Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast

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In eukaryotes, entry into mitosis is induced by cyclin B-bound Cdk1, which is held in check by the protein kinase, Wee1. In budding yeast, Swe1 (Wee1 ortholog) is targeted to the bud neck through Hsl1 (Nim1-related kinase) and its adaptor Hsl7, and is hyperphosphorylated prior to ubiquitin-mediated degradation. Here, we show that Hsl1 and Hsl7 are required for proper localization of Cdc5 (Polo-like kinase homolog) to the bud neck and Cdc5-dependent Swe1 phosphorylation. Mitotic cyclin (Clb2)-bound Cdc28 (Cdk1 homolog) directly phosphorylates Swe1 and this modification served as a priming step to promote subsequent Cdc5-dependent Swe1 hyperphosphorylation and degradation. Clb2-Cdc28 also facilitated Cdc5 localization to the bud neck through the enhanced interaction between the Clb2-Cdc28-phosphorylated Swe1 and the polo-box domain of Cdc5. We propose that the concerted action of Cdc28/Cdk1 and Cdc5/Polo on their common substrates is an evolutionarily conserved mechanism that is crucial for effectively triggering mitotic entry and other critical mitotic events.

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Introduction

Entry into mitosis in eukaryotic organisms is regulated by an intricate network of kinases and phosphatases that coordinately bring about reorganization of various subcellular structures. A key regulatory component of this event is the conserved cyclin B-bound Cdc2. In fission yeast and higher eukaryotes, Cdc2 is phosphorylated at Tyr15 and negatively regulated by Wee1, an event that is reversed by the activity of

Cdc25C phosphatase. These critical steps at mitotic entry appear to be largely conserved throughout evolution.

Studies on the G2/M regulation in genetically amenable organisms such as budding yeast have provided valuable insights into how eukaryotic organisms bring about timely activation of cyclin B-bound Cdc2 activity prior to mitotic entry. In budding yeast (*Saccharomyces cerevisiae*), Swe1 (Wee1 ortholog) negatively regulates mitotic Clb (collectively for the B-type cyclins—Clb1, Clb2, Clb3, and Clb4)-associated Cdc28 (Cdc2 homolog) by phosphorylating the equivalent Tyr19 residue (Booher *et al*, 1993), a modification that is reversed by Mih1 (Cdc25 ortholog) (Russell *et al*, 1989). Swe1 also inhibits Clb2-Cdc28 by direct binding (McMillan *et al*, 1999b), thus providing an additional layer to regulate Clb2-Cdc28 activity at the time of mitotic entry. As with the importance of Swe1 in regulating Clb-Cdc28 activity, disruption of actin organization or defects in septin filament assembly at the bud neck results in hypophosphorylation and stabilization of Swe1 (Sia *et al*, 1998; Shulewitz *et al*, 1999). This stabilized Swe1 imposes a G2 delay by inhibiting Clb-Cdc28, which leads to markedly elongated cells due to the inability of cells to switch from polarized to isotropic growth during budding (Lew and Reed, 1993). Thus, timely phosphorylation and subsequent degradation of Swe1 are critical for proper activation of Clb-Cdc28 and therefore mitotic entry.

During an unperturbed cell cycle, Swe1 begins to accumulate in S phase and becomes hyperphosphorylated as cells proceed through the cell cycle (Shulewitz *et al*, 1999; Sreenivasan and Kellogg, 1999). The hyperphosphorylated Swe1 is susceptible to ubiquitin-mediated degradation (Kaiser *et al*, 1998; Sia *et al*, 1998), which appears to depend, in part, on the ubiquitin ligase (E3) known as the anaphase-promoting complex (APC) (Thornton and Toczyski, 2003). Both genetic and biochemical analyses have revealed several components critical for regulating Swe1. Hsl1 (Nim1/Cdr1-related kinase) appears to be critical for tethering of Hsl7 at the bud neck (Shulewitz *et al*, 1999), which is in turn required for efficient recruitment of Swe1 to the same location (McMillan *et al*, 1999a; Longtine *et al*, 2000). As a result, lack of either Hsl1 or Hsl7 disrupts Swe1 localization to the bud neck. It also greatly diminishes Swe1 phosphorylation *in vivo* (Shulewitz *et al*, 1999) in a manner that is not clearly understood. Studies with the polo-like kinase 1 (Plk1) homolog Cdc5 showed that it plays a critical role in hyperphosphorylating and degrading Swe1 at the late stages (prior to G2/M transition) of the cell cycle (Sakchaisri *et al*, 2004). At a stage earlier than that involving Cdc5 (as early as S and also perhaps G2), a PAK homolog Cla4, which is critical for septin filament assembly and associates tightly with septins (Versele and Thorner, 2004), also phosphorylates Swe1 directly (Sakchaisri *et al*, 2004). In addition, Cdc28 has been shown to contribute to Swe1 phosphorylation (McMillan *et al*, 2002), although how its activity relates to Cdc5- and Cla4-dependent Swe1 regulation is not known.

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In this study, we determined components critical for Cdc5-dependent Swe1 regulation. Our data showed that Cdc5 localization to the bud neck requires Hsl1 and Hsl7 and this step is essential for Cdc5-dependent Swe1 phosphorylation. Mitotic Clb2-Cdc28-dependent Swe1 phosphorylation promoted Cdc5 localization to the bud neck and subsequent Cdc5-dependent Swe1 hyperphosphorylation by enhancing the interaction between Swe1 and the noncatalytic polo-box domain (PBD) of Cdc5. Thus, concerted action of Clb2-Cdc28 and Cdc5 appears to be critical to efficiently trigger Swe1 hyperphosphorylation and degradation prior to mitotic entry.

Results

Both Hsl1 and Hsl7 are required for Cdc5-dependent Swe1 regulation

It has been shown that Cdc5 progressively localizes to the bud neck during the late stages of the cell cycle (Lee *et al*,

2005) and this localization step appears to be important for Cdc5-dependent Swe1 phosphorylation and degradation (Park *et al*, 2004; Sakchaisri *et al*, 2004). Consistent with these observations, overexpression of *GAL1-CDC5* induced Swe1 hyperphosphorylation and degradation efficiently, whereas overexpression to the same extent of a triple point mutant *GAL1-cdc5(FAA)*, which is defective for subcellular localization (Song *et al*, 2000), did not provoke precocious Swe1 downregulation (Figure 1A and Supplementary Figure S1). These data further underline the importance of proper Cdc5 localization in regulating Swe1.

