a). When the *E.coli* extracts were assayed for β-casein kinase activity both full length NIMA and truncated NIMA had kinase activity and no activity was detected in extracts prepared before IPTG induction (Figure 1b). The specific activity of the truncated NIMA was clearly below that of full length NIMA. This suggests an activating function for the C-terminal extension of NIMA. However, it appears that the C-terminus of NIMA is not essential for its kinase activity.

To ascertain the functional importance of the C-terminal extension of NIMA, a truncation series was generated (Figure 2) which was cloned into the *A.nidulans* expression vector pAL5 (Waring *et al.*, 1989; Doonan *et al.*, 1991). The resulting constructs were transformed into strain SO6, which contains the *nimA5* allele, to test for their ability to complement the *nimA5* mutation at the restrictive temperature (Table I). The *alcA* promoter present in pAL5 is repressed when cells are grown on glucose but allows expression during growth on glycerol. For each construct 26 primary transformants, initially grown on glucose

Table I. Complementation of *nimA5* by truncation series and effects of their expression on cell viability[a]

	32°C		42°C	
	Glucose	Glycerol	Glucose	Glycerol
pAL5B	26	25	0	24
pAL5C	26	23	0	24
pAL5D	26	17	0	15
pAL5E	26	4	0	0
pAL5E^{k-}	26	26	0	0
pAL5F	26	22	0	0

[a]A *nimA5*-containing strain was transformed with the plasmid constructs indicated. For each construct, 26 randomly selected transformants were replica plated onto minimal medium plates containing the indicated carbon sources. The number of transformants able to form a colony were scored after 3 days' incubation.

medium, were therefore replica plated onto glucose- or glycerol-containing plates which were then incubated for 3 days at either 32°C or 42°C. Colony formation was then scored (Table I). Transforming DNA normally integrates into the genome of *A.nidulans* and integrated plasmid DNA can be maintained in single copy or multiple copy. Thus these primary transformant colonies were likely to contain a variable copy number of the transforming plasmid DNA (see below). All transformants could grow and form a colony when grown on *alcA*-repressing glucose medium at 32°C. No transformants could grow on the repressing medium at 42°C. This demonstrates, as expected, that in the absence of *alcA* expression none of the *alcA*-driven constructs were able to complement the temperature sensitive *nimA5* mutation. However, growth of the transformants on glycerol-containing medium, which allows expression of *alcA*, enabled at least some transformants to grow at 42°C if they received either pAL5B or pAL5C or pAL5D. The constructs expressing just the kinase domain (pAL5F), or the kinase domain and a region containing two putative nuclear localization sequences (pAL5E), were unable to complement *nimA5* at the restrictive temperature (Table I).

For the pAL5F construct, 22 transformant colonies were obtained at the permissive temperature on glycerol-containing medium. This construct is therefore no more toxic than the full length NIMA construct when expressed but it was unable to complement the *nimA5* mutation. We conclude that the C-terminus of NIMA is required for NIMA function. However, for the pAL5E construct only four colonies were formed at 32°C on glycerol-containing medium. This strongly suggests that tranformants that contain pAL5E which can express the NIMA-E protein were unable to survive when *alcA* expression is allowed. This suggests that the lack of complementation by the pAL5E construct at restrictive temperature could be due to its extreme toxicity when expressed from *alcA*.

We have previously shown that induction of full length NIMA from the *alcA* promoter is toxic to cells due to the premature induction of mitotic events caused by overexpression of NIMA (Osmani *et al.*, 1988). This effect was dependent on the copy number of the *alcA*-driven *nimA* construct integrated into the genome, as a single copy of *nimA* driven by *alcA* is not toxic but multiple copies are (Osmani *et al.*, 1988). One possible explanation

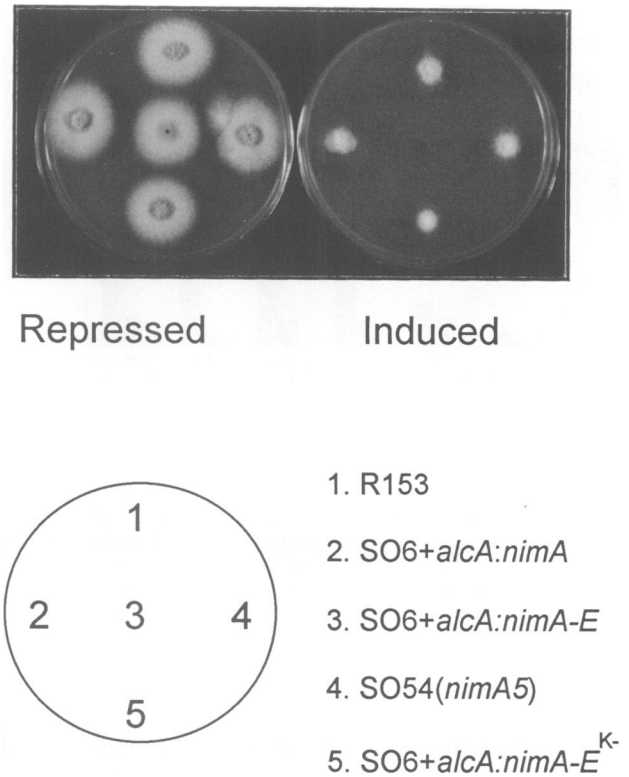

Fig. 3. The toxicity of NIMA-E is due to its kinase activity. The strains indicated in the key were inoculated on repressing (glucose) and inducing (ethanol) media for the *alcA* promoter and grown at 32°C for 2 days. All strains grew at a comparable rate on repressing medium while on inducing medium the strain containing *alcA::nimA-E* could not grow to form a colony. All strains grew less well on inducing medium due to the poor nature of ethanol as a carbon source when compared with glucose.

