A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator

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The protein kinase C of *Saccharomyces cerevisiae*, Pkc1, regulates a MAP kinase, Mpk1, whose activity is stimulated at the G1–S transition of the cell cycle and by perturbations to the cell surface, e.g. induced by heat shock. The activity of the Pkc1 pathway is partially dependent on Cdc28 activity. Swi4 activates transcription of many genes at the G1–S transition, including *CLN1* and *CLN2*. We find that *swi4* mutants are defective specifically in bud emergence. The growth and budding defects of *swi4* mutants are suppressed by overexpression of *PKC1*. This suppression requires *CLN1* and *CLN2*. Inhibition of the Pkc1 pathway exacerbates the growth and bud emergence defects of *swi4* mutants. We find that another dose-dependent suppressor of *swi4* mutants, the novel gene *HCS77*, encodes a putative integral membrane protein. *Hcs77* may regulate the Pkc1 pathway; *hcs77* mutants exhibit phenotypes like those of *mpk1* mutants, are partially suppressed by overexpression of *PKC1* and are defective in heat shock induction of Mpk1 activity. We propose that the Pkc1 pathway promotes bud emergence and organized surface growth and is activated by Cdc28–Cln1/Cln2 at the G1–S transition and by *Hcs77* upon heat shock. *Hcs77* may monitor the state of the cell surface.

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Introduction

The G1 phase of the cell cycle of the budding yeast, *Saccharomyces cerevisiae*, contains a commitment point, termed START, after which the cell is committed to a full round of cell division (reviewed in Reed, 1992). As the cell passes this point and begins the G1–S transition, at least three processes are coordinately initiated; bud emergence/polarized surface growth, DNA synthesis (S phase) and duplication of the spindle pole body (Pringle and Hartwell, 1981). A key regulator of the G1–S transition is the cyclin-dependent kinase, Cdc28, which associates with its regulatory subunits, including the G1 cyclins

Cln1, Cln2 and Cln3, to form active kinase complexes. Mutants defective in any one or pair of these G1 cyclins are viable; mutants lacking all three cannot perform the G1–S transition and arrest in G1 (reviewed in Reed, 1992). It is now clear that the G1 cyclins are not fully redundant but play specialized roles in the wild-type cell, but each can partially compensate for the others when they are absent due to mutation (Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). *CLN3* is expressed throughout the cell cycle and Cdc28–Cln3 kinase is required for proper timing of transcription of many genes at the G1–S transition, including *CLN1* and *CLN2* (Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). *CLN1* and *CLN2* are expressed only as the cell enters the cell cycle and are implicated in promoting bud emergence and polarized cell growth (Lew and Reed, 1993; Benton *et al.*, 1994).

Cell cycle-specific transcription at the G1–S border is mediated by a pair of related transcription factors, one dependent on *SWI4*, the other on *MBP1* (reviewed in Koch and Nasmyth, 1994). Swi4 functions at promoter elements found upstream of many genes, including *CLN1*, *CLN2* and *HO* (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Andrews and Moore, 1992; Nasmyth, 1993), and is the main transcription factor driving expression of *CLN1* and *CLN2*. Swi4 is required for the growth of haploids (S288C strain background) at high temperature and of α/α diploids at all temperatures (Ogas *et al.*, 1991). Mutants lacking *SWI4* exhibit reduced expression of *CLN1* and *CLN2* and appear to arrest in G1 under restrictive conditions. This growth defect is suppressed efficiently by overexpressing *CLN1* or *CLN2* (Ogas *et al.*, 1991), indicating that the activity of the Cdc28–Cln1 and Cdc28–Cln2 kinase complexes is limiting for the growth of *swi4* mutant cells. Mbp1 is a non-essential Swi4-like protein that functions at a related promoter element (McIntosh, 1993) and is responsible for most of the residual expression of *CLN1* and *CLN2* observed in *swi4* mutants (Koch *et al.*, 1993). Swi4 and Mbp1 are activated at the G1–S transition either directly or indirectly by the Cdc28–Cln3 kinase. We know little about the other *in vivo* substrates of the various Cdc28 kinase complexes, although the activity of the yeast protein kinase C (PKC) isozyme, Pkc1, is partially dependent on Cdc28 activity (Marini *et al.*, 1996; Zarzov *et al.*, 1996).

Protein kinase C isozymes (reviewed in Newton, 1995) comprise a family of structurally related serine/threonine protein kinases whose activity is stimulated by diacylglycerol (DAG)/phospholipid and in some cases also by calcium. PKC isozymes are involved in intracellular signaling and have long been implicated in mammalian cell proliferation (Rozengurt *et al.*, 1984; Kaibuchi *et al.*, 1985; Persons *et al.*, 1988), although the mechanism by which they do so is obscure. The *PKC1* gene encodes the budding yeast homolog of mammalian PKC (Levin *et al.*,

1990). Purified Pkc1 displays a serine/threonine protein kinase activity *in vitro* (Antonsson *et al.*, 1994; Wantanabe *et al.*, 1994) that is stimulated by phospholipid when the kinase is complexed with the GTP-bound form of the GTPase Rho1 (Kamada *et al.*, 1996). Genetic evidence also supports the model that Pkc1 is regulated by Rho1 *in vivo* (Nonaka *et al.*, 1995; Kamada *et al.*, 1996).

Pkc1p regulates a kinase cascade that includes the MAP kinase Mpk1. Mpk1 activity is stimulated by at least two signals, the G1-S transition of the cell cycle and perturbations to cell surface induced by heat and hypo-osmotic shocks (Davenport *et al.*, 1995; Kamada *et al.*, 1995; Zarzov *et al.*, 1996). Mutants defective in the Pkc1 pathway lyse preferentially as small-budded cells either at high temperature, in the case of the MAP kinase cascade components, or at all temperatures, in the case of *pkc1Δ* mutants (Lee and Levin, 1992; Levin and Bartlett-Heubusch, 1992; Lee *et al.*, 1993b). These growth defects can be partially suppressed by osmotic stabilization. The primary defect in these mutants is in cell wall growth, organization of cortical actin, or both (Novick and Botstein, 1985; Costigan *et al.*, 1992; Mulholland *et al.*, 1994). A function for the Pkc1 pathway specifically at the G1-S transition has been suggested by a number of observations. First, Mpk1 activity is stimulated at the G1-S boundary, concomitant with bud emergence (Zarzov *et al.*, 1996). Activation appears to be partially dependent on Cdc28 (Marini *et al.*, 1996; Zarzov *et al.*, 1996). Second, *mpk1* mutants and certain *pkc1* alleles exacerbate the growth defects of certain G1-defective *cdc28* alleles (Mozzoni *et al.*, 1993; Marini *et al.*, 1996). Third, Pkc1 pathway mutants can be partially suppressed by overexpression of *BCK2*, a *CLN3*-independent activator of G1/S-specific transcription (Lee *et al.*, 1993b; Di Como *et al.*, 1995).

Here we find that *swi4* mutants are defective primarily in bud emergence and that overexpressing *PKC1* suppresses this growth defect by a Cln1- and Cln2-dependent mechanism. Our observations suggest that the Pkc1 pathway functions downstream of Cln1 and Cln2 to control bud morphogenesis. We also describe a novel dose-dependent suppressor of *swi4* mutations, *HCS77*, which encodes a putative transmembrane protein. Mutants lacking *HCS77* display phenotypes similar to those of mutants defective in the known components of the Pkc1 pathway. *Hcs77* appears to be a novel component of the Pkc1 pathway and is a candidate protein for sensing deformation of the plasma membrane induced by heat shock.

Results

swi4 mutants are defective in bud emergence

Haploid *swi4Δ* mutants (S288C strain background) are temperature sensitive for growth, whereas *a/α swi4Δ/swi4Δ* diploids do not proliferate at any temperature. When an *a/α swi4^{ts}/swi4Δ* strain is shifted to the restrictive temperature, the cells arrest as large, unbudded cells with a single nucleus (mixture of 1N and 2N DNA content) (Ogas *et al.*, 1991). Growth can be restored to these strains by high-copy plasmids carrying one of the G1 cyclin genes, *CLN1* or *CLN2*. It was concluded that *swi4* mutants arrest in the G1 phase of the cell cycle under restrictive conditions (Ogas *et al.*, 1991). In the course of analyzing other dose-dependent suppressors of the *swi4* defect, we

Fig. 1. Haploid *swi4Δ* cells are defective in bud emergence but not DNA synthesis at 25°C. Haploid strains JO31-3A (WT) and JO51-2C (*swi4Δ*) were grown to $A_{600} = 0.4$ in liquid YEPD medium at 25°C, fixed in ethanol (70%) and stained with propidium iodide. The FACS profiles of (A) JO31-3A and (B) JO51-2C cells are shown. The percentage of cells with 1N DNA content ($\pm 4\%$) derived from these plots is shown as a bar (filled) and compared with the percentage of unbudded cells ($\pm 2\%$) in the same samples determined by microscopy, also shown as a bar (hatched) on the same scale.

came to re-examine the terminal phenotype of *SWI4*-defective strains in the S288C background.