In a wild-type background, Cdc28 was phosphorylated at Tyr19 in hydroxyurea-treated (S phase) cells, whereas it was dephosphorylated in nocodazole-arrested (M phase) cells (Figure 1B). As with the importance of Hsl1 and Hsl7 for proper degradation of Swe1, lack of Hsl1 or Hsl7 resulted in accumulation of Cdc28 phosphorylation at Tyr19 even in the nocodazole-treated cells (Figure 1B). Since Mih1 is critical for

Figure 1 Hsl1 and Hsl7 are critical for Cdc5-dependent Swe1 phosphorylation and degradation. (A) Strain JC66 bearing YCplac22, pKL882 (*GAL1-EGFP-CDC5*), or pKL883 (*GAL1-EGFP-cdc5(FAA)*) was cultured in YEP-raffinose medium and arrested in S phase with hydroxyurea for 2.5 h before transferring to YEP-galactose supplemented with hydroxyurea for 3 h. Total cellular proteins were separated by 10% SDS-PAGE and then subjected to immunoblot analyses with anti-Swe1 and anti-GFP (for EGFP-Cdc5) antibodies. The Asterisk indicates a nonspecific protein crossreacting with the anti-Swe1 antibody. (B) Strains KLY1546 (wild type), KLY2868 (*hsl1Δ*), and KLY2872 (*hsl7Δ*) were arrested in S phase with hydroxyurea (S), or in M phase with nocodazole (M). Total cellular proteins were separated by 10% SDS-PAGE for subsequent immunoblotting analyses. (C–E) Strains KLY1546 (C), KLY2868 (D), or KLY2872 (E) were transformed with either YCplac22 (vector) or pKL882 (*pGAL1-EGFP-CDC5*). The resulting transformants were treated with hydroxyurea for 2.5 h and then transferred to YEP-galactose containing hydroxyurea ($t = 0$). Total cellular proteins were separated in 8% low-bis SDS-PAGE. (F) Strains KLY1546 (wild type), KLY2868 (*hsl1Δ*), and KLY2872 (*hsl7Δ*) were transformed with either pSK754 (*EGFP-CDC5*) or pKL880 (*GAL1-EGFP-CDC5ΔN*). The resulting transformants were cultured overnight at 18°C (EGFP-Cdc5 signals were more stable at a low temperature) in YEP-glucose (for pSK754) or YEP-raffinose (for pKL880). Cells were treated with nocodazole at 18°C for 5 h (for pSK754) or for 4 h followed by 1 h induction in YEP-galactose containing nocodazole (for pKL880) before examination under a fluorescent microscope. Error bars indicate standard deviation.

the dephosphorylation of Cdc28 Tyr19 (Supplementary Figure S2A), these results suggest that stabilized Swe1 in *hsl1Δ* or *hsl7Δ* cells over-rides the Mih1 activity and phosphorylates Cdc28 Tyr19. Thus, we investigated whether Hsl1 and Hsl7 are required for Cdc5-dependent Swe1 phosphorylation *in vivo*. In wild-type cells, expression of *GAL1-CDC5*, but not the control vector, rapidly induced Swe1 degradation in a time-dependent manner (hyperphosphorylated Swe1 was not easily detectable because of a rapid induction of Swe1 phosphorylation and degradation by overexpressed Cdc5), while it gradually dephosphorylated Cdc28 Tyr19 as the level of Swe1 decreased (Figure 1C). In contrast, loss of *HSL1* almost completely eliminated the *GAL1-CDC5*-dependent Swe1 phosphorylation and degradation with undiminished Tyr19 phosphorylation (Figure 1D). Loss of *HSL7* also dramatically stabilized Swe1, although slow-migrating forms of Swe1 appeared to be weakly induced following *GAL1-CDC5* expression (Figure 1E). These observations suggest that Hsl1 and Hsl7 are required for Cdc5-dependent Swe1 regulation.

Since Cdc5 localization to the bud neck is critical for Swe1 regulation (Park *et al*, 2004; Sakchaisri *et al*, 2004), we examined whether Hsl1 and Hsl7 are required for proper Cdc5 localization in cells arrested with nocodazole (Cdc5 becomes abundant under this condition). Cdc5 localization to the neck was evident in approximately 42% of wild-type cells. In contrast, *hsl1Δ* or *hsl7Δ* cells exhibited the bud neck-localized Cdc5 in only 12 or 23% of the total population (Figure 1F). Cdc5 localization to the spindle pole body (SPB) was manifest in most of the population (97–100%), with similar levels in all three strains (Figure 1F). The inefficient localization of Cdc5 to the bud neck as opposed to the SPB could be due to a smaller fraction of Cdc5 localizing to the bud neck. Thus, we re-examined Cdc5 localization by using the *GAL1* promoter-controlled *EGFP-CDC5ΔN*, a localization-competent C-terminal PBD of Cdc5 (Song and Lee, 2001). In wild type, expression of *GAL1-EGFP-CDC5ΔN* resulted in distinct fluorescent signals at the bud neck in 98% of the population, but the neck localization was greatly diminished in the absence of Hsl1 or Hsl7 (Figure 1F). Similar results were also obtained when cells were arrested with hydroxyurea (Supplementary Figure S3). Thus, since Hsl1 and Hsl7 are also required for proper localization of Swe1 to the bud neck, the inefficient Cdc5-dependent Swe1 phosphorylation and degradation in *hsl1Δ* or *hsl7Δ* are likely attributable to the impaired localization of both Cdc5 and Swe1 to the bud neck.

Mitotic Cdc28 activity is required for proper Swe1 degradation

We next examined whether Cdc28 regulates Swe1 phosphorylation and stability through a positive feedback loop and, if so, which cyclin-associated Cdc28 complex is critical for this event. An isogenic wild-type and various cyclin mutants were arrested in G1 by α -factor treatment, and then released into YEP-glucose medium containing nocodazole to repress *GAL1* promoter-dependent expression and trap the cells in mitosis. In wild type, Swe1 was progressively phosphorylated and then degraded, as cells proceeded from G1 to M (Figure 2A, first panel). Depletion of G1 cyclins (*cln1-3Δ GAL1-CLN3*) exhibited approximately a 15 min delay in Swe1 phosphorylation, but failed to stabilize Swe1 (Figure 2A, second panel). The delay in Swe1 phosphorylation appeared to be the result of the observed cell cycle delay (Figure 2B) that is

Figure 2 Mitotic cyclin-associated Cdc28 activity is required for proper Swe1 phosphorylation and degradation. (A) Strains 15D (isogenic wild type), SBY458 (*cln1-3Δ GAL1-CLN3*), SBY145 (*clb5-6Δ*), and SBY175 (*clb1-4Δ GAL1-CLB1*) were cultured in YEP-galactose at 30°C, arrested in G1 by α -factor treatment and then released into YEP-glucose containing nocodazole (see Materials and methods for details). Total cellular proteins prepared at each time point after α -factor release were separated by 8% low-bis SDS-PAGE. Asterisks indicate a crossreacting protein with anti-Swe1 antibody. (B) Budding indices were determined to monitor the cell cycle progression from the same samples as in panel A.

likely caused by diminished G1 cyclin-Cdc28 activity during depletion. Without significant changes in Swe1 stability, cells lacking Clb5-6 (*clb5-6Δ*) also exhibited a delay (approximately 10 min) in Swe1 phosphorylation (Figure 2A, third panel), which correlated closely with a comparable delay in S-phase progression (Figure 2B). In contrast, depletion of mitotic cyclins (*clb1-4Δ GAL1-CLB1*) significantly reduced Swe1 phosphorylation and degradation (Figure 2A, fourth panel). These observations suggest that mitotic cyclin (Clb)-associated Cdc28, but not likely the G1 and S cyclin-associated Cdc28, is critical for Swe1 phosphorylation and degradation. However, the existence of multiple tiers of phosphorylated and unstable Swe1 after depletion of mitotic cyclins suggests that other kinase(s) independent of Cdc28 activity contribute to Swe1 phosphorylation and degradation.