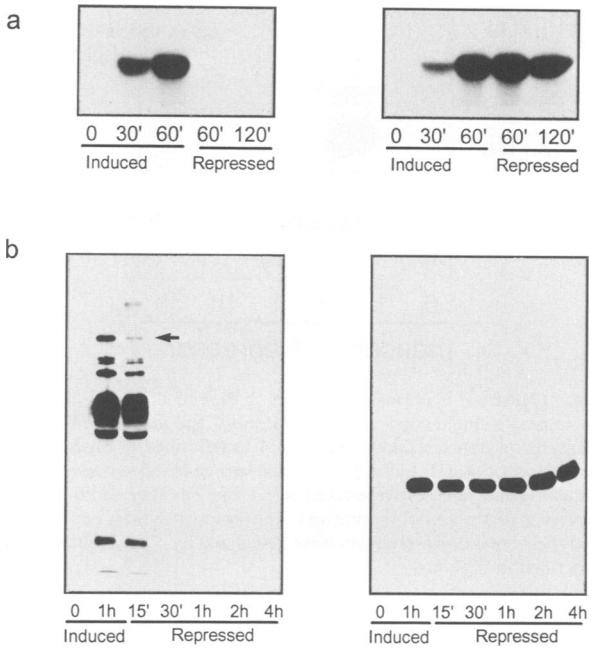

Fig. 4. Full length NIMA is very unstable but truncated NIMA-E is very stable. (**a**) Kinase activity of a strain (B1-24) containing a single copy of plasmid pAL5B (*alcA::nimA-B*) (left panel) and a strain (E2-10) containing a single copy of plasmid pAL5E (*alcA::nimA-E*) (right panel) were grown in *alcA*-repressing medium (time 0), changed to inducing medium for 60 min (Induced 30′ and 60′) before addition of *alcA* repressor (Repressed 60′ and 120′). ^{32}P-labelled β-casein is shown after autoradiography. (**b**). Western blot analysis of the two strains grown as described above for (a). Total protein (2.5 mg) was immunopurified using E-14 antiserum and immunocomplexes were resolved by SDS−PAGE followed by subsequent Western blot analysis using E-14 as the primary antibody and ECL detection of NIMA. Arrow indicates induced full length NIMA.

for the toxicity of the pAL5E construct could therefore be that all transformants containing this construct were high copy number transformants. We therefore identified single copy integrant transformants for pAL5B and pAL5E by Southern blot analysis (data not shown). These strains were inoculated onto *alcA*-repressing or *alcA*-inducing medium to test for their ability to grow when producing either full length or truncated NIMA (Figure 3). Even in single copy, the pAL5E construct proved toxic to cells when grown on *alcA*-inducing (ethanol) medium. The truncated form of NIMA produced from pAL5E is therefore more toxic than the full length protein. We conclude that the lack of complementation by the pAL5E construct at restrictive temperature is due to its extreme toxicity when expressed from *alcA*, even in single copy.

To determine if the toxicity of pAL5E was due to its kinase activity, or some other biological function, we mutated lysine 40 by *in vitro* mutagenesis of pAL5E to inactivate the kinase activity of NIMA-E. Expression of the kinase negative construct pAL5E^{K-} was not toxic to cells when present in single copy (Figure 3), demonstrating that pAL5E is toxic due to the kinase activity of NIMA-E generated from this construct.

C-terminal truncated NIMA is toxic due to its intrinsic stability

As the NIMA-E truncated form of NIMA is more toxic than the full length protein we carried out transient induction experiments. We hoped to determine the level and stability of both full length and truncated NIMA as the truncated NIMA could be stable due to lack of PEST sequences (Rogers *et al.*, 1986) which could then explain its toxicity (Figure 2). Two strains were employed which each contained a single copy of *alcA*-driven *nimA* integrated into the genome. Strain B1-24 contained *alcA*-driven full length NIMA and E2-10 the E-truncated version of NIMA (Figure 2). Both strains were grown in *alcA* repressing medium to log phase. The repressor was removed by filtration and growth continued for 1 h with addition of *alcA* inducer. At this point *alcA* repressor was added back to the cultures. Protein samples were isolated and assayed for NIMA kinase activity (Figure 4a). Upon addition of *alcA* inducer both strains contained elevated NIMA kinase activity at 30 and 60 min. Upon addition of repressor the elevated kinase activity present in strain B1-24 was diminished by 60 min. However, the kinase activity associated with induction of NIMA-E in strain E2-10 was stable for several hours after addition of *alcA* repressor (Figure 4a).