Haploid *swi4Δ* cells growing at permissive temperature (25°C) in rich (YEPD) medium displayed a much higher fraction of unbudded cells (65%) than do wild-type cells (42%) (Figure 1A and B). The unbudded cells contained a single nucleus as judged by 4',6'-diamidino-2-phenylindole (DAPI) staining (data not shown). Fluorescence activated cell sorting (FACS) analysis of these cells revealed, surprisingly, that the majority have 2N DNA content (Figure 1B). Hence a large proportion of *swi4Δ* cells under permissive conditions are unbudded but have undergone DNA synthesis. In contrast, for wild-type cells, the fraction of unbudded cells matches the fraction of cells with 1N DNA content (Figure 1A). These data demonstrate that, even under permissive conditions, *swi4Δ* cells are impaired primarily in bud emergence. When the exponentially growing *swi4Δ* haploid cells in YEPD medium were shifted to high (i.e. restrictive) temperature (37°C), they accumulated with a higher fraction of unbudded cells (79%), again with one nucleus and largely with 2N DNA content (data not shown). We conclude that *swi4Δ* mutants show a bud emergence defect at both permissive and non-permissive temperatures.

High-copy plasmids carrying either *CLN1* or *CLN2* efficiently suppress the growth defects of *swi4Δ* and *swi4^{ts}* mutants (Ogas *et al.*, 1991). We found that these plasmids also corrected the bud emergence defect of haploid *swi4Δ* mutants grown exponentially at low temperature (25°C) on synthetic medium (54% unbudded) relative to the vector control (73% unbudded) (Table I; data not shown).

We investigated if overproduction of other Cdc28-associated cyclins could rescue the *swi4* growth defect efficiently. We found that *CLN3-2* (*DAF1-1*), which encodes a hyperstable allele of Cln3 (Cross, 1988), or a high-copy plasmid bearing *CLB5* are poor suppressors of the *swi4* defect (Figure 2A; data not shown). These findings support the contention that the *swi4* defect is due primarily to a Cln1- and Cln2-dependent process, most likely bud emergence.

Overproduction of Pkc1 suppresses the growth defect of *swi4* mutants

As noted above, haploid *swi4* mutants are temperature sensitive for growth and exhibit a bud emergence defect;

Table I. Overexpression of *PKC1* or *CLN2* partially corrects the budding defect of haploid *swi4Δ* cells growing in liquid Ura drop-out medium at 25°C

Strain	Genotype	Plasmid	% budded cells
JO31-3a	a WT	YEpl352 (vector)	54 (± 3)
JO51-2C	a <i>swi4Δ</i>	YEpl352 (vector)	27 (± 4)
JO51-2C	a <i>swi4Δ</i>	pDL289 (YEpl352. <i>PKC1</i>)	44 (± 4)
JO51-2C	a <i>swi4Δ</i>	YEpl24 (vector)	27 (± 4)
JO51-2C	a <i>swi4Δ</i>	pJO21 (YEpl24. <i>CLN2</i>)	46 (± 4)

a/α *swi4Δ/swi4Δ* diploids display the same defect at all temperatures (Ogas *et al.*, 1991; this work). We found that the presence of a high-copy plasmid carrying the *PKC1* gene efficiently suppressed the growth defects of both temperature-sensitive and deletion mutants of *SWI4* at 37°C on rich (YEPD) and synthetic media (Figure 2B; data not shown) and in both haploid and homozygous a/α diploid mutant strains (Figure 2B; data not shown). The suppression of the *swi4* defect was due to overexpression of *PKC1* and not an adjacent gene, since a plasmid bearing an epitope-tagged version of *PKC1* under control of the *GALI,10* promoter (Watanabe *et al.*, 1994) suppressed the mutant growth defect only when grown on galactose (data not shown). In contrast, expression of Pkc1-K853R, encoding a catalytically inactive version of the kinase (Watanabe *et al.*, 1994), from the *GALI,10* promoter did not suppress the growth defect of haploid *swi4Δ* mutants (data not shown). We conclude that *PKC1* is a dose-dependent suppressor of the *swi4* growth defect. The suppression requires Pkc1 kinase activity and is neither allele specific nor cell type specific.

Pkc1 is believed to control two downstream branches (Lee and Levin, 1992). One involves a kinase cascade culminating in a MAP kinase (Mpk1). The nature of the

Fig. 2. Overproduction of Pkc1 but not a Cln3 strain restores growth to *SWI4*-deficient cells. (A) Haploid strain JO51-2C (*swi4Δ*) was transformed with plasmids YEp50 (vector), pFC101 (*pDAF1-1*; *DAF1-1* in YEp50), pB3.2A (*pSWI4*; *SWI4* in YEp50) and pJO1 (*pCLN1*; *CLN1* in YEp24). Transformants were selected on Ura drop-out plates at 25°C for 3 days and restreaked on fresh drop-out plates which were then incubated at either 25 or 37°C for 3 days. Growth of this strain carrying YEp50 is indistinguishable from the same strain carrying the YEp24 vector (data not shown). (B) High-copy plasmids YEp352 (vector), pDL287 (*pPKC1*; *PKC1* in YEp352), pJO16 (*pSWI4*; *SWI4* in YEp24) or pJO21 (*pCLN2*; *CLN2* in YEp24) were introduced into an a/α *swi4Δ/swi4Δ* strain (JO184) by transformation. The transformants were streaked on Ura drop-out plates and incubated at 25 or at 37°C for 3 days. Note that JO184 carrying YEp24 is indistinguishable in phenotype from JO184 carrying YEp352 (data not shown).

Table II. *PKC1* overexpression does not restore *HO-lacZ* expression to a haploid *swi4Δ* mutant (JO51-2C) growing in liquid Ura drop-out medium at 25°C

Plasmid	Relative <i>HO-lacZ</i> expression
pB3.2A (YCp50. <i>SWI4</i>)	1.00
YEp352 (vector)	<0.001
pDL289 (YEp352. <i>PKC1</i>)	<0.002

second branch is not known (Lee and Levin, 1992). Activation of either or both branches may be required for suppression of the *swi4* defect. A high-copy plasmid bearing *MPK1* or a plasmid carrying the *BCK1-20* allele which encodes a partially activated version of the MEK kinase did not suppress the growth defects of haploid *swi4Δ* mutants or of diploid a/α *swi4^{ts}/swi4Δ* mutants (data not shown) at high temperature. However, these constructs are only weakly activating for the pathway (Lee and Levin, 1992; Lee *et al.*, 1993b; D. Levin, unpublished data), and the *BCK1-20* allele alone confers a growth defect at high temperature. The results are thus inconclusive.

PKC1* overexpression bypasses the essential function of *Swi4

High-copy *PKC1* could suppress the *swi4* mutant growth defect by substituting for or by bypassing the need for *Swi4* function. To discriminate between these two possibilities, we determined whether overexpression of *PKC1* restored transcription to *Swi4*-dependent promoters. The *HO* gene contains multiple SCB elements in its promoter region, and its transcription is absolutely dependent on *Swi4* (reviewed in Nasmyth, 1993). We found that overexpression of *PKC1* did not restore expression to the *HO-lacZ* gene in a haploid *swi4Δ* mutant grown at 25°C (Table II). Furthermore, overexpression of *PKC1* in *swi4* mutants or in wild-type cells did not boost expression of a synthetic reporter gene driven by multimerized SCB (or MCB) elements (data not shown). Elevated Pkc1 pathway activity thus did not simply replace *Swi4* function but rather relieved the growth defect.

The *Pkc1* pathway affects bud emergence

We next tested if overexpression of *PKC1* mimicked overexpression of *CLN1* or *CLN2* (see above) in correcting the bud emergence defect of *swi4* mutants. We found that *swi4Δ* strains harboring a high-copy number *PKC1* plasmid budded more efficiently (44% budded cells) than the vector control (27% budded cells) during exponential growth in synthetic medium under permissive conditions (25°C) (Table I). This effect mirrors that seen when *CLN2* is overexpressed (see above; Table I). The congenic wild-type strain harboring the vector alone budded most efficiently (54% budded cells) (Table I). We conclude that overexpression of *PKC1* mimics overexpression of *CLN2* in partially restoring efficient budding to haploid *swi4Δ* cells.

We tested if inhibition of the Pkc1 pathway exacerbates the bud emergence defect of *swi4* mutants. We first constructed mutant strains in the S288C strain background defective in *SWI4* and *MPK1*. Sporulation of a *SWI4/swi4Δ MPK1/mpk1Δ* diploid strain yielded *swi4Δ* and

mpk1Δ segregants at the expected frequency on YEPD medium at 25°C, but only one in 30 *swi4Δ mpk1Δ* spores formed colonies under these conditions. Microscopic examination showed that the *swi4Δ mpk1Δ* double mutant spores germinated and divided to form microcolonies composed of very large unbudded cells (data not shown). These observations suggested that loss of Mpk1 activity indeed exacerbates the budding defect of *swi4* mutants.

We next determined whether inhibition of the Pkc1 pathway by expression of a catalytically inactive Pkc1 protein inhibited growth of *swi4* mutant cells. Although expression of Pkc1-K853R from the *GAL1,10* promoter did not inhibit growth of wild-type cells (Watanabe *et al.*, 1994; Figure 8A), it strongly inhibited the growth of haploid *swi4Δ* cells at all temperatures (Figure 3A; data not shown). To determine the terminal phenotype of the *swi4Δ* mutant expressing Pkc1-K853R, transformants were grown to mid-log phase at 30°C in synthetic medium containing raffinose (uninduced condition) and then diluted into galactose-containing medium at the same permissive temperature (induced condition). Cells harboring either the vector control or overexpressing wild-type Pkc1 from the *GAL1,10* promoter continued to grow and displayed no obvious phenotype (Figure 3B and C; data not shown). In contrast, cells overexpressing Pkc1-K853R ceased proliferating (Figure 3B), accumulating preferentially as large unbudded cells (78% unbudded compared with 61% unbudded for the vector control) with a single nucleus as judged by DAPI staining (Figure 3B, data not shown). FACS analysis of the arrested cells indicates that most had 2N DNA content (data not shown). We conclude that overexpression of catalytically inactive Pkc1 exacerbates the bud emergence defect of *swi4* mutants.

