Nif1, a novel mitotic inhibitor in *Schizosaccharomyces pombe*

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In *Schizosaccharomyces pombe*, the activity of the M-phase-inducing Cdc2/Cdc13 cyclin-dependent kinase is inhibited by Wee1 and Mik1 tyrosine kinases, and activated by Cdc25 and Pyp3 tyrosine phosphatases. Cdc2/Cdc13 activity is also indirectly regulated by the ~70 kDa Nim1 (Cdr1) serine/threonine kinase, which promotes mitosis by inhibiting Wee1 via direct phosphorylation. To understand better the function and regulation of Nim1, the yeast two-hybrid system was used to isolate *S.pombe* cDNA clones encoding proteins that interact with Nim1. Sixteen of the 17 cDNA clones were derived from the same gene, named *nif1*⁺ (nim1 interacting factor-1). Nif1 is a novel ~75 kDa protein containing a leucine zipper motif. The Nif1–Nim1 interaction requires a small region of Nim1 that immediately follows the N-terminal catalytic domain. This region is required for Nim1 activity both *in vivo* and *in vitro*. Δ *nif1* mutants are ~10% smaller than wild type, indicating that Nif1 is involved in inhibiting the onset of mitosis. Consistent with this proposal, overproduction of Nif1 was found to cause a cell elongation phenotype that is very similar to Δ *nim1* mutants. Nif1 overproduction causes cell cycle arrest in cells that are partly defective for Cdc25 activity, but has no effect in Δ *nim1* or Δ *wee1* mutants. Nif1 also inhibits Nim1-mediated phosphorylation of Wee1 in an insect cell expression system. These observations strongly suggest that Nif1 negatively regulates the onset of mitosis by a novel mechanism, namely inhibiting Nim1 kinase.

Keywords: cell cycle/fission yeast/mitosis/Nim1/Wee1

Introduction

Studies of the fission yeast *Schizosaccharomyces pombe* have played a major role in uncovering how eukaryotic cells regulate the onset of mitosis. The focus of the mitotic control is Cdc2–Cdc13, the M-phase-inducing cyclin-dependent kinase that consists of the 34 kDa Cdc2 catalytic subunit and the ~60 kDa Cdc13 cyclin-B subunit (Booher *et al.*, 1989; Moreno *et al.*, 1989). The activity of Cdc2–Cdc13 is maintained in an inhibited state during interphase mainly through the activities of Wee1 and Mik1 tyrosine kinases, which phosphorylate tyrosine-15 of the Cdc2 subunit (Russell and Nurse, 1987b; Gould and Nurse, 1989; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker *et al.*, 1992). Wee1 is the dominant compon-

ent of the Wee1–Mik1 pair, as shown by the observation that *wee1*[–] mutants have a wee phenotype (Nurse, 1975), undergoing mitosis and cell division at half the length of wild type, whereas the *mik1*[–] mutation has no effect on cell size (Lundgren *et al.*, 1991). However, simultaneous inactivation of *wee1*⁺ and *mik1*⁺ causes a lethal premature mitosis (Lundgren *et al.*, 1991), often referred to as mitotic catastrophe (Russell and Nurse, 1986), showing that inhibitory tyrosine-15 phosphorylation of Cdc2 is essential for viability in *S.pombe*. The inhibitory activities of Wee1–Mik1 tyrosine kinases are counteracted by Cdc25–Pyp3 tyrosine phosphatases, which directly carry out the dephosphorylation of tyrosine-15 of Cdc2 (Russell and Nurse, 1986; Gould and Nurse, 1989; Millar *et al.*, 1991, 1992). Cdc25 is the dominant element of the pair: loss of Cdc25 activity causes G₂ arrest, whereas *pyp3*[–] phenotypes are apparent only in strains that are partially defective for Cdc25 activity (Millar *et al.*, 1992). Genetic studies have established that the timing of mitosis is largely determined by the counteractive activities of Wee1 and Cdc25 (Russell and Nurse, 1986, 1987b), a balance which shifts in the favor of Cdc25 as cells reach the appropriate size to undergo mitosis.

The protein kinases and phosphatases that regulate Cdc2–Cdc13 are themselves controlled by phosphorylation (Dunphy, 1994). Studies in fission yeast, *Xenopus*, humans and other species have established that Cdc25 undergoes activating phosphorylation around the time of the G₂–M transition (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993; Kovelman and Russell, 1996). Although it appears that Cdc2–cyclin B kinases are capable of carrying out at least some of the activating phosphorylations *in vitro* (Hoffmann *et al.*, 1993; Izumi and Maller, 1993), it remains to be resolved whether Cdc2–cyclin B kinases perform these phosphorylations *in vivo*. Indeed, recent studies of *Xenopus* oocyte extracts have led to the identification of a second protein kinase, Plx1, that activates Cdc25 *in vitro* (Kumagai and Dunphy, 1996). In fission yeast, it is clear that Cdc25 remains in a weakly active state in *cdc2* temperature-sensitive mutants that are arrested in late G₂, establishing that the activations of Cdc2–Cdc13 and Cdc25 are mutually dependent *in vivo* (Kovelman and Russell, 1996). Studies of *Xenopus* and human cells have established that Wee1 is also regulated around the time at which cells undergo mitosis, in this case phosphorylation inhibits Wee1 (Tang *et al.*, 1993; McGowan and Russell, 1995; Mueller *et al.*, 1995; Watanabe *et al.*, 1995). As is the case for Cdc25 regulation, Cdc2–cyclin B has been proposed to have a role in the inhibitory phosphorylation of Wee1, although this remains to be proven. These discoveries regarding Cdc25 and Wee1 regulation have led to a model in which activation of Cdc2–cyclin B involves autocatalytic feedback loops, whereby Cdc2–cyclin B promotes its own activation by

Fig. 1. Nif1 specifically interacts with Nim1 in the yeast two-hybrid assay. Strain Y190 was transformed with pACT-Nif1 together with pAS2-Nim1, pAS2-CDK2, pAS2-p53, pAS2-lamin or pAS2-SNF1 as indicated in the left panel. Transformants were incubated either on a SSC-Trp-Leu plate without 3-AT (middle panel), which only selects for the presence of the plasmids, or on a SSC-Trp-Leu-His + 25 mM 3-AT plate, which also selects for transcriptional activation of *HIS3* reporter gene (right panel). Only the combination of pAS2-Nim1 and pACT-Nif1 promoted the formation of colonies on plates containing 25 mM 3-AT.

directly or indirectly catalyzing the activating phosphorylation of Cdc25 and inhibitory phosphorylation of Wee1.