As transient expression of truncated NIMA-E caused stable elevation of NIMA kinase activity we carried out Western blot analysis to see if the truncated NIMA-E protein was also more stable than full length NIMA (Figure 4b). After a 1 h induction, accumulation of full length NIMA could be detected along with many

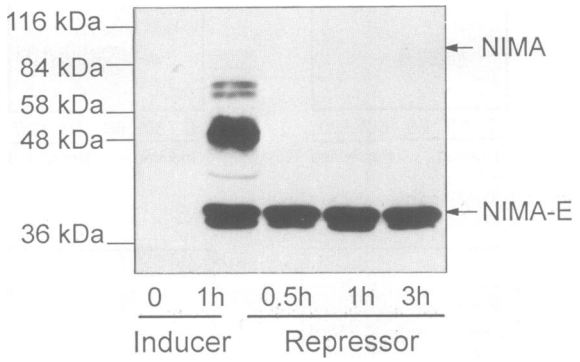

Fig. 5. NIMA-E is intrinsically stable. A diploid strain (RPD1) containing a single copy of both *alcA::nimA* and *alcA::nimA-E* was subjected to transient induction of *alcA* as described in Figure 4. After 1 h of induction (1h Inducer) repressor was added to terminate the induction and samples were taken 0.5, 1 and 3 h after addition of repressor (0.5h, 1h, 3h, Repressor). The levels of NIMA and NIMA-E and their breakdown products were visualized by Western blotting as described in Figure 4.

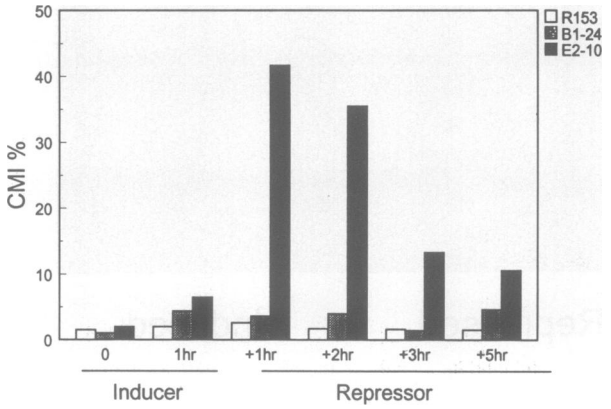

Fig. 6. Transient expression of NIMA-E causes a delayed increase in chromosome mitotic index. Exponentially growing cells containing a single copy of *alcA::nimA-E* (strain E2-10) were subjected to a 1 h induction of *alcA* by switching from repressing to inducing medium for 1 h prior to re-addition of *alcA* repressor. Control strains were wild type (R153) and a strain containing a single copy of *alcA::nimA-B* (B1-24). Samples were taken at the time points indicated, fixed and stained with DAPI to monitor the chromosome mitotic index (CMI) of the cultures.

proteolytic breakdown products. After addition of repressor the amount of full length NIMA was decreased significantly by 15 min and no NIMA, or its breakdown products, could be detected after 30 min. The half life of induced full length NIMA is therefore <15 min. For transient induction of NIMA-E a completely different pattern of stability was obtained. The truncated protein induced by 1 h of induction from *alcA* was completely stable for the entire time course of the experiment. This gives a half life for the truncated NIMA-E protein of >240 min. The NIMA-E truncated NIMA is thus considerably more stable than full length NIMA.

There are two possible explanations for the stability of NIMA-E: either this protein could be intrinsically stable, or induction of NIMA-E could promote a cellular environment in which NIMA is more stable. For instance, we know that mitotic transition is required for degradation of NIMA so induction of a mitosis-like state by NIMA-E could promote mitotic stability to NIMA-E. To distinguish between these two possibilities we generated a diploid between a haploid strain containing *alcA*-driven full length NIMA and another containing *alcA*-driven NIMA-E. This diploid was then transiently induced for *alcA* expression and Western blot analysis was carried out to detect NIMA and NIMA-E. In the diploid state, when both NIMA and NIMA-E were transiently induced, NIMA-E was still stable and full length NIMA still very unstable (Figure 5). This demonstrates that NIMA-E is intrinsically stable and that it does not generate a cellular environment in which NIMA is stable.

Transient induction of stable NIMA prevents normal mitotic progression

To examine the phenotypic consequences of transient expression of the non-degradable NIMA-E on mitotic progression we stained cells with the DNA specific dye DAPI to visualize nuclei. Three strains were employed: strain R153, which does not contain *alcA*-driven NIMA; strain B1-24, which contains a single copy of *alcA*-driven full length NIMA; and strain E2-10, which contains a single copy of *alcA*-driven NIMA-E. These strains were transiently induced on *alcA*-inducing medium for 1 h before addition of *alcA* repressor. At hourly intervals samples were taken and stained with DAPI to monitor mitotic progression (Figure 6). After a 1 h induction a slight increase in the chromosome mitotic index of strains B1-24 and E2-10 was observed. Following a further hour of growth on repressing medium the mitotic index for strain E2-10 was elevated and this elevated mitotic index decreased slowly over the next 3 h of growth. Very little variation in the mitotic index of the other two strains was observed.

The mitotic figures observed after transient induction of NIMA-E were somewhat atypical and many nuclei appeared stretched out as if unable to segregate their chromosomes (Figure 7). This phenotype suggests that cells reached mitosis normally but then had problems completing mitosis. However, although induction of truncated stable NIMA-E for 1 h was not sufficient to promote mitosis, it could be argued that, with time, the stable kinase eventually promoted mitotic events prematurely. The abnormal mitotic figures we observed may therefore not have resulted from cells being unable to exit mitosis but instead be caused by promotion of mitotic events early in the cell cycle.

