

# Phosphorylation of the mitotic regulator Pds1/securin by Cdc28 is required for efficient nuclear localization of Esp1/separase

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Sister chromatid separation at the metaphase-to-anaphase transition is induced by the proteolytic cleavage of one of the cohesin complex subunits. This process is mediated by a conserved protease called separase. Separase is associated with its inhibitor, securin, until the time of anaphase initiation, when securin is degraded in an anaphase-promoting complex/cyclosome (APC/C)-dependent manner. In budding yeast securin/Pds1 not only inhibits separase/Esp1, but also promotes its nuclear localization. The molecular mechanism and regulation of this nuclear targeting are presently unknown. Here we show that Pds1 is a substrate of the cyclin-dependent kinase Cdc28. Phosphorylation of Pds1 by Cdc28 is important for efficient binding of Pds1 to Esp1 and for promoting the nuclear localization of Esp1. Our results uncover a previously unknown mechanism for regulating the Pds1–Esp1 interaction and shed light on a novel role for Cdc28 in promoting the metaphase-to-anaphase transition in budding yeast.

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In all eukaryotic cells, cyclin-dependent kinases (CDKs) are essential for driving cell cycle progression. Periodic activation of different cyclin–CDK complexes is largely responsible for the regulation of various cell cycle processes, such as passage through START, initiation of DNA replication, and entry into mitosis. It is still not clear how cyclin–CDK activity is translated into this complex interplay of events, mainly because many of the critical cyclin–CDK substrates are unknown.

A central step in mitosis is the separation of sister chromatids, which takes place at the metaphase-to-anaphase transition. The proper timing of sister chromatid separation is key for accurate chromosome segregation: both premature separation, prior to the formation of bipolar spindle attachments between the chromatids and the spindle microtubules, and a prolonged delay in separation will result in extensive chromosome missegregation. Sister chromatid separation is triggered by the ubiquitin-dependent degradation of an anaphase inhibitor known as securin. Prior to anaphase, securin associates with and inhibits an anaphase activator known as separase, a protease that cleaves a subunit of the cohesin

complex that mediates the association between the two sister chromatids (Ciosk et al. 1998; Uhlmann et al. 1999; Nasmyth et al. 2000; Cohen-Fix 2001). At the onset of anaphase securin is degraded in a process that involves the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase, acting in conjunction with the Cdc20/Fizzy protein (Visintin et al. 1997; Zachariae and Nasmyth 1999). This, in turn, allows separase to promote anaphase initiation. The activity of the APC/C is also required for mitotic exit, where it acts in concert with the Cdh1 protein to promote the degradation of several proteins, including the mitotic cyclins (Schwab et al. 1997; Visintin et al. 1997). The APC/C is subjected to regulation by the spindle checkpoint pathway (Li et al. 1997; Fang et al. 1998; Alexandru et al. 1999), which inhibits APC/C activation until all chromosomes have formed stable bipolar spindle attachments, thereby preventing the premature dissociation of sister chromatids.

The budding yeast securin is called Pds1. Pds1 is a nuclear protein that is present in cells from late G<sub>1</sub>/early S until the onset of anaphase, when it is degraded in an APC/C<sup>Cdc20</sup>-dependent manner (Cohen-Fix et al. 1996; Visintin et al. 1997). Pds1 physically associates with Esp1, the budding yeast separase, and inhibits its activity (Uhlmann et al. 1999). Although Pds1 is not essential for viability, cells lacking Pds1 are temperature-sensitive for growth and are unable to arrest mitotic progression in

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response to DNA or spindle damage (Yamamoto et al. 1996a). In the presence of DNA damage, Pds1 is required for inhibiting both anaphase initiation and mitotic exit (Yamamoto et al. 1996b). The latter is likely to be mediated by the ability of Pds1 to inhibit mitotic cyclin degradation (Cohen-Fix and Koshland 1999; Tinker-Kulberg and Morgan 1999). The presence of DNA damage leads to Pds1 phosphorylation via the DNA damage checkpoint pathway in a Mec1- and Chk1-dependent manner (Cohen-Fix and Koshland 1997; Sanchez et al. 1999). This phosphorylation is essential for Pds1's ability to inhibit cell cycle progression in the presence of DNA damage, but is not observed in undamaged cells, nor is it required for the response to other types of cellular impairments, such as spindle damage (Wang et al. 2001).

Pds1 plays a dual role in regulating the activity of Esp1. In addition to inhibiting Esp1, Pds1 is also required for the activation of Esp1, by promoting its efficient nuclear localization (Jensen et al. 2001). Cells lacking Pds1 are viable but do not accumulate Esp1 in the nucleus, resulting in the failure to initiate anaphase at elevated temperatures (Yamamoto et al. 1996a; Jensen et al. 2001). It is presently unknown how Pds1 targets Esp1 to the nucleus or how this process is regulated. The requirement for securin in separase's nuclear localization was first observed in fission yeast, where the nuclear localization of the separase/Cut1 was dependent on securin/Cut2 (Kumada et al. 1998). Interestingly, the requirement for securin in separase's activation is not limited to organisms that undergo a closed mitosis (i.e., no nuclear envelope breakdown). In *Drosophila*, cells defective in securin function (*pim* mutants) fail to separate sister chromatids (Leismann et al. 2000), and the absence of the human securin, known as pituitary tumor transforming gene (PTTG), results in reduced levels of separase (Zou et al. 1999; Jallepalli et al. 2001). Thus, the dual role for securins in regulating the activity of separase appears to be conserved throughout evolution.

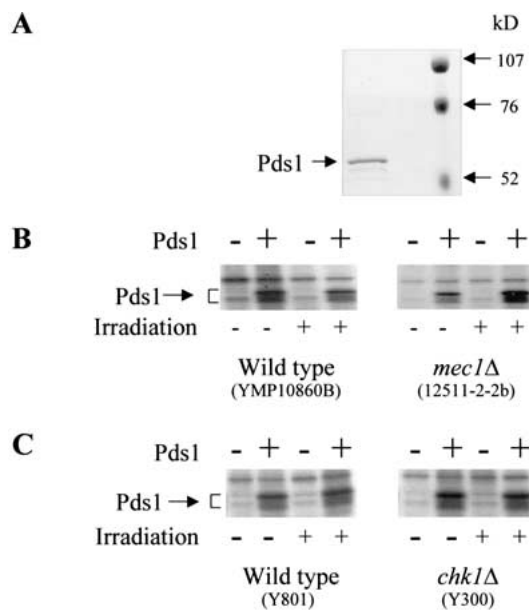
The central role played by Pds1 in regulating mitosis prompted us to examine the upstream pathways that regulate the activity of Pds1. In this study we describe a previously unknown modification of Pds1. We show that Pds1 is phosphorylated by the cyclin-dependent kinase Cdc28, a key regulator of cell cycle progression in budding yeast, and that this phosphorylation is distinct from the previously known DNA damage-induced phosphorylation. We provide evidence that phosphorylation of Pds1 by Cdc28 is important for its efficient binding to Esp1 and for promoting the nuclear localization of Esp1. Our results uncover a previously unknown target of Cdc28 and reveal a novel mechanism for regulating the Pds1-Esp1 interaction.