In fission yeast, there is a second mechanism of regulating Wee1 that is distinct from the M-phase inactivation process mentioned above. The discovery of this regulatory process arose from the cloning of the gene *nim1*⁺ as a high-copy suppressor of *cdc25-22*, a temperature-sensitive mutation of *cdc25*⁺ (Russell and Nurse, 1987a). *cdc25-22* is suppressed by loss of Wee1 activity (Fantès, 1979), and a series of genetic tests strongly suggested that Nim1 overproduction rescued *cdc25-22* by inhibiting Wee1 (Russell and Nurse, 1987a). The identification of Nim1 as a mitotic inducer was confirmed by finding that Δ *nim1* mutants undergo division at an elongated cell length and that loss of Nim1 activity severely enhances the cell cycle delay phenotype of *cdc25-22* cells grown at the permissive temperature (Russell and Nurse, 1987a). Biochemical studies eventually established that Nim1 kinase, also known as Cdr1 (Young and Fantès, 1987; Feilottter *et al.*, 1991), inhibits Wee1 via direct phosphorylation occurring in the C-terminal catalytic domain of Wee1 (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993).

Having established that Nim1 functions as a mitotic inducer by negatively regulating Wee1, we have since extended our studies of Nim1 regulation. In this report, we describe how we have identified proteins that specifically interact with Nim1 by use of the yeast two-hybrid screening system (Durfee *et al.*, 1993). The most common clone isolated in the screen, *nif1*⁺, encodes a novel protein. Nif1 overproduction delays mitosis, whereas mutational inactivation of Nif1 advances mitosis. These findings strongly suggest that Nif1 functions as a mitotic inhibitor via a direct interaction with Nim1 protein kinase.

Results

Isolation of *S.pombe* cDNA clones encoding proteins that specifically interact with Nim1

A yeast two-hybrid screen was carried out to identify proteins that interact with Nim1 (Figure 1). The bait, encoded on plasmid pAS2-Nim1, consisted of full-length Nim1 protein kinase fused to the C-terminal end of the Gal4 DNA binding domain. Gal4-Nim1 was tested for interaction with a *S.pombe* cDNA library fused to the Gal4 activation domain in plasmid pACT (Durfee *et al.*,

1993). Approximately 2000 Y190/pAS2-Nim1 cells transformed with the pACT *S.pombe* cDNA library grew on SSC-Trp-Leu-His + 25 mM 3-amino-triazole (AT) plates. A transformation efficiency test indicated that 2×10^7 Trp⁺ Leu⁺ transformants were obtained in this experiment, thus the 25 mM 3-AT selection provided an $\sim 10^4$ enrichment. Transcriptional activation of the *lacZ* gene was detected in 134 of the His⁺ clones. pACT-cDNA plasmids that retested as positive were recovered from 17 of these transformants. Southern hybridization showed that 16/17 ($\sim 95\%$) of these cDNAs were derived from the same gene, which we named *nif1*⁺ (Nim1 interacting factor 1). The specific interaction of Nif1 with Nim1 as compared with several control bait proteins is shown in Figure 1. Analysis of the second gene, *nif2*⁺, will be presented elsewhere.

The *nif1*⁺ cDNA was used to probe membranes containing overlapping cosmid and P1 clones spanning the complete *S.pombe* genome (Hoheisel *et al.*, 1993). A 6 kb *HindIII* fragment from P1 clone 28C4p, containing the complete genomic sequence of *nif1*⁺, was cloned into pBluescript. *nif1*⁺ encodes a novel protein of 681 amino acids with a predicted mol. wt of ~ 75 kDa (Figure 2). Nif1 has no extensive regions of homology to any proteins with known function. Nif1 contains a leucine zipper motif at amino acids 505–526. The leucine zipper motif has been implicated in protein dimerization. Three PEST regions were found in Nif1 (amino acids 78–107, 234–252 and 280–298). PEST regions are commonly found in proteins that are rapidly degraded (Rogers *et al.*, 1986).

Nif1 associates with Nim1 in *S.pombe*

Experiments were carried out to determine whether Nim1 and Nif1 interact in fission yeast cells. Nim1 was co-expressed with Nif1 fused to glutathione-S-transferase (GST) or unfused GST. *Schizosaccharomyces pombe* cells were lysed under stringent conditions and the supernatants were incubated with glutathione-Sepharose to purify GST fusion proteins. The bound proteins were analyzed by immunoblotting. Nim1 was specifically detected in the GST-Nif1 sample, whereas no Nim1 signal was detected in the GST sample (Figure 3). These findings strongly suggest that Nif1 and Nim1 proteins interact in *S.pombe* cells.

An important region of Nim1 is required for its association with Nif1

The ~ 30 kDa C-terminal non-catalytic domain of Nim1 (amino acids 354–593) is not required for Nim1 function *in vivo* (Russell and Nurse, 1987a; Feilottter *et al.*, 1991). In particular, expression of a truncated form of Nim1 containing amino acids 1–354 from a multicopy plasmid causes a wee phenotype and rescues *cdc25-22* (Russell and Nurse, 1987a). Experiments were carried out to determine whether Nif1 interacts with the functional N-terminal domain of Nim1. In the two-hybrid assay, Nif1 interacted with the Nim1(1–354) construct, but failed to interact with the Nim1(1–291) construct (Figure 4A). These findings suggested that the 291–354 region of Nim1 is required for the interaction with Nif1. Further support for this conclusion was provided by the observation that Nim1(258–593) interacts with Nif1 in the yeast two-hybrid assay.