Pkc1* suppression of the *swi4* growth defect requires *Cln1* and *Cln2

Overproduction of Pkc1, *Cln1* or *Cln2* suppresses the *swi4* growth defect by promoting bud emergence (see above). To explore the possibility that the Pkc1 pathway and these cyclins act in the same pathway, we tested if a subset of the G1 cyclins is required for the ability of *PKC1* overexpression to suppress the *swi4* growth defect. We utilized a congenic series of haploid strains containing a *swi4^{ts}* allele in combination with deletions of one or more of the *CLN* genes and observed that overproduction of Pkc1 could efficiently suppress the growth defects of *swi4^{ts}* strains deleted for any one of the G1 cyclin genes *CLN1*, *CLN2* or *CLN3* (Table III). In contrast, high-copy *PKC1* did not suppress the growth defect of the congenic *swi4^{ts} cln1Δ cln2Δ* triple mutant even at semi-permissive temperature (Table III). We also found that high-copy *PKC1* did not suppress the growth defect of a *swi4^{ts} mbp1Δ* double mutant, although expression of *CLN2* could (Figure 4, Table III). Given that the residual transcription of *CLN1* and *CLN2* in *swi4* mutants is dependent on the Mbp1 transcription factor, we conclude that residual expression of the *CLN1* and *CLN2* genes is absolutely required for *PKC1* overexpression to suppress the *swi4* defect. We have also found that high-copy *PKC1* is an efficient suppressor of the growth defect of *swi4^{ts} pcl1Δ pcl2Δ* mutants (J.V.Gray, unpublished work), indicating that the major Pho85-associated cyclins are not required for suppression by Pkc1 (Ogas *et al.*, 1991; Espinosa

Fig. 3. Overexpression of inactive Pkc1 inhibits the growth of *SWI4*-deficient cells. Haploid *swi4* Δ strain (JO395-1A) was transformed with pBM743 (vector), pDL468 (p*GALPKC1*) and pDL469 [p*GALPKC1*(K853R)], and transformants selected on Ura drop-out plates containing glucose at 25°C. (A) The transformants were streaked on Ura drop-out plates containing galactose and incubated at 30°C for 3 days. The same strain transformed with pB3.2A (p*SWI4*) served as a positive control. (B) Transformants were grown in liquid Ura drop-out medium containing raffinose at 30°C until exponential growth phase. Cells were collected by centrifugation, washed and resuspended in Ura drop-out medium containing galactose and incubated at 30°C. The budding indices of the cultures were determined as a function of time in galactose-containing medium. (C) The morphology of cells containing the vector or expressing inactive Pkc1 after 15 h in galactose-containing medium are shown under Nomarski optics at the same magnification.

et al., 1994; Measday *et al.*, 1994). The above data indicate that the Pkc1 pathway requires the G1 cyclin proteins Cln1 and Cln2 (associated with Cdc28) to suppress the *swi4* defect.

We have determined whether the Pkc1 pathway affects expression of *CLN1* and *CLN2* (and other *Swi4*-dependent genes including *PCL1*, *PCL2* and *FKS1*) and of synthetic reporter constructs bearing *lacZ* under the control of multimerized SCB and MCB elements. In agreement with Igual *et al.* (1996) and Madden *et al.* (1997), we did not find convincing evidence that the Pkc1 pathway directly affected transcription at the G1–S boundary. The expression of some genes was somewhat reduced in *mpk1* Δ mutants, but only under restrictive conditions, probably as a secondary result of cell lysis (J.V.Gray, unpublished work). Furthermore, we found that the transcription factor Rlm1, which may be a target of Mpk1 kinase (Dodou and Treisman, 1997), was not required for overexpression of *PKC1* to suppress the *swi4*^{ts} growth defect efficiently (J.V.Gray, unpublished work). In addition, we found that

Table III. Suppression of the growth defect of haploid *swi4*^{ts} mutants at 37°C by *PKC1* overexpression requires a subset of the G1 cyclins

Strain	Genotype	Suppression of growth defect by 2 μ <i>PKC1</i> (pDL289)
JVG994	a <i>swi4</i> ^{ts}	+++
JVG287	a <i>swi4</i> ^{ts} <i>cln1</i> Δ	+++
JVG286	a <i>swi4</i> ^{ts} <i>cln2</i> Δ	+++
JVG281	a <i>swi4</i> ^{ts} <i>cln3</i> Δ	++
JVG290	a <i>swi4</i> ^{ts} <i>cln1</i> Δ <i>cln2</i> Δ	–
JVG284	a <i>swi4</i> ^{ts} <i>mbp1</i> Δ	–

overexpression of *CLN1* or *CLN2* from high-copy plasmids or from heterologous promoters did not suppress the growth defects of mutants defective in the Pkc1 pathway, including *pkc1* Δ , *pkc1*^{ts}, *bck1* Δ and *mpk1* Δ in either the S288C or EG123 strain backgrounds (data not shown). These negative results are consistent with the view that the Pkc1 pathway does not act upstream of the G1 cyclins.

The Pkc1 pathway is activated at the G1–S transition

Fig. 4. Restoration of growth to *SWI4*-deficient strains at high temperature by *PKC1* overexpression requires Mbp1. The haploid *swi4^{ts} mbp1Δ* strain (JVG284) was transformed with the high-copy plasmids YEp352 (vector), pDL289 (*pPKC1*), pJO21 (*pCLN2*) and pB3.2A (*pSWI4*) as a positive control. The transformants were streaked on Ura drop-out plates and incubated for 3 days at the lowest restrictive temperature (30°C) for this strain.

and functions downstream of Cdc28 kinase activity (Marini *et al.*, 1996; Zarzov *et al.*, 1996). Our findings are consistent with these observations and indicate that the Pkc1 pathway promotes bud emergence in a Cdc28–Cln1- and Cdc28–Cln2-dependent process, at least in a *swi4* mutant background. To test if the Pkc1 pathway and the CLNs play redundant roles in *SWI4*⁺ cells, we examined the consequence of combining mutations in *MPK1*, *CLN1*, *CLN2* and *CLN3*. Sporulation of a *CLN3/cln3Δ* *MPK1/mpk1Δ* diploid yielded healthy single and double mutant spores on YEPD medium at 25°C. Sporulation of a *CLN1/cln1Δ* *CLN2/cln2Δ* *MPK1/mpk1Δ* diploid strain yielded viable single and double mutant spores on YEPD at 25°C (Figure 5B) except for *mpk1Δ cln1Δ cln2Δ* segregants, which grew very slowly at 25°C and not at all at higher temperatures (Figure 5A), even when supplemented with sorbitol (10%). We conclude that *mpk1Δ* mutations are synthetically lethal with *cln1Δ cln2Δ* mutations, consistent with the view that the Pkc1 pathway and Cln1 and Cln2 function in the same process. These data also indicate that Cln1 and Cln2 also have Mpk1-independent functions and that Mpk1 has Cln1- and Cln2-independent functions (Figure 10).

HCS77 is a novel dose-dependent suppressor of swi4 and encodes a putative cell surface type I transmembrane protein

HCS77 was found as a dose-dependent suppressor of the growth defect of *a/α swi4^{ts}/swi4Δ* strains at high temperature (Ogas *et al.*, 1991). Overexpression of *HCS77* suppresses the growth defect of both *swi4^{ts}* and *swi4Δ* alleles in both haploids and *a/α* diploids (Figure 6A; data not shown). In addition, the plasmid suppresses the growth defect of *a/α swi4Δ/swi4Δ* strains at ambient temperature. *HCS77* is thus a dose-dependent, bypass suppressor of the *swi4* defect. The suppression is not allele specific, cell type specific or temperature dependent.

The *HCS77* open reading frame (ORF) was identified by transposon mutagenesis of the original plasmid that suppressed the *swi4*[−] defect and by subcloning into a high-copy vector (see Materials and methods). The DNA sequence spanning *HCS77* was determined and found to be in agreement with that determined by the Yeast Genome Project: locus YOR008C on the left arm of chromosome

Fig. 5. *mpk1Δ* is synthetic with *cln1Δ,cln2Δ*. The diploid strains JVG310 *a/α MPK1/mpk1Δ CLN1/cln1Δ CLN2/cln2Δ* and JVG341 *a/α MPK1/mpk1Δ CLN3/cln3Δ* were sporulated and the spores germinated on YEPD plates supplemented with sorbitol (10%). The resulting single, double and triple mutant haploids were identified by virtue of genetic markers, and the cells were streaked on YEPD plates and incubated at (A) 30°C and (B) 25°C for 3 days.

