Molecular Biology of the Cell
Vol. 9, 3321–3334, December 1998

The Protein Kinase Cdr2, Related to Nim1/Cdr1 Mitotic Inducer, Regulates the Onset of Mitosis in Fission Yeast

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Submitted June 24, 1998; Accepted September 21, 1998
Monitoring Editor: Mitsuhiro Yanagida

Cdc2–Cyclin B, the protein kinase that catalyzes the onset of mitosis, is subject to multiple forms of regulation. In the fission yeast *Schizosaccharomyces pombe* and most other species, a key mode of Cdc2–Cyclin B regulation is the inhibitory phosphorylation of Cdc2 on tyrosine-15. This phosphorylation is catalyzed by the protein kinases Wee1 and Mik1 and removed by the phosphatase Cdc25. These proteins are also regulated, a notable example being the inhibition of Wee1 by the protein kinase Nim1/Cdr1. The temperature-sensitive mutation *cdc25–22* is synthetic lethal with *nim1/cdr1* mutations, suggesting that a synthetic lethal genetic screen could be used to identify novel mitotic regulators. Here we describe that such a screen has identified *cdr2*$^+$, a gene that has an important role in the mitotic control. Cdr2 is a 775 amino acid protein kinase that is closely related to Nim1 and mitotic control proteins in budding yeast. Deletion of *cdr2* causes a G2-M delay that is more severe than that caused by *nim1/cdr1* mutations. Genetic studies are consistent with a model in which Cdr2 negatively regulates Wee1. This model is supported by experiments showing that Cdr2 associates with the N-terminal regulatory domain of Wee1 in cell lysates and phosphorylates Wee1 in vitro. Thus, Cdr2 is a novel mitotic control protein that appears to regulate Wee1.

INTRODUCTION

In eukaryotic organisms the onset of mitosis is brought about by a protein kinase consisting of Cdc2 and Cyclin B. Cdc2 is the catalytic subunit, whereas Cyclin B is an essential regulatory component. The onset of mitosis marks a major transition in the cell cycle, involving a commitment to complete the cycle and undergo cell division. Thus, it is perhaps not surprising that Cdc2–Cyclin B is subjected to many forms of regulation. For example, in the fission yeast *Schizosaccharomyces pombe*, the timing of the onset of mitosis determines cell size; thus the mechanism that controls activation of Cdc2–Cyclin B is apparently able to sense cell size. Similarly, checkpoint controls that monitor DNA replication and repair regulate the activation of Cdc2–Cyclin B (Enoch and Nurse, 1990; Rhind *et al.*, 1997; Rhind and Russell, 1998). These checkpoints ensure that DNA is fully replicated and repaired before the onset of mitosis.

Much effort has been devoted to understanding how the activity of Cdc2–Cyclin B is regulated. In the fission yeast there appear to be at least four major mechanisms of regulating Cdc2–Cyclin B. The first involves the degradation of Cdc13, the major B-type cyclin in *S. pombe*. Cdc13 is specifically proteolyzed after exit from the mitotic (M) phase of the cell cycle (Yamano *et al.*, 1996). The second mechanism involves the protein Rum1, which binds and inhibits Cdc2–Cdc13 (Correa-Bordes and Nurse, 1995). Rum1 is important for regulating Cdc2–Cdc13 during G1 phase in cells that are attempting to undergo mating or meiosis (Moreno and Nurse, 1994). The third mechanism involves phosphorylation of Cdc2 on threonine-167. This phosphorylation is required for Cdc2–Cdc13 protein kinase activity (Gould *et al.*, 1991). Phosphorylation of threonine-167 does not appear to change during the cell cycle; thus it may not have an important role in

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regulating the cell cycle. The fourth mechanism of regulating Cdc2–Cdc13 involves the phosphorylation of Cdc2 on tyrosine-15 (Gould and Nurse, 1989). This phosphorylation is inhibitory and appears to play a crucial role in the cell size and checkpoint mechanisms mentioned above.

In fission yeast, phosphorylation of Cdc2 on tyrosine-15 is catalyzed by the protein kinases Wee1 and Mik1. Inactivation of Wee1 causes a wee phenotype. Thus the temperature-sensitive *wee1–50* mutation cause cells incubated at 35°C to divide at a cell length of ~7.5 μm, approximately half the size of wild-type cells (Russell and Nurse, 1987a). Inactivation of Mik1 has no effect on cell size, but simultaneous inactivation of Wee1 and Mik1 causes a lethal premature mitosis phenotype in which mitosis is initiated before DNA replication is complete (Lundgren *et al.*, 1991). Tyrosine-15 on Cdc2 is dephosphorylated by the phosphatases Cdc25 and Pyp3. Cdc25 contributes the major activity that dephosphorylates tyrosine-15 (Millar *et al.*, 1991). Cdc25 is normally essential for mitosis, although *wee1 cdc25* cells are viable. Pyp3 is required for mitotic induction in a *wee1 cdc25* background (Millar *et al.*, 1992).

There appear to be multiple mechanisms for modulating the protein kinases and phosphatases that regulate phosphorylation of Cdc2 on tyrosine-15. Cdc25 is activated by phosphorylation in M phase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993; Kovelman and Russell, 1996). The identities of the protein kinases that activate Cdc25 are uncertain, but in vitro studies suggest that Cdc2–Cyclin B and members of the Polo kinase family are directly involved (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Kuang *et al.*, 1994; Kumagai and Dunphy, 1996; Descombes and Nigg, 1998). It is thought that the activation of Cdc25 plays an important role in a positive feedback loop that is required for the induction of mitosis, although this hypothesis remains to be proven. Cdc25 also appears to be negatively regulated by Chk1, a protein kinase that is required for the repair checkpoint in fission yeast (Furnari *et al.*, 1997). Recently, the peptidyl-prolyl isomerase Pin1 was shown to bind and inhibit phosphorylated Cdc25 in human cells and *Xenopus* egg extracts (Crenshaw *et al.*, 1998; Shen *et al.*, 1998), suggesting a possible role for Pin1 in the mitotic control.

Wee1 and Mik1 are also regulated by multiple mechanisms. Studies of Wee1 in human cells and *Xenopus* egg extracts have shown that Wee1 is inhibited by phosphorylation during M phase (McGowan and Russell, 1995; Mueller *et al.*, 1995; Watanabe *et al.*, 1995). This regulation might also be part of a positive feedback loop to activate Cdc2–Cyclin B at the transition from G2 to M. Wee1 and Mik1 appear to also be regulated by the replication checkpoint that couples the onset of mitosis with the completion of DNA synthesis (Boddy *et al.*, 1998). Thus Cds1, a protein kinase that is activated by the replication checkpoint, associates with and phosphorylates Wee1 in cell lysates. Cds1 is also required for the large increase in the amount of Mik1 that occurs in cells arrested by the replication checkpoint.

In fission yeast, Wee1 is inhibited by the protein kinase Nim1 (Russell and Nurse, 1987b; Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Deletion of *nim1*$^+$ causes a cell elongation phenotype that is suppressed by *wee1* mutations. Overproduction of Nim1 causes a wee phenotype that is not additive with *wee1* mutations but causes lethal premature mitosis in *mik1* cells (Lundgren *et al.*, 1991). These data argue strongly that Wee1 is the sole target of Nim1. Recently, the *nif1*$^+$ gene was isolated as a gene encoding a protein that physically interacts with Nim1 (Wu and Russell, 1997a). Nif1 is thought to bind and inhibit Nim1 kinase.

Recent studies of fission yeast have revealed that stress-activated protein kinases also influence the mitotic control. Spc1/StyI is a protein kinase that is activated by Wis1 that in turn is activated by Wis4/Wik1/Wak1 (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995; Samejima *et al.*, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). This protein kinase cascade is activated by many forms of stress, including osmotic, heat, and oxidative stress, as well as nutrient limitation (Degols *et al.*, 1995; Degols and Russell, 1997). Mutants that lack elements of this pathway are quite sensitive to stress-induced killing. These mutants are also delayed at the G2–M transition, exhibiting a cell elongation phenotype that is exaggerated by stress (Shiozaki and Russell, 1995). In fact, *spc1* or *wis1* mutations exhibit a synthetic lethal phenotype when combined with the temperature-sensitive *cdc25–22* mutation. Thus, *cdc25–22* cells divide at a moderately elongated cell length at 25°C, whereas *spc1 cdc25–22* or *wis1 cdc25–22* cells undergo cell cycle arrest at 25°C. Curiously, *spc1 cdc25–22 wee1–50* cells are inviable at 35°C, whereas *cdc25–22 wee1–50* cells are viable at this temperature. Thus, Spc1 is able to influence the mitotic control independently of Cdc25 and Wee1.

