Title

Studies on PTC1, a Type 2C phosphatase that regulates cell cycle progression and signal transduction pathways

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Publication Date

1995

Peer reviewed|Thesis/dissertation

Studies on PTC1, a Type 2C Phosphatase that Regulates
Cell Cycle Progression and Signal Transduction Pathways

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

DEDICATION

For my parents:

Nina and Ian

ACKNOWLEDGMENTS

This thesis represents work that I have done in the Herskowitz lab over the last five years. Many people have contributed significantly to this thesis both scientifically and personally. I must first offer my thanks to Ira Herskowitz for allowing me to join the lab without having rotated in it as a first year student. I am grateful for both the resources that he has provided as well as the intellectual environment that forges independent thought. I must also thank all members of the Herskowitz lab past and present for it is their ceaseless toil that is the form and substance of the lab. Several members must receive special acknowledgments: first is my bay mate, Joseph Gray, who has suffered the slings and arrows of having to breathe the same air with me for years. Next are the other members of the Euro-post-doc lunch club, Matthias Peter and Maarten von Lohuizen; it is through endless discussions about the cell cycle and signal transduction with This, Maarten, and Joe that the experiments in this thesis were shaped. Special thanks go to Anita Sil whose undoubting perseverance has been an inspiration and a profound lesson. Thanks also go to Hay-Oak Park who taught me that yeast biochemistry is much about volume, i.e. liters and liters of medium. Ramon Tabtiang also deserves my gratitude for all the time and patience he has given me in helping operate our computers. I wish to thank the members of my thesis committee Dave Morgan, Pat O'Farrell, and Ira Herskowitz for providing insightful commentary in many meetings over the years and especially for

helping me edit and rewrite my rough and woolly manuscript. I extened my thanks to Sue Adams and Jana Toutolmin who have made my navigation through the vicissitudes of UCSF bureaucracy relatively painless. Special thanks go to Josh Niclas whose wry wit and unflagging friendship has helped see me through the blackest days of graduate school. I am indebted to all my friends in and out of graduate school for their friendship and support, especially my old roommates, Ted Haxo and Kristine Maddocks. I give my gratitude to my partner Jillen Doroan whose love and support over the last year of stygian effort has been essential to my well being. Finally, I thank my parents, who always encouraged my interests in science, from paleontology to astrophysics, and provided me with every opportunity for my education.

STUDIES ON PTC1, A TYPE 2C PHOSPHATASE THAT REGULATES CELL CYCLE PROGRESSION AND SIGNAL TRANSDUCTION PATHWAYS

Bruce Cree

ABSTRACT

The regulation of the G1 to S transition in *Saccharomyces cerevisiae* involves the coordination of budding, nuclear, and mitotic cycles. Activity of the Cdc28p/Clnp kinase is essential for these processes. To identify additional components of these cell cycle transitions, a genetic interaction approach was taken. A synthetic lethal screen with *cdc28-1* identified *PTC1*, a gene encoding a type 2C phosphatase. *PTC1* mutants arrest growth at 37°C, with many cells having more than one bud. DAPI staining reveals that each bud inherits a nucleus. This complex arrest phenotype suggests that *PTC1* may have roles in cytokinesis as well as in progression through G1. Mutation in *PTC1* enhances the growth defect of mutations in the G1 cyclins or *CDC28*. Although *CLN2* mRNA levels are normal, protein levels of Cln2p are decreased in *PTC1* mutants growing in semi-permissive conditions. This indicates that Ptc1p functions posttranscriptionally to regulate Cln2p protein levels. Because the phosphorylated forms of Cln2p are less stable, a role for Ptc1p in stimulating the G1 to S transition may be to stabilize Cln2p through dephosphorylation. Stabilized Cln2p presumably leads

to stabilized CDK which promotes progression through G1.

In addition to its role as a positive regulator of the G1 to S transition, *PTC1* interacts with MAP kinase pathways. The temperature-sensitive arrest phenotype of *ptc1* mutants is partially dependent on the MAP kinase *HOG1*, suggesting that *PTC1* negatively regulates the *HOG1* pathway. *HOG1* is involved in the signal transduction response to extracellular high osmolarity. Hyperactivity of Hog1p is incompatible with both gain-of-function and loss-of-function mutations in the *PKC1* pathway, suggesting that the two pathways act in opposition to each other. This opposition can be explained by a model in which *PKC1* and *HOG1* pathways regulate different aspects of cell wall biosynthesis. Because cell wall remodeling is subject to precise cell cycle regulation, and because *PTC1* has a role in promoting the G1 to S transition, *PTC1* may help coordinate the activity of the *HOG1* MAP kinase pathway with cell cycle progression.