To help circumvent the problems of interpretation associated with transient expression of stable NIMA-E in cells progressing non-synchronously through the cell cycle we employed the temperature sensitive *nimT23*^{cdc25} mutation to arrest cells in G_2 prior to transient induction of NIMA-E. The rationale for using this approach was as follows. Induction of NIMA-E at the G_2 arrest point of *nimT23*^{cdc25} allowed us to generate cells arrested in G_2 with transiently expressed NIMA-E. By returning cells to the permissive temperature we could then determine the effects of the non-degradable NIMA-E on the synchronous mitotic progression that occurs after release of the *nimT23*^{cdc25} block. By keeping some control cells at the restrictive temperature and monitoring their chromosome

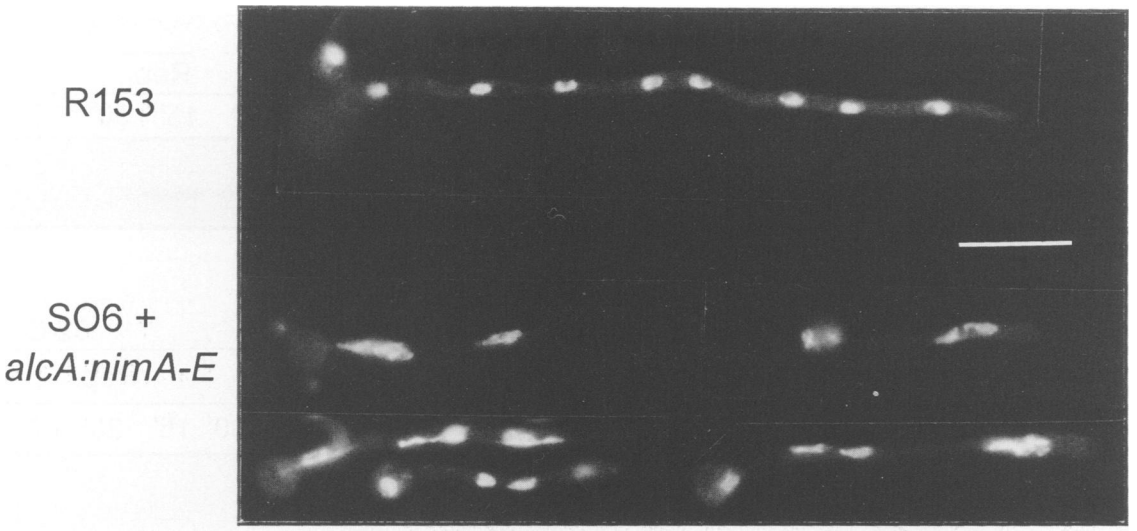

Fig. 7. Mitotic defects observed after transient induction of NIMA-E. Photomicrographs were taken 2 h after the termination of transient induction as described for Figure 6. The upper cell (R153) contains typical interphase (probably G_1) nuclei. The five cells shown below (SO6 + *alcA::nimA-E*) are examples of cells containing disorganized, stretched out nuclear DNA that appear to be unable to complete mitosis correctly after transient induction of *nimA-E*. The bar represents ~10 μm.

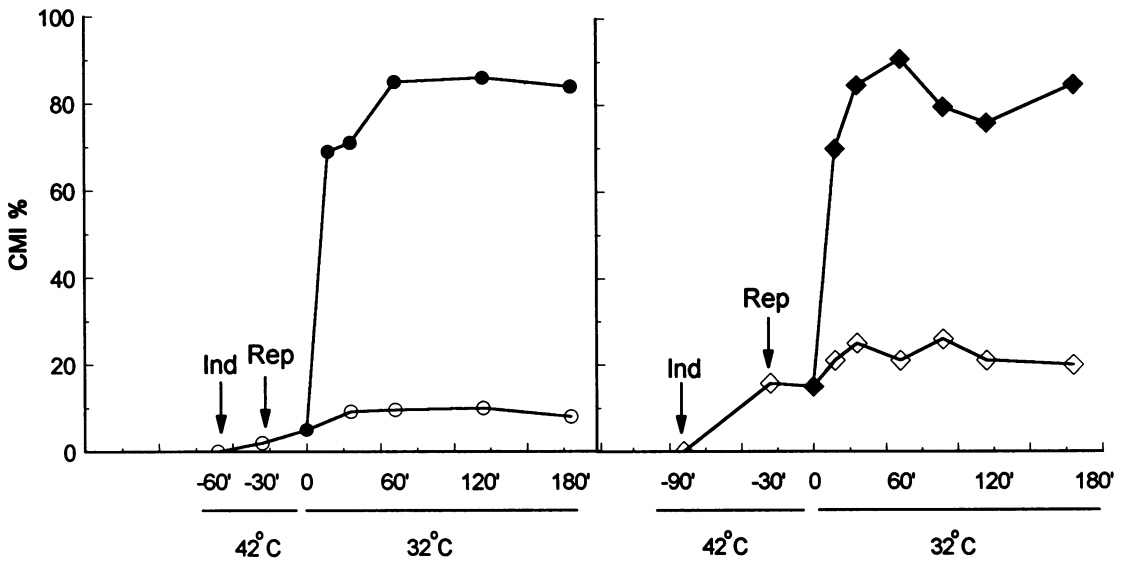

Fig. 8. NIMA-E expression can prevent mitotic exit. Strain RP3 (*nimT23*^{cdc25} + *alcA::nimA-E*) was grown in *alcA*-repressing medium to early log phase at 32°C and then shifted to 42°C for 3 h. *alcA* induction was then carried out for 30 min (left panel) or for 60 min (right panel). *alcA* repressor was then added back and cells were incubated for a further 30 min before returning them to 32°C (● and ◆). Control samples were kept at 42°C (○ and ◇). Induction was carried out by washing with fresh 42°C medium without carbon source and then transferred to inducing medium prewarmed to 42°C for 30 or 60 min. Samples were taken at the times indicated and after DAPI staining the chromosome mitotic index of the samples determined.

mitotic index, we could also establish what level of mitotic initiation was induced by NIMA-E induction alone.