Experiments were carried out to determine whether the

CAT CCC GAT ACT TAA TAT TTG AGT GTC ATG GAG ACC ATG CAA AGC CGT ACT TAC GCA GGA TTG ACC AAG TTG AAT ACA ACT ACG GCT CTA
 M E T M Q S R T Y A G L T K L N T T T A L 21
 TTA AAC AAA AAG GAT GGA AAC GAT GAT GAC AAA GCA GAA CAT AGC AAA CGC AGT GGG TAC CAC GGA CTG TCA CCG CTA GAC GCT TTA GCT
 L N K K D G N D D D K A E H S K R S G Y H G L S P L D A L A 51
 TTA AAA CAT AGA GAC TTG AAT CGA AAG CTC AAT TTA CGA CTG ATG ATG TCC AAA TCT GAA GAC AAC TTA CAA ATT TTG AAA GAA ACA ACA
 L K H R D L N R K L N L R A M M S K S E D N L Q I L K E T T 81
 TCC GGA TCT TCG TCT GAC TTA AAC ATC GAA TCA CCC GCT TCA CCA GCT GAA GCT AGT TCT CCG TTT ACT GTT CGA ACC CCA ACC GTT
 S G S S S D L L N I E S P A S P A E A S S P F T V R T P T V 111
 CAT GAT CCG GAA CAC TAC TTT GTC GCA CAG AAA CTT AGC AGT GTT TTT GGA ACT CCA GAC TTG GAG GAT GAA ACA GAT TTT TTT GAT TAT
 H D P E H Y F V A Q K L S S V F G T P D L E D E T D F F D Y 141
 TTT AGC GCT GCC CCA GAT GTC CAT CCA AAC GAT ATC TTT GAC TCA TAT AAC TCA AAC AAT ATT GCT GAA TCC TTT GAT GAC GAT AAT TAT
 F S A A P D V H P N D I F D S Y N S N N I A E S F D D N Y 171
 TAC AAC TCT CTG TTA CCA CCT AAC GCA CCT TAT TAT CAT GAG ATT GAG CCT CCT AGA ACT GCT TCA AAC ACT TCA CCC ACA CCG AAT TCA
 Y N S L L P P N A P Y Y H E I E P P R T A S N T S P T P N S 201
 ATT AAA AGT GCA CAC CCT GCT GAA CCG CCA AAG AGA CCA GCT TTC AGG AGA AGT GCG ACT TCT CCG GAT AAG ATT TTG CCT ACT AGA ATA
 I K S A H P A E P P K R P A F T R S A T S P D K I L P T R I 231
 AAA TCG AAA GAT ACA GTA TCC TCG GGA GAC AGT ACT CCG CTC TCT GGA TCC TCT TCC AAA GGT ATG TTA ATG TCA ATG TCA ACG TCG
 K S K D T V S S G D S T P L S G S S S K G M L M S M S T S 261
 GAA AAC CAT TCA TTA TCT TCC AAT CCC GAA TTA TCA AAC TCA AAT TTA CTT GCT AAA AAC GAA TCA CCT GCG GAC GTT TCT AAT AAC GAA
 E N H S L S S N P E L S S N L L A K N E S P A D V S N N E 291
 TCT GGC AAC GAA TCA AGC AAG GAG CCT GAC AAG GAG CAC TCT ACC CCT ATC CAC CCA ACT ACT CCC GTT TCT CGC TGT GCT CGT CCA AGT
 S G N E S S K E P D K E H S T P I H P T T P V S R C A R P S 321
 TCT AGA CAA CAA ACC ATT TCC ATT TTG CAG GCA CAG TCT CCT TTT CCA AAA AAG TCA GAT AAG GAA CGA GCA AAT CTG AAC AAA ACT ATG
 S R Q Q T I S I L Q A Q S P F L K K S D K E R A N L N K T M 351
 GTT TCA ATA AAC TCC ATT AAT ATT CAT CAA TCG ATA CAC GAA ATT TCA TGT CCA CAT CAT TCC TCT TCT GAT AAT TGT CTG TTC ATA
 V S I N K S I N I H Q S I H E I S C P H H S S S D N C L F I 381
 CTT ATA TCT TTG ATG GAT AGA TTA CAC AGT CCA GTA TTA AAA CAA CTA GAT GTT TCT TTA CAA TCG CTG ACC ATG ACT GCT ATT CGG TAC
 L I S L M R L H S P V L K Q L D V S L Q S L T M T A I R Y 411
 ATC GAT TTA AAT TAT GTA GAT GTA CAG TAT ACA AAC CTG CGT GGT GGA GCT TAT GGA AAT GGA AAT AAC AGT GAA TCA AGC GAT AAT GCA
 I D L N Y V D V Q Y T N L R G G A Y G N G N N S E S S D N A 441
 CAA ATA AAA GAA GAG CAT TTG AAT CTG GCA ATT CAT TTC CAT CTT CTG AAT GAT CAT GAC AAA TGC TTT TGG CAT ACC GGT ATG GCT
 Q L K K E E H L N L A I H F H L L N D H D K C F W H T G M A 471
 TCT AGT TAC GAA GAT TAT ACG GCA ACC TTT ATC TAT GGT CTT TAT TTG CGT CAT GGA CTT GCT TGT TCA CCG AAA ACC CAT GTG TCC TTT
 S S Y E D Y T A T F I Y G L Y L R H G L A C S P K T H V S F 501
 CTA TTT TTG CTG AAA ACA GCA ACA CAA CTT TTA AAA CTA GTC GCA TGC CTC CAT TCA TCT GAT TTA GGC TTA AAC GAT ACA ACT CCA
 L F L L K T A T O L L N K L V E C L H S S D L G L N D T T P 531
 AAT GAG AAG CTA TCA ACT GAA TAC AAT CAA CAA CGC CTT CTT TTA GCT TTA ATA CTT TAT GAA TTG GGC GTA TGT TTT ATG CAT GGA TGG
 N E K L S T E Y N Q Q R L L L A L I L Y E L G V C F M H G W 561
 GGA ATA ACG CGG GAT CGT TAT CTG GCT TTG CAT TTA ATC AAG TTG AGC GGG GCA TGG GGC GAT GCT GAT GCG CAG TTT GAA GCT GGT CTT
 G I T R D R Y L A L H L I K L S G A W G D A D A Q F E A G L 591
 CAA ATG TCT TTG GGA GCT GTT TCA GAC AAG GAC TCT CAT ATG GCA GCT TAC TAT TAC CGT TTG GCT GGA TTT CAA GGG ATT TCA CCC CCT
 Q M S L G A V S D K D S H M A A Y Y Y R L A G F Q G I S P P 621
 TCG AAA TGC AAG TGG GTT TAT AAA TCG AAA TAT AGC TTA GCT GCT AAT CAT AAA GTG CCT GCT GCA AGC GAA GTG GCA ATY GTT TCG GCC
 S K C K V S Y K S K Y S L A A N H K V P A A S E V A Y V S A 651
 ATT GTA GAA AAT TTA GAA TCT CAC TCA CTG AAG TTT TCT ACA AAG CCA AAA GCT AAA TTA CGA AGT TTG ATT ACT TCT GTA AGG TAT TTG
 I V E N L E S S H S L K F S T T K P K A K L R S L I T S V R Y L 681
 TAA AAT CCC TCT CAT TCT TTT ACC ATT TTT TTT GTC TTT TTT TGT TTT TGT GCT ATT CAT TTA TTT GCA TTT TCC ACA ATA TGT GTT
 TGC TAG TTA GTC C

Fig. 2. The nucleotide sequence of *nif1*⁺ and predicted amino acid sequence of Nif1 protein. *nif1*⁺ encodes a protein of 681 amino acids with a predicted mol. wt of 75 kDa. The in-frame stop codon TAA in the 5' untranslated region is underlined. The boxed TCA codon indicates the 5' end of the 2.2 kb *nif1*⁺ cDNA clones obtained from the screen; the boxed TTT sequence indicates the 5' end of 1.8 kb *nif1*⁺ cDNA clones that were also obtained in the screen. The putative leucine zipper motif in the Nif1 protein sequence is also boxed (amino acids 505–526). The three putative PEST regions are underlined: amino acids 78–107, 234–252 and 280–298. The *nif1*⁺ accession number is U64574.

