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$APC^{ste9/srw1}$ promotes degradation of mitotic cyclins in G_1 and is inhibited by cdc2 phosphorylation

Miguel A.Blanco, Alberto Sánchez-Díaz, José M.de Prada and Sergio Moreno[1]

Instituto de Microbiología Bioquímica, Departamento de Microbiología y Genética, CSIC/Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain

[1]Corresponding author
e-mail: smo@gugu.usal.es

M.A.Blanco and A.Sánchez-Díaz contributed equally to this work

Fission yeast ste9/srw1 is a WD-repeat protein highly homologous to budding yeast Hct1/Cdh1 and *Drosophila* Fizzy-related that are involved in activating APC/C (anaphase-promoting complex/cyclosome). We show that $APC^{ste9/srw1}$ specifically promotes the degradation of mitotic cyclins cdc13 and cig1 but not the S-phase cyclin cig2. $APC^{ste9/srw1}$ is not necessary for the proteolysis of cdc13 and cig1 that occurs at the metaphase–anaphase transition but it is absolutely required for their degradation in G_1. Therefore, we propose that the main role of $APC^{ste9/srw1}$ is to promote degradation of mitotic cyclins when cells need to delay or arrest the cell cycle in G_1. We also show that ste9/srw1 is negatively regulated by cdc2-dependent protein phosphorylation. In G_1, when cdc2–cyclin kinase activity is low, unphosphorylated ste9/srw1 interacts with APC/C. In the rest of the cell cycle, phosphorylation of ste9/srw1 by cdc2–cyclin complexes both triggers proteolysis of ste9/srw1 and causes its dissociation from the APC/C. This mechanism provides a molecular switch to prevent inactivation of cdc2 in G_2 and early mitosis and to allow its inactivation in G_1.

Keywords: APC/C/cell cycle/cyclin/proteolysis/ste9/srw1

Introduction

Ubiquitin-mediated proteolysis plays an important role in the control of cell cycle progression. Targeted protein degradation is necessary for multiple processes in mitosis and at the G_1–S transition (reviewed in Krek, 1998; Peters, 1998; Morgan, 1999; Zachariae and Nasmyth, 1999). Proteolysis of Cut2/Pds1, which triggers sister chromatid separation, is required for the metaphase–anaphase transition, and degradation of mitotic cyclins is essential to exit from mitosis (Glotzer *et al*., 1991; Holloway *et al*., 1993; Irniger *et al*., 1995; Cohen-Fix *et al*., 1996; Funabiki *et al*., 1996).

Ubiquitin is transferred to target substrates through several enzymatic reactions involving the E1, E2 and E3 enzymes. The ubiquitin ligase or E3 interacts with both the substrate and the E2 and determines the substrate specificity and the timing of degradation. Anaphase-promoting complex/cyclosome (APC/C) is the cell cycle-regulated ubiquitin ligase or E3 that mediates the degradation of Cut2/Pds1 and mitotic cyclins. APC/C is a multiprotein complex that it is activated at the metaphase–anaphase transition and remains active until late G_1 (Amon *et al*., 1994; Brandeis and Hunt, 1996). APC/C activity and substrate specificity are regulated by its association with highly conserved regulatory activators that are part of the subfamilies of the WD40 repeat proteins, called Cdc20 and Hct1/Cdh1 in budding yeast (Schwab *et al*., 1997; Visintin *et al*., 1997), slp1 and ste9/srw1 in fission yeast (Yamaguchi *et al*., 1997; Kim *et al*., 1998; Kitamura *et al*., 1998), Fizzy and Fizzy-related in *Drosophila* and *Xenopus* (Dawson *et al*., 1995; Sigrist *et al*., 1995; Sigrist and Lehner, 1997; Lorca *et al*., 1998) and $p55^{CDC}$/CDC20 and HCT1/CDH1 in humans (Weinstein *et al*., 1994; Fang *et al*., 1998; Kramer *et al*., 1998). The APC^{Cdc20} (APC^{slp1} in fission yeast) complex promotes sister chromatid separation by ubiquitylating the anaphase inhibitor (or securin) Pds1 (cut2 in *Schizosaccharomyces pombe*) and by liberating the separin Esp1 (cut1 in *S.pombe*), which in turn causes either cleavage or modification of the cohesin subunit Scc1 (rad21 in *S.pombe*) (Michaelis *et al*., 1997; Ciosk *et al*., 1998; Uhlmann *et al*., 1999; see Yanagida, 2000 for a review). $APC^{Hct1/Cdh1}$ ($APC^{ste9/srw1}$ in *S.pombe*) triggers mitotic exit by targeting mitotic cyclins for destruction (Schwab *et al*., 1997; Sigrist and Lehner, 1997; Visintin *et al*., 1997; Yamaguchi *et al*, 1997; Kitamura *et al*., 1998; Kramer *et al*., 1998). Hct1 interaction with APC/C is negatively regulated by Cdk1–cyclin-dependent phosphorylation (Zachariae *et al*., 1998; Jaspersen *et al*., 1999; Kramer *et al*., 2000) and activated by Cdc14 protein phosphatase (Visintin *et al*., 1998, 1999; Jaspersen *et al*., 1999; Shou *et al*., 1999). APC^{Cdc20} activation controls not only Pds1 degradation but also that of Clb5 and Clb2 (Shirayama *et al*., 1999; Baümer *et al*., 2000; Yeong *et al*., 2000). Recently it has been proposed that degradation of the mitotic cyclin Clb2 occurs in two steps. First, a fraction of Clb2 is degraded by APC^{Cdc20} at the metaphase–anaphase transition and later, in telophase, $APC^{Hct1/Cdh1}$ degrades the rest of Clb2 (Baümer *et al*., 2000; Yeong *et al*., 2000).

Fission yeast ste9/srw1 (ste9 from here on) has been proposed to be involved in the degradation of cdc13 B-type cyclin (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998). Here we show that the main role of APC^{ste9} is to target the mitotic cyclins cdc13 and cig1 for degradation in G_1. ste9 interacts with APC/C only in G_1. Cdk-dependent phosphorylation of ste9 in S-phase and G_2 has a dual effect: it triggers the proteolysis of ste9 and also causes its dissociation from APC/C.

Results

cdc13 and cig1 cyclins are targets for APC^{ste9}

Previous studies have shown that overexpression of ste9 promotes cdc13 degradation and induces multiple rounds of S-phase in the absence of mitosis (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998), equivalent to deletion of the *cdc13*$^+$ gene (Hayles *et al*., 1994). To test whether ste9 induces degradation of other B-type cyclins in addition to cdc13, we overexpressed the *ste9*$^+$ gene from the *nmt1* promoter and measured the levels of cig1, cig2 and cdc13 cyclins in *S.pombe*. As shown in Figure 1A, ste9 overexpression promotes proteolysis of cdc13 and cig1 but not of cig2. In fact, cig2 levels increased in these cells in agreement with previous observations showing that down-regulation of cdc2/cdc13 allows accumulation of cig2 (Jallepalli and Kelly, 1996; C.Martín-Castellanos and S.Moreno, unpublished data). This result provides an explanation for the endoreplication phenotype induced by overproduction of the *ste9*$^+$ gene (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Figure 1B) as inactivation of cdc2/cdc13 will prevent mitosis while activation of cdc2/cig2 will trigger multiple rounds of S-phase.