**Results**

*Pds1 is phosphorylated in vitro in a DNA damage-independent manner*

In response to DNA damage, Pds1 is phosphorylated in a Mec1- and Chk1-dependent manner (Cohen-Fix and

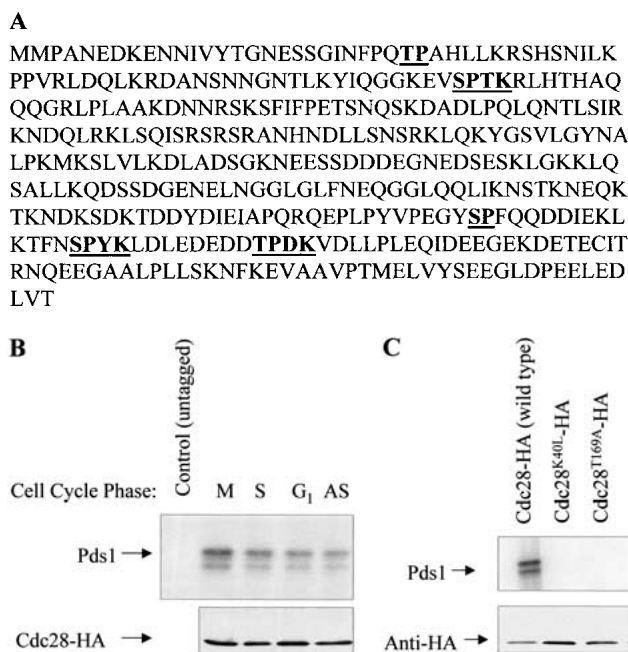
Koshland 1997; Sanchez et al. 1999). In an attempt to reconstitute this pathway in vitro, we examined whether recombinant Pds1 could be phosphorylated when incubated in the presence of a protein extract prepared from wild-type cells that were exposed to  $\gamma$  radiation. We purified bacterially expressed Pds1, the majority of which migrated on SDS-PAGE as a single band with a minor degradation product of slightly faster mobility (Fig. 1A). Analysis by mass spectrometry confirmed that the purified protein was Pds1 (data not shown). Following incubation with [ $\gamma$ -<sup>32</sup>P]ATP and protein extracts from either irradiated or nonirradiated wild-type cells, a radiolabeled doublet specific to Pds1 was observed in all reactions containing purified Pds1, even those that contained extracts from nonirradiated cells (Fig. 1B,C, wild type). It was formally possible that DNA fragments generated during the extraction procedure activated the DNA damage checkpoint pathway and led to Pds1 phosphorylation. To test this, we used extract from strains in which either the *MEC1* or *CHK1* gene was deleted. As seen in Figure 1, B and C, Pds1 was still phosphorylated in the absence of Mec1 or Chk1. Thus, unexpectedly, our results showed that there is a DNA damage-independent pathway for Pds1 phosphorylation.



**Figure 1.** Pds1 is phosphorylated in vitro in a Mec1- and Chk1-independent manner. (A) Purification of Pds1. Pds1 was expressed in *Escherichia coli* and purified as described under Materials and Methods. The purified protein was resolved by 10% SDS-PAGE and visualized by Coomassie blue staining. (B,C) Modification of Pds1 in vitro. In vitro kinase reactions were carried out as described under Materials and Methods in the absence or presence of purified Pds1, as indicated. The protein extract was made from the following strains that were either irradiated or left untreated: (B) wild type and *mec1Δ* (YMP10860b and 12511-2-2b, respectively); (C) wild type and *chk1Δ* (Y801 and Y300, respectively). Radiolabeled proteins were resolved by 10% SDS-PAGE and detected by autoradiography.

### Pds1 can be phosphorylated by Cdc28 in vitro

In search of a kinase that phosphorylates Pds1 in a DNA-damage-independent manner, we analyzed the protein sequence of Pds1 and found five putative cyclin-dependent kinase (CDK) phosphorylation sites (Fig. 2A). CDKs phosphorylate serine and threonine residues found within the consensus S/TPXX/R (single-letter amino acid code), although the K/R at position +3 relative to the phosphoacceptor site is not absolutely necessary (Songyang et al. 1994; Zhang et al. 1994; Srinivasan et al. 1995; Holmes and Solomon 1996). To test whether Pds1 is a substrate for the budding yeast CDK Cdc28 in vitro, cyclin-Cdc28 complexes were immunoprecipitated from either asynchronously growing cells or from cells ar-



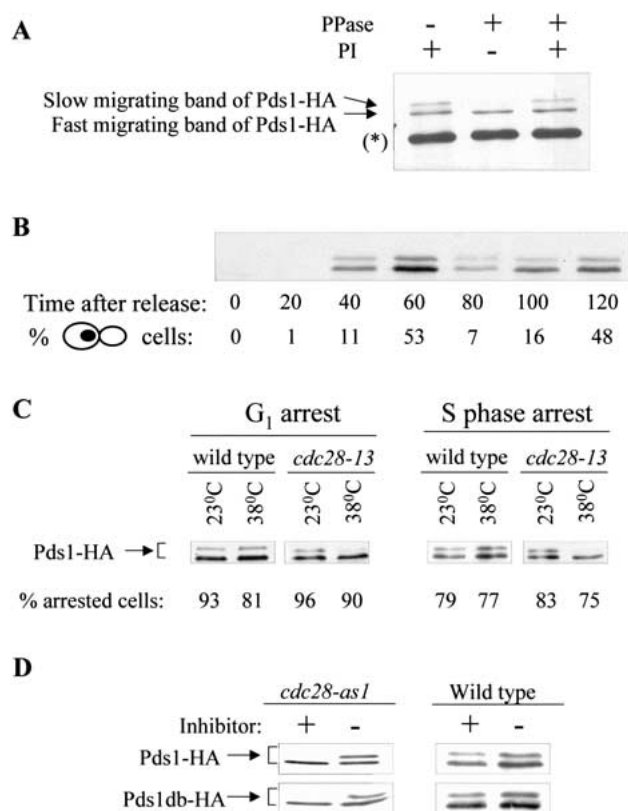
**Figure 2.** Pds1 is a Cdc28 substrate in vitro. (A) The assignment of the putative Cdc28 phosphorylation sites was done according to Songyang et al. (1994), Zhang et al. (1994), and Srinivasan et al. (1995). (B) Pds1 is phosphorylated in vitro by Cdc28 isolated from cells arrested in different cell cycle phases. HA-tagged Cdc28 was immunoprecipitated from protein extracts made from cells that were either grown to mid-log phase (asynchronous, AS), or arrested in G<sub>1</sub> (with  $\alpha$ -factor, 200 nM final concentration), in S phase (with hydroxyurea, 0.2 M final concentration), or in mitosis, M, (with nocodazole, 15  $\mu$ g/mL final concentration). Asynchronous cells expressing untagged Cdc28 were used as a control. Immunoprecipitated Cdc28 was used in the in vitro phosphorylation reaction as described under Materials and Methods. The reaction products were resolved by 10% SDS-PAGE followed by autoradiography to detect <sup>32</sup>P-labeled Pds1 (upper panel) or Western blot analysis with anti-HA antibody to show the relative amounts of the Cdc28 protein in each reaction (lower panel). (C) Pds1 is phosphorylated by wild-type but not by mutant Cdc28. Cells carrying plasmids expressing Cdc28-HA, Cdc28<sup>T169A</sup>-HA, or Cdc28<sup>K40L</sup>-HA were grown to mid-log phase. Protein extraction, immunoprecipitation, and in vitro kinase reactions were carried out and analyzed as described for panel B.

rested in different phases of the cell cycle, and the immunoprecipitates were used in in vitro phosphorylation reactions containing purified Pds1. As seen in Figure 2B, Pds1 could be phosphorylated by HA-tagged Cdc28 immunoprecipitated from different cell cycle stages but not by immunoprecipitates from control cells that express untagged Cdc28. The phosphorylation by Cdc28 purified from G<sub>1</sub>-arrested cells was reproducibly less efficient. This was not a result of less Cdc28 in this reaction as the levels of immunoprecipitated Cdc28 were the same in all reactions (Fig. 2B, lower panel).

To establish that the phosphorylation activity was specific to Cdc28 and not caused by a copurifying kinase, we used two Cdc28 mutants that were previously shown to be defective in their kinase activity: Cdc28<sup>K40L</sup>, in which the ability to bind ATP is abolished, and Cdc28<sup>T169A</sup>, in which activation by CAK is eliminated. Unlike immunoprecipitates containing wild-type Cdc28, those containing either of the two mutant forms were unable to promote Pds1 phosphorylation (Fig. 2C, top panel), although the levels of the different Cdc28 derivatives were the same in all reactions (Fig. 2C, lower panel). Thus Pds1 appears to be a Cdc28 substrate. This conclusion is also consistent with an observation made by David Morgan and Jeff Ubersax (UCSF), who identified Pds1 in a large-scale screen for Cdc28 substrates. In this screen, a series of GST-fused proteins were tested for their ability to undergo in vitro phosphorylation by an altered Cdc28, Cdc28-as1, the ATP-binding site of which was modified to accommodate bulky ATP analogs (see below; Bishop et al. 2000). The labeling reactions were carried out in the presence of a radioactive bulky ATP analog that could be used by no kinase other than Cdc28-as1 (D. Morgan and J. Ubersax, unpubl.). Taken together, these results suggest that Pds1 is specifically phosphorylated by Cdc28 in vitro.