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CHAPTER 1

A Synthetic Lethal Screen with *cdc28-1* Identifies a Mutation in *PTC1*, a Gene Encoding a Type 2C Phosphatase that Regulates Cln2p Protein Level

INTRODUCTION

Progression through the division cycle of *Saccharomyces cerevisisiae* can be thought of as the orchestration of several cyclic mechanisms: the cell must select a site for polarized growth that leads to bud emergence, duplicate its spindle pole body which will later organize the mitotic apparatus, and initiate replication of its DNA. That each of these processes is independent is evidenced by mutations that allow two of the three processes to occur. For example, *cdc4*, *cdc7*, and *cdc34* temperature-sensitive mutants cannot enter S phase at their restrictive temperatures but are competent for bud emergence and spindle pole body duplication (Pringle and Hartwell, 1981; Byers and Goetsch, 1974). In contrast, *cdc31* mutants cannot undergo spindle pole body duplication but can form a bud and initiate DNA synthesis (Baum *et al.*, 1986). *cdc24* mutants are incapable of forming a bud but can replicate their DNA and spindle poles (Sloat *et al.*, 1981).

The coordination of these three processes occurs in the newly replicated cell and requires the activity of cyclin dependent kinases (CDKs), Cdc28p and its various cyclin subunits (reviewed by Nasmyth, 1993). Temperature-sensitive alleles of *CDC28* will arrest at "START", a position in G1 just prior to mitotic commitment where haploid cells arrest in response to mating pheromone (Hereford and Hartwell, 1974; Hartwell *et al.*, 1974; Reid and Hartwell, 1977; Pringle and Hartwell, 1981). Cdc28p forms complexes with an ever growing number of cyclin subunits that are

presumed to direct the activity of the catalytic kinase subunit to specific substrates. Extensive analysis of the cyclins has revealed that although deletion of any one cyclin is not lethal, deletion of various combinations of cyclins can result in specific delays or blocks in the cell cycle (Richardson *et al.*, 1989; Surana *et al.*, 1991; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). These observations indicate that certain cyclins perform overlapping functions required for each of the processes that characterize the cell cycle.

Initial analysis of three G1-specific cyclins, *CLN1*, *CLN2*, and *CLN3,* suggested that they were functionally redundant and were required for passage through "START" because a triple deletion resulted in a *cdc28*-like arrest (Richardson *et al.*, 1989; Cross 1990). Because of this phenotype and their distant relationship to mitotic cyclins, whose oscillating protein levels are associated with entry into and exit from mitosis (Evans *et al.*, 1983; Murray and Kirschner, 1989), it was proposed that cell cycle control in *S. cerevisiae* was unusual because it was regulated at G1 (Wittenberg and Reed, 1989). The emerging picture is far more complex: there are at least nine cyclins that have specialized functions throughout the cell cycle. *CLN1* and *CLN2* are thought to play a critical role in polarity establishment and bud emergence (Lew and Reed, 1995; Lew and Reed, 1993; Benton et al., 1993; Cvrckova and Nasmyth, 1993) whereas *CLB5* and *CLB6* function to promote S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993; Schwob *et al.*, 1994). Spindle pole body duplication requires the G1 cyclins (Cross, 1990) whereas migration, elongation, and maintenance depend on the B

cyclins: *CLB1, CLB2, CLB3, and CLB4* (Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson et al., 1992). Interestingly, *CLB5* may also have a mitotic role since *clb3*Δ *clb4*Δ *clb5*Δ cells arrest in G2 with short unseparated spindles (Schwob and Nasmyth, 1993). Thus cyclins are involved in many if not all major cell cycle events.

