Cell Cycle Control by a Complex of the Cyclin HCS26 (PCL1) and the Kinase PHO85

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The events of the eukaryotic cell cycle are governed by cyclin-dependent kinases (cdk's), whose activation requires association with cyclin regulatory subunits expressed at specific cell cycle stages. In the budding yeast *Saccharomyces cerevisiae*, the cell cycle is thought to be controlled by a single cdk, CDC28. Passage through the G_1 phase of the cell cycle is regulated by complexes of CDC28 and G_1 cyclins (CLN1, CLN2, and CLN3). A putative G_1 cyclin, HCS26, has recently been identified. In **a**/α diploid cells lacking CLN1 and CLN2, HCS26 is required for passage through G_1. HCS26 does not associate with CDC28, but instead associates with PHO85, a closely related protein kinase. Thus, budding yeast, like higher eukaryotes, use multiple cdk's in the regulation of cell cycle progression.

Cyclin-dependent kinases (cdk's) interact with cyclin regulatory subunits to control the major transitions of the cell division cycle. In higher eukaryotes, the cell cycle is governed by multiple cdk's, each of which interacts with a narrow subset of cyclins to promote a limited range of cell cycle events. In budding yeast, it is thought that a single cdk, CDC28, associates with a broad range of stage-specific cyclins to control all major cell cycle events (*1*).

CDC28 associates with the G_1 cyclins CLN1, CLN2, and CLN3 to control passage through the G_1 phase of the cell cycle. Several observations suggest that a recently identified gene, *HCS26*, may also encode a G_1 cyclin. The sequence of the *HCS26* gene product (*2*) is similar to those of the G_1 cyclins CLN1 and CLN2, and transcription of *HCS26*, like that of *CLN1* and *CLN2*, is restricted to G_1 and controlled by the G_1-specific transcription factor SWI4 (*3*). When overexpressed, HCS26, CLN1, and CLN2 are each able to suppress the growth defect of a SWI4-deficient **a**/α diploid strain, suggesting that there is some functional redundancy among the three genes (*2*). Overexpression of HCS26 does not, however, allow growth of a *cln1*Δ *cln2*Δ *cln3*Δ strain, indicating that the redundancy is not complete (*4*).

We explored the possibility that HCS26 participates in G_1 control. We constructed a yeast strain carrying a chromosomal deletion of *HCS26* (*5*). Like strains carrying single (or double) mutations in *CLN1*, *CLN2*, or *CLN3* (*1*), the *hcs26*Δ strain had no discernible growth defect. We also analyzed *hcs26*Δ strains carrying additional mutations in one or two CLN genes. Only *hcs26*Δ *cln1*Δ *cln2*Δ strains exhibited a growth defect: these cells grew slowly as haploids and were inviable as **a**/α diploids. Thus, under conditions where CLN1 and CLN2 functions are compromised, HCS26 is required for viability.

To clarify the essential function of HCS26 in cells lacking CLN1 and CLN2, we characterized the terminal phenotype of the *hcs26*Δ *cln1*Δ *cln2*Δ **a**/α diploid strain (*6*). We rescued this strain with a plasmid expressing *CLN2* under the control of the methionine-repressible *MET3* promoter (*7*). This strain is viable in the absence of methionine (*CLN2* expressed) and inviable in the presence of methionine (*CLN2* repressed) (Fig. 1A). We added methionine to an asynchronous logarithmic phase culture of cells grown in the absence of methionine (Fig. 1, B and C). At time zero, when *CLN2* expression was still maximal, cells were 28% unbudded, and a large fraction exhibited a 4n DNA content; this distribution probably resulted from a G_2 delay induced by constitutive expression of *CLN2* (*8*). Four hours after *CLN2* expression was repressed, 89% of the cells were large and unbudded with a single nucleus (Fig. 1B), and the majority exhibited a 2n DNA content (Fig. 1C), consistent with a G_1 arrest. These observations suggest that the *hcs26*Δ *cln1*Δ *cln2*Δ **a**/α diploid strain is defective in progress through G_1.