Mutations of a number of important genes have synthetic lethal interactions with *cdc25–22* at 25°C. These include null mutations of *nim1* and *spc1* as well as the temperature-sensitive *cdc13–117* mutation (Russell and Nurse, 1987b; Shiozaki and Russell, 1995; our unpublished data). Therefore, we reasoned that a screen for mutations that exhibit synthetic lethal interactions with *cdc25–22* might uncover new genes that influence the mitotic control. These genes might encode elements of the Nim1 or Spc1 pathways or elements of undiscovered control processes. In this article we describe *slm1*, one of the first genes identified in this screen. We show that *slm1* is identical to *cdr2*, a

Table 1. *S. pombe* strains used in this study (all strains are *leu1-32 ura4-D18*)

Strains	Genotype	Source or reference
AB90	*h*$^-$ *mik1::LEU2+ura4*$^+$ *wee1-50*	Lab stock
PR109	*h*$^-$	Lab stock
GL192	*h*$^+$ *cdc2-3w cdc25::ura4*$^+$	Lab stock
JM298	*h*$^-$ *wee1-50*	Lab stock
JM300	*h*$^-$ *cdc2-3w*	Lab stock
PR1319	*h*$^+$ *ade6-210 his7-366*	Lab stock
PR1320	*h*$^-$ *ade6-216 his7-366*	Lab stock
KS1361	*h*$^-$ *cdc25-22 wee1-50 his3-237*	Lab stock
KS1483	*h*$^+$ *cdc25-22*	Lab stock
LW1817	*h*$^-$ *nim1::ura4*$^+$	Lab stock
JK1864	*h*$^-$ *cdc25-22*	This study
JK2240	*h*$^-$ *cdr2::ura4*$^+$	This study
JK2241	*h*$^-$ *cdr2::ura4*$^+$ *nim1::ura4*$^+$	This study
JK2242	*h*$^-$ *cdr2::ura4*$^+$ *wee1-50*	This study
JK2243	*h*$^-$ *cdr2::ura4*$^+$ *cdc25-22 wee1-50*	This study
JK2244	*h*$^-$ *cdr2::ura4*$^+$ *mik1::LEU2 wee1-50*	This study
JK2246	*h*$^-$ *slm9::ura4*$^+$	This study
JK2248	*h*$^-$ *slm9::ura4*$^+$ *cdc25-22 wee1-50*	This study
JK2249	*h*$^+$ *cdr2-1*	This study
JK2258	*h*$^-$ *cdr2::ura4*$^+$ *cdc2-3w*	This study
JK2259	*h*$^-$ *cdr2::ura4*$^+$ *cdc2-3w cdc25::ura4*$^+$	This study
JK2310	*h*$^-$ *cdr2HA6H(ura4*$^+$*)*	This study
JK2312	*h*$^-$ *cdr2HA6H(ura4*$^+$*) cdc25-22*	This study

locus identified in a previous mutant screen (Young and Fantes, 1987). We report that Cdr2 is a serine/threonine protein kinase that shares homology to Nim1/Cdr1. Our studies suggest that Cdr2 modulates phosphorylation of Cdc2 on tyrosine-15. This control appears to be largely accomplished by regulation of Wee1, probably in a manner similar to Nim1/Cdr1.

MATERIALS AND METHODS

Yeast Strains and General Techniques

The *S. pombe* strains used in this study are listed in Table 1. Yeast extract medium YES and synthetic minimal media EMM2 and SSA were used for growing yeast cells. Growth media and basic genetic and biochemical techniques for fission yeast have been described (Alfa *et al.*, 1993).

Isolation of Synthetic Lethal Mutants in a cdc25–22 *Background*

The *cdc25–22 leu1–32 ura4-D18* strain (KS1483) was transformed with p25SS containing the *cdc25*$^+$ gene and the *ura4*$^+$ marker (Russell and Nurse, 1986). The resulting strain was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine at a level sufficient for 70% killing (Uemura and Yanagida, 1984). Surviving cells were grown in EMM2 liquid media overnight and then incubated on a YES plate for 3 d at 25°C. The cells were replicated onto YES plates eight times and then onto YES plates containing 0.5 mg/ml 5-fluoroorotic acid. This screen identified 116 colonies that could not grow on 5-fluoroorotic plates.

Disruption of cdr2

For the first disruption construct, *cdr2-D1* (see Figure 2A), 5′ and 3′ flanking DNA fragments were amplified by PCR with two pairs of primers. The primers were JK18 (5′-TCTACTACTGAGCTCCAA-3′; *Sac*I site underlined), JK19 (5′-CGCGGATCCCAAGGTC-CAACTTC-3′; *Bam*HI site underlined), JK20 (5′-ACAGAATTC-CAGCTGATTT-3′; *Pvu*II site underlined), and JK21 (5′-GATAAC-CTAGATATCCTAC-3′; *Eco*RV site underlined). Wild-type *S. pombe* genomic DNA was used as template. The amplified DNA fragments were cloned into pBlueScript SK (Stratagene, La Jolla, CA), and a 1.8-kb fragment of the *ura4*$^+$ cassette (Grimm *et al.*, 1988) was inserted. The resultant plasmid was digested by *Sac*I and *Xho*I to release the *cdr2::ura4*$^+$ fragment and then used to transform diploid cells. For the second disruption construct, *cdr2-D2* (see Figure 2A), a 1.7-kb *Eco*RI DNA fragment, resulting from the DNA amplified with a pair of primers JK59 and JK60 (see below), was cloned into pBlueScript SK and digested by *Pst*I, and then a 1.8-kb fragment of the *ura4*$^+$ cassette was inserted. The resultant plasmid was digested by *Eco*RI, and the *cdr2::ura4*$^+$ fragment was used for transformation.

Chromosomal Integration of cdr2*HA6H*

To tag genomic *cdr2*$^+$ with a sequence encoding two copies of the HA epitope and hexahistidine at the carboxyl terminus, the *cdr2*$^+$ open reading frame (ORF) was amplified by PCR with primers JK103 (5′-GGAGGAGATCTTATGAGTACAATTTCAGAAGTTGG-3′; *Bgl*II site underlined) and JK104 (5′-AAATATGCGGCCG-CAACTTTGGACGGATTGTCGTTG-3′; *Not*I site underlined) and cloned into pRIP42-HA6H (Shiozaki and Russell, 1997). After the *nmt1* promoter was eliminated from the vector, the resultant plasmid was linearized at the *Xba*I site in *cdr2*$^+$ and used for transformation of wild-type (PR109) or *cdc25–22* (JK1864) strains. Stable integration and tagging were confirmed by Southern blotting and immunoblotting. The function of Cdr2HA6H was confirmed by analysis of cell morphology.

Expression of GST–Cdr2 Fusion Protein in Fission Yeast

The *cdr2*$^+$ ORF was amplified by PCR with primers JK59 (5′-GCGCGCGGATCCTATGAGTACAATTTCAGAAGTTG-3′; *Bam*HI site underlined) and JK60 (5′-GCGCGCGGATCCACGAGTATA-CATTATGTTCAATTA-3′; *Bam*HI site underlined) and cloned into the *Bam*HI sites of pJL205, which expresses GST fusion protein from the *nmt1* promoter (Leatherwood *et al.*, 1996). Plasmids were transformed into *S. pombe* cells, and expression from the *nmt1* promoter was induced by thiamine depletion (Maundrell, 1993). GST fusion proteins were precipitated by using glutathione (GSH)–Sepharose 4B (Pharmacia, Uppsala, Sweden) as described (Shiozaki and Russell, 1997). The *cdr2-K39A* mutation was made by sequential PCR mediated mutagenesis, changing codon 39 from AAA to GCT. After PCR, DNA sequencing confirmed that no additional mutations had been introduced.

Expression of GST–Wee1 Proteins in Bacteria

Construction of GST–Wee1(11–152) has been described previously (Boddy *et al.*, 1998). PCR fragments were made encoding amino acids 153–459 and 460–877 of Wee1, incorporating a 5′ *Nde*I site and a 3′ *Not*I site. Those fragments were cloned into a modified pGEX vector and transformed into the *Escherichia coli* BL21 (DE3) (Shiozaki and Russell, 1997). Preparation of GST–Wee1 proteins bound to GSH–Sepharose was performed as described previously (Boddy *et al.*, 1998).

Kinase Assays

GSH–Sepharose precipitates of GST–Cdr2 were washed three times with lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 50 mM NaF, 0.1 mM Na_3VO_4 supplemented with 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml aprotinin, leupeptin, and pepstatin). The GSH–Sepha-

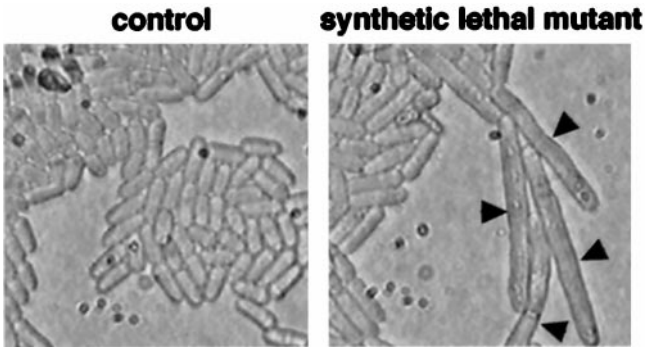


Figure 1. Screen of *slm* mutants. The *cdc25–22* strain (KS1483) transformed with p25SS carrying *cdc25*$^+$ and *ura4*$^+$ was mutagenized and grown on YES (glucose and yeast extract with supplements) agar medium at 25°C. Nonsynthetic lethal mutants can lose p25SS and remain viable because YES medium contains uracil (left panel). On the other hand, synthetic lethal mutants undergo cell cycle arrest after they lose p25SS (indicated by arrowheads in the right panel).

rose precipitates were then washed three times with kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM DTT). The bound complexes were resuspended in 50 μl of kinase buffer containing 50 μM [γ-^{32}P]ATP, 100 μM to 1.5 mM ATP, and 10 mM glutathione. The reaction was incubated at 30°C for 30 min. After the incubation, 50 μl of Laemmli sample buffer was added, samples were boiled for 2 min, and then half of each reaction was analyzed by SDS-PAGE.