This experiment also suggests that cig1 cyclin is another target of APC^{ste9}. In contrast to cdc13 or cig2, analysis of the cig1 amino acid sequence reveals no obvious destruction box (Bueno *et al*., 1991) or KEN box (Pfleger and Kirschner, 2000). We examined cig1 protein levels during the mitotic cell cycle in cells synchronized by centrifugal elutriation and found that cig1 protein is destroyed in mitosis with similar kinetics to cdc13 (Figure 2A). For this experiment, we used the temperature-sensitive *wee1-50* mutant where the G_1 phase of the cell cycle is extended when incubated at the restrictive temperature of 36°C (Nurse, 1975). To confirm this result, we performed an additional experiment using the cold-sensitive *nda3-KM311* β-tubulin mutants that arrest the cell cycle in metaphase. *nda3-KM311* cells were pre-synchronized in early G_2 by centrifugal elutriation. The resulting culture was incubated at the restrictive temperature of 20°C for 4 h and then released to 32°C. Samples were taken at 0, 2 and 4 h during the block and at 5, 15, 30 and 45 min during the release to measure cdc13, cig1 and rum1 protein levels. As shown in Figure 2C, cig1 was completely degraded during mitosis. Degradation of cig1 occurred slightly earlier than that of cdc13. Interestingly, in metaphase cells, cig1 levels started to decrease while cdc13 levels were still high (Figure 2C, t = 4 h); at this time point, the rum1 cdk inhibitor (a target for cdc2/cig1 phosphorylation) is already present at high levels (Figure 2C). This is consistent with previous observations indicating that cdc2/cig1 promotes phosphorylation and degradation of rum1 (Correa-Bordes *et al*., 1997; Benito *et al*., 1998). Taken together, these results indicate that cig1 cyclin is destroyed in mitosis and that cig1 may be an additional target of APC^{ste9}.

APC^{ste9} is required for the degradation of mitotic cyclins in G_1

Next, we wanted to test if ste9 was required in every cell cycle to degrade cdc13 and cig1 in mitosis, in G_1 or both. In yeast, inactivation of cdc2–cyclin complexes in mitosis and G_1 is thought to depend on two mechanisms: cyclin proteolysis by APC/C and cdk inhibition. Budding yeast cells lacking Hct1 and Sic1 are not viable, presumably because Cdc28/Clb2 cannot be down-regulated at the end of mitosis (Schwab *et al*., 1997). In contrast, fission yeast cells deleted for *ste9* and *rum1* are viable. We have found that the double mutant *ste9*Δ *rum1*Δ is wild-type in size and, like the single mutants *ste9*Δ and *rum1*Δ, is unable to arrest the cell cycle in G_1 in response to nitrogen starvation and sterile (data not shown). We compared wild-type, *ste9Δ*, *rum1Δ* and *ste9Δ rum1Δ* cells synchronized in G_2 using a *cdc25-22* mutant and determined levels of cdc13 and cig1 protein as these cells proceeded through mitosis. As shown in Figure 3, degradation of cdc13 and cig1 took place with similar kinetics in wild-type, in the single mutants *ste9Δ* and *rum1Δ* and in the double mutant *ste9Δ rum1Δ*, suggesting that degradation of cdc13 and cig1 at the metaphase–anaphase transition can occur through a

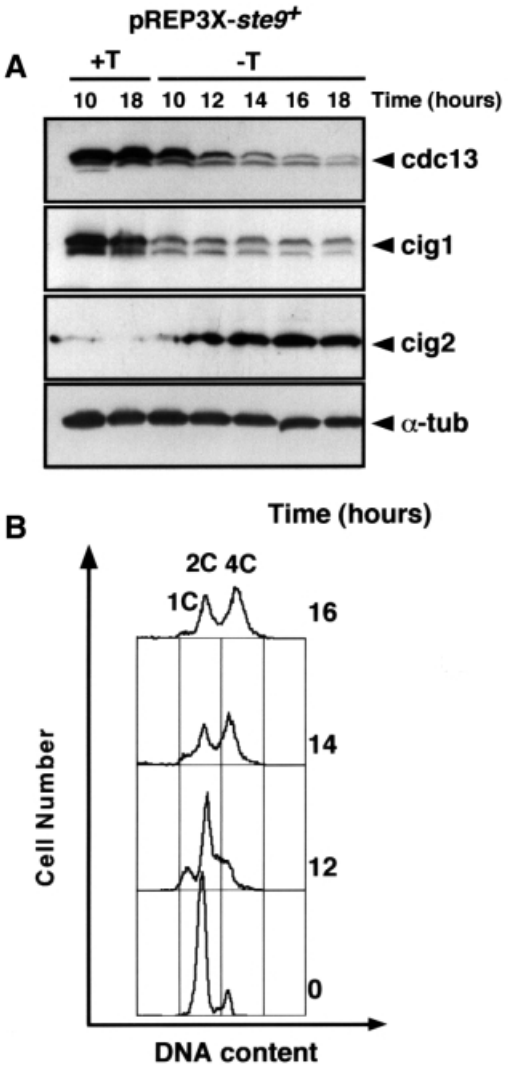


Fig. 1. Overproduction of *ste9*$^+$ promotes degradation of cdc13 and cig1 cyclins but not of cig2. An *S.pombe leu1-32* strain was transformed with the plasmid pREP3X-*ste9*$^+$. Transformants were selected on plates containing minimal medium with thiamine. Cells were grown in the presence (+T, repressed conditions) or absence of thiamine (–T, derepressed conditions) and samples were taken at the indicated times. (**A**) Extracts were prepared from these samples and the amounts of cdc13, cig1, cig2 and α-tubulin were determined. (**B**) FACS analysis of the cells.

ste9-independent mechanism. However, ste9 is absolutely required for the degradation of cdc13 and cig1 in cells arrested in G_1 using the *cdc10-129* mutant (Kitamura *et al*., 1998; see Figure 7A, lanes 2–3 and 5–6). These experiments indicate that ste9 is not the only APC/C activator necessary for the proteolysis of mitotic cyclins as cells exit mitosis but it plays a fundamental role in G_1. Perhaps APC^{slp1} contributes to the degradation of fission yeast mitotic cyclins during anaphase like in *Saccharomyces cerevisiae*, where APC^{Cdc20} can also target Clb2 for degradation (Baümer *et al*., 2000; Yeong *et al*., 2000). We propose that the main role of APC^{ste9} in fission yeast is to target mitotic cyclins for degradation in G_1.

ste9 is regulated by protein phosphorylation

We raised specific antibodies to ste9 and measured ste9 protein levels in extracts of cells blocked at different points during the mitotic cell cycle. Extracts were made