Many cyclins are highly unstable proteins whose transcripts appear and disappear during the cell cycle (Wittenberg *et al.*, 1990; Surana *et al.*, 1991; Ghiara *et al.*, 1991; Richardson *et al.*,1992; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). *CLN1* and *CLN2* transcript levels rise during late G1 and fall during S phase in response to activation by SCB binding factor (SBF), a transcription factor composed of Swi4p and Swi6p that binds to SWI4/SWI6 cell cycle box elements (SCBs) found upstream of *CLN1*, *CLN2*, and *HCS26* (Andrews and Herskowitz, 1989a; Andrews and Herskowitz, 1989b; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Primig *et al.*, 1992). *CLB5* and *CLB6* follow a similar pattern to *CLN1* and *CLN2*, but are activated by MluI binding factor (MBF), composed of Swi6p and Mbp1p, responsible not only for transcription of *CLB5* and *CLB6* but also for many of the genes necessary for DNA synthesis such as *TMP1* and *POL1* (Lowndes *et al.*, 1991; Mcintosh *et al.*, 1991, Dirick *et al.*, 1992; Lowndes *et al.*, 1992; Koch *et al.*, 1993). SBF and MBF are themselves subject to cell cycle regulation and are *CDC28* dependent (Nasmyth, 1985; Breeden and Nasmyth 1987; Marini and Reed, 1992). Transcription of *CLB3* and *CLB4* begins just prior to START, rises during S phase, and falls during G2/M, whereas transcription of *CLB1* and *CLB2* rises during S phase to peak at G2/M and falls following completion of mitosis (Surana *et al.*, 1991;

Ghiara *et al.*, 1991; Richardson *et al.*, 1992). These cyclic oscillations can, in part, be accounted for by autoregulation. Clb1p and Clb2p stimulate their own transcription and inhibit transcription of *CLN1* and *CLN2*, accounting for their own rise and the fall of *CLN1* and *CLN2* (Amon *et al.*, 1993). Furthermore, accumulation of Clb1p and Clb2p proteins is partially dependent on the presence of Cln1p and Cln2p, which function to inhibit the proteolytic machinery that degrades Clb1p and Clb2p through their destruction boxes (Amon *et al.*, 1994; Glotzer *et al.*, 1991). This machinery is activated in anaphase and remains on through G1, preventing Clb1p and Clb2p from accumulating until Cln1p and Cln2p are present (Cln1p and Cln2p are not degraded by this pathway since they lack the "cyclin box" destruction motifs). These findings have led to a crude framework for understanding how some of the oscillations in cyclin expression occur.

One notable exception to this oscillating pattern is Cln3p, which is present at a low and constant level throughout the cell cycle in spite of being highly unstable (Tyers *et al.*, 1993; Cross and Blake, 1993). It is proposed that *CLN3* functions as a monitor of cell size, allowing daughter cells that are smaller than their mothers to grow to an appropriate size before commencing the cell cycle (Sudberry and Goodey, 1980; Cross, 1988; Nash *et al.*, 1988). After a minimum size requirement has been reached, *CLN3* triggers transcription of the cyclins *CLN1*, *CLN2*, and *CLB5* as well as the cyclin-like genes *HCS26* (*PCL1*) and *ORFD* (*PCL2*) (Tyers *et al.*, 1993). Thus transcription of *CLN1* and *CLN2* involves not only *CDC28* and SBF (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991) but also *CLN3*.

Because Cln1p/Cdc28p and Cln2p/Cdc28p kinases can activate *CLN1* and *CLN2* transcription, it was thought that the sudden appearance of their mRNA in late G1 was due to a positive feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth,1991; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). This hypothesis is probably incorrect, because timely activation of late G1 genes under control of SBF, including *HCS26* and *CLN2*, does not depend on *CLN1* or *CLN2* (Dirick *et al.*, 1995). Precise activation of these transcripts does require *CLN3*, reinforcing the idea that *CLN3* functions to promote progression through G1 by activating late G1 transcripts including *CLN1* and *CLN2* (Tyers *et al.*, 1993).

CLN3 may be the "trigger" of the cell cycle—its constant expression enables it to be present at G1 when *CLN1* and *CLN2* transcription is off due to prior inhibition by the Clbps which are also absent because of the proteolytic machinery turned on in anaphase. Cln3p is produced as the cell grows in size in early G1 until, presumably, some threshold level is reached which can efficiently activate late G1 transcription. Perhaps this event is really the "START" of the cell cycle because all the events attributed to passage through START are dependent on the products of these transcripts.