RESULTS

Isolation of Mutations That Are Synthetic Lethal with cdc25–22

To identify novel mitotic control genes, we screened for synthetic lethal mutations (*slm*) in a *cdc25–22* background at 25°C. A *cdc25–22* strain was transformed with a plasmid (p25SS) containing the *cdc25*$^+$ gene and then mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Among 76,500 mutagenized cells, we isolated 116 mutants that were dependent on p25SS for growth on complete YES medium at 25°C. Eleven of these strains underwent dramatic cell elongation or cell cycle arrest when p25SS was lost (Figure 1). Genetic analysis established that three mutations resided in *nim1* and one mutation resided in *wis1*. The other mutations appear to occur in seven other genes. In this article we describe one of these genes, *slm1*.

The slm1 *Gene Is Identical to* cdr2

We were unable to clone *slm1*$^+$ by complementation of the *cdc25–22 slm1–1* synthetic lethal phenotype, so instead we used positional cloning. Mitotic haploidization showed that *slm1* mapped to chromosome I. Genetic linkage analysis indicated that *slm1* was located 9.3 cM to the right of *cut9*. This site was predicted to lie within the genomic DNA fragment cloned in 57a10, a cosmid sequenced as part of the *S. pombe* genome sequencing project at the Sanger Centre (Cambridge, UK). P87050, an ORF in 57a10, rescued *slm1–1* (our unpublished data). The *ura4*$^+$ marker was used to create disruption and deletion mutations of P87050 in diploid strains (Figure 2A). The resulting heterozygous Ura$^+$ diploid cells produced four viable spores that segregated 2:2 for uracil prototrophy, indicating that P87050 was not essential. Genetic crosses established that P87050 and *slm1* were tightly linked. Furthermore, mating of the P87050 disruptant strain to the *cdc25–22* mutant yielded tetratype tetrads in which one of the two Ura$^+$ spores germinated to produce cells that appeared identical to the original *cdc25–22 slm1–1* cells. These data supported the conclusion that the P87050 ORF is indeed *slm1*.

In the process of analyzing *slm1*, we found that it is identical to the *cdr2* gene (Kathy Gould, personal communication). The *cdr2* mutant was originally isolated as a mutant that failed to arrest division at a small size in response to nitrogen starvation (Young and Fantes, 1987). The *cdr2* mutant cells are longer than wild-type cells, and the *cdr2–97 cdc25–22* double mutant is highly elongated at 20°C and cannot grow at 27°C. Hereafter we shall refer to *slm1* as *cdr2*.

Cdr2 Is a Novel Serine/Threonine Protein Kinase Related to Nim1/Cdr1

The *cdr2* gene encodes a novel protein of 775 amino acids with a predicted molecular weight of ~86 kDa. Cdr2 protein contains a serine/threonine kinase motif in the N-terminus region. The deduced amino acid sequence of Cdr2 was compared with databases. It showed substantial similarity to *Saccharomyces cerevisiae* Gin4p (Akada *et al.*, 1997; Altman and Kellogg, 1997) and *S. pombe* Nim1/Cdr1 (Figure 2B). These proteins belong to the SNF1 serine/threonine kinase family. The *S. cerevisiae GIN4* gene was first isolated as a growth inhibitory gene, but it recently has been shown that *GIN4* is not essential and physically interacts with Cdc28p complex (Okuzaki *et al.*, 1997). Another budding yeast homolog of Nim1, *NIK1/HSL1*, is thought to be a negative regulator of the SWE1 kinase, a homolog of fission yeast Wee1 (Ma *et al.*, 1996; Tanaka and Nojima, 1996). DNA sequence analysis revealed that the *cdr2–1* allele changes a glycine codon to aspartic acid at position 186 in subdomain IX. Glycine-186 is conserved among Cdr2, Nim1/Cdr1, Gin4p, and Nik1/Hsl1.

cdr2 *Cells Cannot Properly Arrest in G1 When Starved of Nitrogen*

When starved for nitrogen, *S. pombe* cells undergo several divisions and then arrest in G1 phase with a small cell size. Proper cell size control at the G1–S and G2–M transitions is required to arrest in G1. The *cdr2*

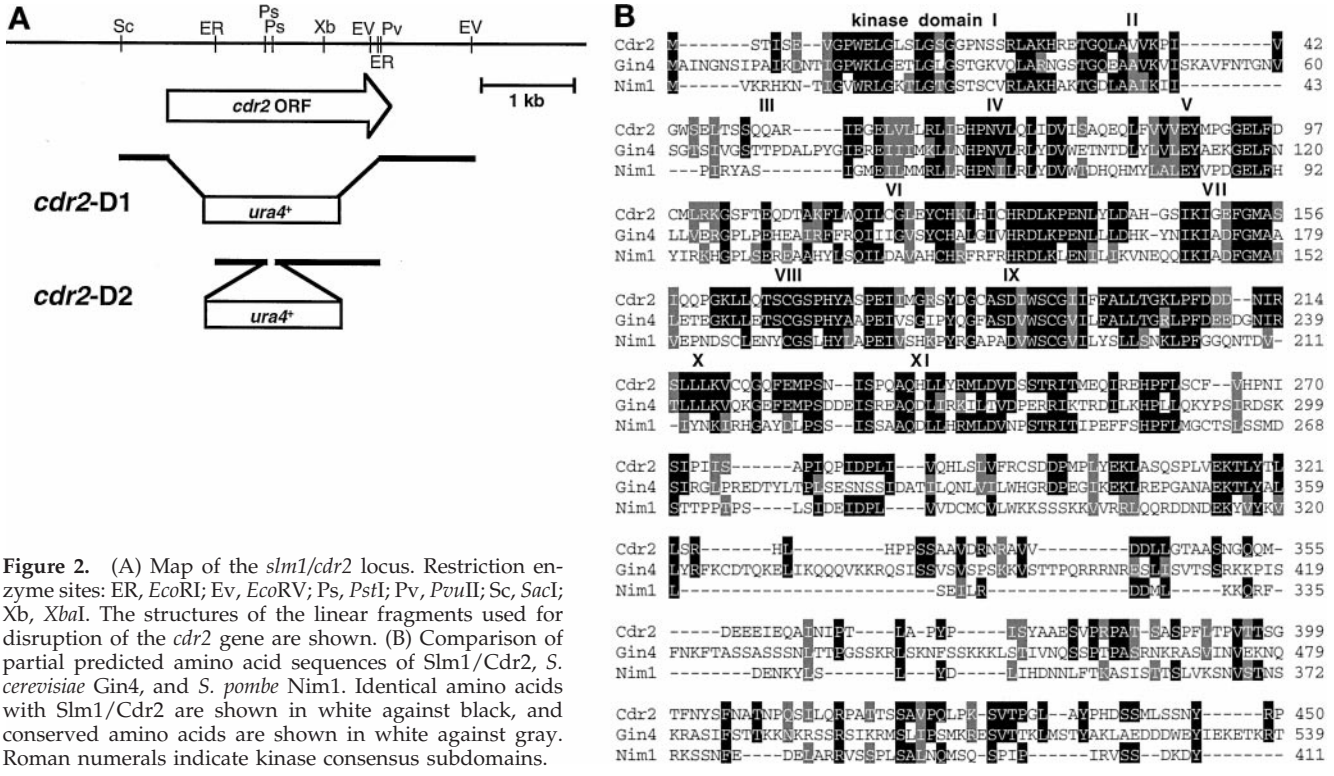


Figure 2. (A) Map of the *slm1/cdr2* locus. Restriction enzyme sites: ER, *Eco*RI; Ev, *Eco*RV; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Xb, *Xba*I. The structures of the linear fragments used for disruption of the *cdr2* gene are shown. (B) Comparison of partial predicted amino acid sequences of Slm1/Cdr2, *S. cerevisiae* Gin4, and *S. pombe* Nim1. Identical amino acids with Slm1/Cdr2 are shown in white against black, and conserved amino acids are shown in white against gray. Roman numerals indicate kinase consensus subdomains.