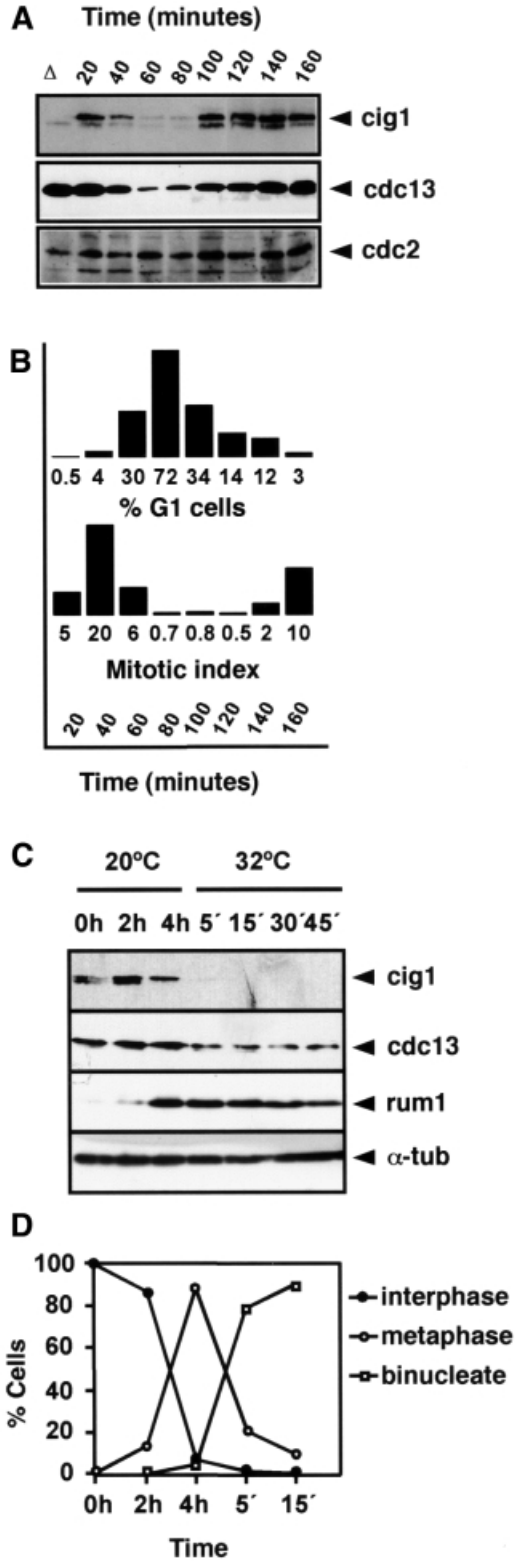


Fig. 2. Cig1 oscillates during the mitotic cell cycle. (**A**) Cig1 protein levels were measured in a synchronous culture of the temperature-sensitive *wee1-50* strain. A homogeneous population of cells in early G_2 was selected by centrifugal elutriation of the *wee1-50* strain at 25°C. This culture was incubated for 20 min at 25°C and then shifted up to 36°C. Samples were taken every 20 min to determine cig1, cdc13 and cdc2 protein levels. Both cig1 and cdc13 protein levels decreased in anaphase and increased at the end of G_1. A *cig1* deletion (Δ) cell extract was used as a negative control. (**B**) Percentage of G_1 cells and mitotic index of the synchronous culture. (**C**) Cig1 is degraded during mitosis. Early G_2 cells of *nda3-KM311* were isolated by centrifugal elutriation at 32°C and blocked in metaphase for 4 h at 20°C. The culture was then released at 32°C. Samples were taken at the indicated times to determine cig1, cdc13, rum1 and α-tubulin protein levels. (**D**) Percentage of cells in interphase, metaphase and anaphase determined by DAPI staining.

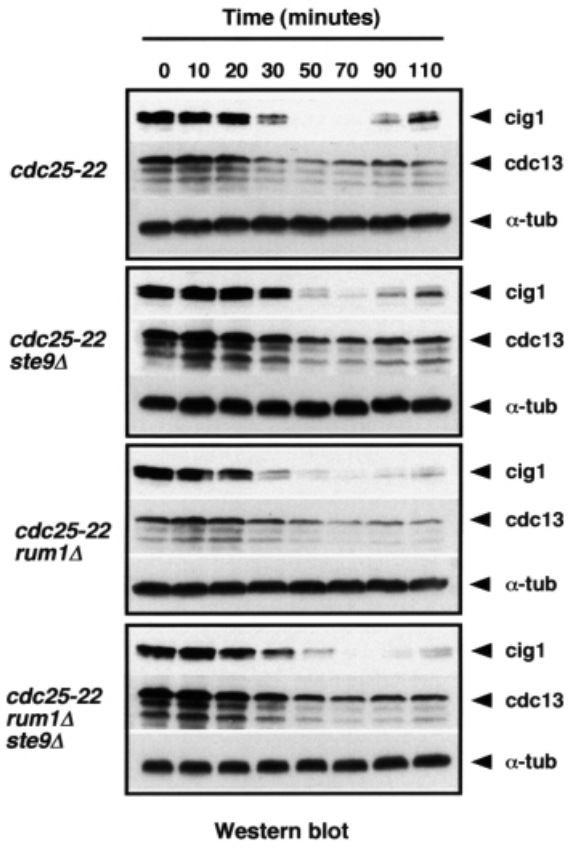
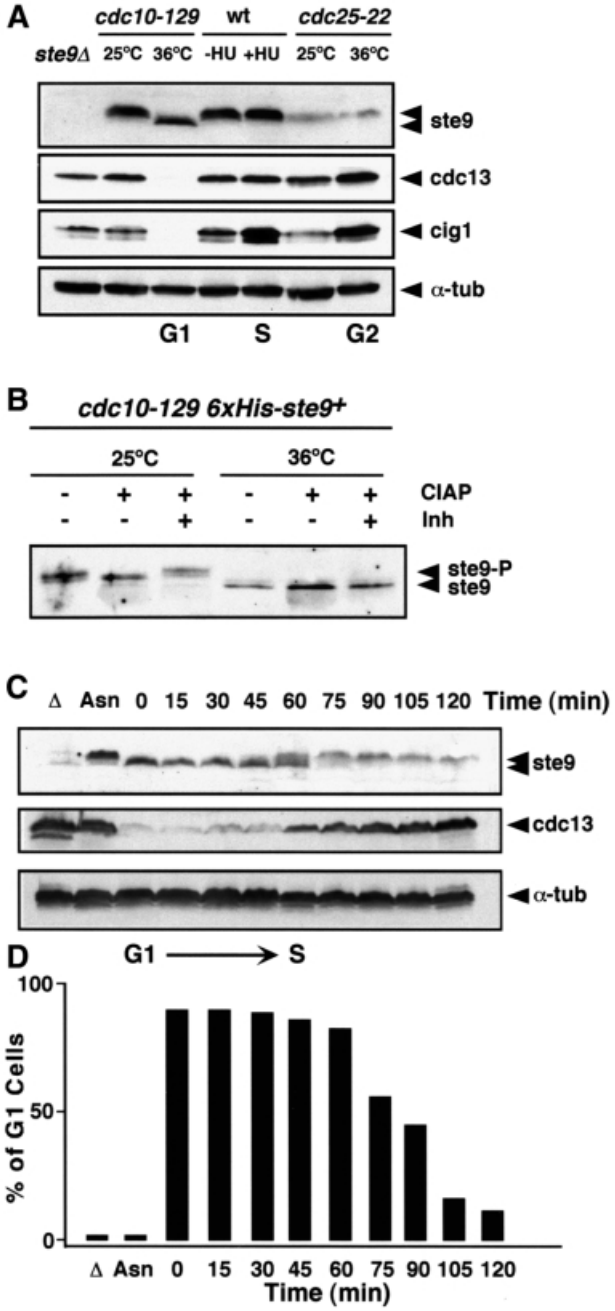


Fig. 3. Degradation of cdc13 and cig1 in mitosis does not require ste9 or rum1. Wild-type, *ste9Δ*, *rum1Δ* and *ste9Δ rum1Δ* cells were synchronized in G_2 using the *cdc25-22* mutant. After 4 h at 36°C, the cultures were release to 25°C and samples were taken to measure cig1, cdc13 and α-tubulin levels.

from cells blocked in G_1 using the *cdc10-129* mutant, in S-phase with the DNA synthesis inhibitor hydroxyurea (HU) and in G_2 using the *cdc25-22* mutant. We observed that ste9 migrated more rapidly in protein extracts from cells blocked in G_1 (*cdc10-129* at 36°C) than in S-phase (wt + HU) or G2 (*cdc25-22* at 36°C) (Figure 4A). Treatment with alkaline phosphatase converted the upper bands into the lower band, indicating that ste9 is phosphorylated *in vivo* (Figure 4B). Phosphorylation of ste9 was then analysed in cells synchronized in G_1 by blocking the *cdc10-129* mutant during 4 h at 36°C and then releasing these cells to 25°C. ste9 was completely dephosphorylated in G_1 (Figure 4C, t = 0) and became phosphorylated ~60 min after the release as cells were undergoing S-phase (Figure 4C and D). Cdc13 cyclin, one of the targets of APC^{ste9}, began to accumulate in these cells at the time when ste9 became phosphorylated. These experiments suggest that, similarly to Hct1/Cdh1 in *S.cerevisiae*, ste9 is negatively regulated during S-phase and G_2 by phosphorylation.