XV; nucleotide sequence under GenBank accession Nos Z74916 and U39481 (Sterky *et al.*, 1996). The gene encodes a putative protein of 378 amino acid residues which lacks striking similarity to any other proteins or translated reading frames. Analysis of the predicted sequence of the Hcs77 protein revealed a hydropathy plot that strongly predicts a Type I transmembrane protein, with an N-terminal signal sequence and a single membrane-spanning domain (Figure 6B). The putative N-terminal

Fig. 6. *HCS77* is a dose-dependent suppressor of *swi4* mutants which encodes a putative transmembrane protein and is required for growth at high temperature at low but not high osmolarity. (A) *a/α swi4Δ* cells (JO184) were transformed with the high-copy plasmids YEp24 (vector) or pJO36 (pHCS77; *HCS77* in YEp24) and transformants selected on Ura drop-out medium at 25°C. Transformants were streaked on fresh plates and incubated at 25 or 37°C. (B) Kyte–Doolittle hydropathy plot of hydropathy versus residue number for the predicted Hcs77 protein. The shaded area at the N-terminus indicates a signal sequence and the shaded area near the C-terminus indicates a predicted single transmembrane-spanning region. (C) *a/α* WT (JO227) and *a/α hcs77Δ/hcs77Δ* (JO347) cells were grown at 25°C on YEPD plates and then streaked on YEPD plates or YEPD plates supplemented with sorbitol (10%) and incubated at 37°C for 3 days.

extracellular/luminal domain is rich in serine and threonine residues and shows the highest sequence similarity to the serine/threonine-rich, extracellular domains of heavily glycosylated proteins on the cell surface, including agglutinins and lectins. The small intracellular C-terminal domain shows limited sequence similarity to predicted intracellular domains of a number of ORFs encoding other putative transmembrane proteins.

Mutants defective in *HCS77* share phenotypes with mutants defective in the *Pkc1* pathway

To determine the role of Hcs77 in cell growth, we constructed an *hcs77* null allele by replacing the majority

of the chromosomal copy of the reading frame with *LEU2* (see Materials and methods). We found that haploid *hcs77Δ* mutants displayed phenotypes like those of mutants defective in the MAP kinase cascade of the *Pkc1* pathway. The *hcs77* mutants grew poorly at 37°C, most noticeably on rich, glucose-containing medium (data not shown). *a/α hcs77Δ/hcs77Δ* diploids displayed a more severe growth defect at high temperature than did the corresponding haploid mutants (as observed for *bck1Δ* and *mpk1Δ* mutants; Lee *et al.*, 1993b; Mozzoni *et al.*, 1993; J.V.Gray, unpublished data), with a restrictive temperature in the S288C background of ~30°C on YEPD (Figure 6C). Haploid *hcs77Δ* mutants also displayed a moderate mating defect, apparently due to death upon exposure to high concentrations of mating pheromones (J.V.Gray, unpublished data). Mutants lacking *HCS77*, like *bck1Δ* mutants (Costigan *et al.*, 1992), showed poor survival in stationary phase (J.V.Gray, unpublished data). Transfer of growing liquid cultures of *a/α hcs77Δ/hcs77Δ* mutants from permissive (25°C) to restrictive temperature (37°C) resulted in a rapid cessation of growth (Figure 7A), with accumulation of small-budded cells (Figure 7C) each with a single nucleus (Figure 7C) and with a DNA content intermediate between 1N and 2N, indicating incomplete replication (Figure 7D). These phenotypes are similar to those of mutants defective in the *Pkc1* pathway (Lee *et al.*, 1993a,b). These small-budded cells also stained intensely with the vital dye methylene blue, indicating that they were dead rather than arrested (Figure 7B). Loss of viability correlated with the appearance of small-budded cells (Figure 7A and B; data not shown). We found that the growth defect of *hcs77Δ* mutants was suppressed efficiently by osmotic stabilization, in particular, by 10% sorbitol (Figure 6C) or by 1 M sorbitol, 0.5 M sodium chloride or 0.5 M potassium chloride (data not shown). All these phenotypes are like those of mutants defective in genes of the *Pkc1* pathway (Lee *et al.*, 1993b; Costigan *et al.*, 1994). We therefore set out to explore further the possibility that Hcs77 functions in the *Pkc1* pathway.

Genetic analysis of *hcs77* mutations is consistent with the view that Hcs77 functions in the *Pkc1* pathway. First, we observed that *hcs77Δ swi4Δ* strains are inviable. In 20 tetrads dissected from an *HCS77/hcs77Δ SWI4/swi4Δ* diploid, no double mutant spores (*His⁺Leu⁺*) grew to form a colony on YEPD medium at room temperature (data not shown). We also constructed *hcs77Δ bck1Δ* and *hcs77Δ mpk1Δ* double mutants. These strains grew normally on YEPD medium at ambient temperature and displayed a temperature-sensitive growth defect that was suppressed efficiently by osmotic stabilization (data not shown). Similarly, we found that although haploid *pkc1Δ* and *hcs77Δ pkc1Δ* double mutants are inviable at all temperatures on YEPD medium, both were suppressed efficiently by osmotic stabilization at ambient temperature (data not shown), the condition where *Pkc1* pathway activity is neither elevated nor required (Kamada *et al.*, 1995). Deletion of *HCS77* does not exacerbate the growth defects of strains lacking known components of the *Pkc1* pathway. These observations support the view that Hcs77 functions in the *Pkc1* pathway.

If *Pkc1* activity is dependent on Hcs77, then we would expect *hcs77* mutants to be very sensitive to reduction in *Pkc1* activity. To test this, we determined whether the

Fig. 7. Phenotype of *HCS77*-deficient cells. Cultures of wild-type (WT) *a/α HCS77/HCS77* (JO227) and *HCS77*-deficient (*hcs77*) *a/α hcs77Δ/hcs77Δ* (JO347) strains were grown in YEPD liquid medium at 25°C. Exponentially growing cultures were then shifted to 37°C and incubated for up to 8 h. (A) Absorbance at 600 nm (A_{600}) of each culture as a function of time at 37°C for WT and mutant strains. (B) Percentage of cells that stain with the vital dye methylene blue (at least 200 cells counted for each time point) as a function of time at 37°C for WT and mutant strains. (C) Morphology and DAPI staining of fixed *HCS77*-deficient cells after 0 and 8 h at 37°C. (D) Flow-cytometric (FACS) analysis of the DNA content of the *HCS77*-deficient culture as a function of time at 37°C. The number of cells (# Cells) is plotted as a function of the intensity of staining with propidium iodide (PI) in arbitrary units. The intensities of PI staining by cells with 1N and 2N DNA content are indicated by vertical lines marked '1N' and '2N' respectively.

growth of *hcs77* mutants is particularly sensitive to expression of catalytically inactive Pkc1 protein. Expression of Pkc1-K853R from the *GAL1,10* promoter did not inhibit growth of wild-type cells (Figure 8A). In contrast, expression of this protein on standard galactose-containing medium strongly inhibited growth of haploid *hcs77Δ* cells at all temperatures (Figure 8A; data not shown). We conclude that on media lacking osmotic stabilization, partial inhibition of Pkc1 activity is detrimental to *hcs77* mutant cells but not to wild-type cells. These findings are consistent with Hcs77 being an upstream regulator of Pkc1 activity.

We next tested if overexpression of *HCS77* could suppress the growth defects of mutations affecting the known components of the Pkc1 pathway. Although high-copy *HCS77* was an excellent suppressor of the *swi4* defect, it did not suppress the growth defects of haploid *pkc1^{ts}*, *pkc1Δ*, *bck1Δ* or *mpk1Δ* mutants (EG123 background) (data not shown). Hcs77 is thus unlikely to act downstream of the Pkc1 MAP kinase cascade. We next tested if overexpression of *PKC1* could suppress the *hcs77Δ* growth defect. Although we found that high-copy *PKC1* was only a weak suppressor of the temperature-sensitive growth defect of *a/α hcs77Δ/hcs77Δ* (data not shown), it did significantly alleviate the lysis defect of the mutant under both permissive and non-permissive conditions (Figure 8B). These data suggest that Pkc1 acts downstream of Hcs77.

We conclude that Hcs77 displays the hallmarks of a component of the Pkc1 pathway that may act upstream of Pkc1. We resolved to address the question directly by measuring the activity of the MAP kinase, Mpk1, in congeneric wild-type and *hcs77Δ* mutant strains.

Basal and heat shock induction of Mpk1 activity is severely compromised in *hcs77* mutants

We examined the heat shock induction of Mpk1 kinase activity in both wild-type and *hcs77* mutants in the S288C background. Wild-type and mutant diploids were transformed with a plasmid encoding a hemagglutinin (HA) epitope-tagged Mpk1 (Kamada *et al.*, 1995). Transformants growing exponentially in liquid synthetic medium at 23°C were shifted to 39°C for 30 min. The Mpk1HA kinase was immunoprecipitated and its activity assayed both before and after heat shock using myelin basic protein as an *in vitro* substrate (Kamada *et al.*, 1995). We found that the wild-type and *hcs77* mutant strains exhibited similar, low-level Mpk1 kinase activity when growing at 23°C, although activity in *hcs77* mutants was consistently lower than in the wild-type strain (Figure 9). However, the heat induction of Mpk1 kinase activity was severely compromised in the *hcs77* mutant relative to wild-type: the mutant exhibited <15% of the wild-type induction (Figure 9). This result strongly suggests that Hcs77 is a component of the Pkc1 pathway, and that it acts upstream of Mpk1.