Cdc28 and Cdc5 phosphorylate Swe1 synergistically *in vitro*

Next, we examined the ability of various Cdc28 complexes to phosphorylate Swe1 *in vitro*. Purified cyclin-bound Cdc28

complexes were reacted with a GST-fused catalytically inactive swe1(K473A) and histone H1 (HH1) as substrates in the same reaction, and the relative activity of these complexes to phosphorylate Swe1 was compared. Clb2-bound Cdc28 phosphorylated Swe1 as efficiently as HH1 and generated multiple tiers of Swe1 species (Figure 3A). Under the same conditions, the relative levels of Swe1 phosphorylation by Cln1-Cdc28 or Clb5-Cdc28 complex were significantly lower than those of HH1 phosphorylation (Figure 3A). Since mitotic Cdc28 activity is critical for proper Swe1 regulation *in vivo* (Figure 2), the Clb2-Cdc28 complex was chosen for further analyses.

It has been reported that both Cdc5 and Cla4 phosphorylate Swe1 and regulate its stability (Sakchaisri *et al*, 2004). Thus, we examined how these kinases function together with Clb2-Cdc28 to phosphorylate Swe1 *in vitro*. To this end, purified Clb2-Cdc28 and Cdc5 were reacted either alone or together to test their ability to phosphorylate GST-swe1(K473A) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Clb2-Cdc28 or Cdc5 alone exhibited an efficient incorporation of ^{32}P into Swe1 in a time-dependent manner (Figure 3B). Strikingly, when both Clb2-Cdc28 and Cdc5 were present in the same reaction mixture, incorporation of ^{32}P into Swe1 was significantly greater than the predicted additivity of each reaction (Figure 3B and D), suggesting that Clb2-Cdc28 and Cdc5 phosphorylate Swe1 synergistically *in vitro*. In contrast to this finding, phosphorylation of Swe1 by Cdc5 and Cla4, or by Clb2-Cdc28 and Cla4, incorporated ^{32}P into Swe1 only in an additive manner (Figure 3C and E, and data not shown).

Priming of Swe1 by Cdc28 enhances Cdc5-dependent Swe1 phosphorylation *in vitro*

It is possible that the synergistic phosphorylation of Swe1 by Clb2-Cdc28 and Cdc5 was the result of priming Swe1 by one

of the two kinases, in which the resulting phosphorylated Swe1 became a better substrate for the other kinase. To examine this possibility, we carried out sequential, two-step, Swe1 phosphorylation reactions *in vitro* using purified recombinant proteins. To prime Swe1 in the first reaction (priming reaction), Swe1 was phosphorylated by a priming kinase with excess ATP. After washing out the remaining ATP from the resulting reaction mixtures, the second kinase was added and then incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (labeling reaction) (Figure 4A). Since both kinases were immobilized (bead-bound enzymes were used because we failed to purify sufficient soluble enzymes), the incorporated radioactivity into Swe1 was the result of the labeling capacity of both the priming and the second kinase during the labeling reaction. When Swe1 was primed by Cdc5 and then subjected to the labeling reaction in the absence of Cdc28, no ^{32}P incorporation into Swe1 was detected (Figure 4B, lane 1), suggesting that Cdc5-dependent Swe1 phosphorylation was already saturated during the priming reaction. Under these conditions, addition of Clb2-Cdc28 into the Cdc5-primed reaction resulted in ^{32}P incorporation into Swe1 at a level similar to that primed by either a catalytically inactive cdc5(N209A) or buffer control (Figure 4B, compare lane 2 with lane 4 or 5), suggesting that Cdc5 did not prime Swe1 for Clb2-Cdc28-dependent Swe1 phosphorylation. In contrast, priming of Swe1 by Clb2-Cdc28, but not by kinase-inactive Clb2-cdc28(D145N) or control buffer, greatly increased the subsequent Cdc5-dependent ^{32}P incorporation into Swe1 (Figure 4B, compare lane 8 with lane 10 or 11), even though the priming phosphorylation of Swe1 by Clb2-Cdc28 was not saturated (Figure 4B, lane 7; as evidenced by the capacity of the remaining Clb2-Cdc28 to incorporate ^{32}P in the labeling reaction). The level of Cdc5-dependent ^{32}P

Figure 3 Clb2-Cdc28 and Cdc5 phosphorylate Swe1 synergistically *in vitro*. (A) Cyclin-associated Cdc28 complexes were reacted with GST-swe1(K473A) and histone H1 (HH1) as *in vitro* substrates in each reaction. The reaction mixtures were separated by 8% low-bis SDS-PAGE (upper gel) to detect Swe1 mobility shift and 10% normal SDS-PAGE (lower gel) to retain HH1 in the gel, and then subjected to autoradiography. WT, GST-Cdc28; DN, GST-cdc28(D145N). (B, C) GST-Cdc28/His6-Cks1/MBP-Clb2, T7-HA-Cdc5-Flag, and T7-HA-Cla4-Flag were reacted with kinase-inactive GST-swe1(K473A) in the presence of 50 μM ATP (10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) at 30°C. Reactions were terminated at the indicated time points and then separated by 8% low-bis SDS-PAGE. After staining with Coomassie, the gel was subjected to autoradiography. Cdc5 WT, T7-HA-Cdc5-FLAG; cdc5NA, kinase-inactive T7-HA-cdc5(N209A)-FLAG; Cla4 WT, T7-HA-Cla4-FLAG; cla4KA, kinase-inactive T7-HA-cla4(K594A)-FLAG. (D, E) The bands corresponding to GST-swe1(K473A) from the reactions were excised and the incorporated ^{32}P was quantified. Error bars indicate standard deviation.

Figure 4 Priming of Swe1 by Clb2-Cdc28 enhances Cdc5-dependent Swe1 phosphorylation *in vitro*. (A) Scheme of the two-step kinase reactions. (B, C) Bead-bound GST-swe1(K473A) was primed by the indicated enzymes in the first reaction. After washing out the remaining ATP, second enzymes were added as indicated to carry out labeling reactions (see Materials and methods for details). Samples were separated by 8% low-bis SDS-PAGE and then analyzed. The asterisk indicates a contaminating protein copurified with Cdc5 and Cla4 from Sf9 cells. Cdc5, T7-HA-Cdc5-FLAG; cdc5(N209A), T7-HA-cdc5(N209A)-FLAG; Cdc28, MBP-Clb2/GST-Cdc28/His6-Cks1 complex; cdc28(D145N), MBP-Clb2/GST-cdc28(D145N)/His6-Cks1 complex; Cla4, T7-HA-Cla4-FLAG; cla4(K594A), T7-HA-cla4(K594A)-FLAG. (D, E) Cdc5 activation assays with Clb2-Cdc28. Reactions were carried out as described in panel D and analyzed as above. Cdc28 W, MBP-Clb2/GST-Cdc28/His6-Cks1 complex; Cdc28 D, MBP-Clb2/GST-cdc28(D145N)/His6-Cks1 complex; Cdc5 W, T7-HA-Cdc5-FLAG; Cdc5 N, T7-HA-cdc5(N209A)-FLAG.

incorporation into the Clb2-Cdc28-primed Swe1 in lane 8 (after subtracting the incorporated 32 P by the remaining Clb2-Cdc28 as detected in lane 7) was approximately three-fold higher than that of control reactions (Figure 4B, lanes 10 and 11). Time-course studies revealed that Swe1 priming by Clb2-Cdc28 could enhance the Cdc5-dependent Swe1 phosphorylation in a time-dependent manner (up to 4.7-fold), although overpriming of Swe1 diminished the effect (Supplementary Figure S4). Since Clb2-Cdc28 failed to activate Cdc5 *in vitro* (Figure 4E) and was not required for Cdc5 activation *in vivo* (Supplementary Figure S5), priming of Swe1 by Clb2-Cdc28 likely promoted the subsequent Cdc5-dependent Swe1 phosphorylation. In contrast, neither Clb2-Cdc28 nor Cla4 exerted any priming effect on Swe1 for the subsequent Cla4- or Clb2-Cdc28-dependent Swe1 phosphorylation, respectively (Figure 4C).