Cdc2–cyclin phosphorylates ste9 at multiple sites

ste9 phosphorylation occurs in S-phase and G_2 when the cdc2–cyclin kinase activity is high. In order to study whether ste9 phosphorylation is dependent on cdc2–cyclin activity, we synchronized the wild-type and the *cdc2-33* mutant in G_1 by nitrogen starvation at 25°C for 12 h. Nitrogen was then added back and the cultures were divided into two. Half of the cells were incubated at the permissive temperature (25°C) and the other half at the restrictive temperature (36°C) for the $cdc2^{ts}$ mutant. Wild-type and *cdc2-33* cells incubated at 25°C underwent S-phase ~4 h after the addition of nitrogen (Figure 5A). At 36°C, wild-type cells completed S-phase after 4 h whereas the *cdc2-33* mutant remained in G_1 for up to 6 h (Figure 5A). As shown in Figure 5B, ste9 phosphorylation in the *cdc2-33* mutant was detected after 4 h at 25°C but not at 36°C, suggesting that phosphorylation of ste9 is dependent on the activation of cdc2–cyclin kinase at G_1/S. We also observed that ste9 protein levels decreased at least 4-fold as it became phosphorylated (Figure 5B); expression of the $ste9^+$ gene is constant under this experimental condition (data not shown), suggesting that phosphorylation of ste9 reduces its stability. We consistently found a reduction in ste9 protein levels in G_2 cells compared with cells in G_1 (see also Figure 4A, compare levels in $cdc25^{ts}$ with levels in $cdc10^{ts}$).

To confirm that phosphorylation of ste9 depends on cdc2 kinase activity, we performed an additional experiment. *cdc2-33* and *cdc25-22* mutant cells growing at 25°C were shifted to 36°C and samples were taken at 0, 2 and 4 h after the shift. ste9 became dephosphorylated in *cdc2-33* cells incubated at the restrictive temperature but not in *cdc25-22* cells (Figure 5C), indicating that active cdc2 kinase is needed to maintain ste9 in its phosphorylated form.

Fig. 4. ste9 is phosphorylated in S-phase and G_2 but not in G_1. (**A**) ste9, cdc13 and cig1 protein levels in cells arrested in G_1 with the *cdc10-129* mutant, in S-phase with hydroxyurea and in G_2 with the *cdc25-22* mutant. As a negative control, we used an extract prepared from the *ste9Δ* mutant and α-tubulin as loading control. (**B**) A His_6-ste9 allele introduced by gene replacement into the *ste9* locus was purified on an Ni^{2+}-NTA column from *cdc10-129* cells growing at 25°C or after 4 h at 36°C. The purified His_6-ste9 was treated with calf intestine alkaline phosphatase (CIAP) in the absence (–) or presence (+) of phosphatase inhibitors (Inh). (**C**) ste9 phosphorylation occurs at the G_1–S transition. A *cdc10-129* culture grown at 25°C to mid-exponential phase in minimal medium was shifted to 36°C for 4 h and then released at 25°C. Samples for western blot and flow cytometry were taken before the shift to 36°C (Asn), 4 h after the shift to 36°C (t = 0 min) and every 15 min after the release to 25°C. The levels of ste9, cdc13 and α-tubulin were determined. ste9 was unphosphorylated at the block point (t = 0 min) and became phosphorylated at the onset of S-phase (t = 60 min). Cdc13 protein levels were low in G_1 cells while ste9 was unphosphorylated and started to accumulate when ste9 became phosphorylated and the cells initiated S-phase. Δ is a negative control from *ste9Δ* and Asn is an extract from the asynchronous culture of *cdc10-129* at 25°C. (**D**) Percentage of cells in G_1 during the course of the experiment.

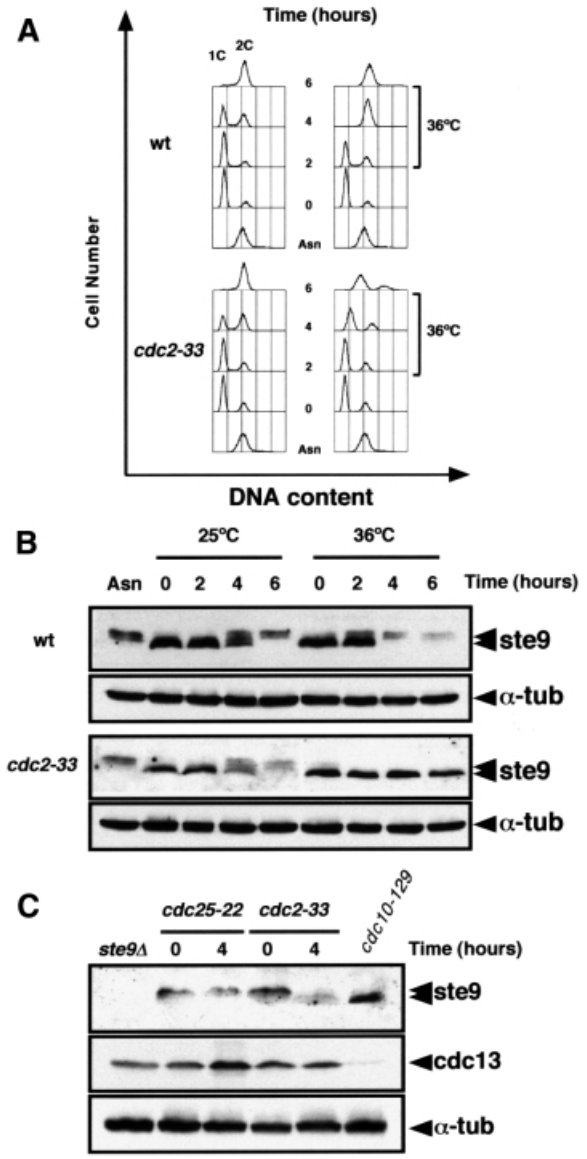


Fig. 5. ste9 phosphorylation depends on cdc2 function. Wild-type and *cdc2-33* cells were nitrogen starved for 12 h at 25°C. NH_4Cl was added to the culture and half of the cells were incubated at 25°C and the rest at 36°C. Samples were taken for flow cytometry (**A**) and for western blots (**B**) at 0, 2, 4 and 6 h after the addition of NH_4Cl to determine ste9 and α-tubulin protein levels. Asn corresponds to a sample taken from the asynchronous culture before nitrogen starvation. (**C**) ste9 mobility in *cdc25-22* and *cdc2-33* extracts prepared from cells growing exponentially at 25°C (t = 0) and then shifted to 36°C for 4 h (t = 4). The levels of cdc13, cdc2 and α-tubulin in these extracts are also shown. As a control, we used an extract from *cdc10-129* cells incubated at 36°C for 4 h.

Examination of the ste9 amino acid sequence revealed the presence of four putative cdc2 phosphorylation sites (S62, T98, T177 and S214) with the consensus S/T-P-X-K/R (where X represents any amino acid). There are nine additional sites (S130, T134, T143, T159, T174, S187, S425, S513 and S547) with the sequence S/T-P of which six are located at the N-terminus and three at the C-terminus of the protein within the seven WD repeats (Figure 6A). We generated three mutant alleles of *ste9*$^+$ by site-directed *in vitro* mutagenesis where these putative phosphorylation sites were mutated to alanine. *ste9-4A* contained the four putative phosphorylation sites with the strict consensus sequence, *ste9-10A* contained in addition the six S/T-P sites at the N-terminus and *ste9-13A* has all the sites mutated to alanine. The three mutant alleles were introduced into the *ste9*$^+$ locus by gene replacement. Expression of these mutant forms of *ste9* were able to rescue fully the sterility defect of *ste9*-deleted cells (data not shown). Cells expressing *ste9-10A* and *ste9-13A* were elongated compared with wild-type cells and, when they were streaked onto YES plates containing phloxin B, many dark red colonies were observed, suggesting that these cells were undergoing diploidization at high frequency. To confirm this observation, we measured the DNA content of these cells by flow cytometry and found that 8% of the cells were diploids in *ste9-4A*, 18% in *ste9-10A* and 38% in *ste9-13A* (Figure 6B). There was a direct correlation between the number of phosphorylatable residues mutated to alanine and the ability of the ste9 mutants to induce diploidization. This result indicates that cdk phosphorylation of ste9 is important to down-regulate ste9 in S-phase and G_2. If ste9 is not phosphorylated in G_2, it can promote cdc13 degradation and, as a consequence, the cells endoreduplicate their DNA. We have also found that the electrophoretic mobility of ste9-10A and ste9-13A was similar to that of unphosphorylated ste9 from cells arrested in G_1 with *cdc10-129* at 36°C (Figure 6C). Thus, ste9 is phosphorylated at multiple sites *in vivo* and this phosphorylation results in its inactivation.