disruptant cells were elongated at division compared with wild-type cells in both rich YES and synthetic EMM2 media. These haploid cells had a 2C DNA content, indicating a G2 cell cycle delay (Figure 3B). Some mutants that undergo mitosis at an elongated cell length are defective at arresting in G1 phase when starved of nitrogen, probably because of the problem with G2–M cell size control. We determined whether the *cdr2* disruptant had a G1 arrest when starved of nitrogen. The *cdr2* and control cells were cultured to midlog phase in EMM2 medium, transferred to nitrogen-free EMM2 medium (EMM2-N), and then subjected to flow cytometry analysis of DNA content. Most of the wild-type cells arrested in G1 with a 1C DNA content after 24 h incubation in EMM2-N medium (Figure 3B). The *nim1* disruptant cells had a defect in response to nitrogen starvation, but they eventually arrested as small cells in G1 phase after 24 h starvation (Figure 3, A and B); however, most of the *cdr2* cells had a 2C DNA content even 24 h after nitrogen starvation, and they were substantially larger than wild-type cells (Figure 3, A and B). Although the optical density of the *cdr2* and the wild-type cultures increased to similar extents, the final cell number in the *cdr2* culture was approximately half the amount of the culture of wild-type cells (Figure 3C). The nitrogen-starved *cdr2* cells were uninucleate and acquired resistance to heat shock (our unpublished data), suggesting that the *cdr2* cells enter a quiescent state from G2 instead of G1. These data suggest that the G2–M size control defect caused the *cdr2* cells to fail to arrest in G1 when starved of nitrogen.

The Amount of Cdr2 Protein Decreases in Nitrogen-starved Cells

To determine whether the expression of *cdr2*$^+$ is regulated during the cell cycle, we first measured the abundance of *cdr2*$^+$ mRNA. Cells were synchronized by a *cdc25–22* block and release protocol, which arrests cells in late G2 phase by incubating at the restrictive temperature of 35.5°C and then induces them to enter M-phase synchronously by shifting the culture to the permissive temperature of 25°C. The *cdr2*$^+$ mRNA was detected at all time points, and no significant periodic change could be seen (Figure 4A). Cell cycle periodicity was confirmed by the oscillation of the *cdc22*$^+$ mRNA signal that appears during S phase (Lowndes *et al.*, 1992). We also examined the level of *cdr2*$^+$ mRNA in nitrogen-starved cells. The *cdr2*$^+$ mRNA signal was unchanged during the course of nitrogen starvation (Figure 4B).

We next determined the amount of Cdr2 protein using strains in which the single chromosomal copy of *cdr2*$^+$ was tagged with a sequence encoding two copies of HA epitope and six consecutive histidine residues. A *cdc25–22* block and release protocol synchronized cells, and whole-cell extracts of each sample

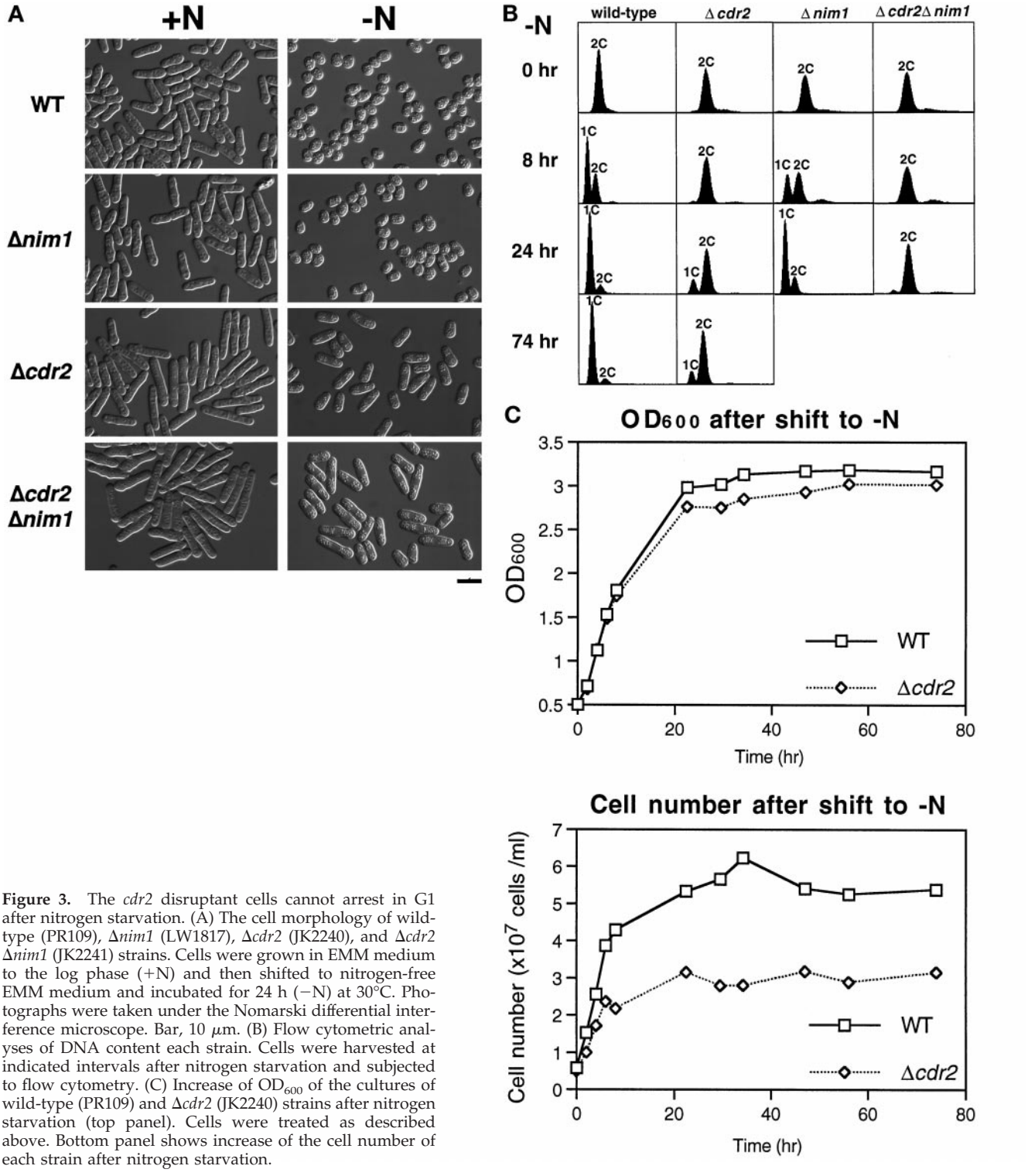


Figure 3. The *cdr2* disruptant cells cannot arrest in G1 after nitrogen starvation. (A) The cell morphology of wild-type (PR109), Δ*nim1* (LW1817), Δ*cdr2* (JK2240), and Δ*cdr2* Δ*nim1* (JK2241) strains. Cells were grown in EMM medium to the log phase (+N) and then shifted to nitrogen-free EMM medium and incubated for 24 h (−N) at 30°C. Photographs were taken under the Nomarski differential interference microscope. Bar, 10 μm. (B) Flow cytometric analyses of DNA content each strain. Cells were harvested at indicated intervals after nitrogen starvation and subjected to flow cytometry. (C) Increase of OD_{600} of the cultures of wild-type (PR109) and Δ*cdr2* (JK2240) strains after nitrogen starvation (top panel). Cells were treated as described above. Bottom panel shows increase of the cell number of each strain after nitrogen starvation.

were used for the detection of Cdr2–HA protein by immunoblotting. Cdr2–HA protein was detected as a band of ~85 kDa. Cdr2 protein was present throughout the cell cycle, with no significant change in abundance (Figure 5A). In contrast, the amount of Cdr2 protein dramatically changed in nitrogen-starved cells. The Cdr2 signal dropped to an undetectable level within 3 h after nitrogen starvation (Figure 5B).