Cdc28 activity is required for Cdc5-dependent Swe1 phosphorylation and degradation *in vivo*

We next investigated the importance of Clb-Cdc28 for Cdc5-dependent Swe1 regulation *in vivo*. To inhibit acutely Cdc28 kinase activity, we used an analog-sensitive (as) *cdc28* allele (*cdc28-as1*) that is sensitive to the cell-permeable inhibitor 4-amino-1-tert-butyl-3-(1-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1NM-PP1) (Bishop *et al*, 2000). Treatment of the

cdc28-as1 mutant with 0.5 μ M of 1NM-PP1 greatly stabilized Swe1 in nocodazole-arrested (M phase) cells and partially inhibited Swe1 phosphorylation in hydroxyurea-arrested (S phase) cells (Supplementary Figure S6), suggesting that 1NM-PP1 treatment is suitable for acute inhibition of mitotic Clb-Cdc28-dependent Swe1 phosphorylation. To test the ability of Clb-Cdc28 and Cdc5 in regulating Swe1, a *cdc28-as1 cdc5 Δ* double mutant kept viable by the expression of a weakly functional *cdc5-1* allele under control of the *GAL1* promoter control was transformed with either control vector or a centromeric plasmid expressing wild-type *CDC5* from its native promoter (p*CDC5*). The resulting strains were arrested in G1 and then released into YEP-glucose medium containing nocodazole to repress *GAL1-cdc5-1* expression and trap the cells in M phase. To inhibit mitotic Cdc28 activity after proceeding through G1, 1NM-PP1 was added to the cultures 20 min after α -factor release. When Cdc5 was provided and Cdc28 was active (Figure 5A, top panel), Swe1 progressively accumulated from 20 min after release and became hyperphosphorylated, generating multiple tiers of Swe1 species. These hyperphosphorylated Swe1 species appeared to become unstable at 80 min, as large-budded cells arose (Figure 5A, top panel, and Figure 5B, first panel). Depletion of Cdc5 prevents Swe1 hyperphosphorylation and degradation after achieving several tiers of phosphorylation (Sakchaisri *et al*, 2004),

Figure 5 Requirement of mitotic Clb-Cdc28 activity for Cdc5-dependent Swe1 phosphorylation and degradation *in vivo*. Strain KLY5426 (*cdc28-as1 cdc5Δ* + *pGAL1-cdc5-1*) transformed with either pKL321 (YCplac22-*CDC5*) or YCplac22 (control vector) was cultured in YEP-galactose, arrested in G₁, and then released into YEP-glucose medium containing nocodazole (see Materials and methods for details). Either control DMSO or 0.5 μM of 1NM-PP1 was added to the cultures as indicated. (**A**, **C**) Total cellular proteins were separated by 8% low-bis SDS-PAGE. Asterisks indicate a nonspecific protein crossreacting with anti-Swe1 antibody. (**B**, **D**) To monitor cell cycle progression, samples harvested in panel A or C were counted for budding indices.

indicating that Cdc5 is critical for the late stages of Swe1 hyperphosphorylation. However, even in the presence of *pCDC5*, inhibition of Cdc28 by 1NM-PP1 was sufficient to

prevent Cdc5 from generating hyperphosphorylated, unstable, Swe1, although a tier of phosphorylated Swe1 was detectable (Figure 5A, middle panel). In addition, the levels of Swe1 phosphorylation and stability under these conditions were similar to those when Cdc28 was inhibited in the absence of Cdc5 (compare the middle panel with the bottom panel in Figure 5A), underscoring the importance of mitotic Clb-Cdc28 activity in priming Swe1. Consistent with this notion, addition of 1NM-PP1 into the cultures with large-budded cells (80 min) also rapidly induced Swe1 dephosphorylation and stabilization (Figure 5C). Since Clb-Cdc28 activity was not required for Cdc5 activation *in vivo* and failed to activate Cdc5 *in vitro* (Figure 4E and Supplementary Figure S5), Cdc5 activity was not likely to be downregulated upon inhibition of Clb-Cdc28 activity. Treatment of 1NM-PP1 did not significantly induce a cell cycle delay (Figure 5B), suggesting that it did not significantly inhibit both the G₁ cyclin- and S cyclin-dependent Cdc28 activities under these conditions. However, a tier of phosphorylated Swe1 was still detectable even in the absence of both Clb-Cdc28 and Cdc5 activities (the Swe1 doublet in Figure 5A, middle and bottom panels), suggesting that another kinase(s) still phosphorylates Swe1 independently of both Clb-Cdc28 and Cdc5.

Cdc28 promotes Cdc5 localization to the bud neck in part by enhancing the Swe1-Cdc5 interaction

The PBD of mammalian polo-like kinase 1 (Plk1) has been shown to interact with phospho-peptide sequences generated by Cdk1 or other Pro-directed kinases (Elia *et al*, 2003). Thus, we investigated whether the Clb2-Cdc28-dependent Swe1 priming phosphorylation enhances the interaction between Swe1 and Cdc5, and subsequently facilitates the Cdc5-dependent Swe1 phosphorylation. To this end, partially purified GST-swe1(K473A) was phosphorylated by either Clb2-Cdc28 or its kinase-inactive Clb2-cdc28(D145N), and then subjected to *in vitro* binding analyses with T7-tagged Cdc5. Precipitation of primed GST-swe1(K473A) by Clb2-Cdc28 (approximately 50% of GST-swe1(K473A) exhibited mobility shift; Figure 6A) co-precipitated T7-Cdc5 efficiently. In contrast, precipitation of GST-swe1(K473A) primed by either kinase-inactive Clb2-cdc28(D145N) or buffer control (no apparent mobility shift of GST-swe1(K473A) was detected under both of these conditions) co-precipitated T7-Cdc5 only marginally (Figure 6A). The interaction between phosphorylated Swe1 and Cdc5 appeared to be through the PBD, since precipitation of primed GST-swe1(K473A) co-precipitated T7-Cdc5, but not the localization-defective *cdc5/FAA* (Song *et al*, 2000) PBD mutant (Figure 6B). These data suggest that priming phosphorylation of Swe1 by Clb2-Cdc28 enhances the interaction between Swe1 and Cdc5, and that the intact PBD of Cdc5 is required for this event.