We investigated whether the HCS26 protein, like the CLN proteins, is associated with CDC28-dependent protein kinase activity. We used antibodies to HCS26 (anti-HCS26), developed against a TrpE-HCS26 fusion protein (*9*), to immunoprecipitate HCS26 protein from yeast lysates (*10*). Anti-HCS26 immunoprecipitates from wild-type cells or cells that overexpressed HCS26 did not contain higher amounts of histone H1 kinase activity than immunoprecipitates from cells lacking HCS26 (Fig. 2A); in contrast, histone H1 kinase activity was readily detectable in control immuno-

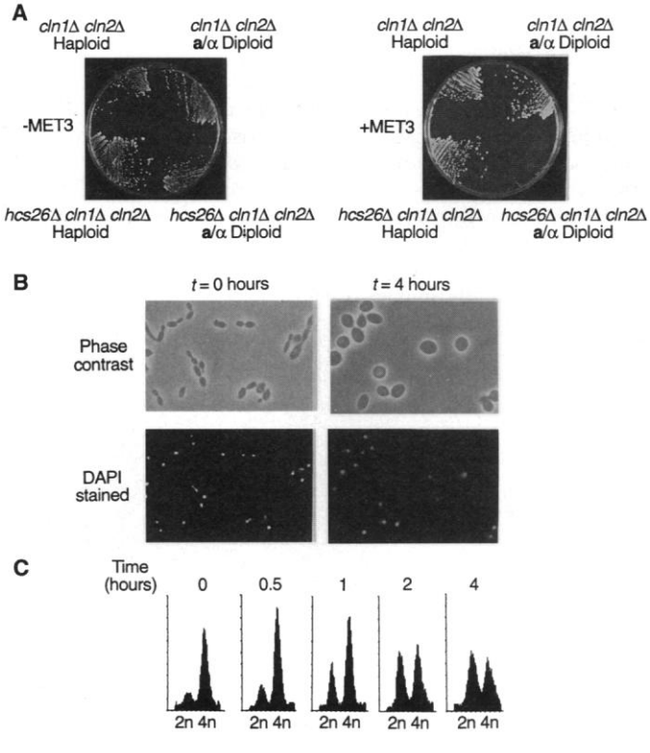

Fig. 1. Arrest of **a**/α diploid *hcs26*Δ *cln1*Δ *cln2*Δ cells in G_1. (**A**) The **a**/α diploid and haploid *cln1*Δ *cln2*Δ (top) and *hcs26*Δ *cln1*Δ *cln2*Δ (bottom) strains (*5*), expressing *CLN2* under the control of the methionine-repressible *MET3* promoter (*7*), were grown in the presence or absence of methionine. (**B**) Phase contrast and fluorescent micrographs of DAPI-stained cells (**a**/α *hcs26*Δ *cln1*Δ *cln2*Δ, pMET3-CLN2) before (t = 0) and 4 hours after addition of methionine (*6*). (**C**) Flow cytometric analysis of DNA content in propidium iodide–stained cells (**a**/α *hcs26*Δ *cln1*Δ *cln2*Δ, pMET3-CLN2) at 0, 0.5, 1, 2, and 4 hours after addition of methionine (*6*).

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precipitates obtained with antibody to CLN3 (Fig. 2B) (*11*). A phosphorylated protein of approximately 32 kD was observed in anti-HCS26 immunoprecipitates (Fig. 2A, lanes 1 and 2); the labeling of this protein was greater in immunoprecipitates from cells overexpressing HCS26 (Fig. 2A, lane 1) and was greatly reduced in cells lacking HCS26 or in control immunoprecipitates lacking anti-HCS26 (Fig. 2A, lanes 3 and 4). Because this protein is the same size as HCS26, and many cdk's phosphorylate their associated cyclin subunit, we suspect that this phosphorylation represents phosphorylation of HCS26 by an associated catalytic subunit.

To determine whether the HCS26-associated kinase activity was dependent on CDC28, we analyzed activity in lysates from cells with a temperature-sensitive defect in CDC28 activity (*11*). HCS26-associated kinase activity was similar in a wild-type strain (Fig. 2C, lane 3), a *cdc28-4* strain (lane 4), and a *cdc28-13* strain (*4*). As expected, CLN3-associated kinase activity was CDC28-dependent (Fig. 2C, lanes 1 and 2). These results suggest that HCS26 associates with a kinase other than CDC28.