Induction of NIMA-E for 30 min or 1 h at the G_2 arrest point of *nimT23*^{cdc25} caused a slight increase in the chromosome mitotic index of the culture (Figure 8). After addition of repressor no further significant increase in NIMA-E kinase activity occurred and the mitotic index of the cultures also remained fairly constant. Thus, at these levels of induction of NIMA-E, the majority of the cells remained arrested in G_2. Upon downshift to the permissive temperature cells rapidly entered mitosis, as monitored by chromosome mitotic index, and they remained in a mitotic state for up to 3 h (Figure 8). A strain in which full length NIMA was induced for an hour prior to release of *nimT23*^{cdc25} into mitosis also caused a delay in mitotic exit but not to the same degree as NIMA-E. After 180 min of release of *nimT23*^{cdc25}, at a time when NIMA-E-induced cells had a mitotic index of >80%, cells transiently induced for full length NIMA had a mitotic index of <30%. These data indicate that progression through mitosis is markedly impaired in the presence of non-degradable NIMA. Control cells not containing *alcA*-driven *nimA*, arrested and then released from the restrictive temperature arrest point of *nimT23*^{cdc25}

under identical conditions, completed mitosis within 60 min of the downshift to permissive temperature (data not shown).

Non-degradable NIMA prevents mitotic exit after degradation of cyclin B

One potential mechanism by which non-degradable NIMA could prevent mitotic exit involves stabilization of cyclin B. In this model the non-degradable NIMA would in some way stabilize cyclin B. As degradation of cyclin B is required for correct mitotic exit this would then in turn arrest mitotic exit. We therefore monitored the level of cyclin B in cultures transiently induced for NIMA prior to release of the *nimT23*^{cdc25} G_2 arrest. G_2 arrest, caused by incubation of a control *nimT23*^{cdc25} strain at restrictive temperature, allowed accumulation of NIME$^{cyclin\ B}$ protein (Ye *et al.*, 1995). The mock process of induction and repression of *alcA* in the control cells did not then affect the level of NIME$^{cyclin\ B}$. However, subsequent downshift to permissive temperature, and mitotic progression, led to a marked decrease in the level of NIME$^{cyclin\ B}$ by 30 min. The level of NIME$^{cyclin\ B}$ then recovered by 120 min as cells progressed into the next cell cycle (Figure 9a).

For cells expressing full length NIMA from the *alcA* promoter, induction of NIMA at the G_2 arrest point of *nimT23*^{cdc25} caused a reduction in the level of NIME$^{cyclin\ B}$ (Figure 9b). This effect may be related to the fact that a 1 h transient induction of full length NIMA at the G_2 arrest point of *nimT23*^{cdc25} was able to cause chromosome condensation in a significant proportion (40%) of the cells. This may then trigger the pathway for NIME$^{cyclin\ B}$ degradation. Upon release to permissive temperature cells progressed through mitosis and, as for the strain not containing induced NIMA, NIME$^{cyclin\ B}$ levels recovered by 120 min as cells progressed into the next cell cycle.

For cells expressing NIMA-E the induction did not lead to a marked degradation of NIME$^{cyclin\ B}$, consistent with these cells being predominantly arrested in G_2 (Figure 9c). Indeed, continued incubation of the cells for 120 min at the restrictive temperature after induction of NIMA-E did not lead to degradation of NIME$^{cyclin\ B}$ (Figure 9c, last lane, 42°C 120′). However, downshift to permissive temperature allowed cells to enter mitosis, but not to exit mitosis due to the presence of non-degradable NIMA-E, and NIME$^{cyclin\ B}$ was degraded (Figure 9c). Thus, these cells were arrested in a mitotic state due to the presence of non-degradable NIMA-E even though NIME$^{cyclin\ B}$ was degraded, though perhaps a little more slowly than control cells (Figure 9a). This indicates that the mitotic arrest caused by NIMA-E was not caused by stabilization of NIME$^{cyclin\ B}$. In addition, it demonstrates that cells can be arrested in a mitotic state after degradation of NIME$^{cyclin\ B}$. The mitotic arrest caused by NIMA-E also prevented the re-accumulation of NIME$^{cyclin\ B}$ (Figure 9c, 180′, Rep. 32°C), which was seen for the two control strains (Figure 9a and b), further indicating that NIMA-E caused arrest of cell cycle progression by preventing correct mitotic progression.