Fig. 8. *HCS77*-deficient mutants are supersensitive to loss of *Pkc1* function and are partially suppressed by overproduction of *Pkc1*. (A) Haploid wild-type (JO369) and *hcs77Δ* strains (JO394-2C) were transformed with pBM743 (vector), pDL468 (pGALPKC1) and pDL469 [pGALPKC1(K853R)], and transformants selected on Ura drop-out plates containing glucose at 25°C. The transformants were streaked on Ura drop-out plates containing galactose and incubated at 30°C for 3 days. The same strain transformed with pB3.2A (p*SWI4*) served as a positive control. (B) a/α *hcs77Δ/hcs77Δ* (JO347) was transformed with YEpl352 (vector) and the high-copy plasmid pDL289 (pPKC1; PKC1 in YEpl352). pJO36 (pHCS77; HCS77 in YEpl24) was also introduced into JO347 by transformation to serve as a positive control. Transformants were grown in Ura drop-out liquid medium at 25°C and then shifted to 37°C at 0 h. The percentage of cells staining positive for the vital dye methylene blue is shown as a function of time at 37°C, with a minimum of 200 cells counted for each sample.

Discussion

The *SWI4* gene appears to be a key regulator of the G1–S transition in budding yeast. It controls the transcription of many genes in that interval of the cell cycle, including *CLN1* and *CLN2*, which encode cyclins for Cdc28, and *PCL1* (previously known as *HCS26*), which encodes a cyclin for the Cdc28-related kinase, Pho85. It had been thought previously that *swi4* mutants in certain strain backgrounds arrest in the G1 phase of the cell cycle under restrictive conditions (Ogas *et al.*, 1991). Here we provide evidence that these mutants are defective in bud emergence but are able to carry out DNA synthesis. Spindle pole body duplication, the third major event at the G1–S transition, has not been examined. Insights into the regulatory steps controlled by *Swi4* have been gleaned by isolating high-copy plasmids that overproduce various products which allow cells to grow in its absence (Ogas *et al.*, 1991; this work). Because of the nature of the *SWI4* deficiency, these products identify targets or affect processes that act after *Swi4* to stimulate bud emergence. Indeed, included in this group of high-copy suppressors are *CLN1* and *CLN2* whose protein products are thought to play particularly important roles in early bud morphogenesis (Lew and Reed, 1993).

Here we describe two other high copy suppressors of the *swi4* growth defect, *HCS77* and *PKC1*, and have shown that they affect a common process, the *Pkc1* MAP kinase pathway. This pathway is activated at the G1–S transition at the time of bud emergence (Zarzov *et al.*, 1996), and its activation in part requires the function of Cdc28 (Marini *et al.*, 1996; Zarzov *et al.*, 1996). The *Pkc1* pathway is also activated by heat shock and other membrane stresses (Kamada *et al.*, 1995; Davenport *et al.*,

1996). The protein encoded by *HCS77* appears to be a component of the *Pkc1* pathway by two criteria. First, *hcs77Δ* mutants behave like mutants defective in components of the *Pkc1* MAP kinase cascade. Second, *hcs77* mutants are defective in heat shock activation of Mpk1, the MAP kinase of the *Pkc1* pathway. We propose that *Hcs77* is an upstream component of the *Pkc1* pathway which may monitor membrane stress. The ability to relieve the bud emergence defect of *swi4* mutants by overexpression of *Pkc1* or *Hcs77* can be explained by the proposal that Cdc28–Cln1 or Cdc28–Cln2 are limiting for activation of the *Pkc1* pathway in these mutants. Apparently, overproduction of Cln1 or Cln2 restores adequate Cdc28–Cln1 or Cdc28–Cln2 function for this activation and consequent bud emergence, and overproduction of *Hcs77* or *Pkc1* provides adequate activation in the presence of low CLN levels.

swi4 mutants are defective in bud emergence

We find that *swi4Δ* mutants are specifically defective in bud emergence at both permissive and restrictive temperature. Budding and DNA synthesis, which are normally coordinately initiated at the G1–S transition, are uncoupled in *swi4Δ* mutants (Figure 1). Although we do not yet fully understand the nature of this uncoupling, we hypothesize that the reduced expression of *CLN1* and *CLN2* in these mutants provides sufficient levels of cyclin proteins to stimulate degradation of Sic1, which then allows DNA synthesis to proceed (Schneider *et al.*, 1996; Tyers, 1996) but provides insufficient levels of cyclins to drive efficient bud emergence.

In both haploid and a/α strains lacking *SWI4*, overexpression of *CLN1* or *CLN2*, whose gene products are

Fig. 9. *HCS77*-deficient cells are defective in heat induction of Mpk1 kinase activity. **a/α** *HCS77/HCS77* (JO227) and **a/α** *hcs77Δ/hcs77Δ* (JO347) strains were transformed with pDL922 expressing Mpk1HA. Transformants were grown at 25°C in Ura drop-out medium and shifted to 39°C for 30 min. Extracts were prepared from each sample and Mpk1HA immunoprecipitated using the 12CA5 antibody (Ab). In one sample a mock precipitation without antibody was performed to determine non-specific kinase activity in the strain background. The *in vitro* kinase activity of the precipitates was determined using myelin basic protein (MBP) as substrate and assaying incorporation of ³²P from radioactive ATP by autoradiography of SDS-PAGE gels. (A) Autoradiograph showing incorporation of radioactivity into MBP for each sample, and (B) immunoblot detection of Mpk1HA on aliquots of these samples.

implicated in promoting bud emergence at the G1-S transition and actin polarization during early bud growth (Lew and Reed, 1993), is sufficient to rescue the growth and budding defects of *swi4Δ* mutants (Ogas *et al.*, 1991; Figure 2B; Table I). In contrast, we find that *CLN3-2* (*DAF1-1*), coding for a hyper-stable version of the Cln3 protein, is a very poor suppressor of the *swi4* growth defect (Figure 2A) even though it is a potent activator of the G1-S transition in *SWI4*⁺ cells (Cross, 1988). These observations indicate that *swi4* mutants are limited by the activity or function of the specific subset of CLNs, Cln1 and Cln2, implicated in promoting bud emergence and morphogenesis.

a/α cells lacking *SWI4* exhibit a more severe growth defect than **a** or **α** haploid *swi4* mutants, being unable to grow at all temperatures (Ogas *et al.*, 1991). The more severe growth defect in **a/α** cells may result in part from reduced transcription of *CLN2* due to repression of the *RME1* gene product, a *SWI4*-independent activator of *CLN2* transcription which is not expressed in **a/α** cells (Toone *et al.*, 1995). The temperature-sensitive growth defect of haploid *swi4Δ* mutants may result from inadequate activity of the Pkc1 pathway (see below), which is required for viability at high temperatures (Kamada *et al.*, 1995).

The budding defect of *swi4* mutants is unique and quite distinct from that of other mutants known to be defective

Fig. 10. Proposed model for the regulation of the Pkc1 pathway. Cdc28-Cln1/2 and Pkc1 play a partially redundant role in promoting bud emergence and bud morphogenesis at the G1-S transition. The activity of Pkc1 is regulated directly or indirectly by Cdc28-Cln1/2 during early bud growth and by Hcs77 in the plasma membrane upon heat shock. Pkc1 regulates bud emergence and surface growth via Mpk1 alone or via Mpk1 and the other unknown target of Pkc1 (?). Aberrant surface growth (e.g. induced by heat shock) activates Hcs77 and thereby stimulates the Pkc1 pathway to try to correct or compensate for the defect.

in bud emergence. In the cases of *cdc24-ts* and *cdc42-ts* mutants, which appear unable to localize surface growth (Drubin and Nelson, 1996), the loss of gene activity results in a severe budding defect and an associated proliferation defect that is not suppressed by overexpression of *CLN1* or *CLN2* (J.V.Gray, unpublished data). *swi4Δ* strains also display secondary phenotypes associated with a budding and morphology defect even under permissive conditions, i.e. large cell size and a mitotic delay. As expected for large cells, some cell lysis is clearly evident in cultures, especially at high temperature (J.V.Gray, unpublished data). Cell lysis appears, however, to be a secondary consequence of the primary budding and morphology defect since arrested cells continue to increase in size for many hours after ceasing proliferation, as seen for other cell cycle arrest mutants (Molero *et al.*, 1993).

The budding and proliferation defects of *swi4* mutants are not due simply to reduced expression of the *CLN1* and *CLN2* genes, since complete deletion of both cyclin genes in otherwise wild-type cells merely increases the average cell size at START, with no dramatic budding or proliferation defects (Reed, 1992; J.V.Gray, unpublished data). *swi4* mutants are defective in the expression of many genes at the G1-S transition, and their growth defects must result from the cumulative sum of these expression defects. However, *swi4* mutants are clearly limited by Cln1 and Cln2 activity (Ogas *et al.*, 1991) and by Pkc1 pathway activity (this work) in promoting bud emergence.