To search for the cdk that associates with HCS26, we added a bacterially expressed glutathione-S-transferase (GST)–HCS26 fusion protein (*12*) to crude yeast lysates and incubated the mixture briefly (*13*). GST-HCS26 and its associated proteins were then adsorbed to glutathione-agarose, washed, and eluted (*13*). GST-HCS26 eluates contained the previously observed HCS26 kinase activity but contained little activity toward histone H1 (*14*). Control eluates with GST-CLB3, a mitotic cyclin known to interact with CDC28, contained strong histone H1 kinase activity (*14*).

The cdk's, including CDC28, contain a conserved amino acid motif, PSTAIRE, near their NH_2-terminus. To determine if HCS26 associates with a cdk containing this motif, we immunoblotted (*15*) with an antibody against the PSTAIRE peptide to analyze proteins associated with GST-HCS26 after incubation in yeast lysates (*13*). Wild-type yeast lysates contained two major immunoreactive proteins of approximately 36 and 34 kD (Fig. 3A, lane 4). GST-CLB3 eluates contained an immunoreactive protein of 34 kD (Fig. 3A, lane 3) that comigrated with the lower protein in the wild-type lysate and reacted with antibodies specific for CDC28 (*14*). Thus, the lower band probably represents CDC28. On the other hand, GST-HCS26 eluates contained a larger (36-kD) immunoreactive protein that comigrated with the upper protein detected in crude lysates (Fig. 3A, lane 1).

Aside from CDC28, the only other yeast protein known to contain the PSTAIRE motif is PHO85, a 36-kD protein kinase that is 51% identical to CDC28 (*16, 17*). We found that crude lysates from cells lacking PHO85 (*pho85*Δ) did not contain the 36-kD protein recognized by antibodies to PSTAIRE (Fig. 3A, lane 5), suggesting that this protein is PHO85. GST-HCS26 eluates prepared from lysates of the *pho85*Δ strain did not contain the 36-kD protein (Fig. 3A, lane 2), suggesting that the GST-HCS26–associated kinase is PHO85.

Identification of PHO85 as the likely cdk partner of HCS26 suggested a possible exogenous substrate for analysis of HCS26-associated kinase activity. PHO4 is a substrate for the cyclin-cdk pair PHO80-PHO85 (*18*). GST-HCS26 eluates prepared from wild-type cells contained PHO4 kinase activity (Fig. 3B, lane 1) (*19*). GST-HCS26 eluates prepared from *pho85*Δ lysates lacked this activity (Fig. 3B, lane 2), further supporting the notion that PHO85 is the cdk partner of HCS26.

To confirm that HCS26 and PHO85 associate in vivo, we constructed an epitope-tagged version of HCS26 containing a triple tandem hemagglutinin (HA) tag at the COOH-terminus (*20*). The epitope-tagged HCS26 was expressed in various yeast strains and immunoprecipitated (*21*) from cell lysates with a monoclonal antibody (mAb) to the HA tag (mAb 12CA5). PHO4 kinase activity (*19*) was present in 12CA5 immunoprecipitates of