### Pds1 is phosphorylated in vivo in a Cdc28-dependent manner

The observation that Pds1 can be phosphorylated in vitro by Cdc28 prompted us to examine whether Pds1 is phosphorylated in vivo in a Cdc28-dependent manner. Overexpressed Pds1 and endogenous HA-tagged Pds1 have been shown to migrate as a doublet by SDS-PAGE (Cohen-Fix et al. 1996), but phosphatase treatment of Pds1 in crude extracts preparation did not yield a change in this electrophoretic mobility (Cohen-Fix and Koshland 1997). We decided to reexamine the in vivo phosphorylation state of Pds1 using a more purified preparation. Pds1-HA was immunoprecipitated from asynchronously growing cells, and the immunoprecipitates were treated with alkaline phosphatase. As can be seen in Figure 3A, the slow-migrating band of Pds1 disappeared upon phosphatase treatment. Moreover, the presence of phosphatase inhibitors prevented this change, indicating that the slower-migrating Pds1 form was caused by phosphorylation. We therefore concluded that Pds1 is phosphorylated in vivo and that the electrophoretic mobility of Pds1 can be used as an indicator for its phosphoryla-



**Figure 3.** Pds1 is phosphorylated in vivo in a Cdc28-dependent manner. (A) Pds1-HA was immunoprecipitated from mid-log-phase wild-type OCF1522 cells using anti-HA antibodies. The immunoprecipitates were subjected to treatment with alkaline phosphatase (PPase) in the presence and absence of phosphatase inhibitors (PI). Pds1 mobility was analyzed by 10% SDS-PAGE followed by Western blot analysis using anti-HA antibodies. The asterisk in brackets indicates the heavy chain of the antibody used for immunoprecipitation. (B) Wild-type cells (OCF1522) expressing Pds1-HA from its native promoter were grown at 30°C, arrested in G<sub>1</sub> with  $\alpha$ -factor mating pheromone and then released into media lacking pheromone. Samples were taken at the indicated time points, processed for Western blot analysis, and scored for cell morphology by DIC and DAPI staining. The percent of large budded cells with a single nucleus, indicative of a post-S phase/pre-anaphase state, is shown. (C) *cdc28-13* and wild-type strains (RA2817-8a and RA2817-1d, respectively), both carrying a plasmid encoding for *PDS1-HA* expressed from a galactose-inducible promoter (pOC42), were grown to mid-log phase at 23°C, arrested in G<sub>1</sub> with  $\alpha$  factor (5  $\mu$ M) or in S phase with hydroxyurea (200 mM), and then shifted to 38°C for 3.5 h. Following the temperature shift, the expression of Pds1-HA was induced for 1 h (still at 38°C), after which protein extracts were prepared and analyzed by Western blot analyses. The percent of cells in G<sub>1</sub> or S phase is indicated. (D) Centromeric plasmids encoding for Pds1-HA or Pds1db-HA (Pds1 lacking a destruction box; Cohen-Fix et al. 1996) expressed from a galactose-inducible promoter (pOC42 and pOC57-HA, respectively) were introduced into either a wild-type strain or a *cdc28-as1* strain (Bishop et al. 2000; see Materials and Methods). Following an arrest in G<sub>1</sub> with  $\alpha$  factor, cells were either mock-treated or treated with 10  $\mu$ M of PP1 (analog 9) for 4.5 h. The expression of Pds1-HA or Pds1db-HA was induced for 1 h, after which protein extracts were prepared and analyzed by Western blot analysis.

tion state. To examine when in the cell cycle Pds1 is phosphorylated, cells expressing Pds1-HA under the native *PDS1* promoter were arrested in G<sub>1</sub> with  $\alpha$ -factor mating pheromone followed by a synchronous release from the arrest. As observed previously, the levels of Pds1 peak prior to anaphase (Fig. 3B; Cohen-Fix et al. 1996), and judging by the mobility shift Pds1 is phosphorylated for as long as it is present in the cell. Using densitometry scanning we estimate that the fraction of the slow-migrating Pds1 form in cycling cells is ~20% of the total Pds1, but under these separation conditions some phospho-Pds1 forms may not show a shift in protein mobility (see below).

To test whether in vivo Pds1 is phosphorylated in a Cdc28-dependent manner, we used a strain carrying a temperature-sensitive allele of *CDC28*, *cdc28-13*, that is inactive for its kinase activity at the nonpermissive temperature of 38°C. Wild-type and *cdc28-13* strains, both expressing HA-tagged Pds1 from a galactose-inducible promoter, were arrested in G<sub>1</sub> with the  $\alpha$ -factor mating pheromone (Fig. 3C, left) or in S phase with hydroxyurea (Fig. 3C, right) and then shifted to the nonpermissive temperature for 3.5 h. The arrest prior to the temperature shift was done to ensure that Pds1 phosphorylation was examined while both strains were in the same cell cycle phase. Following the temperature shift the expression of Pds1-HA was induced for 1 h, and the phosphorylation state of Pds1 was monitored by SDS-PAGE (Fig. 3C). At the permissive temperature, under both arresting conditions, Pds1-HA appeared as a doublet in both the wild-type and *cdc28-13* strains. However, in the *cdc28-13* strain after the shift to the nonpermissive temperature, there was a significant reduction in the relative amount of the slow-migrating Pds1 form, both in G<sub>1</sub> and in S phase, indicating that this Pds1 form is a result of Cdc28-dependent phosphorylation. Parenthetically, it should be noted that despite the pheromone treatment, which induces the Cdc28-inhibitor Far1 and markedly reduces the levels of Cln1 and Cln2 (Peter and Herskowitz 1994; Gartner et al. 1998), we could still detect Cdc28-dependent phosphorylation of Pds1 in G<sub>1</sub>. These results suggest that in the presence of pheromone, Cdc28 may only be partially inactive, and that the G<sub>1</sub> arrest is achieved by keeping the Cdc28 kinase levels below a certain threshold.

We also examined the involvement of Cdc28 in the phosphorylation of Pds1 by using the *cdc28-as1* allele that encodes for a Cdc28 derivative with an altered ATP-binding site that is inactive in the presence of a synthetic inhibitor, PP1 (Bishop et al. 2000; see Materials and Methods). Wild-type and *cdc28-as1* cells carrying plasmids encoding for galactose-inducible *PDS1-HA* or *PDS1-db-HA* (mutated in the Pds1 destruction box; Cohen-Fix et al. 1996) were arrested in G<sub>1</sub> with the  $\alpha$ -factor mating pheromone, and induced with galactose in the presence or absence of the PP1 inhibitor. As can be seen in Figure 3D, in the absence of the inhibitor Pds1-HA and Pds1db-HA appeared as a doublet in both the wild-type strain and the *cdc28-as1* mutant strain. In contrast, in the presence of the inhibitor most of the Pds1 in the