As shown in Figure 5B, phosphorylation of ste9 by cdc2–cyclin complexes correlates with a significant decrease in ste9 protein levels. To investigate the possibility that cdc2 phosphorylation regulates ste9 stability, we compared the half-life of wild-type ste9 with that of ste9-4A, ste9-10A and ste9-13A mutant proteins. Cultures of wild-type and the three mutant strains generated by gene replacement with the *ste9* mutant alleles were grown to mid-exponential phase and the half-lives of ste9, ste9-4A, ste9-10A and ste9-13A were measured after the addition of the protein synthesis inhibitor cycloheximide. Figure 6D shows that ste9 is short-lived (half-life <10 min) under these conditions. The half-lives of the mutant proteins increased considerably, particularly in the case of the ste9-10A mutant (half-life >120 min) (Figure 6D). This result confirms that ste9 protein levels are down-regulated *in vivo* by cdc2-dependent phosphorylation.

ste9 phosphorylation prevents its interaction with APC/C

Previous reports have shown that cdk phosphorylation of Hct1/Cdh1 in budding yeast and animal cells prevents its association with APC/C (Zachariae *et al*., 1998; Jaspersen *et al*., 1999; Lukas *et al*., 1999; Kramer *et al*., 2000). To test whether ste9 associates with APC/C, we used a strain where one of the APC/C subunits cut9 was epitope tagged with three copies of haemagglutinin (HA) (Berry *et al*., 1999). ste9 co-precipited with cut9-HA in extracts prepared from cells arrested in G_1 with the *cdc10-129* mutant (Figure 7A and B, lanes 6). In contrast, ste9 was not associated with APC/C in extracts from cells arrested in G_2 with the *cdc25-22* mutant (Figure 7B, lane 3). Thus ste9 interacts with APC/C only in G_1 when it is not phosphorylated and it does not interact with APC/C in

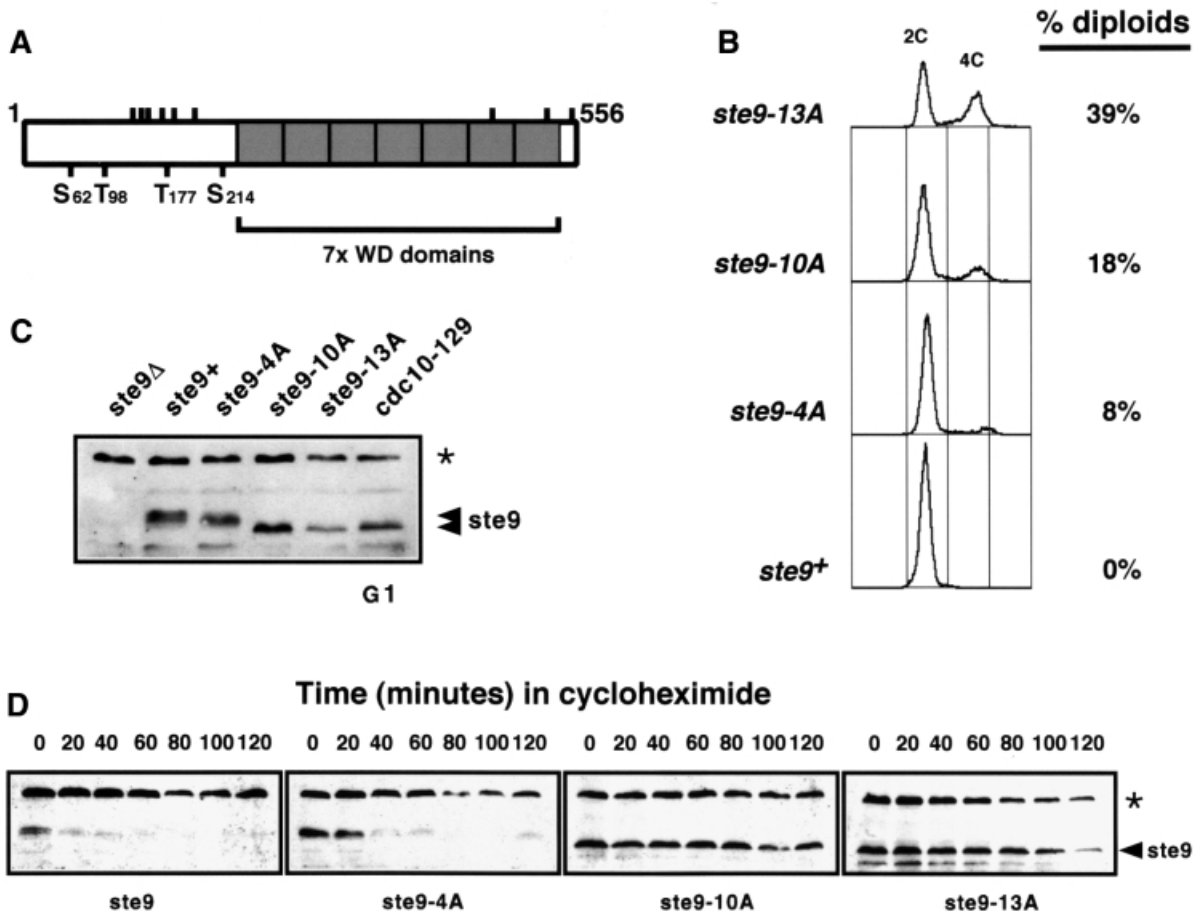


Fig. 6. Expression of ste9 phosphorylation mutants induces diploidization. Three mutants, *ste9-4A*, *ste9-10A* and *ste9-13A*, containing four, 10 and the 13 putative cdk phosphorylation sites were mutated to alanine by site-directed *in vitro* mutagenesis. These mutant alleles were introduced into the fission yeast genome by gene replacement. (**A**) Schematic representation of the ste9 protein with the seven WD repeats and the position of the 13 putative cdk phosphorylation sites. (**B**) FACS profile of cells replaced with the different ste9 mutant alleles. (**C**) Electrophoretic mobility of the different *ste9* alleles. The asterisk corresponds to a non-specific band recognized by the anti-ste9 antibody that it is also detected in *ste9Δ*. (**D**) Half-lives of ste9, ste9-4A, ste9-10A and ste9-13A in exponentially growing cultures after the addition of 100 μg/ml of cycloheximide. The asterisk corresponds to a non-specific band recognized by the anti-ste9 antibody that it is also detected in *ste9Δ* and serves as loading control.

G_2 when it becomes phosphorylated. We then analysed whether the mutant proteins ste9-10A and ste9-13A were able to interact with APC/C in G_2 as this may be the reason for the diploidization phenotype observed in these mutants. To test this, we prepared extracts from cells arrested in G_2 with the *cdc25-22* mutant and then immunoprecipitated APC/C using anti-HA antibodies. ste9 co-precipitated with APC/C in extracts expressing *ste9-10A* and *ste9-13A* but not in extracts expressing wild-type *ste9*$^+$ (Figure 7B, lanes 3–5), suggesting that phosphorylation of ste9 causes its dissociation from APC/C in G_2.