Bud neck-localized Cdc5 is critical for proper Swe1 regulation (Park *et al*, 2004; Sakchaisri *et al*, 2004). Thus, if the Cdc28-dependent Swe1 phosphorylation were important for Swe1-Cdc5 interaction, Cdc28 activity might be important for Cdc5 localization to the bud neck. To examine this possibility, *cdc28-as1* cells expressing *EGFP-CDC5* under endogenous *CDC5* promoter control were arrested in G₁ and then released into nocodazole-containing medium. At 20 min after release, cells were treated with either control DMSO or 0.5 μM of 1NM-PP1 to inhibit mitotic Clb-Cdc28 activity. In samples harvested at two different time points (150 and 180 min after

α -factor release), localization of EGFP-Cdc5 to the SPB was manifest in most of the populations (100% in DMSO-treated cells and 85–88% in 1NM-PP1-treated cells) (Figure 6C, left). Among the cells with distinct EGFP-Cdc5 signals at the SPBs, approximately 42–45% of the DMSO-treated population exhibited the bud neck-localized EGFP-Cdc5 signals, whereas only 5–8% of the 1NM-PP1-treated cells exhibited this localization (Figure 6C, left). Treatment of cells with 1NM-PP1 induced bud elongation due to a delay at mitotic entry but did not cause mitotic exit under these conditions (Figure 6C, right), indicating that the decreased Cdc5 localization to the bud neck by 1NM-PP1 was not the result of Cdc5 delocalization following mitotic exit. Similar results were obtained when cells were arrested with nocodazole and then treated with 1NM-PP1 (Supplementary Figure S7A). As expected, if the PBD was critical for the Clb-Cdc28-dependent Cdc5 localization to the bud neck, inhibition of Clb-Cdc28 activity did not influence the localization efficiency of Cdc5 Δ C-Cdc12 (Park *et al*, 2004; a PBD-deficient fusion that localizes to the bud neck through the function of Cdc12) (Figure 6D and Supplementary Figure S7B). Taken together, these data demonstrate that Clb-Cdc28 activity is critical for promoting the PBD-dependent Cdc5 localization to the bud neck.

Since proper localization of Cdc5 to the bud neck required Hsl1 and Hsl7 (Figure 1F), we next examined whether inactivation of Clb2-Cdc28 influenced the localization of Hsl1-GFP and GFP-Hsl7. Localization of *GAL1* promoter-expressed Swe1-GFP was also examined (we were unable to localize endogenous promoter-controlled Swe1-GFP because its fluorescent signal was below our detection limit). Unlike Cdc5 localization, inhibition of Clb-Cdc28 by 1NM-PP1 did not significantly alter the localization of any of the Hsl1-GFP, GFP-Hsl7, and Swe1-GFP to the bud neck (Figure 6E). These results suggest that delocalization of EGFP-Cdc5 induced by the inhibition of cdc28-as1 was not due to the delocalization of Hsl1, Hsl7, or Swe1, but rather due to a defect after recruiting Swe1 to the Hsl1–Hsl7 platform. Thus, we examined whether Swe1 is required for proper Cdc5 localization to the bud neck. As expected, regardless of the presence or absence of *SWE1*, Cdc5 localization to the SPB was manifest in all the cells examined. In contrast, approximately 38% of the *SWE1* cells exhibited EGFP-Cdc5 signals at the bud neck, whereas about 21% of the *swe1* Δ cells displayed these signals

at the corresponding structure (Figure 6F). These data demonstrate the importance of Swe1 for recruiting Cdc5 to the Hsl1–Hsl7 platform and further support the direct interaction between Swe1 and Cdc5. However, a significant level of EGFP-Cdc5 still localized to the bud neck in *swe1* Δ cells, suggesting that Cdc5 also interacts with other bud neck-localizing components independently of Swe1. Since loss of Hsl1 or Hsl7 drastically diminished the Cdc5-dependent Swe1 phosphorylation (which would not occur if the Cdc5–Swe1 interaction were the only requirement for this event) (Figure 1D and E), the Hsl1–Hsl7 platform could provide additional interaction(s) with Cdc5 to recruit efficiently Cdc5 to the bud neck. In support of this opinion, introduction of *hsl1* Δ and *hsl7* Δ into *swe1* Δ cells further impaired Cdc5 localization to the bud neck (Figure 6G). Delocalization of Cdc5 from the bud neck in *hsl1* Δ *hsl7* Δ cells did not appear to be due to a diminished Clb-Cdc28 activity, since provision of *CDC28Y19F*, which lacks the inhibitory phosphorylation site at Tyr19, failed to remedy this defect (Figure 6H).

Discussion

Hsl1 and Hsl7, but not MIH1, are required for Cdc5-dependent Swe1 regulation

As cells proceed through the cell cycle, Swe1 is progressively phosphorylated to yield hyperphosphorylated forms. These multiply phosphorylated forms of Swe1 are susceptible to ubiquitination and subsequent degradation through a protease-containing complex called the 26S proteasome (Sia *et al*, 1998; McMillan *et al*, 2002). Here, we showed that Hsl1 and Hsl7, which are critical for recruiting Swe1 to the bud neck (Shulewitz *et al*, 1999; Longtine *et al*, 2000), were also required for proper localization of Cdc5 to the bud neck through both a Swe1-dependent and -independent fashion. Moreover, Hsl1 and Hsl7 were critical for Cdc5-dependent Swe1 phosphorylation. These data suggest that Hsl1 and Hsl7 serve as a scaffold to bring Cdc5 and Swe1 in close proximity and promote Cdc5-dependent Swe1 regulation.

In higher eukaryotes, Plk1 phosphorylates and activates Cdc25C, which in turn activates Cdc2 (Kumagai and Dunphy, 1996). Activated Cdc2 then phosphorylates and downregulates Wee1A in a positive feedback loop (Watanabe *et al*, 2004). Similarly, Cdc5 becomes active even after depleting

Figure 6 Clb2-Cdc28 is critical for Cdc5-dependent Swe1 regulation. (A, B) GST-swe1(K473A) primed by either GST-Cdc28/His6-Cks1/MBP-Clb2, catalytically inactive GST-cdc28(D145N)/His6-Cks1/MBP-Clb2, or control buffer was incubated with clarified cellular lysates from either Sf9 cells expressing T7-HA-cdc5(N209A)-FLAG (A) or bacterial cells expressing T7-cdc5(N209A)-His6 or T7-cdc5(FAA)-His6 (B) (see Materials and methods for details). After pull-down, proteins were separated by 10% normal SDS–PAGE to retain the GST ligand in the gel (as a result, the migration pattern of phosphorylated GST-Swe1 appeared slightly different from that in 8% low-bis SDS–PAGE). The migration difference of the GST ligand in panel A does not reflect its actual mobility difference in the gel. W, GST-Cdc28/His6-Cks1/MBP-Clb2; D, GST-cdc28(D145N)/His6-Cks1/MBP-Clb2; GST-Swe1-P, phosphorylated GST-swe1(K473A) (arrows). As input, 1% of the clarified cellular lysates was used. (C, D) Strain KLY5401 transformed with either centromeric pSK754 (pEGFP-CDC5) (C) or centromeric pKL2438 (pYFP-CDC5 Δ C-CDC12) (D) was cultured overnight, arrested by α -factor treatment, and then released into YEP-glucose containing nocodazole. Either DMSO or 0.5 μ M of 1NM-PP1 was added into the culture 20 min after release. Samples harvested at the indicated time points after release were fixed and then examined. Greater than 500 cells were counted from duplicated experiments. (C, D, right panels) DAPI morphology of the 180 min samples in panels C and D was quantified. Treatment of cells with 1NM-PP1 induced elongated bud morphologies. Error bars indicate standard deviation. (E) Strain KLY5401 (*cdc28-as1*) bearing either centromeric YCpT-HSL1-GFP, YCpT-GFP-HSL7, or YCpLG-SWE1-GFP (*GAL1*-promoter controlled) was cultured in YEP-glucose (for Hsl1-GFP and GFP-Hsl7) or YEP- raffinose (for *GAL1*-Swe1-GFP) at 23°C and then arrested with nocodazole for 3 h. Cells were then treated with 0.5 μ M of 1NM-PP1 for 2 h either in the same medium (for Hsl1-GFP and GFP-Hsl7) or after transferring to YEP-galactose containing nocodazole (for *GAL1*-Swe1-GFP). Samples were examined as in panels C and D. A low efficiency of Hsl1-GFP localization in comparison to GFP-Hsl7 localization is likely due to weaker Hsl1-GFP fluorescent signals. Error bars indicate standard deviation. (F) Strain KLY4362 (*swe1* Δ) transformed with either a centromeric pSWE1 or control vector was additionally transformed with pSK754 (pEGFP-CDC5). The resulting transformants were cultured at 23°C and arrested with hydroxyurea for 6 h (the condition where Swe1 is abundant and a significant level of EGFP-Cdc5 signal is detectable at the bud neck) before fixation. Error bars indicate standard deviation. (G, H) Localization of EGFP-Cdc5 in the indicated genetic background was examined as in panel F. Y19F, *CDC28 Y19F* allele.