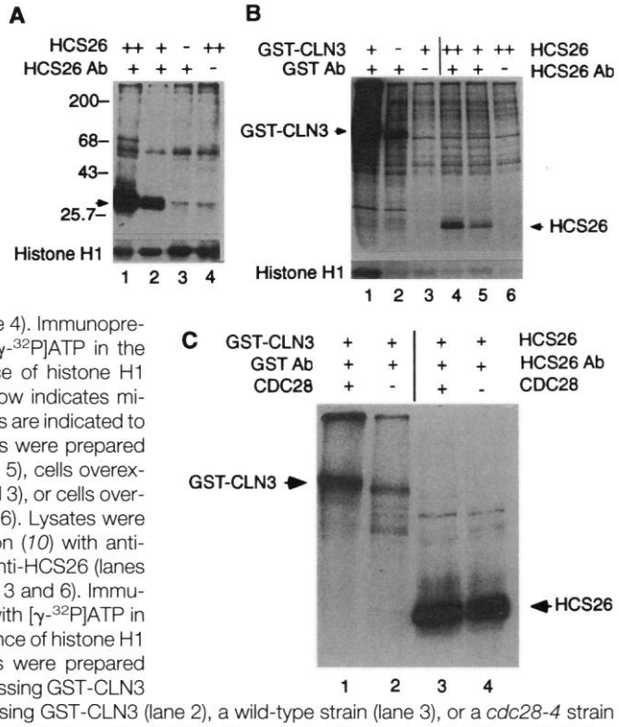

Fig. 2. Kinase activity associated with HCS26 is not dependent on functional CDC28. (**A**) Lysates were prepared from wild-type cells carrying HCS26 on a high copy vector (lanes 1 and 4), wild-type cells without vector (lane 2), or *hcs26*Δ cells (lane 3). Lysates were subjected to immunoprecipitation (*10*) with anti-HCS26 (HCS26 Ab) (lanes 1 to 3) or no antibody (lane 4). Immunoprecipitates were incubated with [γ-^{32}P]ATP in the absence (top panel) or presence of histone H1 (bottom panel) (*10, 11*). The arrow indicates migration of HCS26. Molecular sizes are indicated to the left in kilodaltons. (**B**) Lysates were prepared from wild-type cells (lanes 2 and 5), cells overexpressing GST-CLN3 (lanes 1 and 3), or cells overexpressing HCS26 (lanes 4 and 6). Lysates were subjected to immunoprecipitation (*10*) with anti-GST (GST Ab) (lanes 1 and 2), anti-HCS26 (lanes 4 and 5), or no antibodies (lanes 3 and 6). Immunoprecipitates were incubated with [γ-^{32}P]ATP in the absence (top panel) or presence of histone H1 (bottom panel) (*11*). (**C**) Lysates were prepared from a wild-type strain overexpressing GST-CLN3 (lane 1), a *cdc28-4* strain expressing GST-CLN3 (lane 2), a wild-type strain (lane 3), or a *cdc28-4* strain (lane 4). Immunoprecipitates were prepared with anti-GST (lanes 1 and 2) or anti-HCS26 (lanes 3 and 4) and incubated with [γ-^{32}P]ATP (*10, 11*).

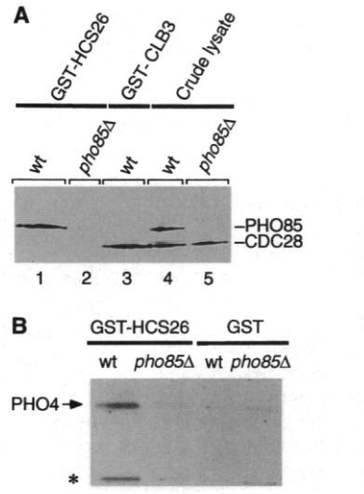

Fig. 3. Association of GST-HCS26 and PHO85 in vitro. (**A**) Crude lysates from a wild-type strain (lane 4) or a *pho85*Δ strain (lane 5) were immunoblotted (*15*) with polyclonal antibodies to the PSTAIRE motif. Purified GST-HCS26 (lanes 1 and 2) or GST-CLB3 (lane 3) were incubated in lysates of wild-type (lane 1 and 3) or *pho85*Δ yeast (lane 2) (*12, 13*). GST fusion proteins and associated proteins were then precipitated and immunoblotted with anti-PSTAIRE. (**B**) Proteins associated with GST-HCS26 or with GST alone were purified from wild-type (lanes 1 and 3, respectively) or *pho85*Δ lysates (lanes 2 and 4) and assayed for PHO4 kinase activity (*12, 13, 19*). The lower band (*) is a degradation product of bacterially expressed PHO4.

lysates from wild-type cells (Fig. 4, lane 1) and from a strain with a defective CDC28 protein (*cdc28-4*, lane 3). PHO4 kinase activity was not detectable in immunoprecipitates from lysates of *pho85Δ* cells (Fig. 4, lane 2) or cells not expressing the epitope-tagged HCS26 protein (*14*). In addition, 12CA5 immunoprecipitates from wild-type lysates contained a 36-kD protein that was recognized by antibodies to PHO85 and was not present in immunoprecipitates from *pho85Δ* lysates (*14*). These data indicate that the major cdk partner of HCS26 in yeast cells is PHO85.