Discussion

In this study, we provide biochemical evidence for a role for ste9 as a negative regulator of cell cycle progression in G_1. ste9 is a member of a highly conserved family of proteins containing seven WD repeat domains of which the prototypes are Hct1/Cdh1 of budding yeast and Fizzy-related of higher eukaryotes (Schwab *et al*., 1997; Sigrist and Lehner, 1997; Visintin *et al*., 1997; Kramer *et al*., 1998). These proteins function as activators of APC/C to promote polyubiquitylation and degradation of mitotic cyclins in mitosis and G_1. Here we show that in fission yeast: (i) APC^{ste9} promotes degradation of the mitotic cyclins cdc13 and cig1 but not of the S-phase cyclin cig2. The fact that cig2 levels are high in cells overexpressing ste9 provides an explanation for the re-replication phenotype associated with these cells. (ii) APC^{ste9} is not necessary for the proteolysis of mitotic cyclins at the end of mitosis because in cells lacking ste9, degradation of cdc13 and cig1 still occurs. However, ste9 is absolutely required to degrade mitotic cyclins completely when cells need to delay or to stop the cell cycle in G_1. This is important for small cells that had to lengthen the G_1-phase until they reach the minimum cell size required to initiate DNA replication or to prevent entry into mitosis from G_1. (iii) ste9 is phosphorylated by the cdc2 kinase *in vivo* at multiple sites. Cdk phosphorylation of ste9 in G_2 has two effects. First, to promote the degradation of ste9 and secondly, to prevent ste9 association with APC/C. APC/C–ste9 interaction occurs only in G_1 when ste9 is in its dephosphorylated form. In S-phase and G_2, ste9 becomes phosphorylated and it does not interact with APC/C.

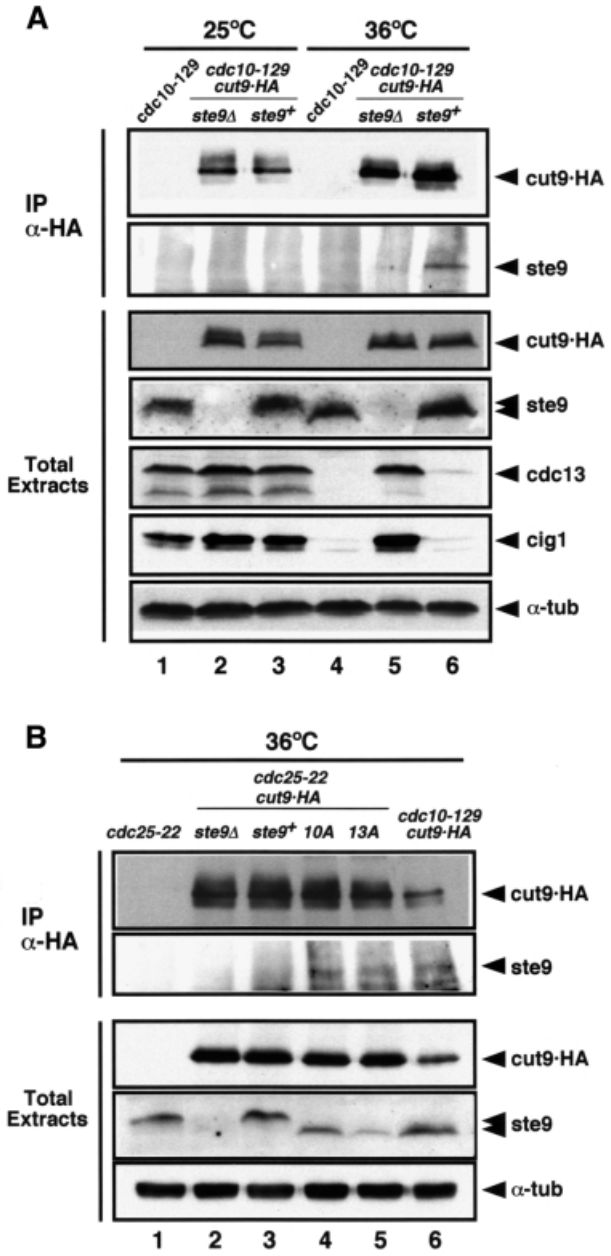


Fig. 7. ste9 associates with APC/C only in G_1. (**A**) Extracts from *cdc10-129*, *cdc10-129 cut9·HA ste9Δ* and *cdc10-129 cut9·HA* mutants grown at 25 or 36°C were immunoprecipitated with anti-HA antibodies (IP α-HA) and then western blotted with anti-HA or anti-ste9 antibodies. Total cell extracts were separated on an SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-HA, anti-ste9, anti-cdc13, anti-cig1 and anti-α-tubulin antibodies. (**B**) ste9 phosphorylation mutants associate with APC/C in G_2. Extracts from *cdc25-22*, *cdc25-22 cut9·HA* and *cdc10-129 cut9·HA* mutants grown at 36°C for 4 h were immunoprecipitated with anti-HA antibodies (IP α-HA) and then western blotted with anti-HA and anti-ste9 antibodies. Total cell extracts were separated on an SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-HA, anti-ste9 and anti-α-tubulin antibodies.

Two APC complexes are involved in the degradation of mitotic cyclins

We have found that cdc13 and cig1 are targets for APC^{ste9} in G_1. Two observations support this idea: first cdc13 and cig1 protein levels decrease when ste9 is overproduced. In addition, cig1 and cdc13 are not degraded when a *cdc10-129 ste9Δ* mutant is incubated at the restrictive temperature. The fact that both cyclins are destroyed during mitosis in cells lacking ste9 suggests that another APC complex is responsible for the degradation of cig1 and cdc13 at the metaphase–anaphase transition. Perhaps slp1, the fission yeast homologue of Cdc20, in association with APC/C may trigger the degradation of cig1 and cdc13 as cells exit mitosis. This is reminiscent of the situation in budding yeast where proteolysis of Clb2 by the APC/C occurs in two stages. First, a fraction of Clb2 is destroyed during anaphase by APC^{Cdc20} and the rest is degraded at the end of mitosis by APC^{Hct1} (Baümer *et al*., 2000; Yeong *et al*., 2000). It is interesting to note that cig1 does not have a clear destruction box sequence as is the case with cdc13, nor does it have a KEN box (Pfleger and Kirschner, 2000). However, cig1 is destroyed in mitosis a bit earlier than cdc13. Future experiments will address the existence of a non-canonical destruction box in cig1.

Inhibition of Cdk–cyclin B in G_1 and cell differentiation

In fission yeast, down-regulation of cdk activity is important for the G_1 arrest upon nitrogen starvation or after treatment with mating pheromone (Moreno and Nurse, 1994; Stern and Nurse, 1997; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998). APC^{ste9} and rum1 play a pivotal role in decreasing levels and activities of the cdc2–cdc13 complex during G_1 below a threshold level, which allows cells to initiate the differentiation programmes such as mating or meiosis. Therefore, APC^{ste9} and rum1 provide the molecular switch between cell proliferation and cell differentiation (Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Stern and Nurse, 1998). We have observed an increase in *rum1*$^+$ and *ste9*$^+$ mRNA and protein levels in cells that exit the mitotic cell cycle because of nutrient limitation (Martín-Castellanos *et al*., 2000; M.A.Blanco and S.Moreno, unpublished results). There are a number of reports in the literature suggesting that mitotic cyclins need to be kept under tight control in differentiating cells. For example, fission yeast cells lacking rum1 or ste9 are unable to undergo cell differentiation (Moreno and Nurse, 1994; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Stern and Nurse, 1998). In budding yeast, *hct1* mutants are resistant to mating pheromone, suggesting that APC^{Hct1} is important for the mating response (Schwab *et al*., 1997). *Drosophila fizzy-related* (*fzr*) is expressed and required at specific stages of embryogenesis when cells stop proliferating (Sigrist and Lehner, 1997). In animal cells, *HCT1/CDH1* is highly expressed in tissues composed predominantly of differentiated cells, such as adult brain where APC$^{Hct1/Cdh1}$ is very active (Gieffers *et al*., 1999). In the plant *Medicago sativa*, expression of the *ste9* homologue *ccs52* is turned on when nodule primordium differentiates and for the formation of large differentiated cells that polyploidize (Cebolla *et al*., 1999). This is consistent with results in *Drosophila* and fission yeast where down-regulation of mitotic cyclins caused by high levels of *fzr* or *ste9*$^+$ expression induces endoreduplication (Sigrist and Lehner, 1997; Yamaguchi *et al*., 1997; Kitamura *et al*., 1998; Figure 1B). All these findings suggest that mitotic cyclins need to be destroyed in G_1 to allow cell differentiation, and abnormal degradation of mitotic cyclins in G_2 will lead to re-replication.