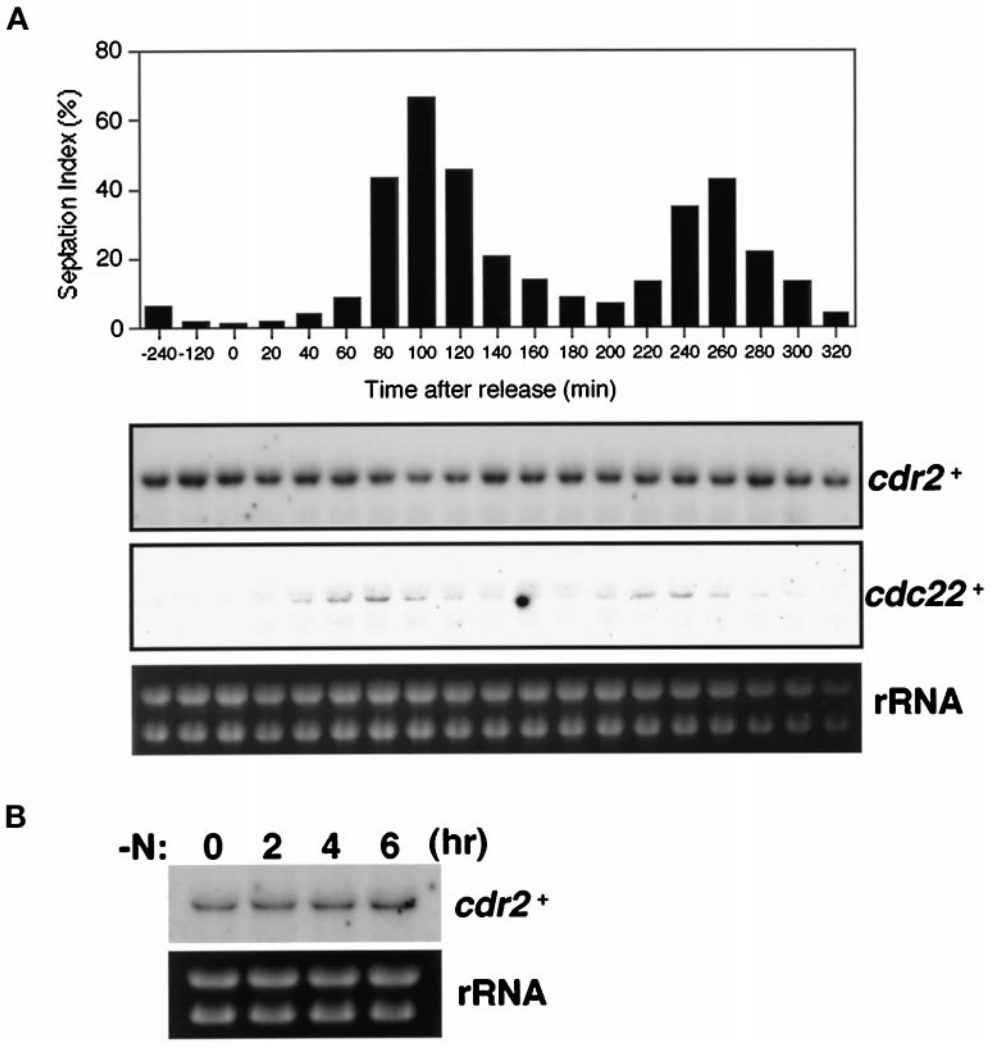


Figure 4. *cdr2*$^+$ mRNA is constant during the cell cycle and in nitrogen starvation conditions. (A) Cells of a *cdc25–22* strain (JK1864) were arrested in late G2 by a temperature shift to 35.5°C for 4 h and then released from the arrest by a temperature shift to 25°C. Samples were taken during the block and every 20 min after the shift to permissive temperature. The percentage of cells with septa was determined by counting ~200 cells at each time point. Northern blot analysis of *cdr2*$^+$ and *cdc22*$^+$ mRNA was performed. Ethidium bromide staining of ribosomal RNA shown below indicates that an approximately equal amount of RNA was loaded in each lane. (B) Cells of a wild-type strain (PR109) were grown to midlog phase in EMM2 and washed and resuspended in EMM2-N. At intervals after starvation, samples were taken and Northern blot analysis was performed.

These properties of Cdr2 are similar to those of Nim1 (Wu *et al.*, 1996; Wu and Russell, 1997b).

Genetic Studies Suggest That Cdr2 Regulates Phosphorylation of Cdc2 on Tyrosine-15

To investigate the basis of the *cdr2* cell elongation phenotype, we transformed *cdr2* cells with the pREP3-*cdc25* plasmid. This plasmid contains *cdc25*$^+$ regulated by the *nmt1* promoter. Overexpression of *cdc25*$^+$ rescued the cell elongation phenotype of *cdr2*, indicating that *cdr2* regulates the activity of Cdc2 directly or indirectly (Figure 6A). This supposition was supported by the observation that a *wee1* mutation suppresses the *cdr2 cdc25–22* synthetic lethality at 25°C. Double-mutant *cdc25–22 wee1–50* cells are viable at the restrictive temperature of 35°C, because the *wee1–50* mutation bypasses the requirement for Cdc25 activity. Triple mutant *cdr2 cdc25–22 wee1–50* cells also grew well at 35°C (Figure 6B). In fact, at 35°C these cells were indistinguishable from *cdc25–22 wee1–50* cells (Figure 6C). Thus, loss of Cdr2 apparently has no effect in cells that lack Cdc25 and Wee1 activity. These findings are reminiscent of the effect of the *nim1* mutation in *cdc25–22 wee1–50* cells. Deletion of *nim1* has no effect in *cdc25–22 wee1–50* cells incubated at 35°C. These findings contrast with the effect of the *spc1* mutation or some of the other *slm* mutations such as *slm9*. Loss of Spc1 activity causes cell-cycle arrest in *cdc25–22 wee1–50* cells incubated at 35°C (Shiozaki and Russell, 1995). A very similar effect was seen with the *slm9* mutation (Figure 6, B and C).

Cdc25 Is Not the Primary Target of Cdr2

These data suggested that the G2 delay caused by *cdr2* deletion might involve the regulation of tyrosine-15 phosphorylation of Cdc2. We performed two experi-

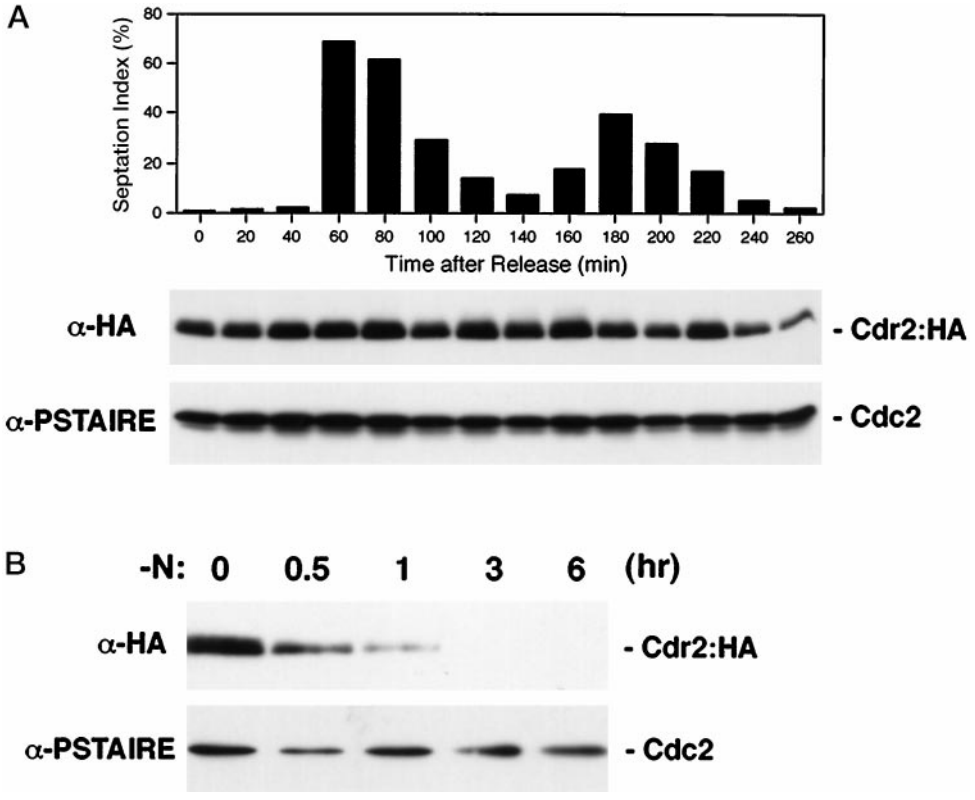


Figure 5. The amount of Cdr2 protein is constant during the cell cycle but decreases in nitrogen-starved cells. (A) Strain JK2312 (h^- *cdr2HA6H cdc25–22*) in which the genomic copy of *cdr2*$^+$ encodes an HA epitope-tagged form of Cdr2 at the carboxyl terminus, was grown in EMM medium to midlog phase, and cells were synchronized as described in Figure 4. Samples were taken every 20 min after the shift to permissive temperature, and whole-cell extracts were prepared. Immunoblotting was performed with anti-HA antibodies for Cdr2–HA protein and with anti-PSTAIRE antibodies for Cdc2 protein (control). (B) Strain JK2310 (h^- *cdr2HA6H*) was grown to midlog phase in EMM2 and then resuspended in EMM2-N. Samples were taken at intervals after nitrogen starvation.

ments to evaluate whether Cdr2 was regulating Cdc25 activity. In the first experiment we examined the phenotype of the Δ*cdr2 cdc2–3w* Δ*cdc25* strain. The *cdc2–3w* mutation activates Cdc2, thereby bypassing the requirement for Cdc25. If Cdr2 functions exclusively to activate Cdc25, we would expect the *cdr2* mutation to have no effect in a *cdc2–3w* Δ*cdc25* background; however, we observed that the Δ*cdr2 cdc2–3w* Δ*cdc25* cells were elongated relative to *cdc2–3w* Δ*cdc25* cells (Figure 7A). These data indicate that Cdr2 regulates cell size at division in the absence of Cdc25 activity. In the second experiment we measured the effect of the *cdr2* deletion on the rate of induction of mitosis after inactivation of Wee1 and Mik1. This experiment used a *wee1–50* Δ*mik1* strain that lacked Mik1 and expressed temperature-sensitive Wee1 protein. Cells with the wild-type *cdr2*$^+$ or mutant Δ*cdr2* allele were synchronized in G2 by centrifugal elutriation and then shifted from 25 to 35°C. In this experiment the rate of division after the temperature shift is determined by the amount of Cdc25 activity (Furnari *et al.*, 1997). We found that *cdr2*$^+$ and mutant Δ*cdr2* cells underwent division at equal rates (Figure 7B). These data argue strongly that Cdr2 does not regulate Cdc25 activity.