Our observations that HCS26 functions in progression of the cell through G_1, and that HCS26 is associated with PHO85, suggest that PHO85 contributes to progression through G_1 as well. To investigate this possibility, we examined the requirement for PHO85 function in cells lacking CLN1 and CLN2. We constructed a haploid *cln1Δ cln2Δ pho85Δ* strain (*5*) carrying a plasmid containing *CLN2* under the control of the *MET3* promoter (*7*). In the absence of methionine, CLN2 was expressed and the strain was viable, whereas in the presence of methionine, CLN2 was repressed and the strain was inviable (Fig. 5). Thus, like HCS26, PHO85 is required for cell viability in the absence of CLN1 and CLN2.

Our results indicate that HCS26 is a G_1 cyclin that associates with the cdk PHO85. HCS26 is thus a member of a small family of PHO85 cyclins that includes ORFD (*22*) and PHO80 (*18*). To better reflect its biochemical function, we propose renaming HCS26 as PCL1 (PHO85 cyclin 1). With this nomenclature, ORFD becomes PCL2 (*22*).

The precise role of HCS26 (PCL1) in regulating the cell cycle remains unclear. Deletion of *HCS26* (*PCL1*) has a greater effect in **a**/α diploid cells than in haploid cells. The effects of *HCS26* (*PCL1*) deletion are, in this respect, similar to those of a deletion of SWI4. Haploid *swi4Δ* cells are viable, whereas **a**/α diploid *swi4Δ/swi4Δ* cells are inviable (*2*). Perhaps HCS26 plays a specialized role in promoting G_1 progression in **a**/α diploid cells. Alternatively, **a**/α diploid cells may express a smaller complement of cyclins and may therefore be more dependent on those cyclins that are expressed. HCS26 (PCL1) is required for G_1 progression in haploid cells under some conditions: for example, a *cln1Δ cln2Δ pcl1Δ pcl2Δ* haploid strain is unable to exit G_1 (*22*).

Our observations suggest that budding yeast, like higher eukaryotes, use multiple cdk systems to regulate cell cycle progression. The roles of CDC28 and PHO85 are clearly not equivalent. Overexpression of PHO85 cannot complement the lethality in CDC28-deficient strains (*16*). CDC28 is absolutely required for progression through multiple cell cycle transitions in wild-type cells (*1*), whereas PHO85 is nonessential (*17*) and required for passage through G_1 only in the absence of CLN1 and CLN2. PCL-PHO85 complexes and CLN-CDC28 complexes have different substrate specificities in vitro (Fig. 2), suggesting that the targets of PHO85 in vivo are probably different from the targets of CDC28.

PHO85 was originally identified as a negative regulator of phosphatase (PHO5) gene expression (*23*). When phosphate concentrations are high, an active complex of PHO85 and the cyclin PHO80 represses phosphatase expression by phosphorylating the transcription factor PHO4; decreased phosphate concentrations inactivate the complex (*18*). The participation of PHO85 in both phosphate metabolism and cell cycle progression provides an example of how the same cdk may be directed to distinct biological functions by association with different cyclin subunits. Perhaps PHO85 serves to coordinate nutritional state and cell cycle progression. Phosphate starvation, in addition to inhibiting the activity of the PHO80-PHO85 complex, may also control other PCL-PHO85 complexes. Because PHO85 is not essential for G_1 progression, except in the absence of CLN1 and CLN2, inhibition of PHO85 activity would not cause cell cycle arrest but instead could provide more subtle regulation of progression through G_1 in response to nutritional requirements.

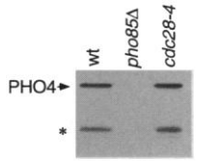

Fig. 4. Association of HCS26 and PHO85 in vivo. Epitope-tagged HCS26 (*20*) was expressed in wild-type (lane 1), *pho85Δ* (lane 2), or *cdc28-4* yeast (lane 3). Lysates were subjected to immunoprecipitation (*21*) with mAb 12CA5, and immunoprecipitates were assayed for PHO4 kinase activity (*19*). The lower band (*) is a degradation product of bacterially expressed PHO4.