Table I. *Schizosaccharomyces pombe* strains

Strain	Genotype	Source
PN1	*972 h⁻*	P.Nurse
PN22	*leu1-32 h⁻*	P.Nurse
PN7	*cdc25-22 h⁻*	P.Nurse
PN8	*cdc2-33 h⁻*	P.Nurse
S391	*wee1-50 cig2-HA h⁻*	S.Moreno
S18	*nda3-KM311 leu1-32 h⁺*	S.Moreno
S683	*cig2HA leu1-32 h⁺*	S.Moreno
S627	*ste9Δ::ura4⁺ leu1-32 ura4d18 h⁻*	H.Okayama
S10	*cdc25-22 leu1-32 h⁻*	P.Nurse
S11	*cdc10-129 leu1-32 h⁻*	P.Nurse
S868	*cdc25-22 rum1Δ::ura4⁺ leu1-32 ura4d18*	this study
S869	*cdc25-22 ste9Δ::ura4⁺ leu1-32 ura4d18 h⁻*	this study
S870	*cdc25-22 ste9Δ::ura4⁺ rum1Δ::ura4 leu1-32 ura4d18*	this study
S871	*cdc10-129 ste9::His$_6$-ste9 leu1-32 ura4d8 h⁻*	this study
S872	*ste9::ste9-4A leu1-32 ura4d18 h⁻*	this study
S873	*ste9::ste9-10A leu1-32 ura4-d18 h⁻*	this study
S874	*ste9::ste9-13A leu1-32 ura4-d18 h⁻*	this study
KGY1365	*cut9::cut9-HA/Kan^r h⁺*	K.Gould
S875	*cdc10-129 cut9::cut9-HA/Kan^r leu1-32 ura4d18*	this study
S876	*cdc10-129 cut9::cut9-HA/Kan^r ste9Δ::ura4⁺ leu1-32 ura4d18*	this study
S877	*cdc25-22 cut9::cut9-HA/Kan^r leu1-32 ura4d18*	this study
S878	*cdc25-22 cut9::cut9-HA/Kan^r ste9Δ::ura4⁺ leu1-32 ura4d18*	this study
S879	*cdc25-22 cut9::cut9-HA/Kan^r ste9::ste9-10A leu1-32 ura4d18*	this study
S880	*cdc25-22 cut9::cut9-HA/Kan^r ste9::ste9-13A leu1-32 ura4d18*	this study

Phosphorylation of ste9 promotes its proteolysis and dissociation from APC/C

ste9 is phosphorylated in all phases of the cell cycle except in G_1. Low Cdk–cyclin activity in G_1 is generated by the combined effects of cyclin proteolysis (through APC^{ste9}) and cdk inhibition (through rum1). Cdc2–cyclin complexes negatively regulate both ste9 and rum1 (Benito *et al*., 1998 and this study). In early G_1, ste9 and rum1 are dephosphorylated and active. As the cell grows during G_1, cig1, cig2 and cdc13 cyclins begin to accumulate. Cdk activity eventually predominates during S-phase resulting in the phosphorylation of both ste9 and rum1. Phosphorylation of ste9 causes its dissociation from APC/C and also affects its stability. In *S.cerevisiae* and animal cells, it has been reported that Hct1/Cdh1 is a very stable protein and phosphorylation of Hct1/Cdh1 only affects its association with APC/C (Kramer *et al*., 1998; Prinz *et al*., 1998; Zacchariae *et al*., 1998; Jaspersen *et al*., 1999; Lukas *et al*., 1999). This is based on experiments showing that Hct1/Cdh1 protein levels do not change throughout the cell cycle. However, no half-life experiments using wild-type levels of Hct1 and mutant proteins expressed under its own promoter, like that described in Figure 6D, has been performed. In fission yeast, we find that ste9 is an unstable protein. ste9 protein levels decrease in G_2, specially in cells re-entering the mitotic cell cycle from starvation conditions (Figure 5B). We have observed an increase in *ste9⁺* mRNA and protein levels in cells arrested in G_1 after nitrogen starvation (M.A.Blanco and S.Moreno, unpublished data). This might provide a mechanism to supply plenty of ste9 protein in cells that arrest the cell cycle in G_1 because of nutrient limitation and to reduce the ste9 protein levels in proliferating cells.

A mutant strain expressing a *ste9* allele where 10 putative Cdk phosphorylation sites were mutated to alanine (*ste9-10A*) showed a very clear gain-of-function phenotype. This mutant protein is very stable and associates with APC/C in G_2. These cells showed a high frequency of diploidization. This phenotype could be explained if unregulated association of the mutant protein with APC/C in G_2 reduces the level of the cdc2/cdc13 activity below a certain threshold level required to prevent initiation of another round of S-phase within the same cell cycle (Hayles *et al*., 1994). Thus, the APC^{ste9} complex, which normally is present only in G_1, could induce extra rounds of S-phase when present in S-phase or G_2.

Cdc2–cyclin complexes and their inhibitors (rum1 and APC/C) antagonize each other's activity during the cell cycle. Phosphorylation of ste9 and rum1 at G_1/S by cdc2–cyclin provides a molecular switch to prevent inactivation of cdc2 during S-phase and early mitosis. A key unresolved issue in fission yeast cell cycle research is which is the protein phosphatase that reactivates ste9 and rum1 during mitosis. Very recently, a fission yeast protein with significant homology to budding yeast Cdc14 has been deposited in the fission yeast sequencing project (A.Bueno and V.Simanis, personal communication). Functional analysis of this protein will be necessary in order to determine its role at the metaphase–anaphase transition or in cell cycle exit.

Materials and methods

Fission yeast strains and methods

The *S.pombe* strains used in this study are listed in Table I. Growth conditions and strain manipulations were as described by Moreno *et al*. (1991). The *h⁺ cut9::cut9-HA/Kan^r* strain was described by Berry *et al*. (1999). The *srw1/ste9Δ::ura4⁺ ura4d18 leu1-32 h⁻* strain was described by Yamaguchi *et al*. (1997), and involves deletion of the entire open reading frame. Since deletion of *ste9⁺* causes sterility, all the crosses involving a deletion of *ste9⁺* were done by transforming with pREP3X-*ste9⁺*, so that *ste9⁺* is expressed from a plasmid, and the double mutants

were checked subsequently to ensure that the plasmid had been lost. Protoplast fusion and tetrad analysis was performed to construct double *rum1Δ ste9Δ* mutants, and the identity of these mutants was confirmed by Southern blotting. Yeast transformation was carried out using the lithium acetate transformation protocol (Norbury and Moreno, 1997).

All experiments in liquid culture were carried out in essential minimal medium (EMM) containing the required supplements, starting with a cell density of 2–4 × 10^6 cells/ml, corresponding to mid-exponential phase growth. Temperature shift experiments were carried out using a water bath at 36.5°C.

To induce expression from the *nmt1* promoter, cells were grown to mid-exponential phase in EMM containing 5 μg/ml thiamine, then spun down and washed four times with minimal medium lacking thiamine at a density calculated to produce 4 × 10^6 cells/ml at the time of peak expression from the *nmt1* promoter.