Genetic Studies Suggest That Cdr2 Regulates Wee1

The high sequence homology between Cdr2 and Nim1 suggested that the two protein kinases might have similar functions. If this hypothesis were true we would expect that *cdr2* and *nim1* mutations should have additive effects. This hypothesis was confirmed in the nitrogen starvation experiment that showed that the Δ*cdr2* Δ*nim1* double mutant was longer than either single mutant (Figure 3A). The G1 arrest defect was also more severe in the double mutant (Figure 3B). The cell elongation and G2 delay caused by the *nim1* mutation is completely suppressed by *wee1–50*, a finding that supports the conclusion that Nim1 acts solely as an inhibitor of Wee1. In view of the similarity between Nim1 and Cdr2, we tested whether the *cdr2* phenotype is suppressed by the temperature-sensitive *wee1–50* mutation. We observed that Δ*cdr2 wee1–50* cells were slightly elongated at the permissive temperature of 25°C. The Δ*cdr2 wee1–50* cells became much smaller at the restrictive temperature of 35°C, showing that the Δ*cdr2* cell elongation phenotype was fully suppressed by inactivation of Wee1 protein (Figure 8). These data indicate that Wee1 is likely to be the main target of Cdr2.

Autophosphorylation of Cdr2 Protein

To characterize Cdr2 protein, a protein kinase assay of GST–Cdr2 purified from fission yeast was performed. GST–Cdr2^{K39A}, in which the lysine-39 residue in the kinase domain is changed to alanine, was made to

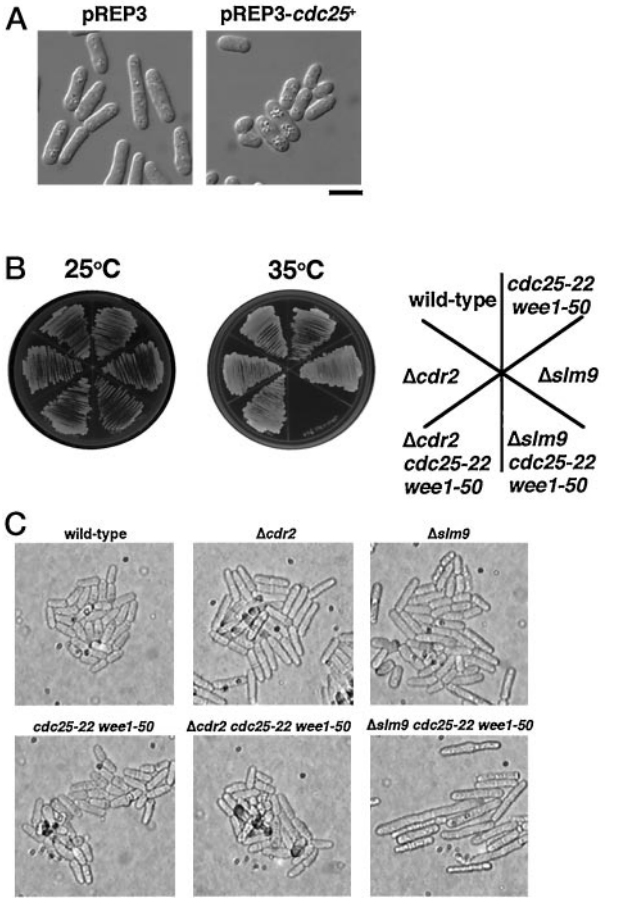


Figure 6. (A) Overexpression of *cdc25*$^{+}$ rescues the cell elongation phenotype of Δ*cdr2*. Δ*cdr2* (JK2240) cells were transformed with pREP3 (vector) or pREP3-*cdc25*$^{+}$. The transformants were grown in EMM2 medium without thiamine at 30°C to induce the *nmt1* promoter of the vector. Cells were photographed by differential interference contrast microscopy. Bar, 10 μm. (B) Growth of Δ*cdr2 cdc25–22 wee1–50* cells at restrictive temperature. Wild-type (PR109), *cdc25–22 wee1–50* (KS1361), Δ*cdr2* (JK2240), Δ*cdr2 cdc25–22 wee1–50* (JK2243), Δ*slm9* (JK2246), and Δ*slm9 cdc25–22 wee1–50* (JK2248) cells were grown on YES media for 2–3 d at 25°C permissive temperature and 35°C restrictive temperature. The strains JK2246 and JK2248 were used for the negative control. (C) Cells grown on the plate at 35°C in B were photographed.

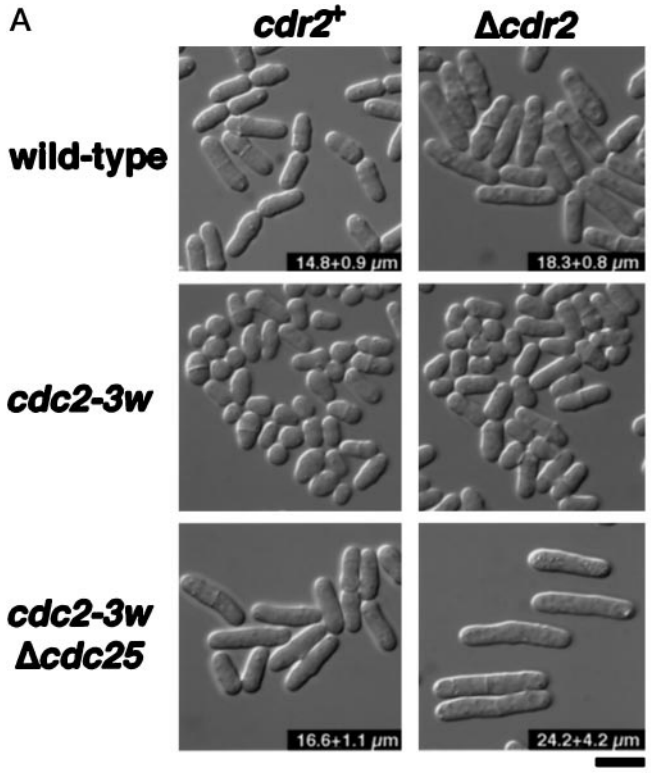
inactivate kinase activity of Cdr2 protein. The kinase activity of GST–Cdr2^{K39A} was substantially diminished relative to wild-type GST–Cdr2 (Figure 9A). In these assays, both autophosphorylation and phosphorylation of the myelin basic protein were greatly decreased in the GST–Cdr2^{K39A} samples. These findings were reflected in studies of GST–Cdr2 phosphorylation in vivo. As assayed by immunoblotting, GST–Cdr2 migrated as a diffuse protein that had a slower mobility than GST–Cdr2^{K39A}. After treatment with calf intestinal alkaline phosphatase (CIAP), GST–Cdr2 migrated as a less diffuse band that had the same mobility as GST–Cdr2^{K39A} (Figure 9B). These data indicate that GST–Cdr2 autophosphorylates in vivo. The fact that some kinase activity was detected when we used GST–Cdr2^{K39A} suggests that GST–Cdr2^{K39A} is not fully kinase deficient or that some Cdr2-associated kinases exist.

Cdr2 Binds and Phosphorylates N-Terminus of Wee1 In Vitro

Genetic analyses indicated that Cdr2 regulates Wee1. Experiments were performed to explore whether Cdr2 is able to interact directly with Wee1. Lysates from cells in which genomic *cdr2* was tagged with two copies of HA at the C-terminal end of ORF were prepared, incubated with GST–Wee1 or GST proteins, washed, and analyzed by immunoblotting (Figure 10A). Cdr2–HA protein was precipitated specifically with GST–Wee1(11–152), which contains amino acids 11–152 of Wee1. Next, we performed an in vitro kinase assay using GST–Wee1(11–152) as a substrate. GST–Cdr2 protein, but not GST–Cdr2^{K39A}, efficiently phosphorylated GST–Wee1(11–152) in vitro (Figure 10B, lanes 1–3). Interestingly, GST–Cdr2 did not phosphorylate GST–Wee1^{70}, a truncation product of GST–Wee1(11–152), whereas GST–Cds1 did (Figure 10B, lane 4). Cds1 protein kinase was shown to bind and phosphorylate GST–Wee1(1–72) in vitro after treatment with hydroxyurea (Boddy *et al.*, 1998). These data indicate that Cdr2 and Cds1 phosphorylate different sites of Wee1 in vitro.