If *CLN3* is primarily the trigger, then what are the functions of *CLN1* and *CLN2*? Although they do not appear to participate in inducing their own transcription, they are essential for correct timing of budding and entry into S phase (Benton *et al.*, 1993; Cvrckova and Nasmyth, 1993; Lew and Reed, 1993; Dirick *et al.*, 1995). *CLN1* and *CLN2* are also required for acquisition of

pheromone resistance and spindle pole body duplication—essentially all the events associated with passage through START. In their absence these events do occur but only after considerable delay. This delay may result in activation of *CLB5* and *CLB6*, which are capable of complementing the G1 functions of the *CLNs* (Epstein and Cross, 1992). One line of evidence for this hypothesis is that the delayed timing of entry into S phase in *cln1*Δ *cln2*Δ strains is dependent on *SIC1*, the Cdc28p/Clb5p and Cdc28p/Clb5p inhibitor (Dirick *et al.*, 1995). This observation suggests that one role of Cln1p and Cln2p is to relieve inhibition by Sic1p, thereby allowing Clb5p and Clb6p to function (Schwob and Nasmyth, 1993). The observation that *CLB5* and *CLB6* can substitute for *CLN1* and *CLN2* does not mean that they do. Even if the functions of Cln1p and Cln2p are to activate *CLB5* and *CLB6* by inhibiting Sic1p, it is unclear how any of these cyclins stimulate progression through START.

It is somewhat surprising that the physiological targets presumed to be regulated by *CDC28* have yet to be identified. The one exception to this is Swi5p, a transcription factor that participates in the regulation of *HO*, the endonuclease that regulates mating type switching (Moll *et al.*, 1991). Perhaps it is even more surprising that only two other genes have mutant phenotypes similar to *cdc28* : *SIT4* and *CDC37*. It is interesting that these genes are both involved in *CLN* function: *SIT4*, a unique 2A-like phosphatase, participates in transcriptional activation of *CLN1* and *CLN2* and has a role in bud emergence (Sutton *et al.*, 1991; Fernandez-Sarabia *et al.*, 1992). Cdc37p seems to promote cyclin association with Cdc28p (Gerber *et al.*, 1995). Their identification indicates that there is

much to learn about *CLN* expression and CDK complex formation and suggests that there are probably more gene products required for passage through START.

Additional genes involved in the G1 to S transition may not have been found because of the methodologies used to probe the cell cycle. The first genetic screens that looked for cell cycle genes utilized the criteria of uniform arrest and single gene heritability (Hartwell *et al.*, 1974; Hereford and Hartwell, 1974; Reed, 1980). Work on the CDKs has raised the specter of redundancy and dramatically enforced the idea that one gene, *CDC28*, can function in many parts of the cell cycle. Indeed, it is astonishing that most of the alleles of *CDC28* arrest in G1 rather than all over the cell cycle—association of Cdc28p with the Clnps and Clbps is required for every aspect of growth and division. Of course, that *CDC28* mutants have a uniform arrest has been a blessing to yeast cell biology since *CDC28* would not have been identified otherwise.

The challenges of "redundancy" could also apply to entire pathways whose outputs are required for a shared essential function. For example, several lines of evidence indicate that the *PKC1* pathway is also involved in G1 progression although mutations in this pathway result in a small budded terminal phenotype (Levin *et al.*, 1990; J. Gray, personal communication). Redundant pathways may also cross regulate each other to ensure that their activity is properly coordinated. Mutations in genes that function to interface two or more pathways could have complex phenotypes although their functions may be very specific. Furthermore, perhaps some of the targets of *CDC28* are redundant and when mutated may not cause

arrest. Alternatively, some targets of*CDC28* may be required at various points in the cell cycle and, therefore, may have heterogeneous arrests. Therefore, in the search for additional genes whose products are involved in cell cycle regulation, alternative approaches that do not demand uniform arrest and essentiality should be employed.

I have undertaken such an approach utilizing genetic interactions to identify additional genes involved in cell cycle regulation. Mutations in *PTC1*, a gene encoding a type 2C phosphatase, genetically interact with mutations in *CDC28* and the *CLNs* and have complex and intriguing phenotypes. The isolation and characterization of *PTC1* is the subject of this thesis.

RESULTS

Identification of a Novel Gene Involved in the G1 to S Transition

A genetic screen was designed to identify mutants that were synthetically lethal with *cdc28-1*, a G1 arrest allele (Bender and Pringle, 1991; Costigan *et al.*, 1992; Frank *et al.*, 1992). Because *CDC28* is involved in coordinating DNA synthesis with bud emergence and spindle pole body duplication (reviewed in Nasmyth, 1993), I reasoned that mutations in genes regulating these processes could be synthetically lethal with *cdc28-1*. Alternatively, synthetic lethal mutants could be activators of *CDC28* or reveal processes parallel to *CDC28* (Fig. 1). The details of this screen are described in Appendix I.