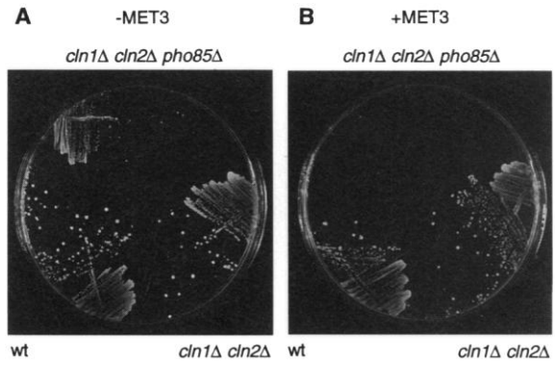

Fig. 5. Requirement for PHO85 in the absence of CLN1 and CLN2. Haploid *pho85Δ cln1Δ cln2Δ* cells (*5*), rescued by *CLN2* under the control of the methionine-repressible *MET3* promoter (*7*), were grown in (**A**) the absence (*CLN2* on) or (**B**) the presence (*CLN2* off) of methionine.

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5. All strains were derived from YPH274 (**a** *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1*) through a series of one-step gene replacements [R. Rothstein, *Methods Enzymol.* **101**, 202 (1983)] and crosses. All gene replacements were checked by Southern (DNA) blot analysis and demonstrated to exhibit 2:2 segregation. The *hcs26::HIS3* allele was created by replacing an internal fragment of *HCS26*, coding for amino acids 12 to 161, with the nutritional marker *HIS3*. The *cln1::TRP1* allele contains a disruption of *CLN1* in which the *Tn10-LUK* transposon is inserted into *CLN1* 38 nucleotides 3′ of the start codon. The *URA3* marker of *Tn10-LUK* was disrupted by insertion of the nutritional marker *TRP1*. The *cln2::LEU2* allele is a disruption of *CLN2* at the Xho I site with the nutritional marker *LEU2* [J. A. Hadwiger, C. Wittenberg, H. E. Richardson, M. D. B. Lopes, S. I. Reed, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6255 (1989)]. The *cln3*$^-$ allele is *daf1-2*, a deletion of *CLN3* from the 5′ Xho I site to the internal Nco I site replaced with the nutritional marker *URA3* [F. R. Cross, *Mol. Cell. Biol.* **8**, 4675 (1988)]. PHO85 was deleted with pYU19 (*17*).
6. Expression of *CLN2* in logarithmic phase [optical density at 600 nm (OD_{600}) ≈ 0.4] **a**/α *cln1::TRP1/cln1::TRP1 cln2::LEU2/cln2::LEU2 hcs26Δ:HIS3/hcs26Δ:HIS3*, pMET3-CLN2 cells grown in SD-met-ura at 30°C was shut off by addition of L-methionine to 100 μg/ml. At each time point, cells were washed once with 50 mM tris (pH 7.5), sonicated briefly, and fixed in 50 mM tris (pH 7.5) and 70% ethyl alcohol for 1 hour at 24°C. Cells were washed twice with 50 mM tris (pH 7.5), and a portion (1/10) was removed for microscopic analysis. Remaining cells were treated with ribonuclease A (1 mg/ml) for 2 hours at 37°C and then with proteinase K (0.04 mg/ml) for 1 hour at 50°C. Cells were washed with phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4 (pH 7.3)] and stained with propidium iodide (50 μg/ml) in PBS for 1 hour at 24°C. Cells were diluted 1:50 with PBS and sonicated briefly (10 s at setting 30 in a Fisher Sonic Dismembrator). Flow cytometry was done in a Becton Dickinson cell sorter, and results were analyzed with LYSIS II software. For microscopic examination, cells were treated with 4,6-diamino-2-phenylindole (DAPI) (1 μg/ml) in 50% glycerol for 1 min, washed twice with PBS, and visualized with a Nikon MICROPHOT FXA microscope at ×40 magnification.
7. The plasmid pMET3-CLN2 (pFHE52) was constructed by insertion of a fragment containing *CLN2* under the control of the *MET3* promoter (from p2663) into pRS316 [described in R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)].