291–354 region is important for Nim1 mitotic inducer activity. This analysis revealed that the 291–354 region is essential for the mitotic induction activity of Nim1 (Figure 4A). Specifically, the Nim1(1–354) construct rescued the *cdc25-22* mutation and caused a wee phenotype when expressed in wild-type cells, consistent with previous studies (Russell and Nurse, 1987a), whereas the Nim1(1–291) construct failed to rescue *cdc25-22* and had no effect on cell size in wild-type cells. These findings were followed by an analysis of whether the 291–354 region of Nim1 is important for Nim1 *in vitro* protein kinase activity. GST–Nim1(1–354) and GST–Nim1(1–291) fusion proteins were expressed in bacteria and purified (Figure 4B). GST–Nim1(1–354) retained a vigorous auto-phosphorylation activity, whereas GST–Nim1(1–291) was inactive in this assay (Figure 4C).

The truncated forms of Nim1 were also tested for their ability to phosphorylate Wee1. Histidine-tagged Wee1 was expressed in insect cells and isolated by Ni²⁺-NTA affinity purification. The purified Wee1 was incubated with the GST–Nim1 fusion proteins in kinase assay conditions. The electrophoretic mobility of Wee1 was then analyzed by immunoblotting. Wee1 incubated with GST–Nim1(1–

354) exhibited reduced electrophoretic mobility (Figure 4D), this was due to phosphorylation of Wee1 carried out by Nim1 (Wu and Russell, 1993). In contrast, the mobility of Wee1 was unaffected by incubation with GST–Nim1(1–291), indicating that GST–Nim1(1–291) was unable to phosphorylate Wee1 (Figure 4D). These results show that the Nif1–Nim1 interaction is dependent on a short region of Nim1 protein, amino acids 291–354, that is crucial for Nim1 function.

Chromosomal disruption of *nif1*⁺ causes a reduction of cell size at division

A *nif1*⁺ gene disruption was performed in a diploid strain (Figure 5A). Two constructs were made, differing in the orientation of the *ura4*⁺ marker used for the disruption. Sporulation of the disrupted diploid cells gave rise to Ura⁺ haploid cells, indicating that *nif1*⁺ is not essential. The disruption of *nif1*⁺ was confirmed by Southern hybridization (Figure 5B). The growth rate of $\Delta nif1$ cells was indistinguishable from that of wild type (data not shown). However, as shown in Figure 5C, the $\Delta nif1$ cells underwent division at a reduced cell length (13.7 ± 0.7

Fig. 3. Nif1 associates with Nim1 in *S.pombe* cells. A *S.pombe* strain (PR109) was co-transformed with pREP1-Nim1 and pREP2-GST-Nif1 or pREP1-Nim1 and pREP2-GST. Transformants were grown in EMM2 – B₁ for 24 h to induce protein expression. Cells were lysed as described in Materials and methods. (A) Nim1 associates with GST–Nif1 in *S.pombe*. Cell extracts were incubated with glutathione beads. After washing with stringent buffer, bound proteins were analyzed by immunoblotting using affinity-purified α -Nim1 antibody. Nim1 was detected in association with GST–Nif1 (lane 1), but not with GST (lane 2). Lysates from Δ *nim1* cells (PR387) were used as an additional control (lane 3). (B) Immunoblot using total cell extracts showed that Nim1 was expressed in cells expressing Nim1 and GST–Nif1 (lane 1) and Nim1 and GST (lane 2), but not in Δ *nim1* cells (lane 3). (C) The same membrane in (A) was probed with α -GST antibody. GST–Nif1 (lane 1) and GST (lane 2) were detected. A large amount of the ~23 kDa GST was also detected in cells expressing GST–Nif1 (lane 1), perhaps indicating that GST–Nif1 is cleaved *in vitro* or is otherwise unstable. No proteins related to GST were detected in the Δ *nim1* control sample (lane 3).

and $13.6 \pm 0.9 \mu\text{m}$) as compared with an isogenic wild-type strain ($15.3 \pm 0.8 \mu\text{m}$).

Nif1 overexpression phenotypes strongly suggest that Nif1 inhibits Nim1

The discovery that mutational inactivation of Nif1 causes cells to undergo mitosis at a reduced cell size suggested that Nif1 may function as a mitotic inhibitor, perhaps by inhibiting Nim1. This possibility was explored further by examining whether Nif1 overexpression affected mitotic timing. Expression of the *nif1*⁺ open reading frame was placed under the control of the thiamine-repressible *nmt1* promoter in plasmid pREP1 (Maundrell, 1993). High expression of Nif1 in a wild-type background had no effect on growth rate or colony-forming ability (data not shown). However, high Nif1 expression caused significant cell elongation, with cells dividing at $19.4 \pm 0.9 \mu\text{m}$ (Table I), a size that is very similar to Δ *nim1* cells (Russell and Nurse, 1987a). Cells transformed with control pREP1 vector divided at $14.5 \pm 0.8 \mu\text{m}$ (Table I). These results strongly suggest that Nif1 functions as a mitotic inhibitor.

This finding was followed by an investigation of the effect of Nif1 overproduction in a *cdc25-22* strain. Cells carrying the *cdc25-22* mutation, which are moderately elongated at the permissive temperature of 25°C, are very sensitive to mutational inactivation of Nim1 (Feilottter *et al.*, 1991). Thus, if Nif1 inhibits Nim1, then overproduction of Nif1 should exacerbate the *cdc25-22* cell cycle delay phenotype. Clonal isolates of *cdc25-22* cells transformed with pREP1-Nif1, which carries the *nmt1:nif1*⁺ construct, were grown in *nmt1*-repressing medium (EMM2 + B₁) or in *nmt1*-inducing medium (EMM2 – B₁) for 24 h at 20°C and then incubated for 4 h at 32°C. As predicted by the model in which Nif1 acts as a Nim1 inhibitor, overproduction of Nif1 caused the *cdc25-22*