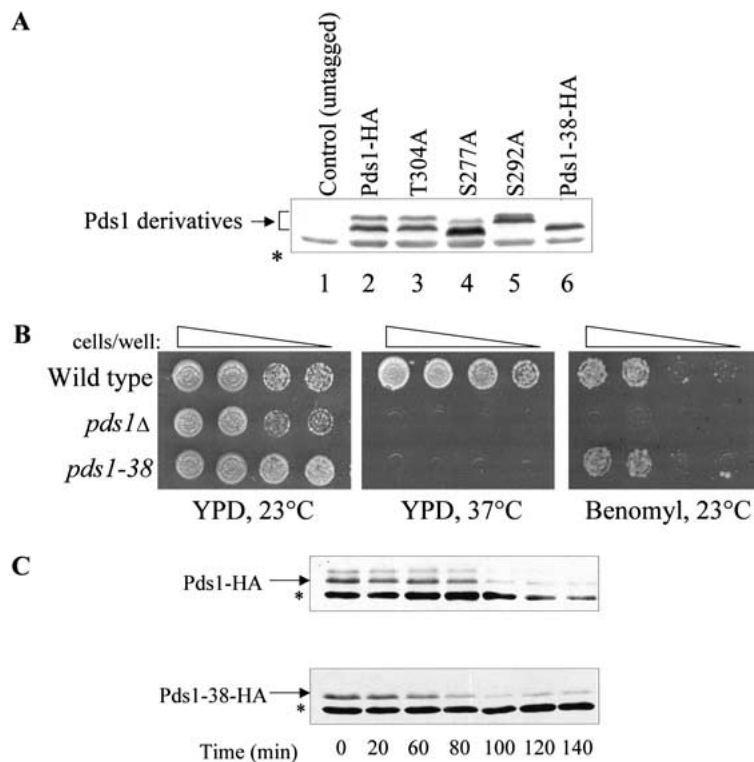
*cdc28-as1* strain was in the fast-migrating form. Taken together, these results show that in vivo Pds1 is phosphorylated in a Cdc28-dependent manner.

*The ability of Pds1 to interact efficiently with Esp1 depends on the phosphorylation of Pds1 by Cdc28*

To determine the role of the Cdc28-dependent phosphorylation of Pds1, we wished to generate Pds1 mutants that could not undergo this type of phosphorylation. To this end we searched for the Pds1 residues that when mutated abolished the mobility shift observed by SDS-PAGE. As mentioned earlier, Pds1 has five consensus sites for Cdc28 (Fig. 2A). Three of these sites reside in the C terminus of the protein, a deletion of which severely compromises the ability of Pds1 to act as a mitotic regulator (R. Agarwal and O. Cohen-Fix, unpubl.). We therefore substituted the serines or threonines of these three sites in *PDS1-HA* with alanines, either individually or in combination, and examined the effect on protein mobility by SDS-PAGE and the growth phenotype conferred when expressed from the native *PDS1* promoter. Pds1 is essential for growth at elevated temperatures and under conditions that induce a spindle checkpoint response. Thus, the functionality of *pds1* alleles can be assessed by examining their ability to promote growth at 37°C or in the presence of spindle poisons such as benomyl. The S277A and S292A amino acid substitution led to changes in the migration of the two Pds1 forms, whereas the T304A mutant form migrated as wild-type Pds1 (Fig. 4A, cf. lanes 3, 4, and 5 to lane 2). None alone or in a pairwise combination conferred a growth phenotype that was dis-

tinguishable from that of the wild-type control strain (data not shown). However, when all three single amino acid substitutions were combined to yield the *pds1-38-HA* allele, the resultant protein behaved by SDS-PAGE as the fast-migrating Pds1 form, consistent with its inability to undergo Cdc28-dependent phosphorylation (Fig. 4A, lane 6), and a strain carrying this allele as its sole source of Pds1 was temperature-sensitive but not benomyl-sensitive (Fig. 4B). The fact that the growth phenotype on benomyl of the *pds1-38* strain was distinct from that of the *pds1Δ* strain suggested that the three substitutions affected a specific Pds1 function rather than completely eliminated all of its activity. The phenotype conferred by a *pds1* allele in which the serines or threonines of all five Cdc28 consensus sites were substituted with alanines was indistinguishable from that conferred by the *pds1-38* allele (data not shown). Owing to the lack of a detectable change in mobility in the T304A mutant protein we currently do not know whether threonine 304 is phosphorylated in vivo, but it should be noted that not all protein phosphorylations result in an altered mobility (e.g., see Ross et al. 2000).

One reason for the temperature sensitivity of the *pds1-38-HA* strain could have been that the Pds1-38-HA protein is unstable at the elevated temperature. To examine this possibility, wild-type and *pds1-38-HA* strains were released synchronously from an S-phase arrest into media containing the  $\alpha$ -factor mating pheromone at 37°C, and the Pds1 levels were monitored over time. In both the strains, the Pds1 protein levels started to decline after 80–100 min following the release (Fig. 4C). Thus, at 37°C, Pds1-38-HA is not unstable nor is there a defect in



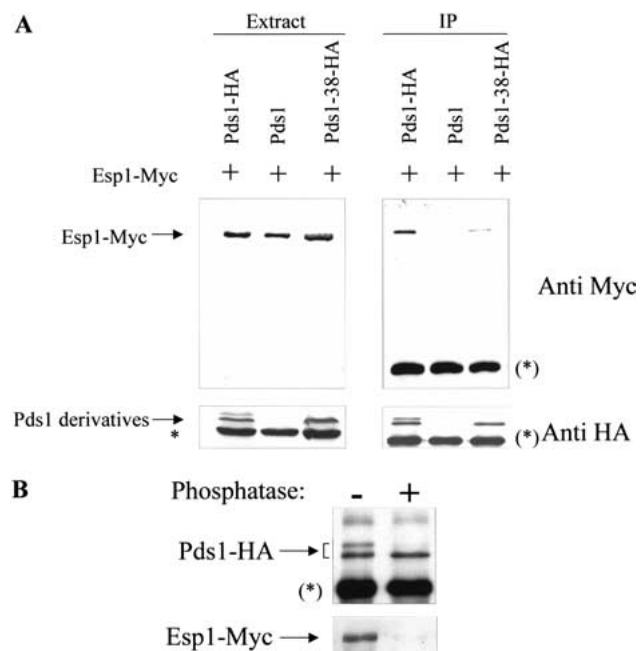
**Figure 4.** The effect of alanine substitutions at putative Cdc28 phosphorylation sites on the electrophoretic mobility of Pds1. (A) Protein extracts from wild type (lane 2) or strains carrying mutations in *PDS1* as indicated (lanes 3–6) were prepared from logarithmically growing cells, resolved by 10% SDS-PAGE, and analyzed by Western blot analysis using anti-HA antibodies. The Pds1-38-HA protein (lane 6) contains all three mutations (S277A, S292A, and T304A). (B) The *pds1-38* strain is temperature-sensitive but not benomyl-sensitive. Wild-type, *pds1Δ*, and *pds1-38* strains were grown to log phase and spotted by serial dilutions onto YPD plates incubated at 23°C or 37°C and onto YPD plates containing benomyl (12.5  $\mu$ g/mL). (C) Pds1-38 stability at 37°C. Cells expressing Pds1-HA or Pds1-38-HA were synchronized in S phase using hydroxyurea, temperature-shifted for 1 h after a full arrest was achieved, and then released into prewarmed YPD (37°C) containing  $\alpha$  factor to arrest the cells at the subsequent G<sub>1</sub>. Protein extracts were prepared every 20 min and analyzed by Western blot analysis. The asterisk represents an anti-HA cross-reacting band in the extracts.

its degradation. We therefore conclude that the temperature sensitivity of the *pds1-38-HA* strain was due to a partial defect in Pds1 function and that Cdc28 phosphorylation plays an important role in regulating the activity of Pds1.