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9. A bacterially expressed TrpE-HCS26 fusion protein was purified from inclusion bodies by denaturing gel electrophoresis, electroeluted from gel slices, and injected into rabbits (BabCO, Berkeley, CA). Antibodies to HCS26 were affinity-purified as described [B. J. Andrews and I. Herskowitz, *Nature* **342**, 830 (1989)].
10. For immunoprecipitations, cells were grown in logarithmic phase (30°C for *CDC28* strains and 25°C for *cdc28-4* strains) to an OD_{600} of 0.4 to 0.6. Cells (30 OD_{600} units) were collected by centrifugation and resuspended in 1 ml of ice-cold buffer A [50 mM NaCl, 50 mM tris (pH 7.5), 0.1% NP-40, and 0.1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, leupeptin (0.1 μg/ml), 1 μM calyculin A, and aprotinin (2 μg/ml). All manipulations were done at 4°C. Resuspended cells were combined with 0.5 ml of glass beads and lysed with three 30-s pulses of a mini beadbeater (BioSpec). The lysate was clarified by centrifugation (10 min at 10,000*g*). Affinity-purified anti-HCS26 or antibody to GST (anti-GST) was added at a 1:100 dilution for 1 hour, and the mixture was then incubated for 45 min with 40 μl of a 1:1 slurry of goat antibodies to rabbit immunoglobulin G coupled to agarose beads (Sigma) suspended in buffer A. Immunoprecipitates were washed three times with 1 ml of buffer A, once with 1 ml of PBS, and once with 1 ml of kinase buffer [50 mM tris (pH 7.5) and 10 mM $MgCl_2$].
11. To determine kinase activity in anti-HCS26 immunoprecipitates, we resuspended beads in 50 μl of kinase buffer supplemented with 1 mM dithiothreitol (DTT), 0.5 mM PMSF, aprotinin (2 μg/ml), leupeptin (0.1 μg/ml), and 10 μCi of [γ-^{32}P]adenosine triphosphate (ATP). Histone H1 (Boehringer Mannheim) was added at 0.5 mg/ml. Reactions were incubated for 20 min at 30°C (or 20 min at 25°C for the experiment shown in Fig. 2C) and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.
12. The NH_2-terminal GST-HCS26 fusion construct (pFHE29) was made by ligating the open reading frame of *HCS26* into the polylinker of pGEX1 [D. B. Smith and K. S. Johnson, Gene **67**, 31 (1988)]. Expression of GST-HCS26 was induced in logarithmic phase ($OD_{600} \approx 0.6$) DH5α cells containing pFHE29 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for 3 hours at 30°C. Cells were collected; frozen (−70°C); thawed into PBS with lysozyme (0.5 mg/ml), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF at 4°C; and lysed by sonication (three pulses of 1 min each at setting 35). The lysate was adjusted to 250 mM KCl and 15 mM DTT and clarified by centrifugation (85,000*g*) for 1 hour. The lysate was loaded onto a glutathione-coupled agarose (Sigma) column and washed extensively with PBS with 250 mM KCl and 0.5 mM DTT, and the GST-HCS26 was eluted with elution buffer [50 mM tris (pH 8.1), 250 mM KCl, and 5 mM reduced glutathione]. Peak fractions were pooled and dialyzed extensively against 50 mM Hepes (pH 7.6), 50 mM KCl, and 30% glycerol. GST-CLB3 and GST were prepared in the same way with the plasmids pGEX-CLB3 and pGEX1, respectively.
13. Logarithmic phase cells ($OD_{600} \approx 0.6$) were mechanically lysed with glass beads in a mini beadbeater (BioSpec) (two 50-s pulses) in buffer A containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin (1 μg/ml), and aprotinin (2 μg/ml). Lysates were clarified by centrifugation (10 min at 10,000*g*). Then 50 μg of purified GST-HCS26 protein was added to 900 μg of lysate containing ATP-regenerating mix [50 mM Hepes (pH 7.5), 40 μM creatine phosphate, creatine phosphate kinase (20 μg/ml), 1 mM $MgCl_2$, and 1 mM ATP] and incubated for 15 min at 24°C. GST-HCS26–associated proteins were recovered by adding 50 μl of a 1:1 slurry of glutathione-coupled agarose beads (Sigma) in buffer A and incubating for 1 hour at 4°C. Beads were washed twice with buffer A, once with PBS, and once with kinase buffer. Associated complexes were eluted from the beads by incubation in kinase elution buffer [50 mM tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5 mM reduced glutathione] for 15 min at 24°C.
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15. Immunoblotting was done as described [S. M. Murphy, M. Bergman, D. O. Morgan, *Mol. Cell. Biol.* **19**, 5290 (1993)]. Where horseradish peroxidase–coupled secondary antibodies were used, the immunoreactive proteins were visualized with the Enzyme-Linked ChemiLuminescence (ECL) Western Blotting Detection Kit (Amersham).
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19. For the analysis of PHO4 kinase activity, samples were incubated in 50 mM tris (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP, 800 ng of PHO4, and 10 μCi of [γ-^{32}P]ATP. Reactions were incubated for 15 min at 30°C and analyzed by SDS-PAGE and autoradiography. Purified, recombinant PHO4 was a gift of E. O'Shea (*18*).
20. An in-frame Not I site was added to the 3′ end of *HCS26* by oligonucleotide-directed mutagenesis. A Not I fragment containing three tandem copies of the HA epitope (YPYDVPDYA) was then ligated into the Not I site to generate HCS26-3XHA. A Hind III–Xho I fragment containing HCS26-3XHA was then ligated into a 2-μm-based plasmid carrying the *HCS26* promoter region to generate pFHE40. A, Ala; D, Asp; P, Pro; V, Val; Y, Tyr.
21. Lysates for immunoprecipitations with 12CA5 were prepared as in (*13*). We added 1 to 7 μg of mAb 12CA5 to lysates (900 μg) and incubated the mixture on ice for 30 min. Protein A–coupled Sepharose beads (Sigma) were then added and the mixture incubated with constant agitation for 1 hour at 4°C. Beads were then washed twice with buffer A, once with PBS, and once with kinase assay buffer.
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The PCL2 (ORFD)–PHO85 Cyclin-Dependent Kinase Complex: A Cell Cycle Regulator in Yeast