51 mutants were identified that enhanced the growth defect of *cdc28-1* and hence were tentatively designated *slc* for synthetic lethal with *cdc28*. 10 of these mutants exhibited temperature-sensitive phenotypes due to single gene traits that segregated independently from *cdc28-1*. One such mutant *slc32*, like *cdc28-1*, is viable at temperatures below 37°C but in combination with *cdc28-1* is inviable at temperatures above 30°C (Fig. 2). Since the double mutant is viable at 25°C, it is better described as being synthetically sick with *cdc28-1*. Because *cdc28-1* and *slc32* are independently temperature-sensitive, it is possible that the combination of the two mutations could result in the synthetic phenotype without being involved in the same process. Thus

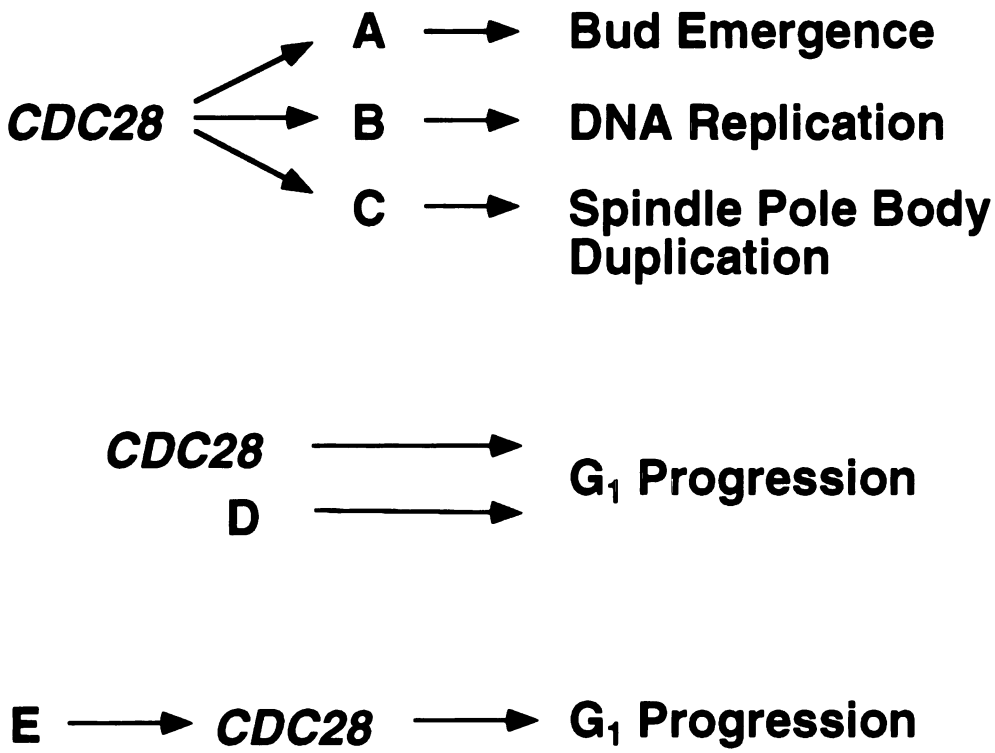


Figure 1. Mutations in A-E in conjunction with a weakened allele in *CDC28* could result in synthetic phenotypes, whereby the double mutant has a more severe phenotype than either single mutant alone. A, B, and C represent downstream targets of *CDC28;* D functions with or in parallel to *CDC28*; and E is a positive regulator of *CDC28.*