Earlier work has shown that the Pds1–Esp1 interaction is required for two purposes: (1) to inhibit the activity of Esp1 (Ciosk et al. 1998) and (2) to promote the nuclear localization of Esp1 (Kumada et al. 1998; Jensen et al. 2001). To examine how the *pds1-38* mutations affect the ability of this protein to interact with Esp1, coimmunoprecipitation experiments were conducted. Cells expressing Esp1-Myc, with Pds1-HA, Pds1-38-HA, or untagged Pds1 were grown at 23°C and subjected to coimmunoprecipitation as described under Materials and Methods. As shown in Figure 5A, the ability of the HA-tagged Pds1-38 to associate with Esp1 was significantly reduced compared with HA-tagged wild-type Pds1. To further examine the effect of Pds1 phosphorylation on its ability to interact with Esp1, Pds1-HA immunoprecipitated from wild-type cells was either mock treated or subjected to phosphatase treatment, and then mixed with a protein extract prepared from a *pds1Δ ESP1-Myc* strain. This strain was chosen as a source for Esp1-Myc to ensure that Esp1-Myc was not already associated with endogenous Pds1. The mixing reactions were carried out in the absence of ATP to prevent rephosphorylation of the treated Pds1-HA. The ability of both phosphatase-treated and mock-treated Pds1 to interact with Esp1-Myc was then examined. As can be seen in Figure 5B, the phosphatase treatment significantly reduced the ability of Pds1 to interact with Esp1. Taken together our results strongly suggest that the phosphorylation of Pds1 by Cdc28 is required for the Pds1–Esp1 interaction.

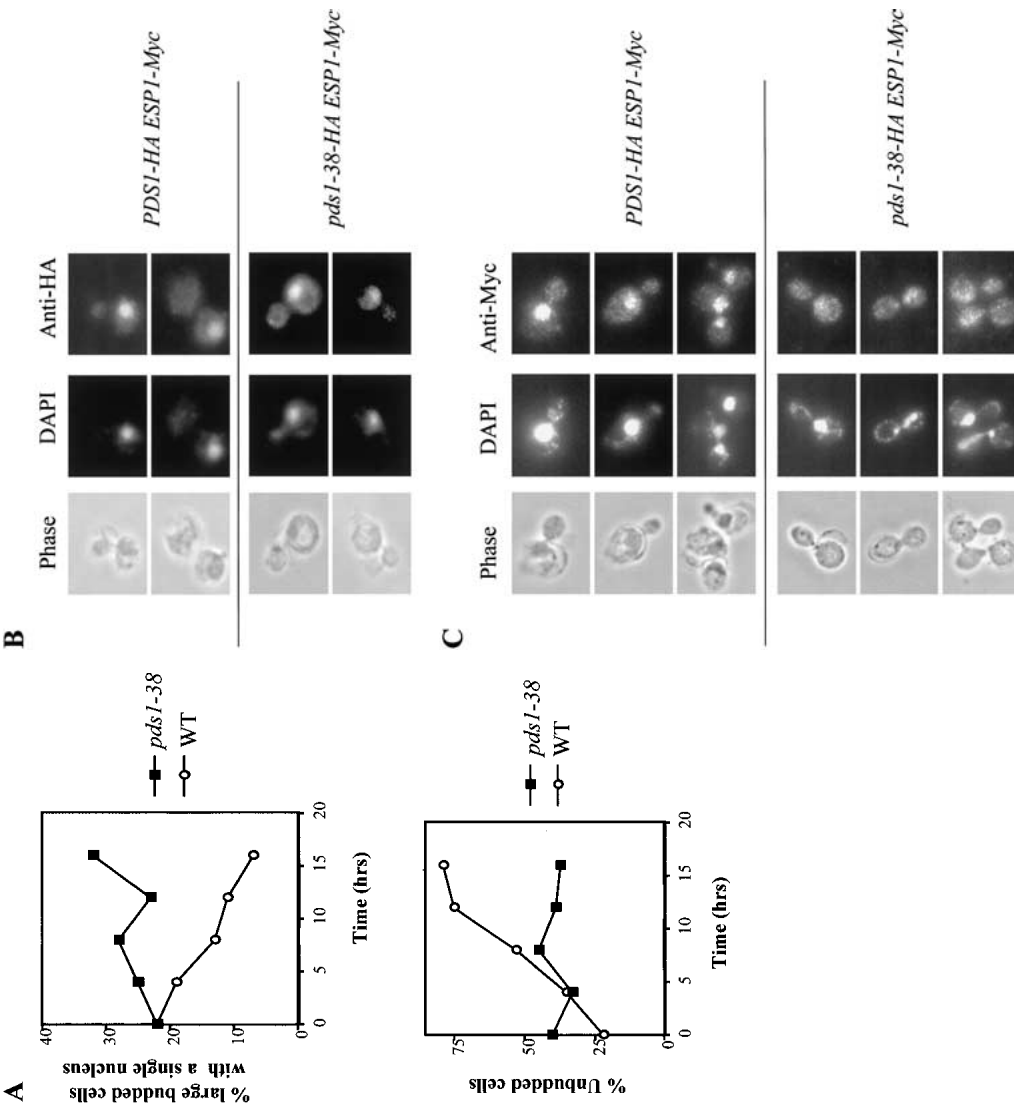
#### *The nuclear localization of Esp1 is dependent on the Cdc28-dependent phosphorylation of Pds1*

The picture that emerges from the data presented above is that mutating the Cdc28 consensus sites in Pds1 results in the reduced binding of Pds1 to Esp1. The fact that *pds1-38* mutants are not benomyl-sensitive suggested that the absence, or partial absence, of Cdc28-dependent phosphorylation did not severely affect the ability of Pds1 to act as a mitotic inhibitor. When grown at 37°C for several hours, *pds1Δ* cells show a cut phenotype (i.e., an unequal distribution of the DNA mass between the two daughter cells; Yamamoto et al. 1996a) and wild-type cells accumulate in G<sub>1</sub> as they enter stationary phase (Fig. 6A). In contrast, the *pds1-38* strain grown at 37°C for several hours accumulated as large budded cells with a single nucleus and a 2N DNA content (Fig. 6A; data not shown), suggesting that these cells have difficulties in carrying out anaphase. Such a phenotype would be expected from a mutant strain that is defective in promoting Esp1 activity but has retained the ability to inhibit mitotic progression. Given the known role of Pds1 in the activation of Esp1, we examined the ability of Pds1-38 to promote the nuclear localization of Esp1. In wild-type cells, Pds1 is concentrated in the



**Figure 5.** Pds1 phosphorylation is required for its efficient binding to Esp1. (A) Pds1-38 has reduced affinity for Esp1. Protein extracts were prepared from strains expressing Esp1-Myc and untagged Pds1 (OCF1548-6D), Pds1-HA (OCF1548-5C), or Pds1-38-HA (RA2806-2b), all grown to mid-log phase at 23°C. The extracts were subjected to immunoprecipitation using anti-HA antibodies. Total extracts (*left panel*) and immunoprecipitates (IP, *right panel*) were separated by SDS-PAGE followed by Western blot analyses using anti-HA antibodies (*bottom panel*) or anti-myc antibodies (*top panel*). Densitometric scans showed that the ratio of the immunoprecipitated Pds1-HA to Pds1-38-HA was ~1.2, whereas the ratio of the Esp1-Myc that coimmunoprecipitated with Pds1-HA to the Esp1-Myc that coimmunoprecipitated with Pds1-38-HA was ~6.8. The asterisk indicates an anti-HA cross-reacting band in the extracts, whereas an asterisk in brackets represents the cross-reacting band of the heavy chain of the antibody used for immunoprecipitation. (B) Nonphosphorylated Pds1 binds weakly to Esp1 in vitro. Pds1-HA was immunoprecipitated from extracts made from mid-log phase wild-type cells expressing Pds1-HA (OCF1522), using anti-HA antibodies. The immunoprecipitates were split in two and were either treated with alkaline phosphatase or mock-treated, after which they were mixed with an extract prepared from the *pds1Δ ESP1-Myc* strain (RA2804-25c). The immunoprecipitates were then washed extensively, and the bound proteins were eluted using SDS sample buffer. The proteins were resolved by SDS-PAGE followed by Western blot analyses of Pds1-HA (*top panel*) and Esp1-Myc (*bottom panel*).

nucleus for as long as it is present, namely, from late G<sub>1</sub>/early S phase to the time of anaphase initiation. Esp1, on the other hand, is present throughout the cell in G<sub>1</sub> and is first seen in the nucleus only as cells enter mitosis (Jensen et al. 2001). To monitor the localization of Pds1 and Esp1, strains expressing Esp1-Myc and either Pds1-HA or Pds1-38-HA were used. Both strains were grown at 37°C for 4 h, and the localization of Pds1 and Esp1 was monitored by indirect immunofluorescence. Western blot analysis revealed that there was no difference be-