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Cyclin-dependent kinase (cdk) complexes are essential activators of cell cycle progression in all eukaryotes. In contrast to mammalian cells, in which multiple cdk's contribute to cell cycle regulation, the yeast cell cycle is largely controlled by the activity of a single cdk, CDC28. Analysis of the putative G_1 cyclin PCL2 (ORFD) identified a second cyclin-cdk complex that contributes to cell cycle progression in yeast. PCL2 interacted with the cdk PHO85 in vivo and in vitro and formed a kinase complex that had G_1-periodic activity. Under genetic conditions in which the Start transition was compromised, PHO85 and its associated cyclin subunits were essential for cell cycle commitment. Because PHO85 and another cyclin-like molecule, PHO80, also take part in inorganic phosphate metabolism, this cdk enzyme may integrate responses to nutritional conditions with the cell cycle.

In eukaryotes, cdk's are essential activators of cell cycle transitions. The activity of cdk's is regulated in part by their association with essential positive regulatory subunits called cyclins. Passage through a given transition is thought to require a threshold amount of appropriate cdk activity. In metazoans, multiple cdk's and associated cyclin subunits control the cell cycle (*1*). In contrast, in the budding yeast *Saccharomyces cerevisiae*, CDC28 is the only cdk enzyme known to be required for cell cycle progression (*2*). Specificity of CDC28 function at different points in the cell cycle is achieved through association with different classes of cyclin subunits. Three G_1 cyclins, CLN1, CLN2, and CLN3, are rate-limiting activators of CDC28 in late G_1 that promote irreversible commitment to a new division cycle; this event is called Start and is analogous to the restriction point for mammalian cells (*3*). The cell cycle machinery is regulated at Start in part by modulation of the abundance of cyclins in response to environmental cues such as glucose availability (*4*) and mating pheromones (*5*, *6*).

Expression of the genes *CLN1* and *CLN2* (but not *CLN3*) is largely controlled by the activity of two transcription factors, SWI4 and SWI6, which form a heterodimeric complex, SCB-binding factor (SBF), that binds to SWI4,6–cell cycle box

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