Fig. 4. The Nif1–Nim1 two-hybrid interaction requires an essential region in the non-catalytic domain of Nim1 protein kinase. (A) Amino acids 291–354 of Nim1 are required for the Nif1–Nim1 interaction and for Nim1 mitotic inducer activity. The organization of Nim1 protein kinase is illustrated in the left panel. The protein kinase homology domain is located in amino acids 1–257. Full-length Nim1(1–593), Nim1(1–354) and Nim1(258–593) interacted with Nif1 in the two-hybrid assay, whereas Nim1(1–291) failed to interact with Nif1. Likewise, full-length Nim1 and Nim1(1–354) rescued *cdc25-22*, whereas Nim1(1–291) failed to rescue *cdc25-22*. The results of the kinase assay experiments are also summarized here. (B) Expression of GST–Nim1(1–354) and GST–Nim1(1–291) in bacteria. The soluble lysates of bacteria expressing GST–Nim1(1–354) (lane 1) or GST–Nim1(1–291) (lane 2) were incubated with glutathione beads. Bound proteins were separated by SDS–PAGE and visualized by Coomassie blue staining. Note that a small amount of truncated or degraded GST–Nim1(1–354) that runs near the position of GST–Nim1(1–291) was detected in lane 1. (C) *In vitro* autophosphorylation of GST–Nim1. Purified GST–Nim1(1–354) and GST–Nim1(1–291) were incubated in kinase assay buffer and [γ -³²P]ATP as described (Wu and Russell, 1993). Autoradiography revealed that only GST–Nim1(1–354) became phosphorylated in this assay. (D) Phosphorylation of Wee1 by GST–Nim1 *in vitro*. Wee1 was produced in Sf9 insect cells and purified on Ni²⁺-NTA beads as described (Wu and Russell, 1993). Wee1 was incubated alone or with the purified GST–Nim1 fusion proteins in kinase assay conditions. Phosphorylation of Wee1 by Nim1 causes Wee1 to migrate with reduced electrophoretic mobility (Wu and Russell, 1993). The electrophoretic mobility of Wee1 was analyzed by immunoblotting using affinity-purified α -Wee1 antibody 7373. Only GST–Nim1(1–354) caused Wee1 to migrate with reduced electrophoretic mobility (lane 3), indicating that GST–Nim1(1–354) phosphorylates Wee1, whereas GST–Nim1(1–291) is unable to phosphorylate Wee1.

cells to be highly elongated as compared with *cdc25-22* cells transformed with pREP1 (Figure 6A). In fact, *cdc25-22* cells transformed with pREP1-Nif1 were unable to form colonies when incubated on EMM2 – B₁ plates at 25°C, whereas pREP1 transformants of *cdc25-22* cells exhibited excellent growth in the same conditions (Figure 6B). Both pREP1 and pREP1-Nif1 transformants of *cdc25-22* cells readily formed colonies when incubated in media that repressed the activity of the *nmt1* promoter. These data confirmed that overexpression of Nif1 causes a delay of mitosis.

Fig. 5. $\Delta nif1$ mutation causes a reduction of cell size at division. (A) Map of the *nif1::ura4⁺* disruption constructs. (B) Southern hybridization analysis of genomic DNA from a *nif1⁺/nif1⁺* diploid (lane 1), a *nif1⁺/nif1::ura4⁺(1)* diploid (lane 2), a *nif1⁺* haploid (lane 3) and a *nif1::ura4⁺(1)* haploid (lane 4), confirming the *nif1⁺* disruption. DNA was digested with *EcoRV* and probed with the 6 kb *nif1⁺* *HindIII* fragment. (C) Cell division size data for *nif1::ura4⁺* disruptant haploids and an isogenic *nif1⁺* control.

Table I. Cell size at division

Strain	Plasmid	Length at division (μm)
<i>h⁻ leu1-32 ura4-D18</i>	pREP1	14.5 ± 0.8
<i>h⁻ leu1-32 ura4-D18</i>	pREP1-Nif1	19.4 ± 0.9
<i>h⁻ leu1-32 ura4-D18 Δnim1</i>	pREP2	19.3 ± 1.4
<i>h⁻ leu1-32 ura4-D18 Δnim1</i>	pREP2-Nif1	20.4 ± 1.5

If Nif1 delays mitosis by a mechanism that exclusively involves Nim1, then Nif1 overexpression should cause no phenotype in a $\Delta nim1$ background. Consistent with this prediction, we found that Nif1 overexpression had no effect on the ability of $\Delta nim1$ cells to form colonies. Cell size measurements showed that there was no significant difference in cell division length between $\Delta nim1$ cells harboring the pREP2 control vector ($19.3 \pm 1.4 \mu m$) and $\Delta nim1$ cells carrying pREP2-Nif1 ($20.4 \pm 1.5 \mu m$) when grown in EMM2 – B₁ (Table I). These results suggest that Nif1 and Nim1 operate through the same pathway.

Overproduction and inactivation of Nim1 have no effect in a $\Delta wee1$ background, a finding consistent with the conclusion that Wee1 is the exclusive target of Nim1 regulation (Russell and Nurse, 1987a). If Nif1 specifically inhibits Nim1, then overproduction of Nif1 should have no effect in $\Delta wee1$ cells. This prediction was confirmed: $\Delta wee1$ cells transformed with pREP1-Nif1 or pREP1 plasmids exhibited identical wee phenotypes when grown in EMM2 – B₁ (data not shown).

Mik1 and Wee1 are redundant protein kinases that phosphorylate Cdc2 on tyrosine-15. Simultaneous inactivation of Wee1 and Mik1 causes a lethal mitotic catastrophe phenotype (Lundgren *et al.*, 1991). If Nif1 specifically inhibits Nim1, then overexpression of Nif1 should not rescue the lethality of *wee1-50 Δmik1* cells at 35°C. Indeed, Nif1 overexpression failed to rescue *wee1-50 Δmik1* mitotic catastrophe (Figure 6C), providing

Fig. 6. Nif1 overproduction delays mitosis. (A) A *cdc25-22* strain (PR196) transformed with pREP1-Nif1, which contains a *nmt1::nif1⁺* construct, was grown on *nmt1*-repressing medium (EMM2 + B₁) or *nmt1*-inducing medium (EMM2 – B₁) for 24 h at 20°C and then incubated for 4 h at 32°C. Cells expressing Nif1 (right panel) became highly elongated as compared with those which repressed Nif1 expression (left panel). (B) Nif1 overexpression is lethal in *cdc25-22* cells incubated at 25°C. Transformant *cdc25-22* cells described in (A) were incubated on EMM2 + B₁ and EMM2 – B₁ media at 25°C. The *cdc25-22* cells expressing Nif1 were unable to form colonies. (C) Overexpression of Nif1 does not rescue the lethal mitotic catastrophe caused by inactivation of Wee1 and Mik1. A *wee1-50 Δmik1* strain (PR754) was transformed with pREP1 or pREP1-Nif1. At the permissive temperature of 25°C, cells are viable regardless of the expression of Nif1 (upper panel). Nif1 failed to rescue lethality when cells were incubated at the restrictive temperature of 35°C (lower panel).

further support for the conclusion that Nif1 overexpression delays mitosis by inhibiting Nim1.