**Pds1 phosphorylation by Cdc28**

**Figure 6.** Cdc28-dependent phosphorylation of Pds1 is required for Esp1 accumulation in the nucleus. (A) The distribution of cell types in a wild-type versus a *pds1-38* strain grown at 37°C. Wild-type (OCF1522) and *pds1-38* mutant (RA2806-2b) strains were grown to log phase at 23°C and then shifted to 37°C. Samples were collected every 4 h and fixed in 70% ethanol; cell morphology was analyzed by microscopy. The percentage of large budded cells with a single nucleus (upper panel) and unbudded cells (lower panel) in wild-type cells (circles) and *pds1-38* mutant cells (squares) is shown. (B,C) Esp1 does not accumulate in the nucleus in the *pds1-38* strain. An HA-tagged Pds1 strain (OCF1548-5C, upper panels) and an HA-tagged Pds1-38 strain (RA2806-2b, lower panels), both of which also carried Myc-tagged Esp1 expressed from its own promoter, were grown to early-log phase and then shifted to 37°C for 4 h. Cells were fixed with 4% formaldehyde and processed for indirect immunofluorescence as described under Materials and Methods using anti-HA antibodies for detecting Pds1-HA or Pds1-38-HA (B) and anti-Myc antibodies for detecting Esp1-Myc (C). In each case several typical examples are shown. (D,E) The nuclear localization of Esp1 is Cdc28-dependent. Wild-type (RA2817-8a, upper panel) or *cdc28-13* (RA2817-1d, lower panel) cells carrying Myc-tagged Esp1 expressed from its own promoter and Pds1-HA expressed from a galactose-inducible promoter were grown to early log phase at 23°C in media containing raffinose, after which they were arrested in G<sub>1</sub> with  $\alpha$  factor (5  $\mu$ M). After a complete arrest the cultures were shifted to 37°C for 3.5 h. Pds1 expression was then induced from a galactose promoter for 2 h. Cells were fixed and processed for indirect immunofluorescence as described under Materials and Methods using anti-Pds1 antibodies to detect Pds1 (D) and anti-Myc antibodies to detect Esp1 (E). (F) The levels of Esp1-Myc in wild-type and *cdc28-13* cells at 23°C and 37°C.

tween the wild-type and *pds1-38* strains in the Esp1-Myc levels (data not shown). Pds1-38 itself showed a normal nuclear localization at both permissive and nonpermissive temperatures (Fig. 6B; data not shown). However, in the *pds1-38* strain Esp1 failed to concentrate in the nucleus at 37°C (Fig. 6C), much like the phenotype observed in a *pds1Δ* strain (Jensen et al. 2001). This suggests that Cdc28-mediated phosphorylation of Pds1 is needed for efficient recruitment of Esp1 into the nucleus. In a *pds1Δ* strain the failure to accumulate Esp1 in the nucleus was observed at 23°C as well (Jensen et al. 2001; data not shown). The *pds1-38* mutant strain has no detectable defect in the nuclear accumulation of Esp1 at 23°C, although even at this temperature the ability of Pds1-38 to bind Esp1 is significantly reduced (Fig. 5A). These observations suggest that the low-affinity interaction between Esp1 and Pds1-38 may be sufficient to promote the nuclear localization of Esp1 at 23°C but not at 37°C, possibly because of the slower growth rate at 23°C, allowing more time for Esp1 to accumulate (also see Discussion). This further suggests that other factors contribute to the low-affinity Pds1–Esp1 interaction.

To eliminate the possibility that the Pds1-38 defect is unrelated to its inability to undergo Cdc28-dependent phosphorylation, we also examined the ability of overexpressed Pds1 to promote the nuclear localization of Esp1 in wild-type or *cdc28-13* cells. It was shown previously that in  $G_1$ , Esp1 does not concentrate in the nucleus but that ectopic expression of Pds1 can induce its nuclear localization (Jensen et al. 2001). If the phosphorylation of Pds1 by Cdc28 is required for this process, one would predict that *cdc28-13* cells would be impaired in Esp1's nuclear accumulation when assayed at the nonpermissive temperature even in the presence of high Pds1 levels. To test this, wild-type and *cdc28-13* strains, both carrying *ESP1-Myc* and galactose-inducible *PDS1-HA*, were arrested in  $G_1$  and shifted to 38°C for 3.5 h, followed by the induction of *Pds1-HA* expression for 2 h. In both strains 50% of the  $G_1$  cells showed nuclear Pds1-HA staining, again showing that Cdc28 activity is not required for nuclear localization of Pds1 (Fig. 6D). Whereas >30% of the wild-type  $G_1$  cells showed the accumulation of Esp1 in the nucleus, this was observed in <10% of the *cdc28-13*  $G_1$  cells (Fig. 6E). Under these conditions both strains expressed similar levels of Esp1-Myc (Fig. 6F). Taken together, our data strongly suggest the Cdc28-dependent phosphorylation of Pds1 is required for Pds1 to promote the nuclear localization of Esp1.

## Discussion

Cell cycle progression is the net result of regulatory mechanisms that drive the cell cycle and those that exert an inhibitory effect. The latter are often required to ensure that late cell cycle events are initiated only after early ones have been successfully completed. During mitosis, it is imperative that sister chromatids separate only after all duplicated chromosomes have formed bi-

polar spindle attachments. Because sister chromatid separation is driven by the proteolytic cleavage of one of the cohesin complex subunits, it is conceivable that the protease that drives this process, separase, would be subjected to multiple levels of regulation. In budding yeast, the activity of the Esp1/separase is inhibited by Pds1/securin, making Pds1 a target for numerous regulatory mechanisms that influence the timing of anaphase initiation. These include the spindle checkpoint pathway, acting via the inhibition of APC/C activity (Fang et al. 1998; Wassmann and Benezra 1998), and the DNA damage checkpoint pathway, stabilizing Pds1 through Chk1-mediated phosphorylation (Wang et al. 2001). Pds1 not only inhibits Esp1 but also acts as an Esp1 activator by driving its nuclear accumulation (Jensen et al. 2001). Despite the diverse roles played by Pds1 in mitotic regulation, little is known about the molecular mechanism of its action.

Mitotic CDKs are known to be required for progression through mitosis. In budding yeast, Clb–Cdc28 complexes are necessary for spindle formation and regulation of the APC/C (Richardson et al. 1992; Irmiger et al. 1995; Amon 1997). In this study we present several lines of evidence suggesting that Pds1 is a target of Cdc28: (1) Pds1 was phosphorylated in vitro by immunopurified wild-type Cdc28. This phosphorylation was specific to Cdc28, because mutant forms of Cdc28 impaired in their catalytic activity did not promote Pds1 phosphorylation. (2) The phosphorylation of Pds1 in vivo was abolished by mutations or conditions that inactivated Cdc28. These included a temperature-sensitive allele of *CDC28*, *cdc28-13*, and a chemically repressible Cdc28 derivative, Cdc28-as1. (3) Mutagenesis of three of the Cdc28 consensus sites in Pds1 altered the mobility of Pds1 as detected by SDS-PAGE and abolished the slow-migrating Pds1 form, similar to the change in mobility observed after phosphatase treatment. Taken together, our data suggest that Pds1 is a bona fide Cdc28 substrate.