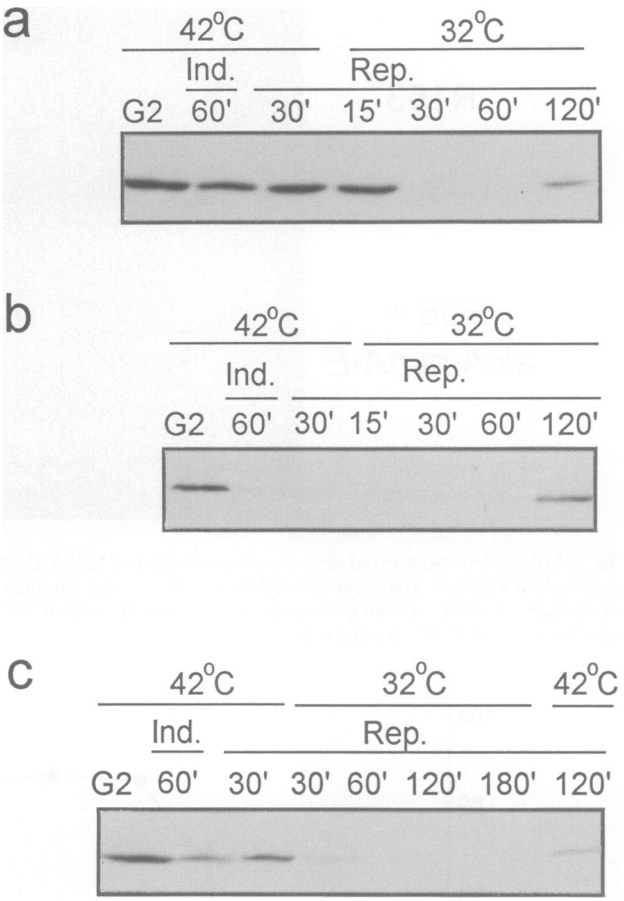

Fig. 9. NIMA-E blocks cells in mitosis without detectable cyclin B. Western blot analysis to determine the level of cyclin B in cells arrested in G_2 using the *nimT23*^{cdc25} mutation and after release into mitosis with (**a**) no induction of extra NIMA, (**b**) induction of full length NIMA and (**c**) induction of truncated NIMA-E. Induction of *alcA::nimA* constructs by induction and repression of *alcA* was as described for Figure 6. Two hundred micrograms of total protein from strains SO53 (a), RP6 (b) and RP3 (c) were separated by SDS–PAGE and standard Western blot procedures were followed using anti-cyclin B antisera (E8) (Osmani *et al.*, 1994) as primary antibody. The temperature of incubation and the induction status of *alcA* is indicated for each strain.

Discussion

NIMA (Osmani *et al.*, 1988) and related kinases (Jones and Rosamond, 1990; Barton *et al.*, 1992; Letwin *et al.*, 1992; Schweitzer and Philippsen, 1992; Gale and Parsons, 1993; Schultz and Nigg, 1993; Levedakou *et al.*, 1994; Schultz *et al.*, 1994) have an N-terminal kinase domain and a C-terminal extension. We have shown that the catalytic domain, like the full length protein, has kinase activity when expressed in *E.coli*. The catalytic domain of mouse Nek1 has also been shown to have catalytic activity (Letwin *et al.*, 1992). However, truncation of the C-terminus of NIMA lowers the specific activity of the kinase, suggesting that the C-terminus may play an activating role for NIMA kinase activity. Although active as a kinase, the catalytic domain of NIMA was unable to complement the *nimA5* mutation when expressed from the *alcA* promoter whereas the full length protein was able to complement *nimA5* when similarly expressed. This demonstrates that the C-terminal extension of NIMA is required for its *in vivo* function although it is not essential for its kinase activity.

To analyze further the functional domains of the C-terminal region of NIMA a truncation series was produced and tested for ability to complement *nimA5*. From

this analysis it appeared that progressive C-terminal deletion of NIMA generated a protein kinase that became more and more toxic to the cell and it therefore lost its ability to complement *nimA5*. However, the kinase domain alone was not more toxic than untruncated NIMA. The most toxic construct identified had both of the PEST sequences (Rogers *et al.*, 1986) removed but still contained two putative nuclear localization motifs (Dingwall and Laskey, 1991). When the nuclear localization signals were removed, to yield just the kinase domain, then the truncated protein was no longer toxic but it was unable to complement *nimA5*. This indicates that the toxicity of truncated NIMA could be related to both its stability and its ability to enter the nucleus.

The half life of full length NIMA was found to be <15 min but the half life of the most toxic C-terminal truncated NIMA (NIMA-E) was found to be >240 min. As the NIMA-E protein lacks all PEST sequences it is likely that the PEST sequences normally maintain NIMA as an unstable protein causing rapid degradation of overexpressed NIMA. Lack of the PEST sequences would prevent rapid proteolysis of NIMA-E causing it to then be toxic.

How the PEST-mediated instability relates to the normal cell cycle accumulation of NIMA has not yet been directly addressed. However, we have shown that NIMA protein accumulates during G_2 at the arrest point of both *nimA5* and *nimT23*^{cdc25}. It is also maintained at an elevated level during mitosis and remains relatively stable if cells are continuously held in a mitotic state by cell cycle specific mutants or by addition of microtubule poisons (Ye *et al.*, 1995; X.S.Ye and S.A.Osmani, unpublished). It is possible to detect large increases in the level of NIMA protein in the absence of a comparable increase in the level of *nimA* mRNA during G_2 arrest (S.L.McGuire and S.A.Osmani, unpublished). This indicates that transcriptional activation is not responsible for accumulation of NIMA at G_2. Whether there is a system which prevents degradation of NIMA during G_2 and mitosis, or whether increased levels of NIMA are a result of increased translation of *nimA* mRNA during G_2 and M, remains to be seen.