Inhibition of Nim1 by Nif1 in an insect cell expression system

These studies suggested that Nif1 may have a direct role in regulating the ability of Nim1 to phosphorylate and inhibit Wee1. As a first step in exploring this mechanism of regulation, we asked whether Nif1 was capable of inhibiting Nim1 in an insect cell expression system. Previous studies showed that Nim1 is able to phosphorylate and inactivate Wee1 in insect cells (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). The phosphorylation of Wee1 is accompanied by a reduction of the mobility of Wee1 in SDS–PAGE. A recombinant baculovirus was constructed to direct the expression of HA epitope-tagged Nif1 protein. Infection of Sf9 cells with this virus led to the production of Nif1 as a protein that migrated with the apparent mol. wt of ~97 kDa

Fig. 7. Nif1 inhibits the ability of Nim1 to phosphorylate Wee1 in Sf9 insect cells. **(A)** Expression of Nif1 in Sf9 cells. Sf9 cells were infected with a recombinant baculovirus expressing Nif1-6His2Ha as described in Materials and methods. Cells were harvested 55 h post-infection and lysed in SDS sample loading buffer. Total protein extract from control cells (lane 1) or cells expressing Nif1 (lane 2) was separated by SDS-PAGE and analyzed by immunoblotting using anti-HA monoclonal antibody 12CA5. Nif1-6His2Ha was detected as a series of bands migrating at the ~97 kDa region of the gel. **(B)** Nif1 inhibits Nim1's ability to phosphorylate Wee1 in Sf9 cells. Sf9 cells were infected with recombinant baculoviruses expressing Wee1 alone (lane 1), Wee1 and Nim1 (lane 2), or Wee1, Nim1 and increasing amounts of Nif1 as indicated (lanes 3–5). The multiplicity of infection (MOI) was 10 for Wee1 and 20 for Nim1 in all infections. For Nif1, the MOI was 20 (1×, lane 3), 40 (2×, lane 4) and 80 (4×, lane 5). Cells were harvested at 55 h post-infection. Samples were treated as described above. Wee1 was detected by immunoblotting using affinity-purified anti-Wee1 antibody 7451. Infection of cells with viruses expressing Wee1 and Nim1 caused Wee1 phosphorylation, as indicated by the slower mobility of Wee1 in SDS-PAGE (lane 2). Infection of cells with increasing amounts of recombinant viruses expressing Nif1 inhibited the ability of Nim1 to phosphorylate Wee1, as indicated by the fast mobility of Wee1 in SDS-PAGE (lanes 3–5).

(Figure 7A). Co-infection of Sf9 cells with viruses encoding Wee1 and Nim1 caused the Wee1 to migrate with reduced mobility (Figure 7B, lanes 1 and 2), confirming earlier findings. This effect was largely abolished by the additional infection of cells with the virus encoding Nif1 (Figure 7B, lanes 3–5). The ability of Nif1 to block the Nim1-mediated phosphorylation of Wee1 was dependent on the dose of Nif1 virus. These findings strongly suggest that Nif1 is able to inhibit Nim1 by a direct mechanism, a suggestion consistent with the detection of an *in vivo* physical interaction involving Nif1 and Nim1.

Discussion

We have used the yeast two-hybrid system to identify a protein, Nif1, that specifically interacts with Nim1 protein kinase. A series of findings strongly suggest that the Nif1–Nim1 interaction is specific and has functional significance in regulating Nim1 activity *in vivo*. First, in the two-hybrid assay, Nif1 cDNA clones were repeatedly isolated, accounting for ~95% of all positive clones. Second, GST–Nif1 fusion protein expressed in *S.pombe* was found to co-precipitate with Nim1. Third, the Nif1–Nim1 interaction is dependent on a short region of Nim1 protein adjacent to the kinase homology domain that is essential for *in vitro* and *in vivo* activities of Nim1. Fourth, gene disruption of *nif1*⁺ causes advancement of mitosis at a small cell size, while Nif1 overproduction causes a delay of mitosis. Fifth,

by a variety of genetic tests, Nif1 overproduction causes a phenotype that is phenotypically equivalent to the Δ *nim1* mutation. Specifically, Nif1 overproduction is synthetically lethal with *cdc25-22*, has no genetic interaction with Δ *nim1* or Δ *wee1* mutations, and fails to rescue *wee1-50* Δ *mik1* mitotic catastrophe. Sixth, in an insect cell expression system, Nif1 reduces the phosphorylation of Wee1 by Nim1. These findings strongly support a model in which Nif1 functions as an inhibitor of mitosis via a direct interaction with Nim1.

It remains to be determined exactly how Nif1 regulates Nim1 activity *in vivo*. Perhaps the simplest possibility is that Nif1 directly inhibits Nim1 kinase by interacting with a domain required for kinase activity or substrate interaction. As noted above, the Nif1–Nim1 interaction is dependent on a small region of Nim1 that follows the kinase homology domain. This region of Nim1, occurring in amino acids 291–354, is predicted to have an amphihelix structure (Feilolter *et al.*, 1991). This region is crucial for Nim1 autophosphorylation activity and for phosphorylation of Wee1.

Another possibility is that Nif1 functions by sequestering Nim1 or otherwise modulating the cellular localization of Nim1. We suggest this possibility because recent studies have shown that Nim1 protein is predominantly localized in the cytoplasm, while its substrate Wee1 is predominantly localized in the nucleus (Wu *et al.*, 1996). It remains to be determined how Nim1 is able to phosphorylate Wee1 when the two proteins predominantly reside in different cellular compartments. One explanation could be that Nim1 shuttles between the nucleus and the cytoplasm. If Nif1 were to act as a cytoplasmic anchor for Nim1, then one might expect Nif1 to regulate Nim1 negatively by restricting its access to Wee1. However, it seems unlikely that this mechanism of regulation would be faithfully replicated in an insect cell expression system, so we favor the possibility of direct inhibition of Nim1 by Nif1.

What is the relationship between Nif1 and mitotic control in other organisms? In general, the mitotic control mechanism appears to be highly conserved between divergent species. For example, human and *Xenopus* homologs encoding Cdc2, cyclin-B, Cdc25 and Wee1 have been cloned and shown to have functionally equivalent roles to the *S.pombe* homologs (Dunphy, 1994). Likewise, homologs of these four genes have been cloned from the highly divergent budding yeast *Saccharomyces cerevisiae*, where they are known as Cdc28, Clb1 through Clb6, Mih1 and Swe1, respectively (Russell *et al.*, 1989; Reed, 1992; Booher *et al.*, 1993; Nasmyth, 1993). Amongst the broad variety of eukaryotic species in which the mitotic control has now been studied, *S.cerevisiae* is exceptional in that the level of inhibitory tyrosine phosphorylation of Cdc28 is normally quite low and of no consequence (Amon *et al.*, 1992; Sorger and Murray, 1992), except in the situation in which a novel morphogenesis checkpoint is activated in response to impaired bud formation (Lew and Reed, 1995). Consistent with the conclusion that inhibitory tyrosine phosphorylation of Cdc28 is normally of little importance, disruption of *MIH1* has very little effect in otherwise wild-type cells (Russell *et al.*, 1989). These findings have been rather puzzling, suggesting that perhaps Swe1 is normally only weakly active.