The Cdc28-dependent phosphorylation of Pds1 occurred at any phase of the cell cycle in which Pds1 was present. Given that not all phosphorylations may result in a mobility shift, the fraction of Pds1 molecules that is phosphorylated by Cdc28 at any given time and the number of Cdc28 phosphorylation sites occupied per molecule are currently unknown. Nonetheless, Cdc28-mediated phosphorylation appears to be critical for the ability to interact efficiently with Esp1 and to promote the nuclear localization of Esp1. This conclusion is based on the following observations: (1) Pds1-38, in which three Cdc28 consensus phosphorylation sites at the C terminus of Pds1 were converted to alanines, had a significantly reduced affinity for Esp1 at the permissive temperature and was unable to promote the nuclear localization of Esp1 at elevated temperatures. (2) Dephosphorylation of in vivo phosphorylated Pds1 reduced its ability to interact with Esp1 in vitro. (3) The ability of high levels of Pds1 to drive Esp1 into the nucleus in  $G_1$  was dependent on Cdc28 activity. Our results also show that phosphorylation by Cdc28 is not required for the nuclear localization of Pds1 itself. Moreover, phosphory-



lation at Ser 277, Ser 292, and possibly Thr 304 is not essential for the role of Pds1 as a mitotic inhibitor, as the *pds1-38* mutant strain, unlike the *pds1Δ* strain, was not sensitive to microtubule depolymerizing drugs. The ability of Pds1-38 to inhibit mitotic progression suggests that it is still capable of binding to Esp1, albeit at much a lower affinity. The difference between the ability to promote the nuclear localization of Esp1 and to inhibit mitotic progression may lie in the different cellular compartments in which these functions take place. Inhibition of mitotic progression is likely to take place in the nucleus, where the levels of Pds1-38, or wild-type Pds1, are high and therefore low-affinity interactions may be compensated by the overall high Pds1-38 concentration. In contrast, the Pds1–Esp1 interaction that is required to promote the nuclear localization of Esp1 may take place in the cytoplasm, where Pds1-38 levels are exceedingly low, and low-affinity interactions may be insufficient to drive Esp1 into the nucleus. Moreover, Pds1 is likely to reside in the cytoplasm for only a short period of time, whereas in the nucleus there is more opportunity to maintain a Pds1–Esp1 interaction. At present we do not know why the defect in Esp1 nuclear localization in the *pds1-38* strain is detected only at elevated temperatures. It is possible that the affinity between Pds1-38 and Esp1 is further reduced at the elevated temperature and that the low-affinity state, detected by the immunoprecipitation assay at 23°C, is sufficient to promote nuclear accumulation at low but not high temperatures.

Our results suggest that following Pds1 phosphorylation by Cdc28, Pds1 associates with Esp1 and promotes its nuclear accumulation (Fig. 7). This mechanism ensures that prior to anaphase, nuclear Esp1 will be inactive because of its association with Pds1, and only after Pds1 inactivation, either by APC/C-mediated degradation or by the activity of a putative phosphatase (see below), will Esp1 become active. Interestingly, the phosphorylation of securins by mitotic CDKs appears to be conserved, as the human securin homolog of Pds1, PTTG, has been shown to be phosphorylated by Cdc2 (Ramos-Morales et al. 2000). In this case the CDK-dependent phosphorylation of securin is unlikely to be required for the nuclear localization of separase due to nuclear envelope break-down upon mitotic entry, but a requirement for targeting to a subnuclear structure (e.g., chromatin) is still possible. In both humans and *Drosophila*, the loss of securin function is accompanied by reduced separase activity, suggesting that the involvement of securin in separase activation is conserved.

Whether the CDK-dependent phosphorylation is required for the positive function of securin in *Drosophila* and humans remains to be determined.

The dependence of the Pds1–Esp1 interaction on Cdc28-mediated phosphorylation suggests that the association between the two proteins could be dissolved not only by APC/C-mediated degradation of Pds1 but also by the action of an as yet unidentified phosphatase (Fig. 7). A possible phosphatase is the Cdc14 protein that was shown to remove Cdc28-mediated phosphorylation from the Cdh1, Sic1, and Swi5 proteins (Jaspersen et al. 1999), and overexpression of Cdc14 resulted in the disappearance of the slower-migrating Pds1 form (Visintin et al. 1998). At the time, the nature of this form was not known, but the results presented here suggest that this effect could be caused by the dephosphorylation of Pds1. Why would a cell need two mechanisms to counteract Pds1's inhibitory grip on Esp1? Having redundant mechanisms for regulating key cell cycle transitions is not uncommon. For example, Clb–Cdc28 inactivation at the exit from mitosis occurs via at least two pathways, the APC/C<sup>Cdh1</sup>-dependent degradation of the mitotic cyclins and the Sic1-dependent inhibition of Clb–Cdc28 activity (Schwab et al. 1997; Visintin et al. 1998). In addition, there may be a pool of Esp1-bound phosphorylated Pds1 that is resistant to APC/C<sup>Cdc20</sup>-mediated degradation and for which dephosphorylation may be the only way to release Esp1. Tinker-Kulberg and Morgan (1999) proposed a role for Esp1 at the exit of mitosis, and at that late cell cycle stage, when the nuclear Pds1 levels are low, dephosphorylation may be an effective way to free Esp1. Dephosphorylation may also render Pds1 more susceptible to degradation, either because dephosphorylated Pds1 is a better substrate for the APC/C or simply because by increasing the Pds1–Esp1 dissociation rate Pds1 may become more accessible to the ubiquitination machinery. Finally, it has been shown recently that Esp1 regulates the early release of Cdc14 from the nucleolus (Stegmeier et al. 2002). It is tempting to speculate that if Cdc14 is indeed the Pds1 phosphatase there may be a positive feedback loop where released Cdc14 dephosphorylates Pds1. As the nuclear concentrations of Pds1 drop owing to APC/C activity, dephosphorylation could play a significant role in dissociating the Esp1–Pds1 complex, thereby further activating Esp1 and allowing more Cdc14 to be released from the nucleolus. Thus, the Cdc28-mediated phosphorylation of Pds1 adds another layer of complexity to the regulation of mitotic progression.

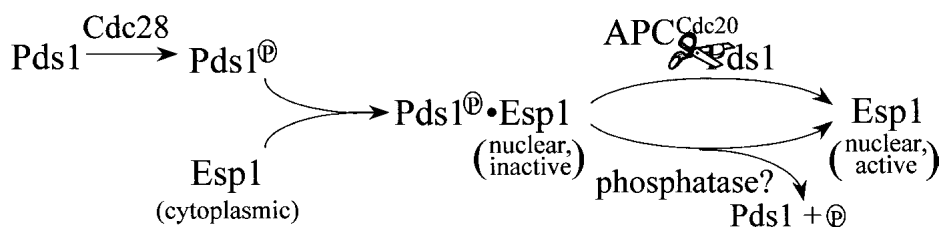


Figure 7. A model for the regulation of the Pds1–Esp1 interaction by Cdc28. See text for details.

## Materials and methods

### Plasmids and strains

The yeast strains used are described in Table 1. All strains were W303 derivatives except when indicated otherwise. The plasmids used were pOC57, pOC52 (Cohen-Fix et al. 1996), and pOC42 (*CEN GAL1-PDS1-HA:URA3*). PKB174 (*CEN CDC28-HA TRP1*), PKB173 (*CEN cdc28<sup>K40L</sup>-HA TRP1*), and PKB175 (*CEN cdc28<sup>T169A</sup>-HA TRP1*) were a generous gift from Philipp Kaldis (National Cancer Institute, Frederick, MD; originally plasmids CWB174, CWB173, and CWB186, respectively, from C. Wittenberg, The Scripps Research Institute, La Jolla, CA).

### Media and reagents

YPD media contained 1% yeast extract, 2% bactopectone, adenine (2.5 mg/L), and 2% glucose; YPGal and YPRaf contained 2% galactose or 2% raffinose instead of glucose, respectively.  $\alpha$ -Factor mating pheromone (Sigma) was used at 0.1  $\mu$ M for *bar1* strains and 5  $\mu$ M for *BAR1<sup>+</sup>* strains. Hydroxyurea (Sigma) was used at 0.2 M. Nocodazole (Aldrich) was used at 15  $\mu$ g/mL. [ $\gamma$ -<sup>32</sup>P]ATP was from NEN life science products (3000 Ci/mmole). Enterokinase was from Invitrogen. Calf intestine alkaline phosphatase was from Roche. Anti-HA antibodies (16B12), anti-Myc antibodies (9E10), and protein A sepharose linked to anti-HA antibodies were from Covance. Anti-Pds1 antibodies were a generous gift from Doug Koshland (The Carnegie Institution of Washington, DC).