Although we have not addressed the role of mitotic stability of NIMA in the initiation of mitosis we have investigated the role of NIMA degradation during mitotic exit. We have shown that NIMA is normally destroyed upon mitotic exit in a manner analogous to cyclin B degradation (Ye *et al.*, 1995). As the truncated NIMA-E version of NIMA is stable during mitotic progression we have been able to ask if there is a functional relationship between degradation of NIMA and progression through, and then out of, mitosis. By transiently inducing a level of stable NIMA-E protein at G_2, which was unable to promote cells into mitosis, before allowing them to enter mitosis synchronously, we have been able to demonstrate that cells are unable to complete mitosis correctly in the presence of non-degradable NIMA-E. In addition, the mitotic arrest caused by non-degradable NIMA-E is at a stage after the mitotic degradation of cyclin B (NIME$^{cyclin\ B}$) has occurred. Therefore, mitotic arrest caused by non-degradable NIMA occurs independently of p34^{cdc2}/cyclin B kinase activity. These data, and that of the accompanying paper (Ye *et al.*, 1995), demonstrate that it is necessary to activate both NIMA and p34^{cdc2}/cyclin B coordinately to initiate mitosis and then to inactivate both during mitosis in order to complete mitosis in *A.nidulans*.

We have been unable to stain cells successfully to visualize microtubules in cells arrested in mitosis by non-degradable NIMA-E due to technical difficulties with immunofluorescence which are associated with temperature shift and carbon source changes during these experiments. We have therefore been unable to determine if arrest of mitotic progression caused by non-degradable NIMA-E in the absence of p34^{cdc2}/cyclin B causes both chromosome condensation and persistent assembly of the mitotic spindle.

The pathway responsible for cyclin B degradation and that required for NIMA degradation during mitosis are apparently not identical. For instance, NIME$^{cyclin\ B}$ is degraded before NIMA is degraded during mitotic progression (Ye *et al.*, 1995). Secondly, although the truncated NIMA-E protein contains a motif similar to a cyclin destruction box (RxxLxxxxN) this protein is not degraded at a time when we have demonstrated that NIME$^{cyclin\ B}$ is degraded. This suggests that NIMA and NIME$^{cyclin\ B}$ are degraded by different mechanisms during mitotic transition. Indeed, the degradation pathway of NIMA during mitosis may not in fact be 'cell cycle specific' if regulation occurs at the level of variable rates of translation during entry into and exit from mitosis. In this scenario a constant rate of degradation of NIMA mediated by the PEST sequences would be overcome by an increase in the production of the protein during G_2 and mitosis. Alternatively, the PEST-mediated degradation of NIMA may be dampened in a cell cycle specific way during G_2 and the early part of mitosis. We are currently trying to determine if NIMA is degraded by a novel cell cycle specific pathway distinct from that which degrades cyclin B.

Protein kinases related to *A.nidulans* NIMA have been isolated from humans, one of which, Nek2, is regulated through the cell cycle in a manner similar to NIMA as it reaches maximal levels during G_2 (Schultz *et al.*, 1994). Mitotic degradation of NIMA homologs, or related kinases, may therefore be required for correct mitotic progression in higher eukaryotes. These potential NIMA homologs may be related to the proteins that need to be degraded, in addition to cyclin B, for *Xenopus* mitotic extracts to exit mitosis (Holloway *et al.*, 1993).

Materials and methods

Aspergillus nidulans strains, media and general techniques

Strains used include: FGSC122 (*riboA1; yA2; nicB8*), R153 (*wA3; pyroA4*), SO6 (*nimA5; wA2; pyrG89; cnxE16; choA1; sC12; yA2; chaA1*), SO26 (*nimT23; pyrG89; wA2; biA1; pabaA1; sC12*), SO27 (*nimT23; choA1; methG1; cnxE16; lacA1; sC12; pabaA1*) and SO54 (*nimA5; wA2*).

SO6 transformant strains B1-24 (*wA2; nimA5; cnxE16; choA1; sC12; yA2; chaA1; pyrG89; pyr4+; alcA::nimA-B*), and E2-10 (*wA2; nimA5; cnxE16; choA1; sC12; yA2; chaA1; pyrG89; pyr4+; alcA::nimA-E*), and EKM3 (*wA2; nimA5; cnxE16; choA1; sC12; yA2; chaA1; pyrG89; pyr4+; alcA::nimA-E*$^{k-}$) were generated by transforming SO6 strain with pAL5B, pAL5E and pAL5E^{k-} and selected after Southern blot analysis as single copy integrants at the H2A locus.

To generate RP6, strain B1-24 (*wA2; nimA5; cnxE16; choA1; sC12; yA2; chaA1; pyrG89; pyr4+; alcA::nimA-B*) was first crossed to FGSC122 (*riboA1; yA2; nicB8*) to isolate RP5 (*yA2; riboA1; nicB8; sC12; pyrG89; pyr4+; alcA::nimA-B*) which was then crossed to SO26 (*nimT23; pyrG89; wA2; biA1; pabaA1; sC12*) to generate RP6 (*nimT23;*

yA2; riboA; pyrG89; pyr4+; alcA::nimA-B). To generate RP3 (*nimT23; yA2; pyrG89; pyr4+; alcA::nimA-E*) strain E2-10 was crossed to SO27.

Diploid strain RD1 was generated from strain RP1 (*yA2; riboA1; alcA::nimA-E*) and strain B1-24 (*wA2; nimA5; cnxE16; choA1; sC12; yA2; chaA1; alcA::nimA-B*) using standard techniques. Growth conditions, media and transformation were as described previously (Morris, 1976; Osmani *et al.*, 1987).

General molecular techniques

Standard molecular cloning techniques were employed (Sambrook *et al.*, 1989). T4 DNA ligase was obtained from New England Biolabs (Beverly, MA). Restriction enzymes were obtained from Promega (Madison, WI). Probes for Southern blot were made using a Promega 1 'Prime a gene' kit.