The Pkc1 MAP kinase pathway promotes bud emergence in a Cln1- and Cln2-dependent process

Mutants lacking Swi4 activity behave like mutants partially defective in the Pkc1 pathway. Their growth defect is

rescued by activation of the Pkc1 pathway (Figure 2B) and is exacerbated by partial inhibition of the pathway, e.g. by mutation and by expression of a catalytically inactive Pkc1 protein (Figure 3A–C). We have found that *swi4* mutants are supersensitive to staurosporine, an inhibitor of PKC isozymes in both mammalian and yeast cells (J.V.Gray, unpublished data). We have also observed that the high temperature growth defect of haploid *swi4Δ* mutants, but not the growth defect of *a/α swi4Δ/swi4Δ* mutants, is partially rescued by a high osmolarity growth medium (J.V.Gray, unpublished data). The mechanism of this partial suppression has not been examined in detail but may result from osmotic stabilization of large arresting cells or from activation of the HOG pathway (Brewster *et al.*, 1993). Interestingly, high osmotic strength also suppresses the growth defects of mutants deficient in the Pkc1 pathway, at least in part because this pathway is not active and hence not required under high osmolarity growth conditions (Kamada *et al.*, 1995). All these observations are consistent with *swi4* mutants being in part defective in Pkc1 pathway activity.

We have presented two lines of evidence indicating that the Pkc1 pathway can activate bud emergence in yeast, as revealed most clearly in the context of *swi4* mutants. First, activation of the Pkc1 pathway (e.g. by overexpression of *PKC1*) efficiently suppresses both the budding and growth defects of *swi4Δ* mutants (Figure 2B; Table I). Second, inhibition of signaling through the Pkc1 MAP kinase pathway by various means (mutation in *MPK1* or a dominant-negative *pkc1* mutation) greatly exacerbates the budding defect of *swi4* mutants (Figure 3A–C). These observations support the view that the Pkc1 MAP kinase pathway plays a role at the G1–S transition (Mozzoni *et al.*, 1993; Marini *et al.*, 1996; Zarzov *et al.*, 1996) and that role is as an activator of bud emergence.

Earlier studies showed that activity of the Pkc1 pathway is stimulated specifically at the G1–S transition concomitant with bud emergence (Zarzov *et al.*, 1996) and that its activity is partly compromised in *cdc28* mutants (Marini *et al.*, 1996; Zarzov *et al.*, 1996). These observations suggest that Cdc28–cyclin activity can directly or indirectly activate the Pkc1 pathway at the G1–S transition. Our findings provide genetic support for this view and further indicate that activity of the Pkc1 pathway is dependent specifically on Cdc28–Cln1 and Cdc28–Cln2. In particular, we have shown that overproduction of either Pkc1 or the G1 cyclins (Cln1 or Cln2, but not Cln3) suppresses the bud emergence and growth defects of *swi4Δ* mutants (Figure 2A and B). Hence, Cln1, Cln2 and Pkc1 must affect a common limiting function in *swi4* mutants and thus may act in a common pathway. We have also found that suppression of the growth defect of *swi4* mutants by overexpressing *PKC1* absolutely requires either *CLN1* and *CLN2* (Table III) or *MBP1*, the transcription factor responsible for residual expression of *CLN1* and *CLN2* in the absence of Swi4 (Figure 4). These observations indicate that the role of the Pkc1 pathway in promoting bud emergence, revealed in the context of *swi4* mutants, is absolutely dependent on residual Cln1 and Cln2. We have found no convincing evidence to suggest that the Pkc1 pathway affects *CLN1* or *CLN2* expression in either the presence or absence of Swi4 (J.V.Gray, unpublished work), in agreement with Igual *et al.* (1996)

and Madden *et al.* (1997). Our observations, therefore, support our proposal that the Pkc1 pathway functions downstream of Cln1 and Cln2 in promoting bud emergence and morphogenesis (Figure 10).

We have further presented evidence that Cln1 and Cln2 share a common function with the Pkc1 pathway in cells carrying a functional *SWI4* gene. In particular, we have observed that mutants lacking the *CLN1* and *CLN2* genes in combination with the *MPK1* gene display a growth defect much more severe than that of any single or double mutant combination (Figure 5). These observations support the idea that these Clns and the Pkc1 pathway play a partially redundant role in cellular morphogenesis at the G1–S transition and in *SWI4*⁺ cells (Figure 10).

It has been reported that *mpk1Δ* mutants display reduced expression of a number of genes encoding cell wall components (Igual *et al.*, 1996), although the effects are modest and may be secondary consequences of cell lysis. Dodou and Treisman (1997) recently have suggested that Mpk1 may in part regulate the transcription factor Rlm1, whose sites may be found upstream of many of these potential target genes. However, we have found that overexpression of *PKC1* can suppress *swi4* mutants when *RLM1* is deleted (J.V.Gray, unpublished data). Thus the mechanism by which the Pkc1 pathway promotes bud emergence and morphogenesis does not involve Rlm1-dependent effects on gene expression.

Madden *et al.* (1997) have suggested that Mpk1 may activate Swi4 to stimulate *PCL1* and *PCL2* expression, although the effects are again modest and may not be direct. We have shown that the Pkc1 pathway stimulates bud emergence when *SWI4* is deleted (Figure 2; Table I) and independently of *PCL1* and *PCL2* (J.V.Gray, unpublished data). Therefore, the Pkc1 pathway promotes bud emergence and morphogenesis by a mechanism different from that proposed by Madden *et al.* (1997) (Figure 10).

The Pkc1 pathway appears to be involved in two processes during vegetative growth; modulating cell surface growth in response to surface stresses and modulating bud emergence and polarized cell surface growth in response to Cln1 and Cln2 activity (Figure 10). Given our ignorance of the upstream regulators and downstream targets of the Pkc1 pathway, we cannot yet comment on the detailed mechanisms involved or say whether these functions derive from the same or distinct sets of regulators and effectors.

HCS77 encodes a putative cell surface receptor for the Pkc1 pathway

Several observations indicate that Hcs77 functions in the Pkc1 pathway and may be an upstream activator of the pathway. First, we found that overexpression of *HCS77* mimics overexpression of *PKC1* by suppressing the growth defect of *swi4* mutants. Second, we observed that *hcs77* null mutants display phenotypes like those of mutants defective in the Pkc1 pathway, notably an osmotic-remedial cell lysis defect with a terminal phenotype of small-budded cells. In addition, *hcs77* mutants are inviable when exposed to α -factor (J.V.Gray, unpublished data), which has been noted for *mpk1* mutants (Errede *et al.*, 1995; J.V.Gray, unpublished data), and are synthetically lethal in combination with *swi4*, as are *mpk1* mutants. Third, *hcs77* mutants are hypersensitive to partial inhibi-

tion of Pkc1 function, suggesting that Pkc1 may not be fully active in the mutant (Figure 8A).

We have made two observations that support the view that Hcs77 functions upstream of Pkc1. First, we have found that the lysis defect of *hcs77* mutants at high temperature is partially suppressed by overexpression of *PKC1* (Figure 8B). The partial suppression may reflect that in the absence of Hcs77, overproduction of Pkc1 may be less activating than in its presence or that Hcs77 activates other processes in addition to the Pkc1 pathway. Second, we have found that heat-induced activation of the Pkc1 pathway, as monitored by assaying the activity of Mpk1 *in vitro*, is severely compromised in *hcs77* mutants (Figure 9). These data strongly support the model that Hcs77 is an upstream activator of the Pkc1 pathway and that it is necessary for response to a heat-induced signal (Figure 10).

It has been suggested that the heat activation of the Pkc1 pathway is mediated by the cell sensing the state of the cell surface (Davenport *et al.*, 1995; Kamada *et al.*, 1995). Hcs77 is an excellent candidate for such a cell surface receptor. The *HCS77* ORF contains all the hallmark sequences of a heavily glycosylated, integral membrane, cell surface protein. Hcs77 appears to sediment with cellular membrane fractions, and its retarded migration on SDS-PAGE gels suggests that it is indeed a heavily glycosylated protein when isolated from wild-type cells (J.P.Ogas, unpublished data). Indeed, Hcs77-green fluorescent protein (GFP) fusion proteins have been found to localize to the cell periphery (R.Ballester, personal communication). We propose that a weakened cell surface activates Hcs77 directly or indirectly to stimulate Pkc1 and the MAP kinase cascade, leading to remodeling and reinforcement of the cell surface (Figure 10). The sequence of Hcs77 does not provide any clues as to how it might signal to Pkc1. The putative cytoplasmic domain is small (90 amino acid residues) and is thus unlikely to contain an intrinsic enzymatic activity. It might, therefore, associate with another protein such as a phospholipase, an ion channel, the Rho1 GTPase or its regulators (Rom1 and Rom2) (Ozaki *et al.*, 1996).

Protein kinase C in cell proliferation

Much of signal transduction and the machinery that regulates cell proliferation is conserved between yeast and larger eukaryotes. Although there has long been evidence linking activation of various PKCs and proliferation in a variety of mammalian cell lines, the plethora of isozymes has greatly complicated definitive analysis (Newton, 1995). Recently, the RHO-like GTPases have been strongly implicated in regulating both cell morphogenesis and proliferation in many mammalian cell types (Ridley, 1996). One subclass of these GTPases directly activates a subset of PKC-like kinases, reflecting the regulation of yeast Pkc1 by Rho1 (Ridley, 1996). The parallels between mammalian cells and yeast are striking, and offer hope that studies of PKC isozymes in a variety of systems will shed common light on how cell morphogenesis and proliferation are coupled.

Materials and methods

Materials and chemicals

All chemicals were from Sigma Chemical Company (St Louis, MO), unless otherwise stated. Components of growth media were from Difco Laboratories (Detroit, MI). Restriction enzymes and buffers were from New England Biolabs (Beverly, MA) or from Boehringer Mannheim (Indianapolis, IN). 12CA5 monoclonal antibody against the HA epitope was from BabCo. (Berkeley, CA). [γ - 32 P]ATP (16 μ Ci/nmol) was from ICN.