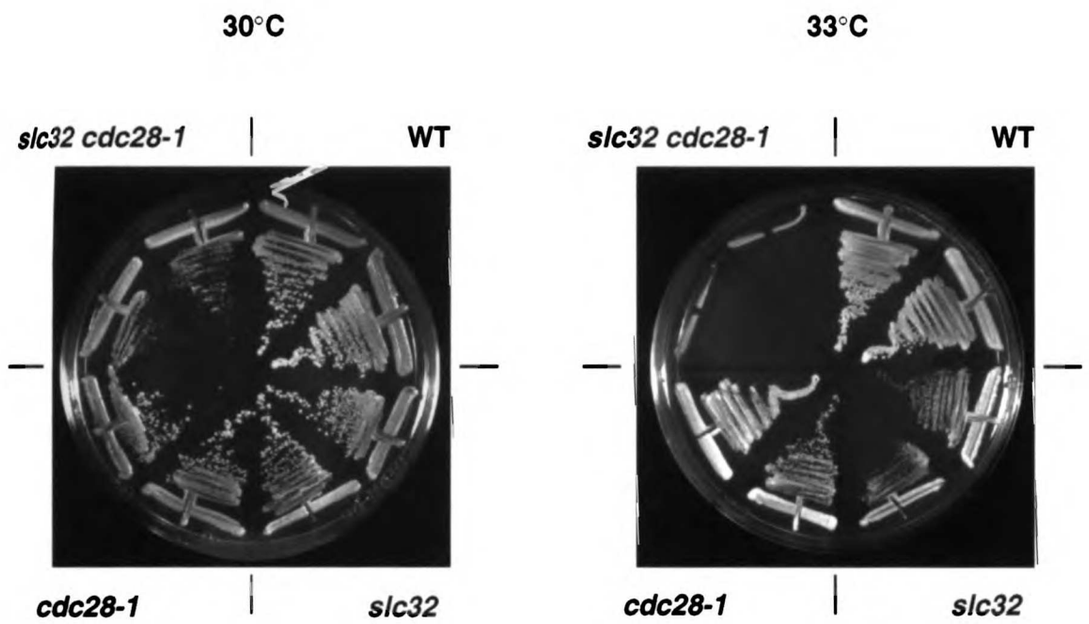


Figure 2. *slc32* is synthetically sick with *cdc28-1*. Although the double mutant will form colonies at 30°C, it is inviable at 33°C. The top half of each plate shows a non-parental ditype tetrad while the bottom half shows a parental ditype tetrad from a *slc32* X *cdc28-1* cross. The tetrad on the top half is BC178-9; and the tetrad on the bottom half is BC178-10. The strains were streaked onto rich medium and incubated for two days.

additional criteria are required to establish that the mutant identified by the synthetic lethal screen is involved in the G1 to S transition. These criteria include a temperature-sensitive cell cycle phenotype independent of *cdc28-1* and genetic interactions with the G1 cyclins.

Phenotype of *slc32* mutants

The *slc32* mutant (BC93-3C) has an unusual terminal phenotype: the mutant grows normally at room temperature but when shifted to 37°C, approximately 20% of cells arrest without a bud, 50% of cells arrest with a bud, and 30% of cells arrest with two or more buds. The buds frequently have an elongated morphology which becomes more pronounced when the cells are maintained at the non-permissive temperature for several hours. DAPI staining reveals that each bud usually contains a nucleus (Fig. 3).

To understand how the double-budded cells are generated, unbudded cells from a culture (BC93-3C) growing at 25°C were transferred to YEPD slabs at 37°C where their growth was followed for several hours. By observing single cells arresting at the non-permissive temperature, I ascertained that mother cells could give rise to at least two daughter cells. However, the daughter cells do not subsequently divide. Because the daughter cells do not continue to bud, they are presumed to arrest in G1, although their nuclear content has not been assessed directly. The cells with the same

characteristics that are observed in asynchronous liquid cultures are presumed to have had the same pedigree.[1]

Because the multibudded phenotype is semi-penetrant and temperature-sensitive, it may be due to partial loss of function; hence, it is important to determine whether deletion of the gene mutated in the *slc32* mutant would result in a more severe phenotype. Furthermore, if the gene were required for cell cycle progression, it should be essential. Thus cloning the gene that corresponds to the mutation is necessary to define its cell cycle role. In addition, sequence analysis could determine whether the gene is novel and might suggest its function.

Cloning and sequencing of a complementing clone of *SLC32*

Low copy suppressors of the growth defect of the *slc32* mutant were isolated. Mutant cells were transformed with a *URA3*-marked centromeric (YCP50) yeast genomic library (Rose *et al.*, 1987), and 1 X 10^6 transformants were screened for colonies able to grow at 37°C. Of the transformants that grew at 37°C, four were plasmid dependent: plasmids were rescued from each of these isolates. Two of the four plasmids had identical restriction enzyme digest patterns. The three distinct plasmids were retransformed into the mutant: two fully complemented and one partially complemented the temperature-sensitive growth defect. A

[1] Visual comparison of arrested daughter cells to cells arrested with α-factor and nocadazole suggest that the size of the nucleus of the daughter cells is similar to the size of the nucleus of α-factor arrested cells, which are known to arrest in G1 and have a 1N DNA content. Cells arrested with nocadazole arrest at G2/M and have nuclei with a 2N DNA content that are larger than either α-factor treated cells or arrested daughter cells.