DISCUSSION

In this article we have presented the discovery and initial analysis of *cdr2*$^{+}$, a new gene that appears to be involved in the regulation of mitosis in fission yeast. Loss of Cdr2 activity causes cells to grow to a larger cell size before initiating cell division. These findings suggest that Cdr2 has a positive role in the induction of mitosis. Consistent with this notion, we discovered *cdr2–1* as a mutation that causes cell-cycle arrest when the activity of Cdc25 is reduced by the *cdc25–22* mutation. These phenotypes are highly reminiscent of the effects of *nim1/cdr1* mutations (Russell and Nurse, 1987b; Feilotter *et al.*, 1991; Wu and Russell, 1993). Like *nim1/cdr1* mutations, *cdr2* mutations are suppressed by *wee1* mutations and appear to have no effect in a *wee1–50 cdc25–22* background. The latter property distinguishes *nim1/cdr1* and *cdr2* mutations from mutations in the Spc1/StyI stress-activated kinase cascade, which cause cell-cycle arrest in a *wee1–50 cdc25–22* background. The similarities between the phenotypes caused by *nim1/cdr1* and *cdr2* mutations are even more striking when one realizes that Cdr2 and Nim1/Cdr1 are closely related members of the serine/threonine protein kinase family. Genetic epistasis studies indicate that Cdr2 and Nim1/Cdr1 function indepen-



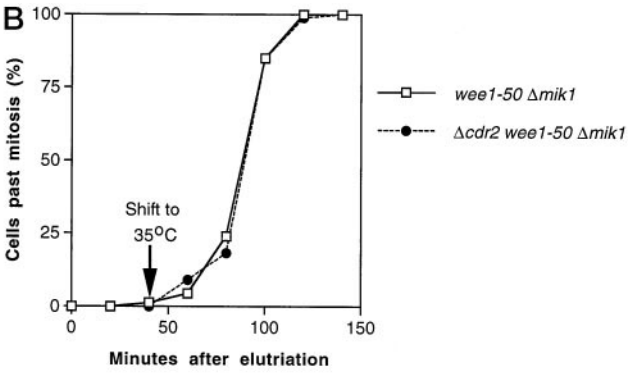


Figure 7. (A) Wild-type (PR109), Δ*cdr2* (JK2240), *cdc2–3w* (JM300), Δ*cdr2 cdc2–3w* (JK2258), *cdc2–3W* Δ*cdc25* (GL192) and Δ*cdr2 cdc2–3W* Δ*cdc25* (JK2259) strains were grown in YES media at 30°C. Photographs were taken using differential interference contrast optics. Bar, 10 μm. Numbers in panels refer to cell length at division. (B) *cdr2* deletion does not cause delay of mitosis after inactivation of Wee1 and Mik1 kinases. Synchronous cultures of Δ*mik1 wee1–50* (AB90) or Δ*cdr2* Δ*mik1 wee1–50* (JK2244) strains in early G2 phase were grown in YES media at 25°C and then shifted to 35°C to inactivate Wee1–50 protein. The cultures were examined microscopically to determine the percentage of the cells that had attempted mitosis.

dently because the phenotypes of *nim1/cdr1* and *cdr2* mutations are additive.

A combination of genetic and biochemical studies has shown that Nim1/Cdr1 directly inhibits Wee1. Details of the mechanism are lacking, but it is well established that Nim1/Cdr1 inhibits Wee1 in an in vitro assay that uses purified proteins (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). The similar genetic properties of *nim1/cdr1* and *cdr2* mutations, coupled with the sequence homology of Cdr2 and Nim1/Cdr1 proteins, strongly suggest that the two protein kinases function by similar mechanisms. A priority in future studies will be to formally establish that Cdr2 directly inhibits Wee1. Alternatives include the possibility that Cdr2 indirectly regulates Wee1, perhaps by phosphorylating Nim1/Cdr1, or that Cdr2 regulates Mik1, Cdc25, or Pyp3. In theory, the first possibility could be determined by examining the effect of Cdr2 overproduction in a *nim1/cdr1* mutant, but there are difficulties with this experiment that are discussed below. If Cdr2 acted primarily by inhibiting Mik1, then we would expect that the *cdr2* cell elongation phenotype would be suppressed by an *mik1* mutation; however, the *cdr2* cell elongation phenotype is not dependent on Mik1. Thus it appears that Mik1 is not a major target of Cdr2. The possibility that Cdr2 is an activator of Cdc25 also appears to be unlikely. Loss of Cdr2 activity delays mitosis in strains that have no active Cdc25 protein (i.e., *cdc2–3w* Δ*cdc25*). Therefore we can certainly conclude that Cdc25 is not the primary target of Cdr2 regulation. This conclusion is supported further by the observation that a *cdr2* mutation does not delay mitosis after inactivation of Wee1 protein in a Δ*mik1* background. The fourth possibility is that Cdr2 is required for Pyp3 activity; however, this proposition appears unlikely to be correct because loss of Pyp3 has very little effect on cell size in a wild-type background (Millar *et al.*, 1992). Moreover, Pyp3 activity is crucial for viability in a *wee1–50* Δ*cdc25* strain, whereas *cdr2* mutations have no effect in this strain background.

The idea that Cdr2 targets Wee1 is also supported by in vitro biochemical studies. We have found that Cdr2 physically interacts with and phosphorylates the N-terminal domain of Wee1 in vitro. Specifically, we found that Cdr2 phosphorylates a GST fusion protein containing amino acids 11–152 of Wee1, but not the 11–70 region. In a previous study we found that Cds1, a protein kinase that is activated by the DNA replication checkpoint that prevents mitosis, also associates with the N-terminus of Wee1 in cell lysates (Boddy *et al.*, 1998). These studies showed that Cds1 phosphorylates the 11–70 region of Wee1. Thus Cdr2 and Cds1 phosphorylate different regions of Wee1. Interestingly, the previous studies of Cds1 also revealed that in cell lysates another protein kinase (or kinases) associates with and phosphorylates the 11–152 region of Wee1, but not the 11–70 region. Moreover, this activity of this

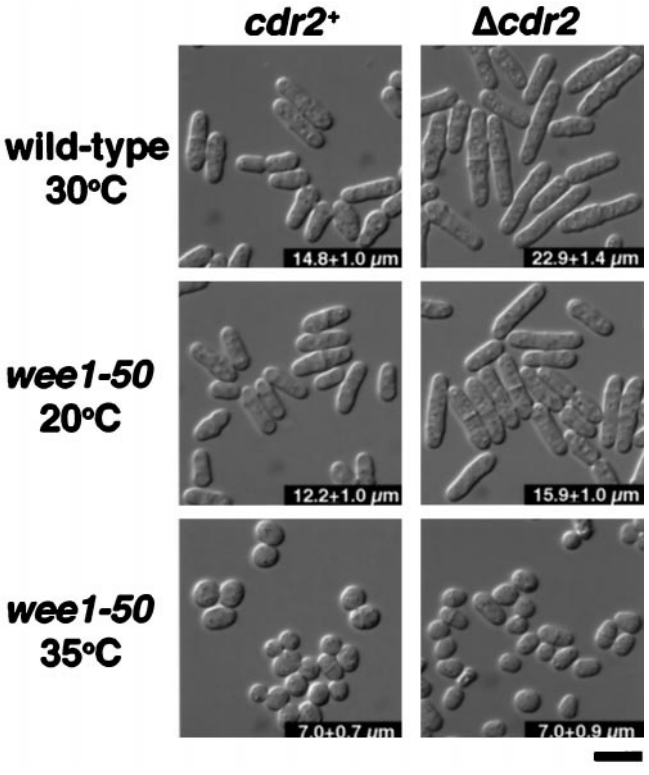


Figure 8. Inactivation of Wee1 fully suppresses the cell elongation phenotype caused by the Δ*cdr2* mutation. Wild-type (PR109) and Δ*cdr2* (JK2240) strains were grown in EMM2 media at 30°C (top panels). The *wee1–50* (JM298) and Δ*cdr2 wee1–50* (JK2242) strains were grown in EMM media at 20°C to log phase (middle panels) and then shifted to 35°C for 15 h (bottom panels). Bar, 10 μm. Numbers in panels refer to cell length at division.