Plasmids, yeast strains and manipulations

Liquid media, both rich (YEPD) and minimal (SD), were prepared and supplemented as described in Ogas *et al.* (1991). YEPgal and YEPraffinose media were prepared as for YEPD, except that glucose was replaced by D-galactose (2%) and sucrose (0.2%) or D-raffinose (2%) and sucrose (0.5%) respectively. Solid media contained an additional 2% agar.

Experiments were performed in the yeast S288C (Siliciano and Tatchell, 1984) strain background (Table IV). Most plasmids containing genes of the Pkc1 MAP kinase pathway were: pDL289 (2 μ PKC1), pDL582 (2 μ MPK1), pDL922 (2 μ MPK1^{HA}), pDL636 (CENBCK1-20), pBM743 (GALPKC1HA₃) and pBM743 [GALPKC1(K853R)HA₃] (Lee and Levin, 1992; Lee *et al.*, 1993a,b; Watanabe *et al.*, 1994; Kamada *et al.*, 1995). pJO1(2 μ CLN1) and pJO21(2 μ CLN2) have been described before (Ogas *et al.*, 1991). pB3.2A (*SWI4* in YCp50; Andrews and Herskowitz, 1989) and pFC101 (*CLN3-2* in YCp50; Cross, 1988) were kind gifts of B.Andrews and F.Cross respectively. All constructs were checked by complementation of the appropriate mutant phenotypes and

Table IV. Strain list

Name	Genotype	Source
S288C background:	<i>ura3-52 trp1Δ-63 lys2-801 ade2-101 his3Δ200 leu2-Δ1</i>	
JO31-1A	a	Ogas (1992)
JO51-2C	α <i>TRP1 ho::lacZ swi4Δ::HIS3</i>	this study
JO227	a / α <i>TRP1/TRP1</i>	Ogas <i>et al.</i> (1991)
JO184	a / α <i>TRP1/TRP1 swi4^{ts}/swi4Δ::HIS3 BARI/bar1Δ::LEU2</i>	Ogas <i>et al.</i> (1991)
JO347	a / α <i>TRP1/TRP1 hcs77Δ::LEU2/hcs77Δ::LEU2</i>	this study
JO369	a <i>GAL2</i>	Ogas (1992)
JO394-2C	a <i>TRP1 hcs77Δ::LEU2 GAL2</i>	this study
JO395-1A	a <i>swi4Δ::HIS3 GAL2</i>	this study
JVG310	a / α <i>MPK1/mpk1Δ::TRP1 CLN1/cln1Δ::TRP1 CLN2/cln2Δ::LEU2</i>	this study
JVG341	a / α <i>MPK1/mpk1Δ::TRP1 CLN3/cln3Δ::ura3Δ::TRP1</i>	this study
JVG994	a <i>swi4^{ts} TRP1</i>	this study
JVG287	a <i>swi4^{ts} cln1Δ::TRP1</i>	this study
JVG286	a <i>swi4^{ts} cln2Δ::LEU2</i>	this study
JVG281	a <i>swi4^{ts} cln3Δ::TRP1</i>	this study
JVG290	a <i>swi4^{ts} cln1Δ::TRP1 cln2Δ::LEU2</i>	this study
JVG284	a <i>swi4^{ts} mbp1Δ::TRP1</i>	this study

by restriction mapping. The chromosomal *MPK1* gene in the S288C background was replaced with *mpk1Δ::TRP1* by transformation of a haploid strain with linearized pDL759, as previously described (Lee *et al.*, 1993b). The presence of the *mpk1Δ* was checked by testing for temperature-sensitive growth that could be complemented by transformation with a CEN*MPK1* plasmid (J.V.Gray, unpublished work). The plasmid, pCK41, containing *mbp1Δ::URA3*, was a kind gift of K.Nasmyth (Koch *et al.*, 1993). Deletions of *MBP1* were created by gene replacement in haploid S288C using linearized pCK41. Gene replacement was checked by PCR and by synthetic lethality in combination with the *swi4Δ* mutation. The chromosomal *mbp1Δ::URA3* was converted to an *mbp1Δ::TRP1* allele by gene replacement (*TRP1* for *URA3*) using pJO122 as described elsewhere (Ogas, 1992).

pJO36 is the original YEp24-based plasmid carrying *HCS77* that was isolated as a dose-dependent suppressor of the JO184 growth defect at 37°C. Insertion mutagenesis of pJO36 with mini-Tn10-LUK was performed as described by Huisman *et al.* (1987) with the exception that the plasmid DNA was pooled from the original λ -infected colonies and transformed into MH6 [$\Delta(lac)X74$ *galK strA hsr⁻ hsm⁺ leuB600 pyrF::Tn5*]. Plasmids that contained transposons in *HCS77* were identified by their inability to correct the growth defect of JO184 at 37°C. The *hcs77Δ::LEU2* allele was created by replacing the internal fragment of *HCS77* in pJO36 coding for amino acids 9–369 with the nutritional marker *LEU2*, resulting in plasmid pJO143 (Ogas, 1992). Cells were transformed with pJO143 linearized with *Pst*I and *Bam*HI to effect a one-step gene replacement of the chromosomal *HCS77* locus.

Genetic crosses and standard manipulations of yeast were performed essentially as described by Sherman *et al.* (1982). In the case of tetrad dissection on osmotically stabilizing media, micromanipulations were performed on YEPD agar slabs (4%) supplemented with sorbitol (10% w/v). Dissected slabs were removed from the coverslip and placed on YEPD plates to allow germination and colony growth. Microscopic examination of microcolonies resulting from germinated spores was performed by transferring the slabs from YEPD plates back onto coverslips.

Microscopy

Cells were examined microscopically (both by Nomarski optics and fluorescence) using a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY). Methylene blue staining of cells was performed as described previously (Rose, 1975). Cells were stained for DNA using the dye DAPI after ethanol (70%) fixation (Ogas *et al.*, 1991). Budding indices were determined on azide-treated yeast samples that were fixed in formaldehyde (3.7%) or ethanol (70%), washed and resuspended in TE buffer and sonicated immediately prior to examination by microscopy. A total of at least 200 cells were counted for each sample and classified as either unbudded or budded.

β -galactosidase assays

Solution assays of the activity of the lacZ protein, encoding bacterial β -galactosidase, were performed essentially as described by Sterns *et al.* (1984). Reactions were performed at 30°C using *o*-nitrophenyl- β -D-galactopyranoside as substrate, and were quenched with sodium bicarbonate. All reactions were performed either in duplicate or in triplicate. The experiments were performed independently at least twice, using cultures grown from different colonies/transformations.

Flow cytometry

Flow cytometry analysis was performed on a Becton Dickinson FACScan at the Howard Hughes Medical Institute at U.C.S.F. Cells were prepared essentially as described in Vai *et al.* (1987), by fixing mid-log cultures in ethanol (70%). The cultures were examined microscopically to check the morphology of the cells and the quality of the sample. The cells were then digested with RNase overnight and stained with propidium iodide for 1 h. The cells were diluted into phosphate-buffered saline (PBS) buffer and sonicated immediately prior to FACS analysis. Each FACS scan was performed on 10 000 cells, with the intensity of the propidium iodide staining and the forward light scattering (FSC) collected for each cell. To estimate relative cell size, the distribution of the FSC was determined for the G1 population of cells (as determined by propidium iodide intensity).

Mpk1 immune precipitation, activity assays and immunoblots

These procedures were carried out exactly as described in Kamada *et al.* (1995).

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References

- Andrews,B.J. and Herskowitz,I. (1989) Identification of a DNA binding factor involved in cell-cycle control of the yeast *HO* gene. *Cell*, **57**, 21–29.
- Andrews,B.J. and Moore,L. (1992) Mutational analysis of a DNA sequence involved in linking gene expression to the cell cycle. *Biochem. Cell Biol.*, **70**, 1073–1080.
- Antonsson,B., Montessuit,S., Friedli,L., Payton,M.A. and Paravicini,G. (1994) Protein kinase C in yeast. Characterization of the *Saccharomyces cerevisiae* *PKC1* gene product. *J. Biol. Chem.*, **269**, 16821–16828.
- Benton,B.K., Tinkelenberg,A.H., Jean,D., Driscoll Plump,S. and Cross,F.R. (1994) Genetic analysis of Cln/Cdc28 regulation of cell morphogenesis in budding yeast. *EMBO J.*, **12**, 5267–5275.
- Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E. and Gustin,M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science*, **259**, 1760–1763.
- Costigan,C., Gehrung,S. and Snyder,M. (1992) A synthetic lethal screen identifies Slk1, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. *Mol. Cell. Biol.*, **12**, 1162–1178.
- Cross,F.R. (1988) *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, **8**, 4675–4684.
- Davenport,K.R., Sohaskey,M., Kamada,Y., Levin,D.E. and Gustin,M.C. (1995) A second osmosensing signal transduction pathway in yeast. *J. Biol. Chem.*, **270**, 30157–30161.
- Di Como,C.J., Chang,H. and Arndt,K.T. (1995) Activation of *CLN1* and *CLN2* G1 cyclin gene expression by *BCK2*. *Mol. Cell. Biol.*, **15**, 1835–1846.
- Dirick,L., Böhm,T. and Nasmyth,K. (1995) Roles and regulation of Cln–Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.*, **14**, 4803–4813.
- Dodou,E. and Treisman,R. (1997) The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. *Mol. Cell. Biol.*, **17**, 1848–1859.
- Drubin,D.G. and Nelson,W.J. (1996) Origins of cell polarity. *Cell*, **84**, 335–344.
- Errede,B., Cailic,R.M., Yashar,B.M., Kamada,Y., Levin,D.E., Irie,K. and Matsumoto,K. (1995) Dynamics and organization of MAP kinase signal pathways. *Mol. Reprod. Dev.*, **42**, 477–485.
- Espinosa,F.H., Ogas,J., Herskowitz,I. and Morgan,D.O. (1994) Cell cycle control by a complex of the cyclin Hcs26 (Pcl1) and the kinase Pho85. *Science*, **266**, 1388–1391.
- Huisman,O., Raymond,W., Froehlich,K., Errada,P., Kleckner,N., Botstein,D. and Hoyt,M.A. (1987) A *Tn10-lacZ-kanR-URA3* gene fusion transposon for insertion mutagenesis and fusion analysis of yeast and bacterial genes. *Genetics*, **116**, 191–199.
- Igual,J.C., Johnson,A.L. and Johnston,L.H. (1996) Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J.*, **15**, 5001–5013.
- Kaibuchi,K., Takai,Y. and Nishizuka,Y. (1985) Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. *J. Biol. Chem.*, **260**, 1366–1369.
- Kamada,Y., Jung,U.S., Piotrowski,J. and Levin,D.E. (1995) The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev.*, **9**, 1559–1571.