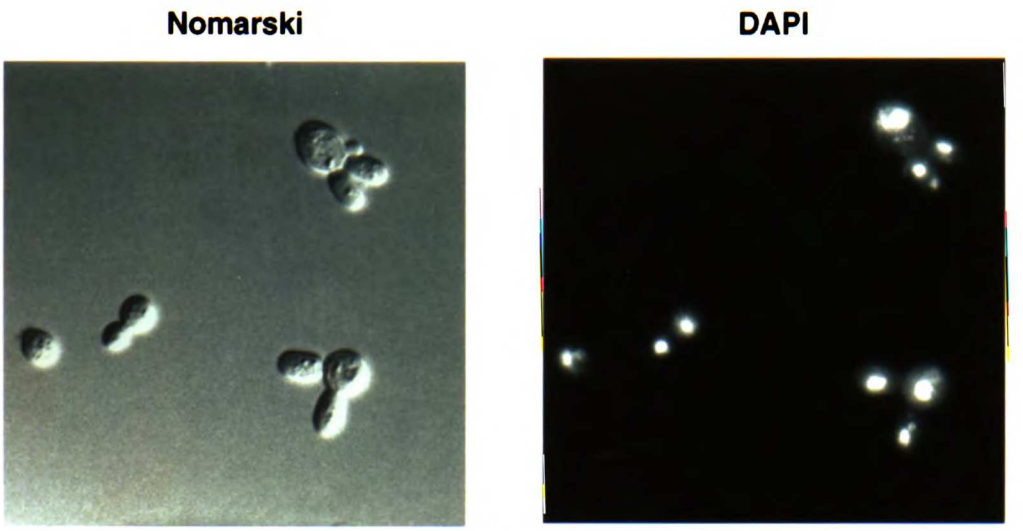


Figure 3. Many *slc32* cells arrest with more than one bud. DAPI staining reveals that each bud has a nucleus. A mid-log culture of *slc32* (BC93-3C) grown at 25°C in liquid, rich medium was shifted to 37°C for 4 hrs.

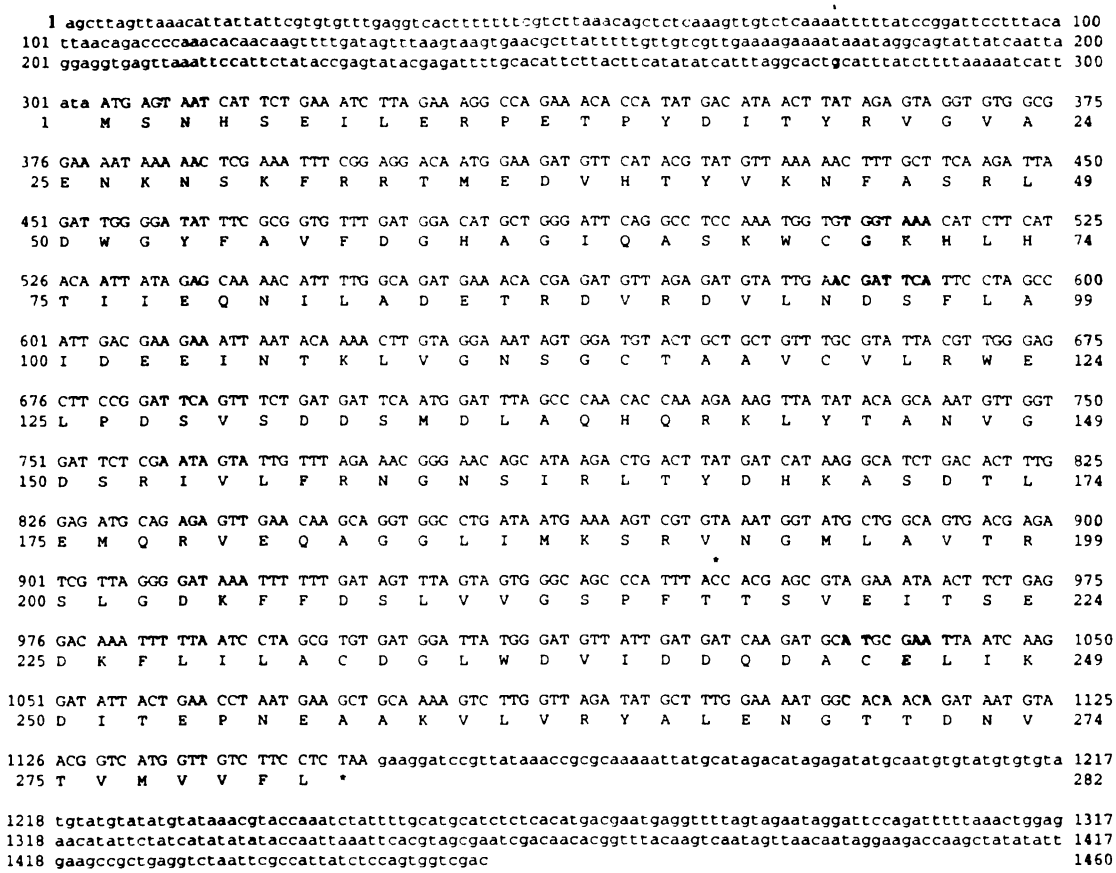
restriction map of the 9.6 kb insert of one of the fully complementing plasmids (pBC7) was prepared.