Generation of truncation series and in vitro mutagenesis

For the truncation B, C, D, E and F series of *nimA* (Figure 2) the same forward primer, 5′-GAAGATCTATGGCAATCGCACTGGCG-3′, and different reverse primers: RPR2, 5′-GAAGATCTCCCGGGTCACCTC-AAGTTGCGAAT-3′ (for B); AR4, 5′-GAAGATCTATGGTCGAGT-GGGCGATGG-3′ (for C); AR5, 5′-GAAGATCTAGTTCGTCGGCG-ACTCAAG-3′ (for E); and AR6 5′-GAAGATCTAACGATCCTGAACC-TCGCA-3′ (for F) were used to amplify and clone the *nimA* coding region as a *KpnI–BglII* fragment into the pAL5 expression vector (Waring *et al.*, 1989) to give rise to pAL5B, pAL5C, pAL5D, pAL5E and pAL5F, respectively.

Bacterially produced full length NIMA-B was as described (Ye *et al.*, 1995). The forward primer RPF1 and reverse primer RPR1 were used to amplify and clone NIMA kinase domain into pET11a as a *BglII* fragment to give rise to pET11a-*nimA-F. Escherichia coli* cells harboring this construct were induced by adding IPTG to generate a cell extract containing the kinase domain. Both full length NIMA-B- and NIMA-F-containing soluble fractions of *E.coli* extracts were used for kinase assays and for Western blotting using T7 specific antisera.

The codon encoding lysine 40 was changed to a methionine codon by *in vitro* site directed mutagenesis using the Clontech transformer kit and following the manufacturer's protocols. The plasmids pET21a-nimA, pAL5B and pAL5E were mutated using a mutagenesis oligonucleotide (K40M) and a selection oligonucleotide (S-SmaI or S-NotI). The resulting plasmid sequences were confirmed and were named pET21a-nimA^{k-}, pAL5B^{k-} and pAL5E^{k-}. Mutagenesis oligonucleotides used were: K40M (5′-ATACTATGTCGAATGGAAATCAACTAT-3′). Selection oligonucleotides: S-SmaI (5′-AACTTGACCTGAACCGGGAGATCC-3′), S-NotI (5′-AACTTGAGGGTGGCCGCACTC-3′).

Preparation of cell extracts

Aspergillus mycelia grown in minimal medium were harvested and ground in HK extraction buffer as described previously (Osmani *et al.*, 1991b) except that 1 μM okadaic acid was included in the extraction buffer and the concentration of sodium vanadate was increased to 1 mM.

Immunoprecipitation of NIMA, kinase assay and Western blotting

Immunoprecipitation of NIMA for kinase assays was carried out as described (Osmani *et al.*, 1991b) using modified substrate concentrations (Lu *et al.*, 1993). Briefly, total protein extracts ranging from 500 μg to 1 mg were incubated with excess NIMA antiserum (E-14) on ice for 2 h with mixing. Protein A–Sepharose beads (Pharmacia, 1 mg per μl of antisera) was then added and mixed for 30 min at 4°C on a shaker. The immunoprecipitation complex was then centrifuged and washed four times in extraction buffer and twice with KAB buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol) before performing kinase assays.

Western blot analysis was carried out to determine NIMA protein levels after cell extracts had been immunoprecipitated by E-14 antiserum generated against full length NIMA. For immunopurification 2.5–5.0 mg protein were used. After immunoprecipitation, protein complexes were resolved by SDS–PAGE and transferred to nitrocellulose membrane for detection using E-14 as primary antibody followed by incubation with either secondary antibody or protein A-conjugated horseradish peroxidase for ECL detection. For cyclin B Western blot, 200 μg of total protein extract was run on SDS–PAGE, transferred to nitrocellulose membrane and probed with specific cyclin B antiserum generated against bacterially produced NIME$^{cyclin\ B}$ protein (Osmani *et al.*, 1994).

Transient induction of alcA::nimA constructs

Conidia were inoculated at 2×10^6/ml in one liter of *alcA*-repressing medium (sodium acetate as the sole carbon source). After growing at 32°C for 24–30 h with shaking at 170 r.p.m. until the packed cell volume reached 0.3–0.5 ml/10 ml culture after centrifuging in an ICE bench top centrifuge at top speed for 1 min, mycelia were filtered by vacuum through Miracloth to remove repressor with 1.5–2 l of fresh medium containing no carbon source. Mycelia were then transferred to fresh medium containing *alcA* inducer (1% ethanol). Termination of induction was achieved by adding repressor back to the medium. For morphological examination, samples were taken into fix solution (4% glutaraldehyde, 0.2% NP-40 and 50 mM K phosphate pH 8.0) at 1:1 ratio (v:v). After at least 1 h of fixation, samples were washed several times and sonicated to disperse clumped mycelia prior to staining with DAPI (Oakley and Osmani, 1993).

For induction of *alcA::nimA* constructs at the G_2 arrest point of *nimT23*^{cdc25}, appropriate strains were grown as described above on repressor medium. After overnight growth, temperature upshift was achieved by shaking the flask in a 55°C water bath until the temperature of the medium reached 42°C. Further incubation at 42°C for 3 h imposed the G_2 block. Mycelia were washed with 42°C fresh medium (no carbon source) as described above by filtration and immediately transferred into inducing medium kept at 42°C to initiate induction. The transient induction was terminated by addition of repressor 30 min before downshift to 32°C to reverse the *nimT23*^{cdc25} G_2 arrest.

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