- Kamada, Y., Qadota, H., Python, C.P., Anraku, Y., Ohya, Y. and Levin, D.E. (1996) Activation of yeast protein kinase C by Rho1 GTPase. *J. Biol. Chem.*, **271**, 9193–9196.
- Koch, C. and Nasmyth, K.A. (1994) Cell cycle regulated transcription in yeast. *Curr. Opin. Cell Biol.*, **6**, 451–459.
- Koch, C., Moll, T., Neuberg, M., Ahorn, H. and Nasmyth, K.A. (1993) A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science*, **261**, 1551–1557.
- Lee, K.S. and Levin, D.E. (1992) Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. *Mol. Cell. Biol.*, **12**, 172–182.
- Lee, K.S., Hines, L.K. and Levin, D.E. (1993a) A pair of functionally redundant yeast genes (*PPZ1* and *PPZ2*) encoding type I-related protein phosphatases function within the Pkc1-mediated pathway. *Mol. Cell. Biol.*, **13**, 5843–5853.
- Lee, K.S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K. and Levin, D.E. (1993b) A yeast mitogen-activated protein kinase homolog (Mpk1) mediates signalling by protein kinase C. *Mol. Cell. Biol.*, **13**, 3067–3075.
- Levin, D.E., Fields, F.O., Kunisawa, R., Bishop, J.M. and Thorner, J. (1990) A candidate protein kinase C gene, *PKC1*, is required for the *S.cerevisiae* cell cycle. *Cell*, **62**, 213–224.
- Levin, D.E. and Bartlett-Heubusch, E. (1992) Mutants in the *S.cerevisiae* *PKC1* gene display a cell-cycle-specific osmotic stability defect. *J. Cell Biol.*, **116**, 1221–1229.
- Lew, D.J. and Reed, S.I. (1993) Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J. Cell Biol.*, **120**, 1305–1320.
- Madden, K., Sheu, Y.-J., Baetz, K., Andrews, B. and Snyder, M. (1997) SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science*, **275**, 1781–1784.
- Marini, N.J., Meldrum, B.B., Hubberstey, A.V., Stone, D.E., Traynor-Kaplan, A. and Reed, S.I. (1996) A pathway in the yeast cell division cycle linking protein kinase C (Pkc1) to activation of Cdc28 at START. *EMBO J.*, **15**, 3040–3052.
- McIntosh, E.M. (1993) MCB elements and the regulation of DNA replication in yeast. *Curr. Genet.*, **24**, 185–192.
- Measday, V., Moore, L., Ogas, J.P., Tyers, M. and Andrews, B. (1994) The Pcl2 (OrfD)–Pho85 cyclin-dependent kinase complex: a cell cycle regulator in yeast. *Science*, **266**, 1391–1395.
- Molero, G., Yuste-Rojas, M., Montesi, A., Vazquez, A., Nombela, C. and Sanchez, M. (1993) A *cdc*-like autolytic *Saccharomyces cerevisiae* mutant altered in budding site selection is complemented by *SPO12*, a sporulation gene. *J. Bacteriol.*, **175**, 6562–6570.
- Mozzoni, C., Zarov, P., Rambourg, A. and Mann, C. (1993) The Slr2(Mpk1) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. *J. Cell Biol.*, **123**, 1821–1833.
- Mulholland, J., Preuss, D., Moon, A., Wong, A., Drubin, D. and Botstein, D. (1994) Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J. Cell Biol.*, **125**, 381–391.
- Nasmyth, K.A. (1993) Regulating the HO endonuclease in yeast. *Curr. Opin. Genet. Dev.*, **3**, 286–294.
- Nasmyth, K.A. and Dirick, L. (1991) The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast. *Cell*, **66**, 995–1013.
- Newton, A.C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem.*, **270**, 28495–28498.
- Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995) A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J.*, **14**, 5931–5938.
- Novick, P. and Botstein, D. (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*, **40**, 405–416.
- Ogas, J.P. (1992) Identification and analysis of regulators of G1 progression in *Saccharomyces cerevisiae*. Ph.D. Thesis, University of California, San Francisco.
- Ogas, J., Andrews, B.J. and Herskowitz, I. (1991) Transcriptional activation of *CLN1*, *CLN2*, and a putative new G1 cyclin (*HCS26*) by Swi4, a positive regulator of G1-specific transcription. *Cell*, **66**, 1015–1026.
- Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y. and Takai, Y. (1996) Rom1 and Rom2 are GDP/GTP exchange proteins (GEFs) for the Rho1 small GTP binding proteins in *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 2196–2207.
- Persons, D.A., Wilkinson, W.O., Bell, R.M. and Finn, O.J. (1988) Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. *Cell*, **52**, 447–458.
- Pringle, J.R. and Hartwell, L.H. (1981) *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 97–142.
- Reed, S.I. (1992) The role of p34 kinases in the G1 to S-phase transition. *Annu. Rev. Cell Biol.*, **8**, 529–561.
- Ridley, A.J. (1996) Rho: theme and variations. *Curr. Biol.*, **6**, 1256–1264.
- Rose, A.H. (1975) Growth and handling of yeast. *Methods Cell Biol.*, **12**, 1–15.
- Rosengurt, E., Rodriguez-Pena, A., Coombs, M. and Sinnett-Smith, J. (1984) Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: role of Ca^{2+} -sensitive phospholipid-dependent protein kinase. *Proc. Natl Acad. Sci. USA*, **81**, 5748–5752.
- Schneider, B.L., Yang, Q.H. and Fletcher, A.B. (1996) Linkage of replication to start by the Cdk inhibitor Sic1. *Science*, **272**, 560–562.
- Sherman, F., Fink, G.R. and Hicks, J.B. (1982) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Siliciano, P.G. and Tatchell, K. (1984) Transcription and regulatory signals at the mating type locus in yeast. *Cell*, **37**, 969–978.
- Sterky, F., Holmberg, A., Pettersson, B. and Uhlen, M. (1996) The sequence of a 30 kb fragment on the left arm of chromosome XV from *Saccharomyces cerevisiae* reveals 15 open reading frames, five of which correspond to previously identified genes. *Yeast*, **12**, 1091–1095.
- Sterns, M., Jensen, R. and Herskowitz, I. (1984) Five *SWI* genes are required for expression of the *HO* gene in yeast. *J. Mol. Biol.*, **178**, 853–868.
- Stuart, D. and Wittenberg, C. (1995) Cln3, not positive feedback, determines the timing of *CLN2* gene expression in cycling cells. *Genes Dev.*, **9**, 2780–2793.
- Toone, W.M., Johnson, A.L., Banks, G.R., Toyn, J.H., Stuart, D., Wittenberg, C. and Johnston, L.H. (1995) Rme1, a negative regulator or meiosis, is also a positive activator of G1 cyclin gene expression. *EMBO J.*, **14**, 5824–5832.
- Tyers, M. (1996) The cyclin-dependent kinase inhibitor p40^{Sic1} imposes the requirement for Cln G1 cyclin function at start. *Proc. Natl Acad. Sci. USA*, **93**, 7772–7776.
- Vai, M., Popolo, L. and Alberghina, L. (1987) Effect of tunicamycin on cell cycle progression in budding yeast. *Exp. Cell Res.*, **171**, 448–459.
- Watanabe, B., Chen, C.-Y. and Levin, D.E. (1994) *Saccharomyces cerevisiae* *PKC1* encodes a protein kinase C (PKC) homolog with a substrate specificity similar to that of mammalian PKC. *J. Biol. Chem.*, **269**, 16829–16836.
- Zarov, P., Mozzoni, C. and Mann, C. (1996) The Slr2(Mpk1) MAP kinase is activated during periods of polarized cell growth in yeast. *EMBO J.*, **15**, 83–91.

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