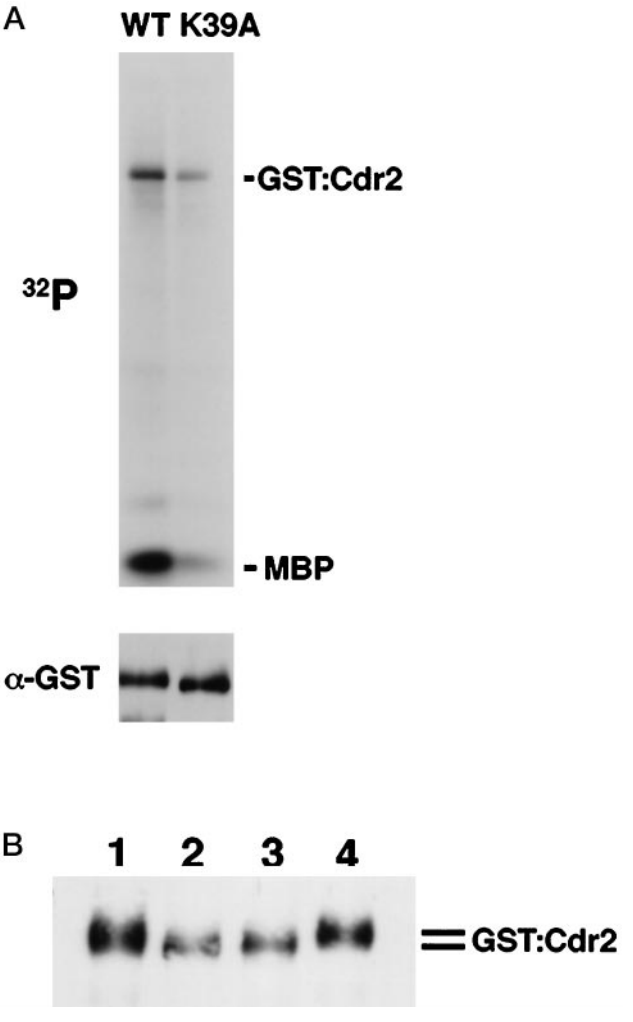


Figure 9. GST–Cdr2 appears to undergo autophosphorylation in vivo. (A) Wild-type strain transformed with pJL205-*cdr2* or pJL205-*cdr2*^{K39A} was grown in the absence of thiamine, and the cell extracts were prepared. GST fusion proteins were precipitated by using GSH–Sepharose and assayed for kinase activity in the presence of myelin basic protein (MBP). Anti-GST immunoblotting shows that approximately equal amounts of GST–Cdr2 protein were used in this assay (bottom panel). (B) Phosphatase treatment causes a mobility shift of GST–Cdr2. Cell extracts of asynchronous wild-type cells carrying pJL205-*cdr2* or pJL205-*cdr2*^{K39A} were prepared, and GST fusion proteins were precipitated by using GSH–Sepharose and subjected to calf intestinal alkaline phosphatase (CIAP) treatment. Lane 1, wild-type Cdr2 with no CIAP treatment; lane 2, Cdr2^{K39A} with no CIAP; lane 3, wild-type Cdr2 with CIAP; lane 4, wild-type Cdr2 with CIAP and phosphatase inhibitors. Western blotting with antibodies to GST is shown.

unknown kinase appears to increase at or shortly before mitosis. This pattern of activation suggests that this kinase might have a role in a feedback loop in which Cdc2 catalyzes its own activation by inhibiting Wee1 directly or indirectly. This model is consistent with studies that have shown that deletion of the N-terminal region of Wee1 causes cell elongation (Aligue *et al.*, 1997). Thus, the N-terminus of Wee1 possibly has the inhibitory effect on Wee1 activity. The unknown kinase that is specific for the 70–152 region of Wee1 is not Cdc2 (Boddy and Russell, unpublished studies), but Cdc2 might regulate the kinase. It will be of interest to determine whether Cdr2 contributes to this activity.

Overall, genetic and biochemical studies support the idea that Nim1/Cdr1 and Cdr2 function by similar mechanisms. There is, however, one important difference between Nim1/Cdr1 and Cdr2. Overproduction of Nim1/Cdr1 causes a wee phenotype, exactly as expected for a rate-limiting inhibitor of Wee1 (Russell and Nurse, 1987b). In contrast, overproduction of Cdr2, even at a relatively moderate level, is toxic (our unpublished data). This fact probably explains why we were unable to clone *cdr2*$^{+}$ by functional complementation from libraries made with multi-copy plasmids. Overproduction of Cdr2 causes cells to elongate and form multiple division plates. This result might be taken as evidence against a mechanism in which Cdr2 inhibits Wee1, but the interpretation of the Cdr2 over-

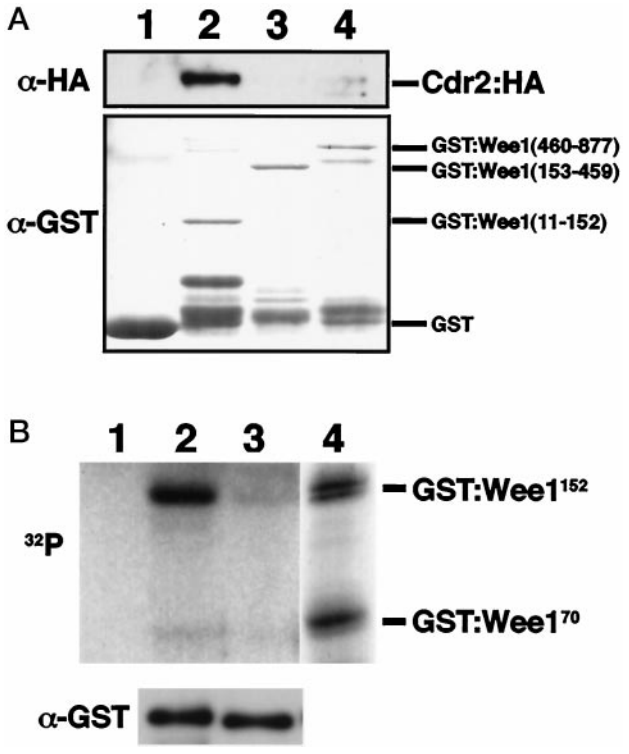


Figure 10. Cdr2 binds and phosphorylates the N-terminus of Wee1 in vitro. (A) Lysates from cells expressing HA epitope-tagged Cdr2 were incubated with GST or GST–Wee1 proteins purified from bacteria with GSH–Sepharose. After extensive washing, samples were subjected to immunoblotting with anti-HA or anti-GST antibodies. Lane 1, GST; lane 2, GST–Wee1(11–152); lane 3, GST–Wee1(153–459); lane 4, GST–Wee1(460–877). (B) GST–Cdr2, GST–Cdr2^{K39A}, and GST–Cds1 proteins were prepared from fission yeast and bound to GSH–Sepharose. Each protein was mixed with GST–Wee1(11–152) protein bound to GSH–Sepharose and incubated in a kinase reaction. Samples were resolved by 12% SDS-PAGE. Lane 1, GST–Wee1 only; lane 2, GST–Cdr2; lane 3, GST–Cdr2^{K39A}; lane 4, GST–Cds1. An anti-GST blotting shows that almost equal amounts of GST–Cdr2 and –Cdr2^{K39A} proteins were used in lane 2 and in lane 3. Note that a mobility shift of GST–Wee1(11–152) caused by phosphorylation by Cds1 was detected in lane 4.

production phenotype is complicated. We observed that overproduction of a kinase inactive version of Cdr2 also caused the same phenotype. In fact, the Cdr2^{K39A} protein appeared to be undiminished in its ability to cause cell elongation. We cannot be certain that Cdr2^{K39A} protein was without kinase activity, but our in vitro kinase assays demonstrated that the kinase activity of Cdr2^{K39A} was substantially decreased relative to wild-type Cdr2. Moreover, Cdr2^{K39A} protein was clearly defective at promoting autophosphorylation in vivo. These findings suggest that cell elongation caused by overproduction of Cdr2 is not due to increased phosphorylation of its substrate. It is plausible that Wee1 was inhibited by overproduction of Cdr2 but that this phenotype was obscured by a kinase-independent effect caused by Cdr2 overproduction. At this point we do not know whether the Cdr2 overproduction phenotype is indicative of Cdr2 having a substrate other than Wee1 or whether Cdr2 interacts with another protein that is not a substrate but is important for cell division. Of course, it is possible that the Cdr2 overproduction phenotype is an artifact in the true sense of the word.

In summary, our studies indicate that the protein kinase Cdr2 has a positive regulatory influence on mitotic control. Genetic findings argue strongly against the possibility that Cdr2 regulates Mik1, Cdc25, or Pyp3, but they are fully consistent with a model in which Cdr2 regulates Wee1 in a negative manner. This model is further supported by studies showing that Cdr1 associates with Wee1 in cell lysates and phosphorylates Wee1 in vitro. Future studies will be aimed at determining whether Wee1 is a physiologically significant substrate of Cdr2.

ACKNOWLEDGMENTS

We are grateful to all the lab members and the Scripps Cell Cycle Group for helpful advice and support. We especially thank Kazuhiro Shiozaki, Beth Furnari, and Nick Boddy for insightful suggestion and encouragement, Yukiko Yamashita for helpful advice, and Susan Forsburg for strains. We also thank Kathy Gould for sharing unpublished data. J.K. was supported by a long-term fellowship of the Human Frontier Science Program. This work was supported by National Institutes of Health.

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