Clb-Cdc28 activity (Supplementary Figure S5), thus placing Cdc5 in a position to phosphorylate and activate Mih1. This finding raises the possibility that Cdc5 might also regulate Swe1 through an analogous Mih1-Cdc28-Wee1 feedback

loop. However, loss of *MIH1* only marginally diminished the endogenous Swe1 phosphorylation and failed to significantly influence the *GAL1-CDC5*-dependent Swe1 phosphorylation even though Cdc28 was highly phosphorylated at

Tyr19 in these cells (Supplementary Figure S2). These data suggest that Cdc5 does not critically require the *MIH1*-mediated feedback loop to downregulate Swe1.

Clb2-Cdc28: a critical element for Cdc5 localization to the bud neck and Cdc5-dependent Swe1 phosphorylation

Studies in cultured mammalian cells have shown that the PBD of Plk1 is sufficient for targeting the enzymes to specific subcellular locations and that this step is critical for its function (Lee *et al*, 1999). The PBD of Plk1 binds to phosphorylated peptide sequences (Elia *et al*, 2003), suggesting that priming phosphorylations by a kinase may provide a phospho-recognition module that recruits Plk1 in close proximity with its substrates. Recent studies on two vertebrate proteins, Cdc25C phosphatase and the peripheral Golgi protein Nir2 (Elia *et al*, 2003; Litvak *et al*, 2004), demonstrate the importance of priming phosphorylation in their interaction with the PBD of Plk1. We showed that priming of Swe1 by Clb2-Cdc28 enhanced both the interaction between Swe1 and the PBD of Cdc5 and the Cdc5-dependent Swe1 phosphorylation, suggesting that Clb-Cdc28-dependent Swe1 phosphorylation provides a docking site for the PBD of Cdc5 and, as a result, promotes the Cdc5-dependent Swe1 phosphorylation. In this regard, it is worthy to note that Swe1 bears five potential Cdc28-dependent PBD-binding sites (i.e., Ser-pThr/pSer-Pro starting at amino-acid residues 44, 262, 293, 372, and 417). However, it is interesting to note that Cdc5 can phosphorylate Swe1 *in vitro* in the absence of a Clb2-Cdc28-dependent priming event (Sakchaisri *et al*, 2004), although this priming step clearly enhances Cdc5-dependent Swe1 phosphorylation (Figure 4). Similarly, vertebrate Cdc25, which is normally primed by Cdc2 prior to G2/M transition (Elia *et al*, 2003), can be directly phosphorylated and activated by *Xenopus* polo-like kinase Plx1 *in vitro* (Kumagai and Dunphy, 1996). These observations suggest that priming phosphorylation is not required, but important, for the polo kinase-dependent phosphorylation of its substrates.

How phosphorylated Swe1 is degraded is not clearly understood at present. During unperturbed cell cycle, Swe1 becomes hyperphosphorylated concurrently with, or shortly after, accumulation of Clb2 and the majority of Swe1 is degraded following hyperphosphorylation (Shulewitz *et al*, 1999; Supplementary Figure S9). These observations together with our data presented here suggest that phosphorylation of Swe1 by mitotic kinases such as Clb-Cdc28 and Cdc5 is critical for triggering its degradation through ubiquitin-mediated proteolysis. In support of this view, Cdc2 and Plk1 appear to cooperate to induce phospho-dependent ubiquitination and degradation of vertebrate Wee1A through the action of an E3 ubiquitin ligase, SKP1/Cul1/F-box protein (SCF) complex (SCF ^{β -TrCP}) (Watanabe *et al*, 2004). However, a large fraction of Swe1 was still degraded in the absence of Clb-Cdc28 activity (a condition that significantly diminished Swe1 phosphorylation; compare the level of Swe1 in 50 min to that in the later time points in Figure 2A), suggesting the possibility of phosphorylation-independent Swe1 degradation. In addition, loss of APC, which does not normally require phospho-degron (signal for degradation) for ubiquitination and is activated during mitosis, substantially stabilizes Swe1 (Thornton and Toczyski, 2003). Since a fraction of

Swe1 persists at the bud neck even after mitotic entry, it is tempting to speculate that APC may be responsible for the phosphorylation-independent Swe1 degradation in mitosis, whereas SCF is accountable for the degradation of the hyperphosphorylated Swe1 prior to mitotic entry.

Our data showed that Clb-Cdc28 activity was required for proper Cdc5 localization to the bud neck, an event that occurred after assembly of the Hsl1-Hsl7 platform at the corresponding location. Subsequent recruitment of phosphorylated Swe1 to the platform appeared to promote Cdc5 localization to this site. Consistent with these results, Swe1 localizes to the bud neck in cells with a medium-size bud (S phase) (Longtine *et al*, 2000), whereas localization of Cdc5 to the bud neck occurs as early as G2 and becomes progressively more abundant as cells enter mitosis (Lee *et al*, 2005). Interestingly, analyses with individual mitotic cyclin mutants revealed that cells lacking Clb2, but not other mitotic cyclins, significantly impaired Cdc5 localization to the bud neck (Supplementary Figure S8) with elongated bud morphologies (data not shown), suggesting that Clb2-associated Cdc28 activity is largely accountable for Swe1 phosphorylation and Cdc5 localization to the bud neck. However, inhibition of Clb-Cdc28 greatly diminished, but did not eliminate, the Cdc5 localization to the neck, while it did not significantly influence the Cdc5 localization to the SPB (Figure 6C and Supplementary Figure S7A). Thus, a significant level of Cdc28 priming-independent interaction(s) also exists between Cdc5 and its target proteins at the SPB and, to a less extent, at the bud neck. It is possible that some of the critical PBD-interacting proteins are phosphorylated by kinase(s) other than Cdc28. Alternatively, they may possess a negatively charged residue to mimic phosphorylation and bypass the requirement of Cdc28-dependent phosphorylation.