Synchronous cultures

wee1-50 h$^-$ cells were grown at 25°C in EMM. Cells were synchronized at 25°C using a JE-5.0 elutriation system (Beckman Instruments, Inc.) and then shifted to 36°C, resulting in entry into mitosis at a reduced cell size. Samples were taken every 20 min for making protein extracts and for flow cytometry analysis.

ste9 phosphorylation site mutants

A 4.6 kb genomic fragment containing the *ste9*$^+$ gene was cloned into pTZ18R. This plasmid was used to obtain the different *ste9* mutants by site-directed mutagenesis using the Muta-gene phagemid *in vitro* mutagenesis kit (Bio-Rad).

All three mutants, pTZ18R-ste9-4A (S62, T98, T177 and S214), pTZ18R-ste9-10A (S62, T98, S130, T134, T143, T159, T174, T177, T187 and S214) and pTZ18R-ste9-13A (S62, T98, S130, T134, T143, T159, T174, T177, T187, S214, S425, S513 and S547) were sequenced after the mutagenesis. The 4.6 kb genomic fragments containing these mutations were then transformed into a *ste9Δ::ura4*$^+$ *ura4d18* strain, and 5-fluoro-orotic acid (5-FOA) was used to select ura$^-$ colonies (Boeke *et al*., 1984). PCR analysis and Southern blotting of DNA isolated from these colonies confirmed that the *ste9Δ::ura4*$^+$ locus had been replaced specifically with *ste9* mutant alleles by homologous recombination. To make the constructions in pREP3X and pREP81X, the coding region of *ste9*$^+$ was amplified by PCR using the Expand High Fidelity PCR System (Roche) and sense (TGAAGTCAGGGATCCTAACG) and antisense (GAGTGAATGGGATCCATTAC) oligonucleotide primers. To facilitate cloning, *Bam*HI sites (underlined sequence) were introduced upstream of the initiation codon and downstream of the termination codon. The 1.67 kb PCR products were digested with *Bam*HI and subcloned into pREP3X and pREP81X vectors.

His_6 tagging of ste9

pTZ18R-*ste9*$^+$ plasmid containing the 4.6 kb genomic fragment was used to introduce a His_6 tag just after the initiation codon by site-directed mutagenesis using the Muta-gene phagemid *in vitro* mutagenesis kit (Bio-Rad) and the oligonucleotide 5′-GGGCCTAACGTGAAATTATGCATCACCATCACCATCACGAATTTGATGGGTTTACTAG-3′ (where the initiation codon and His_6-encoding codons are underlined). The 4.6 kb genomic fragment containing *His_6-ste9* was then transformed into a *ste9Δ::ura4*$^+$ *ura4d18* strain, and 5-FOA used to select ura$^-$ colonies (Boeke *et al*., 1984). Southern blotting, PCR analysis of DNA isolated from these colonies and sequencing of the PCR products confirmed that the *ste9Δ::ura4*$^+$ locus had been replaced specifically with the *His_6-ste9* allele by homologous recombination. In order to demonstrate that *His_6-ste9* is functional, we checked the ability of the *His_6-ste9* strain to conjugate and sporulate. Whilst *ste9Δ* cells are sterile, *His_6-ste9* cells are fertile to the same extent as the wild-type.

Preparation of rabbit polyclonal antibodies against ste9

A peptide spanning the C-terminal 14 residues of ste9 (CSTMSSPFDPTMKIR) was coupled to keyhole limpet haemocyanin (KLH) and injected into a rabbit with Freund's adjuvant followed by standard procedures for raising polyclonal antibodies (Harlow and Lane, 1988).

Preparation of rabbit polyclonal antibodies against Cig1

A 471 bp DNA fragment encoding the first 157 residues of cig1 protein was subcloned into pGEX-KG (Pharmacia). The GST–cig1-157N fusion protein was produced in *Escherichia coli*, purified with glutathione–Sepharose 4B (Pharmacia Biotech) and used to raise anti-cig1 polyclonal antibodies as indicated above.

Protein extracts and western blots

Total protein extracts were prepared from 3×10^8 cells collected by centrifugation, washed in Stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 pH 8.0) and resuspended in 25 μl of RIPA buffer (10 mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 150 mM NaCl pH 7.0) containing the following protease inhibitors, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 100 μM 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), 100 μM *N*-tosyl-L-phenyalanine chloromethyl ketone (TPCK), 100 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM phenanthroline and 100 μM *N*-acetyl-leu-leu-norleucinal. Cells were boiled for 5 min, broken using 750 mg of glass beads (0.4 mm Sigma) for 15 s in a Fast-Prep machine (Bio101 Inc.), and the crude extract was recovered by washing with 0.5 ml of RIPA buffer. Protein concentration was determined by the BCA protein assay kit (Pierce).

For western blots, 50 μg of total protein extract was run on a 10% SDS–polyacrylamide gel, transfered to nitrocellulose and probed with rabbit affinity-purified anti-ste9-C-terminus (1:200), SP4 anti-cdc13 (1:250) or anti-cig1 (1:250) polyclonal antibodies, or with the monoclonal anti-HA antibody 12CA5 (0.15 μg/ml). Goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (HRP; Amersham) (1:3500) was used as secondary antibody. Mouse TAT1 anti-tubulin monoclonal antibodies (1:500) and HRP-conjugated goat anti-mouse antibody (1:2000) as secondary antibody were used to detect tubulin as loading control. Immunoblots were developed using the ECL kit (Amersham) or Super Signal (Pierce).

Alkaline phosphatase treatment

His_6-ste9 protein was purified from total protein extracts as described by Shiozaki and Russell (1997). Briefly, 3 × 10^8 cells were lysed in 6 M guanidine hydrochloride, 0.1 M sodium phosphate, 50 mM Tris–HCl pH 8.0. The lysate was clarified by centrifugation for 10 min at room temperature, and the supernatant was mixed with Ni^{2+}-NTA–agarose (Qiagen) and incubated in a rotating wheel for 60 min at room temperature. The Ni^{2+}-NTA–agarose beads were washed with an 8–0.5 M urea reverse gradient in 0.1 M sodium phosphate, 50 mM Tris–HCl pH 8.0. After washing twice with alkaline phosphatase buffer (5 mM Tris–HCl pH 8.0, 0.5 mM $MgCl_2$), beads were incubated with 50 U of alkaline phosphatase (Roche) for 30 min at 37°C in the presence or absence of phosphatase inhibitors. The final concentrations of phosphatase inhibitors were 1 mM sodium pyrophosphate, 5 mM EDTA and 0.1 mM orthovanadate. The reactions were stopped by boiling for 3 min after the addition of 2× SDS sample buffer, and samples were run on a 10% SDS–polyacrylamide gel, followed by western blot analysis.

Co-immunoprecipitation of ste9 and cut9-HA

Total protein extracts were prepared from 3×10^8 cells using HB buffer (Moreno *et al*., 1991). Cell extracts were spun at 4°C in a microcentrifuge for 15 min, and the protein concentration was determined by the BCA protein assay kit (Pierce). A 3 mg aliquot of total protein extracts was subjected to immunoprecipitation by consecutive incubation with the monoclonal anti-HA 12CA5 (2 μg) for 1 h in ice and protein A–Sepharose (Phrmacia-Biotech) for 30 min at 4°C in a rotating wheel. Immunoprecipitates were washed six times with 1 ml of HB buffer. Lysates and immunoprecipitates were separated on a 10% SDS–polyacrylamide gel, followed by western blot analysis as above.

Flow cytometry and microscopy

About 10^7 cells were spun down, washed once with water, fixed in 70% ethanol and processed for flow cytometry or 4′,6-diamidino-2-phenylindole (DAPI) staining, as described previously (Sazer and Sherwood, 1990; Moreno *et al*., 1991). A Becton-Dickinson FACScan was used for flow cytometry. To estimate the proportion of G_1 cells, we determined the percentage of cells with a DNA content less than a value midway between 1C and 2C. The mitotic index was determined by counting the percentage of anaphase cells (cells with two nuclei and without a septum) after DAPI staining.

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