Table II. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Reference
PR109	<i>h⁻ leu1-32 ura4-D18</i>	lab stock
PR170	<i>h⁻ leu1-32 ura4-D18 wee1-50</i>	lab stock
PR196	<i>h⁺ leu1-32 ura4-D18 cdc25-22</i>	lab stock
PR387	<i>h⁻ leu1-32 ura4-D18 nim1::LEU2</i>	lab stock
972	<i>h⁻</i>	lab stock
PR754	<i>h⁻ leu1-32 ura4-D18 wee1-50 mkl1::ura4⁺</i>	lab stock
PR1677	<i>h⁻ ura4-D18 nif1::ura4⁺(1)</i>	this study
PR1678	<i>h⁻ ura4-D18 nif1::ura4⁺(2)</i>	this study

Very recently, a new *S.cerevisiae* gene has been described, *HSL1*, mutations of which cause a phenotype which is very similar to that observed when *S.pombe* *wee1⁺* or *S.cerevisiae* *SWE1* are overexpressed in budding yeast (Ma *et al.*, 1996). The key features of the phenotype are a greatly lengthened G₂ phase and highly elongated buds. Importantly, the protein sequence of Hsl1 is most similar to *S.pombe* Nim1. These findings suggest that Hsl1 and Nim1 are functional homologs, and that Hsl1 is quite effective at inhibiting Swel in wild-type cells. The discovery of Hsl1 is the first evidence that Nim1 is conserved amongst divergent eukaryotes. This suggests that Nif1 may also be conserved. However, in this regard, we note that sequence homology searches have failed to identify obvious candidates for Nif1 homologs in the recently completed *S.cerevisiae* genome, showing that Nif1 is not highly conserved at the sequence level in budding yeast.

In summary, we have identified a novel mitotic inhibitor, Nif1, which functions by inhibiting or otherwise negatively regulating the activity of Nim1 protein kinase. Nim1 acts by inhibiting Wee1 tyrosine kinase, which in turn inhibits Cdc2–Cdc13 cyclin-dependent kinase. It is striking that the mitotic control should include such a long cascade of negative regulation of protein kinases. Future experiments will be aimed at better understanding the biochemical properties of the Nif1–Nim1 interaction.

Materials and methods

Yeast strains, media and library screening

Fission yeast strains used in this study are listed in Table II. Standard procedures for genetic studies of *S.pombe* were carried out as described (Moreno *et al.*, 1991). YES and synthetic EMM2 media were used to grow *S.pombe* cells (Moreno *et al.*, 1991). Individual cell size measurements were performed using an eyepiece micrometer attached to a Zeiss Axioskop 20 microscope with a 100× objective. At least 20 cells from each culture were measured. Standard procedures for genetic studies in *S.cerevisiae* have been described (Guthrie and Fink, 1991). YEPD and synthetic SSC media were used to grow *S.cerevisiae*. Library screening and the filter lift assay were performed as described (Durfee *et al.*, 1993), except that nitrocellulose membranes were substituted for nylon membranes.

Expression of GAL4–Nim1 fusion proteins in *S.cerevisiae*

The *nif1⁺* open reading frame was cloned into the *Bam*HI/*Sal*I sites of plasmid pAS2 by polymerase chain reaction (PCR), using pREP1–Nim1 as the template. The 3′ primer for pLW14 [pAS2–Nim1(1–593)] and pLW114 [pAS2–Nim1(258–593)] was LW9312: 5′-CCAGGATCCA AACCGTCGAC GAATTCGTTA ATCCTTCCGA AAGAATGA-3′. The 3′ primer for pLW109 [pAS2–Nim1(1–291)] was LW9405: 5′-CCGTCGACGT TAACACATGC AATCAACTAC TAA-3′. The 3′ primer for pLW110 [pAS2–Nim1(1–354)] was LW9406: 5′-CCGTCGACGT TAGGTGAAAA GATTATTGTC GTG-3′. The 5′ PCR primer used for construction of pLW14, pLW109 and pLW110 was LW9206: 5′-CCGAA-

TTCGG GATCCTCATG GTGAAGCGAC AAAAAAT-3′. The 5′ primer used for construction of pLW114 was LW9403: 5′-CCGGGG-ATCC TCATGGGCTG TACATCATTG AGC-3′.

Plasmid recovery from *S.cerevisiae* and cloning of genomic *nif1⁺*

Y190 cells containing pAS2–Nim1 and pACT–cDNA plasmids were grown in patches on SSC–Leu plates for 1–2 days, allowing loss of pAS2–Nim1 and enrichment for pACT–cDNA plasmids. Cells were collected into a 1.5 ml microfuge tube containing 0.2 ml of DNA extraction buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% SDS]. After resuspending the cells, 0.2 ml of phenol/chloroform (1:1) was added to the tube, followed by the addition of glass beads to the meniscus. The cells were vortexed vigorously at room temperature for 30 s. The supernatant was re-extracted with phenol/chloroform two more times, the DNA was then precipitated with ethanol and resuspended in 20 µl of water. One microliter of the DNA was used to transform *Escherichia coli* by electroporation using a Cell-Porator™ Voltage Booster according to the manufacturer's instructions (BRL Life Technologies, Inc.). Cells were allowed to recover for 40–60 min in 1 ml of LB medium at 37°C with shaking and then plated on LB + 50 µg/ml Amp plates. Plasmid DNA was prepared from several transformants. pACT–cDNA was identified by *Xho*I digestion.

Membranes containing overlapping cosmid or P1 clones of *S.pombe* genomic DNA (Hoheisel *et al.*, 1993) were probed with the *nif1* sequence present in the 2.2 kb *Xho*I fragment of pACTII–cDNA#1. The coordinates of the positive clones revealed that P1 clone 28C4p contains the complete *nif1⁺* genomic sequence. The plasmid form of 28C4p was obtained and analyzed by restriction enzyme mapping. A 6 kb *Hind*III fragment containing *nif1⁺* was cloned into pBluescript.

Expression of Nif1 in *S.pombe* cells

The complete open reading frame of *nif1⁺* was cloned into pREP1 by PCR. Plasmid pLW81, which contains the 6 kb genomic *Hind*III sequence of *nif1⁺*, was used as template. The sequences of the PCR primers were: (40PCR1) 5′-GAGACTAGAT CTATGGAGAC CATGCAAAGC CGT-3′ and (40PCR2) 5′-CCGCGGCCGC CCAAATACCT TACAGAAAGTA ATC-3′. The PCR products were digested with *Bgl*II/*Not*I and cloned into the *Bgl*II/*Not*I sites of plasmid pREP1–Wee1–Δ1, creating plasmid pLW111 (pREP1–Nif1). This plasmid contains the complete *nif1⁺* open reading frame fused at its C-terminus to the Ha6H sequence cassette encoding two copies of the Ha epitope followed by six consecutive histidine residues. Expression of *nif1⁺*–Ha6H is regulated by the thiamine-repressible *nmt1* promoter (Maundrell, 1993). The *Pst*I/*Sst*I fragment of pLW111 was cloned into the same sites of pREP2 (Maundrell, 1993), creating plasmid pREP2–Nif1 (pLW121). To express Nif1 as a GST–Nif1 fusion protein in fission yeast, the *wis1⁺* *Bam*HI/*Sst*I fragment of pREP2–KZ–Wis1 (Shiozaki and Russell, 1995) was replaced with the *Bgl*II/*Sst*I *nif1⁺* fragment of pLW111. Expression of Nif1 protein from these constructs was confirmed by immunoblot analysis using monoclonal α-Ha antibody 12CA5.