Cooperative actions of multiple kinases and Hsl1-Hsl7 scaffold in the regulation of Swe1

Previous studies and the data provided here suggest that Swe1 is regulated by the coordinated actions of multiple kinases and regulatory elements, as cells proceed through the cell cycle (McMillan *et al*, 1999a; Shulewitz *et al*, 1999; Sakchaisri *et al*, 2004) (Figure 7). At least three kinases (Cla4, Clb-Cdc28, and Cdc5) contribute to Swe1 phosphorylation in a distinct manner. During the early stage of bud emergence, Cla4 is targeted to septin ring structures at the bud neck (Versele and Thorner, 2004) sufficiently early to encounter Swe1 and contribute to its phosphorylation in S phase (Sakchaisri *et al*, 2004). Thus, Cla4 is likely responsible for phosphorylating the early bud neck-localized Swe1 population (Figure 7A), although it may also contribute to Swe1 phosphorylation later in the cell cycle. Since Cla4 phosphorylates Swe1 independently of Clb-Cdc28 and Cdc5 (Figures 3 and 4), a tier of modified Swe1 detected in cells after eliminating both the Clb-Cdc28 and Cdc5 activities (Figure 5) could be attributable to the undiminished Cla4 activity under these conditions. Interestingly, the *hsl1 Δ cla4 Δ* double mutant exhibits synthetically elongated bud morphologies that can be remedied by *swe1 Δ* (Sakchaisri *et al*, 2004), suggesting that at least a part of the Cla4 and Hsl1 functions in Swe1 regulation is independent of each other.

Later in the cell cycle but prior to the G2/M transition, Clb-Cdc28 and Cdc5 function in a concerted manner to phosphorylate Swe1 synergistically (Figure 7B). Both Clb2 and Cdc28

Figure 7 Model illustrating the multi-kinase-dependent Swe1 phosphorylation and degradation during the cell cycle. **(A)** In S phase, Cla4 is primarily responsible for Swe1 phosphorylation. Swe1 is less abundant at this stage, and thus is shown in plain letters. Swe1-dependent Cdc28 Tyr19 phosphorylation occurs as early as S phase. **(B)** In G₂, Swe1 becomes abundant (Swe1 in bold letters) and more potently inhibits Clb-Cdc28. Prior to mitotic entry, a rise in Clb-Cdc28 activity as the level of mitotic Clb increases tips the balance to trigger Clb-Cdc28-dependent Swe1 phosphorylation. This step is critical both for promoting Swe1-Cdc5 interaction and for the subsequent Cdc5-dependent Swe1 phosphorylation at the bud neck (see text for details). Dotted lines indicate interactions among the proteins. Triple dots between Hsl1-Hsl7 and neck-Cdc5 indicate a putative interaction between these proteins (see online version for color figure).

predominantly localize to the nucleus, although a fraction of them also localize to the bud neck (Hwang and Silver, 2001). Thus, as the level of Clb2 rises in G₂, Cdc28 gains sufficient activity to overcome early Swe1 inhibition and phosphorylate Swe1 presumably in the nucleus, although the possibility of phosphorylating Swe1 by the bud neck-associated Clb2-Cdc28 cannot be excluded. In support of this view, the *swe1Δ1* mutant that primarily localizes to the nucleus because of its failure to interact with Hsl7 still displays multiple tiers of phosphorylation (McMillan *et al*, 2002). Once tethered to the Hsl1 and Hsl7 platform, this Cdc28-primed Swe1 promotes Cdc5 localization to the bud neck through interaction with the PBD (dotted line in Figure 7B between the phosphorylated Swe1 and the bud neck-localized Cdc5). Cdc5 then exerts its kinase activity to generate hyperphosphorylated Swe1 that is susceptible to ubiquitin-mediated degradation (Figure 7B). In line with the importance of the Cdc5-dependent Swe1 phosphorylation, the *cdc5-3 CDC14^{TAB6-1}* mutant (*CDC14^{TAB6-1}*) compensates for the mitotic exit defect, but not the G₂/M delay, of the *cdc5-3* allele; Park *et al*, 2003) exhibited a significant delay in both downregulating Swe1 and activating Clb2-Cdc28, thus resulting in the generation of elongated bud morphologies (Supplementary Figure S9).

Our model delineates how Swe1 regulation is orchestrated by multiple components as cells progress through the cell cycle. Cla4-dependent septin filament formation early in the cell cycle (Versele and Thorner, 2004) permits assembly of the Hsl1-Hsl7 platform, a critical step that is required for the recruitment of Clb2-Cdc28-phosphorylated Swe1 and Cdc5 later in the cell cycle. Phosphorylated Swe1 further promotes Cdc5 localization to the platform by providing a docking site for the PBD of Cdc5. Our data showed that both the Hsl1-Hsl7 platform and the primed Swe1 are two crucial elements for Cdc5-dependent Swe1 hyperphosphorylation and subsequent degradation at the bud neck. This coordinated, multi-step, Swe1 regulation clearly provides a means to monitor the completion of earlier cell cycle events and to effectively bring about Swe1 destruction at the time of mitotic entry. Once unleashed from the Swe1-imposed G₂ delay, Clb-Cdc28 can induce mitotic entry unimpeded.

Materials and methods

Strains, plasmids, growth conditions, and immunoblotting analyses

Yeast strains and plasmids used in this study are listed in Supplementary Tables 1 and 2. Detailed information on the strains, plasmids, growth conditions, and antibodies used for immunoblotting analyses is provided in Supplementary data.

Purification of recombinant proteins from Sf9 cells and E. coli

To prepare recombinant proteins, Sf9 cells infected with baculoviruses expressing GST-Cdc28/His6-Cks1, GST-cdc28(D145N)/His6-Cks1, Cln1, Clb5 (gifts of J Wade Harper, Harvard Medical School, Boston, MA), T7-HA-Cdc5-FLAG, T7-HA-cdc5(N209A)-FLAG (Sakchaisri *et al*, 2004), T7-HA-Cla4-FLAG, and T7-HA-cla4(K594A)-FLAG (Sakchaisri *et al*, 2004) were lysed, and then subjected to pull-down with either glutathione (GSH)-agarose (Sigma) or anti-FLAG M2-agarose (Sigma). GST-swe1(K473A) and MBP-Clb2 were prepared from *E. coli* BL21 bearing pGEX-3X-*swe1*(K473A) and MC1061 bearing pMAL-CLB2 (a gift of R Deshaies, Caltech, Pasadena, CA), respectively. MBP-Clb2 bound to amylose resin (New England Biolab, Beverly, MA) was eluted with 10 mM maltose (Sigma) before reconstituting with an equal amount of purified GST-Cdc28/His6-Cks1 complex on ice for 30 min. CAK was not provided to the GST-Cdc28/His6-Cks1 complex, because it weakly phosphorylated GST-swe1(K473A) and obscured the GST-Cdc28/His6-Cks1-dependent Swe1 phosphorylation (data not shown).

Kinase assays and two-step reactions

Kinase assays were carried out as described previously (Sakchaisri *et al*, 2004). Samples were then separated by SDS-PAGE as indicated, stained with Coomassie, and the incorporated ³²P was detected by autoradiography. The protein bands were excised from the dried gels and the incorporated ³²P was measured by liquid scintillation counter.