### Purification of Pds1

Pds1 was amplified by PCR using a 5' primer that placed a *Bam*HI site at the start codon and a 3' primer that placed an *Xba*I site at the stop codon. The *Bam*HI-*Xba*I fragment was cloned into a GST tagging vector (pGST, a generous gift from Peter Sheffield, University of Virginia, Charlottesville; Sheffield et al. 1999). GST-Pds1 was expressed in *Escherichia coli* strain BL21 codon plus (Stratagene). Bacteria transformed with the GST-Pds1 plasmid were grown to an OD<sub>600</sub> of 0.5, and the expression of Pds1 was induced by IPTG (1 mM) for 16 h at 14°C.

The cells were harvested, lysed in buffer A (50 mM Tris-HCl at pH 7.5 containing 0.15 M NaCl, and a protease inhibitors mix from Roche). After centrifugation, the supernatant was passed through a glutathione-sepharose column. The column was washed extensively with buffer A, and Pds1 was eluted by cleaving off the GST tag with enterokinase (dissolved in 50 mM Tris-HCl at pH 7.5 containing 1 mM DTT). Mass spectrometry was done by the Keck Foundation, Yale University (New Haven, CT).

### Cell lysis and immunoprecipitation

Yeast cell lysates were prepared by breaking cells with glass beads buffer B (0.05 M Tris-HCl at pH 7.5, 0.15 M NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM sodium pyrophosphate, and protease inhibitors mix from Roche). The extract (2 mg of protein) was incubated at 4°C for 2 h with anti-HA antibody prebound to protein A sepharose. The immunoprecipitates were washed four times with 1 mL of extraction buffer. Bound proteins were eluted by boiling in 2 $\times$  SDS loading buffer. Proteins were separated by SDS-PAGE (10% gel) and analyzed by Western blot analysis as indicated.

### Kinase assays

For radioactive kinase assays, 2.5  $\mu$ g of purified Pds1 was mixed with 2.5  $\mu$ L of yeast extract (4  $\mu$ g of protein), 1 mM ATP, 50  $\mu$ g/mL creatine kinase, 35 mM phosphocreatine, 1.3  $\mu$ Ci/ $\mu$ L [ $\gamma$ -<sup>32</sup>P]ATP, and 5 mM MgCl<sub>2</sub> in buffer C (0.05 M Tris-HCl at pH 7.5, 0.1 M NaCl, 1.25 mM EGTA, 1.25 mM EDTA, protease inhibitors [Roche], and phosphatase inhibitors [Sigma]). The reaction was stopped by adding 2 $\times$  SDS sample buffer. The yeast extracts used above were made from cells that were either left untreated or subjected to a radiation dose of 12 krad using a  $\gamma$ -irradiator.

### Phosphatase treatment

Immunoprecipitates prepared as described above were resuspended in 50  $\mu$ L of buffer (50 mM Tris-HCl at pH 7.5) containing 100 U of calf intestine phosphatase in the absence or presence of

**Table 1.** Strains used in this study

Strain	Genotype	Source
YMP10860 <sup>a</sup>	<i>MAT<math>\alpha</math>RNR1-LEU2 sml1d::KAN his3 leu2 trp1 ura3</i>	L. Hartwell
12511-2-2b <sup>a</sup>	Same as YMP10860b but <i>mec1<math>\Delta</math>::TRP1</i>	L. Hartwell
Y300	<i>MAT<math>\alpha</math> can1-100 ade2 his3 leu2 trp1 ura3</i>	S. Elledge and Y. Sanchez
Y801	Same as Y300 but <i>chk1<math>\Delta</math>::HIS3</i>	S. Elledge and Y. Sanchez
PKY 238	<i>MAT<math>\alpha</math> CDC28-HA::URA3 leu2 trp1 his3 ade2</i>	M. Solomon and P. Kaldis
OCF 1522	<i>MAT<math>\alpha</math> bar1 PDS1-HA::URA3 leu2 trp1 his3 ade2ura3</i>	(Cohen-Fix and Koshland 1997)
OCF 1525	Same as 1522 but <i>pds1<math>\Delta</math>::LEU2</i>	(Cohen-Fix and Koshland 1997)
RA2817-1d	<i>MAT<math>\alpha</math> cdc28-13 ESP1-Myc::TRP1 ura3 leu2 his3</i>	This study
RA2817-8a	<i>MAT<math>\alpha</math> ESP1-Myc::TRP1 ura3 leu2 his3</i>	This study
cdc28-as1	<i>MAT<math>\alpha</math> bar1 cdc28d::cdc28-as1 ura3 trp1 ade2 can1-100</i>	David Morgan and Jeff Ubersax
RA2808	Same as 1522 but <i>pds1<sup>T304A</sup>-HA::URA3</i>	This study
RA2810	Same as 1522 but <i>pds1<sup>S277A</sup>-HA::URA3</i>	This study
RA2811	Same as 1522 but <i>pds1<sup>S292A</sup>-HA::URA3</i>	This study
RA2815	Same as 1522 <i>pds1<sup>S277AS292T301A</sup>-HA::URA3</i> (also referred to as <i>pds1-38</i> )	This study
OCF1548-6D	<i>MAT<math>\alpha</math> bar1 ESP1-Myc::TRP1 his3 leu2 ura3 ade2</i>	This study
OCF1548-5C	<i>MAT<math>\alpha</math> PDS1-HA::URA3 ESP1-Myc::TRP1 his3 leu2 ade2</i>	This study
RA2806-2b	<i>MAT<math>\alpha</math> pds1-38-HA::URA3 ESP1-Myc::TRP1 leu2 his3 ade2</i>	This study
RA2804-25c	<i>MAT<math>\alpha</math> pds1<math>\Delta</math>::LEU2 ESP1-Myc::TRP1 ura3 his3 ade2</i>	This study

<sup>a</sup>A derivative of the A364a background.

the phosphatase inhibitors mix (100× dilution of Cocktail 1 and 2 from Sigma). After incubation at 30°C for 30 min, the immunoprecipitated complexes were washed three times in lysis buffer B and analyzed by Western blot analysis.

#### Inhibition of the *cdc28-as1* strain

Wild-type and *cdc28-as1* strains (a generous gift from David Morgan and Jeff Ubersax, University of California, San Francisco) carrying plasmids encoding galactose-inducible Pds1-HA or Pds1db-HA were arrested in G<sub>1</sub> at 23°C using  $\alpha$ -factor mating pheromone. The cultures were then split into two parts; one was treated with the 10  $\mu$ M final concentration of synthetic inhibitor PPI derivative (C 3-1-naphthylmethyl PPI [PPI derivative 9]; Bishop et al. 2000), and the other was mock-treated. The cultures were incubated at 23°C for 4.5 h followed by the galactose induction of Pds1-HA or Pds1db-HA for 1 h. Protein extracts were then prepared and examined by Western blot analysis using anti-HA antibodies.

#### Protein techniques

Protein extracts were prepared as described (Cohen-Fix et al. 1996).

#### Site-directed mutagenesis

The Quik-Change XL site-directed mutagenesis kit (Stratagene) was used to mutate serines/threonines in *PDS1-HA* to alanines, and the complete *pds1* mutant allele was sequenced. The template used was pOC52. A *ClaI*-*SacI* fragment containing the mutagenized *PDS1* with a neighboring *URA3* marker was used to transform the *pds1* $\Delta$  strain, OCF1525.

#### Microscopy

Fluorescence and DIC microscopy were performed using an E800 Microscope (Nikon) linked to a cooled CCD camera. To visualize Pds1-HA or Esp1-Myc, cells were fixed for 20 min or 1 h with 4% formaldehyde at room temperature and processed as described (Cohen-Fix et al. 1996). The primary antibodies used were as described above. The nuclei were visualized by DAPI.

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