Interaction of GST–Nif1 with Nim1 in *S.pombe* cells

Schizosaccharomyces pombe strain PR109 was co-transformed with pREP1–Nim1 and pREP2–GST–Nif1 or with pREP1–Nim1 and pREP2–GST. Transformants were grown in liquid EMM2 + B₁ (EMM2 supplemented with thiamine) to mid-log phase. Cells were collected on membrane filters, washed several times with fresh EMM2, and then resuspended in EMM2 at ~0.2 OD₆₀₀ and incubated at 30°C. Cells were diluted into EMM2 after 12 h to keep them in log phase. After 24 h of induction, samples were collected and ~20 OD₆₀₀ of cells were lysed in a 1.5 ml microfuge tube with 0.5 ml of lysis buffer [50 mM Tris (pH 8.0), 50 mM NaF, 500 mM NaCl, 1% NP-40, 5 mM EDTA, 10% glycerol, 5 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)]. Glass beads were added to the samples and the tubes were vortexed vigorously in the cold room for 5 min. The supernatant was collected after a high-speed centrifugation (10 min at 14 000 g). The protein concentration in each sample was estimated by measuring the OD₂₈₀ of diluted samples. Equal amounts of proteins were incubated with 20 µl of glutathione–Sepharose 4B (Pharmacia) that had been washed with the same lysis buffer. Binding was performed at 4°C for 1 h on a rolling platform. The beads were washed several times with lysis buffer. Samples were boiled at 100°C for 3 min. Nim1, GST–Nif1 or GST were detected by immunoblot analysis using affinity-purified α-Nim1 antibody 9805 or α-GST sera.

Nim1 and Wee1 kinase assays

Escherichia coli strain DH5 α was transformed with plasmids pLW1 (pGEX-KG-Nim1-cata) (Wu and Russell, 1993) or pLW6 [pGEX-KG-Nim1(1–294)] to express recombinant GST–Nim1 fusion proteins. pLW6 was created by PCR, using pLW1 as the template. The sequences of the PCR primers were LW9206 5'-CCGAATTCGG GATCCTCATG GTGAAGCGGAC ACAAAAAT-3' and LW9302 5'-TATCAATGCGGCCGCCCAA AGCACACACA TGCAA-3'. Wee1 was expressed in Sf9 cells as described (Wu and Russell, 1993). GST–Nim1 fusion proteins were purified using glutathione–Sephadex 4B and Wee1 protein was purified using Ni²⁺-NTA matrix (Quiagen). Kinase assays were performed as described (Wu and Russell, 1993).

Gene disruption of *nif1*⁺

The 1.8 kb *HindIII ura4*⁺ fragment was treated with Klenow DNA polymerase to blunt the DNA ends and cloned into the *SmaI* site of pSP72 in both orientations, creating plasmids pLW135 and pLW136. The *HindIII/SalI* fragment of pLW81, containing the *nif1*⁺ open reading frame, was cloned into the *HindIII/SalI* sites of plasmid pGEM9Z, creating pLW107. The *ura4*⁺ *KpnI/XbaI* fragments of pLW135 and pLW136 were cloned into the *KpnI/XbaI* sites of pLW107. The resulting plasmids, pLW137 and pLW138, contain *nif1::ura4*⁺(1) and *nif1::ura4*⁺(2), respectively. A diploid strain was made by mating CHP428 (*h*⁺ *leu1-32 ura4-D18 ade6-210 his7-366*) to CHP429 (*h*⁺ *leu1-32 ura4-D18 ade6-216 his7-366*), kindly provided by C.Hoffman. After 24 h, the mating mix was streaked out on EMM2+LUH (EMM2 supplemented with leucine, uracil and histidine) to select for diploid cells. After identifying diploids with high sporulation efficiency, a single diploid colony was transformed with pLW137 and pLW138 digested with *HindIII* and *SalI*. Transformants were grown on EMM2+LH plates supplemented with 3% glucose to select for *Ura*⁺ colonies and repress sporulation. The cells were then replica printed to 5' fluoroorotic acid (FOA) plates, which are toxic to *Ura*⁺ cells. Genomic DNA was extracted from 20 of the 5'FOA-sensitive colonies and Southern hybridization was performed using the 6 kb *nif1*⁺ genomic fragment from pLW81 as the probe. Eighteen of these clones had one wild-type and one disrupted allele of the *nif1* gene. Several of the *nif1*⁺/*nif1::ura4*⁺ diploids were sporulated and *Ura*⁺ colonies were easily obtained. Genomic DNA Southern hybridization confirmed that these haploid *Ura*⁺ colonies were *nif1::ura4*⁺. In the course of this analysis, it was discovered that *nif1*⁺ is tightly linked to the mating type locus.

Inhibition of Nim1 by Nif1 in insect cells

The recombinant baculoviruses that direct the expression of Nim1 and Wee1 have been described (Wu and Russell, 1993). Nif1-6His2Ha was expressed in Sf9 cells using the Bac-To-Bac™ Baculovirus Insect cells Expression System (Gibco BRL). The *BglIII-SmaI* fragment of pLW111 (pREP1-Nif1-6His2Ha) was cloned into the *BglIII-SmaI* sites of plasmid SP72, creating plasmid pLW227. The *BglIII-KpnI* fragment of pLW227 was replaced with a DNA fragment which contained the Kozac sequence before the translational initiation codon. The sequences of the PCR primers were: (Nif1-5ACC) 5'-GAGACTAGAT CTACCATGGA GACCATGCAA AGCC-3' and (40-1) 5'-GTGAAGCGGG TGATTTCGAT-3'. The resulting plasmid was named pLW230. The *BglIII-HindIII* fragment of pLW230 was cloned into the *BamHI-HindIII* sites of pFASTBAC1 to create plasmid pFASTBAC1-Nif1-6His2Ha(pLW231). A recombinant baculovirus expressing Nif1-6His2Ha was generated following the manufacturer's instruction. Expression of recombinant Nif1 was performed as described. Nif1 was detected using α -Ha antibody.

Sf9 insect cells were infected by recombinant baculoviruses expressing Wee1, Nim1 and Nif1 alone or in combinations as indicated in various experiments. Cells were harvested 55 h post-infection and lysed in SDS sample loading buffer. Total protein extract was separated on 7.5% SDS–PAGE. Wee1 protein was detected by affinity-purified α -Wee1 antibody 7451.

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