In order to carry out two-step reactions, immobilized, catalytically inactive, GST-swe1(K473A) was first reacted with an immobilized priming kinase as indicated in a kinase cocktail (TBMD) (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 2 mM EGTA, 0.5 mM Na₃VO₄, 20 mM PNPP) containing 500 μM ATP for 60 min. The reaction mixtures were then washed with PBS three times to remove remaining ATP and then subjected to subsequent labeling reaction in TBMD containing 5 μM ATP (10 μCi of [^γ-³²P]ATP; 1 Ci = 37 GBq) for 15 min at 30 °C. To facilitate reactions for bead-bound proteins, continuous agitation was provided. Reactions were terminated by the addition of SDS-sample buffer, heated at 95 °C for 5 min, and then subjected to 8% low-bis SDS-PAGE (96:1 acrylamide:bis-acrylamide mixture) for autoradiography.

In vitro protein-protein interaction studies

To determine the phosphorylation-dependent Swe1-Cdc5 interaction, bead-bound, catalytically inactive, GST-swe1(K473A) was first primed by immobilized GST-Cdc28/His6-Cks1/MBP-Clb2 or GST-

cdc28(D145N)/His6-Cks1/MBP-Clb2 in TBMD containing 500 μ M ATP for 60 min at 30°C, washed, and then resuspended in a binding buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 3 mM KCl, 430 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, pH 7.4). Clarified cellular lysates (15 000 g spin, 10 min) prepared from either Sf9 cells expressing T7-HA-cdc5(N209A)-FLAG or *E. coli* BL21 (DE3) expressing T7-cdc5(N209A)-His6 or T7-cdc5(FAA)-His6 were added, and then incubated for 2 h at 4°C. The resin was washed five times with the binding buffer before eluting the bound proteins by boiling in SDS-PAGE sample buffer. Samples were separated by 10% SDS-PAGE, and then subjected to anti-T7 immunoblotting to detect co-precipitated T7-Cdc5. The same membrane was stained with Coomassie to detect the immobilized GST-Swe1 and GST-Cdc28/MBP-Clb2 proteins.

References

- Bishop AC, Ubersax JA, Petsch DT, Matheos DP, Gray NS, Blethrow J, Shimizu E, Tsien JZ, Schultz PG, Rose MD, Wood JL, Morgan DO, Shokat KM (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* **407**: 395–401
- Booher RN, Deshaies RJ, Kirschner MW (1993) Properties of the *Saccharomyces cerevisiae* wee1 and its differential regulation of p34^{cdc28} in response to G₁ and G₂ cyclins. *EMBO J* **12**: 3417–3426
- Elia AE, Cantley LC, Yaffe MB (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science* **299**: 1228–1231
- Hwang WW, Silver PA (2001) The *Saccharomyces cerevisiae* cyclin Clb2p is targeted to multiple subcellular locations by *cis*- and *trans*-acting determinants. *J Cell Sci* **114**: 589–597
- Kaiser P, Sia RAL, Bardes EGS, Lew DJ, Reed SI (1998) Cdc34 and the F-box protein Met30 are required for degradation of the Cdk-inhibitory kinase Swe1. *Genes Dev* **12**: 2587–2597
- Kumagai A, Dunphy WG (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* **273**: 1377–1380
- Lee KS, Park JE, Asano S, Park CJ (2005) Yeast polo-like kinases: functionally conserved multitask mitotic regulators. *Oncogene* **24**: 217–229
- Lee KS, Song S, Erikson RL (1999) The polo-box-dependent induction of ectopic septal structures by a mammalian polo kinase, Plk, in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **96**: 14360–14365
- Lew DJ, Reed SI (1993) Morphogenesis in the yeast cell cycle: regulation by Cdc28 and the cyclins. *J Cell Biol* **120**: 1305–1320
- Litvak V, Argov R, Dahan N, Ramachandran S, Amarilio R, Shainskaya A, Lev S (2004) Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. *Mol Cell* **14**: 319–330
- Longtine MS, Theesfeld CL, McMillan JN, Weaver E, Pringle JR, Lew DJ (2000) Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Mol Cell Biol* **20**: 4049–4061
- McMillan JN, Longtine MS, Sia RA, Theesfeld CL, Bardes ES, Pringle JR, Lew DJ (1999a) The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Mol Cell Biol* **19**: 6929–6939

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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- McMillan JN, Sia RA, Bardes ES, Lew DJ (1999b) Phosphorylation-independent inhibition of Cdc28p by the tyrosine kinase Swe1p in the morphogenesis checkpoint. *Mol Cell Biol* **19**: 5981–5990
- McMillan JN, Theesfeld CL, Harrison JC, Bardes ES, Lew DJ (2002) Determinants of Swe1p degradation in *Saccharomyces cerevisiae*. *Mol Biol Cell* **13**: 3560–3575
- Park CJ, Song S, Lee PR, Shou W, Deshaies RJ, Lee KS (2003) Loss of CDC5 function in *Saccharomyces cerevisiae* leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. *Genetics* **163**: 21–33
- Park J-E, Park CJ, Sakchaisri K, Karpova T, Asano S, McNally J, Sunwoo Y, Leem S-H, Lee KS (2004) A novel functional dissection of the localization-specific roles of budding yeast polo kinase Cdc5p. *Mol Cell Biol* **24**: 9873–9886
- Russell P, Moreno S, Reed SI (1989) Conservation of mitotic controls in fission and budding yeast. *Cell* **57**: 295–303
- Sakchaisri K, Asano S, Yu LR, Shulewitz MJ, Park CJ, Park J-E, Cho YW, Veenstra TD, Thorner J, Lee KS (2004) Coupling morphogenesis to mitotic entry. *Proc Natl Acad Sci USA* **101**: 4124–4129
- Shulewitz MJ, Inouye CJ, Thorner J (1999) Hsl7 localizes to a septin ring and serves as an adapter in a regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**: 7123–7137
- Sia RA, Bardes ES, Lew DJ (1998) Control of Swe1p degradation by the morphogenetic checkpoint. *EMBO J* **17**: 6678–6688
- Song S, Grenfell TZ, Garfield S, Erikson RL, Lee KS (2000) Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. *Mol Cell Biol* **20**: 286–298
- Song S, Lee KS (2001) A novel function of *Saccharomyces cerevisiae* CDC5 in cytokinesis. *J Cell Biol* **152**: 451–469
- Sreenivasan A, Kellogg D (1999) The Elm1 kinase functions in a mitotic signaling network in budding yeast. *Mol Cell Biol* **19**: 7983–7994
- Thornton B, Toczyski DP (2003) Cycling without the cyclosome: securin and B-type cyclins are the only essential targets of the APC in budding yeast. *Nat Cell Biol* **5**: 1090–1094
- Versele M, Thorner J (2004) GTP binding and direct phosphorylation by the PAK, Cla4, regulate septin collar assembly in budding yeast. *J Cell Biol* **164**: 701–715
- Watanabe N, Arai H, Nishihara Y, Taniguchi M, Watanabe N, Hunter T, Osada H (2004) M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCF β -TrCP. *Proc Natl Acad Sci USA* **101**: 4419–4424