The plasmid was subjected to LukTn10 transposon mutagenesis (Huisman *et al.*, 1987), and five plasmids which had a transposon insert that failed to complement the mutant were isolated. Restriction mapping these plasmids indicated that four of the five plasmids had a transposon hop into the same region of the insert. A 2.8 kb EcoRI/KpnI fragment from the region identified by the transposon hops was subcloned and found to fully complement the mutant. Various subclones of this insert were introduced into M13 vectors and sequenced (Sanger *et al.*, 1977). Sequencing revealed that there were two potential open reading frames (ORFs) on the 2.8 kb EcoRI/KpnI fragment. Each ORF was subcloned into pRS316 (*URA3*, *CEN*) and reintroduced into the mutant: one of the two ORFs complements the mutant and lies on a 1.4 kb HindIII/SalI fragment (pBC14).

The complementing ORF has sequence similarity to 2C phosphatases

Sequence similarity searches (using the BLAST electronic mail service provided by the National Center for Biotechnology Information) showed that the predicted translation product of the complementing ORF shares 34% identity with mammalian type 2C ser/thr protein phosphatases (Fig. 4a and 4b). The gene was independently cloned at the same time and has been named *PTC1* for protein phosphatase two c (Maeda *et al.*, 1993).

A.



B.

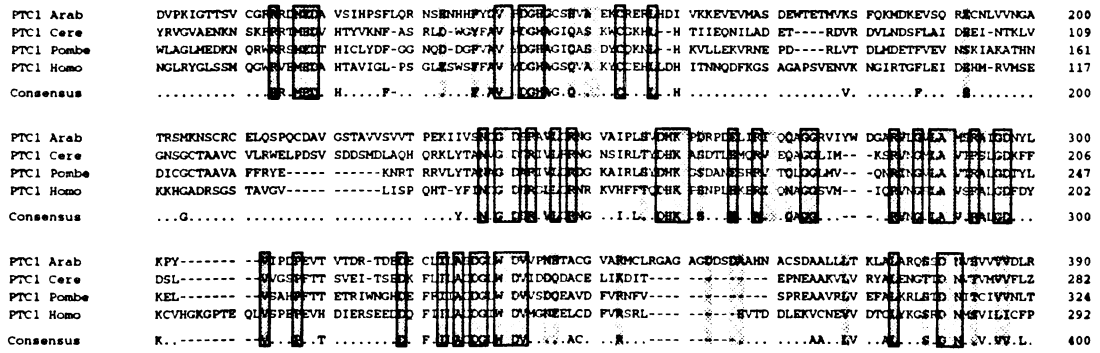


Figure 4A. DNA sequence of *PTC1* including promoter region and 3'UTR. This fragment fully complements a *ptc1*Δ mutant. The *ptc1-1* mutation is a C⇨T change at nucleotide 951 which converts T^{219} to I.

B. Alignment of regions of protein sequence similarity between *PTC1* and type IIc phosphatases from *S. pombe*, *H. sapiens*, and *A. thaliana*. Identities are boxed and similarities are shaded.

PTC1 encodes the gene defective in the *slc32* mutant

Sequence analysis of pBC7 revealed that *PTC1* is near the *TRP1* locus on chromosome IV. If the mutation is closely linked to the *TRP1* locus, then it is likely that the cloned *PTC1* gene corresponds to the mutation rather than being a low copy suppressor. The *TRP1* *ptc1-1* mutant was crossed to a *trp1*Δ strain, the **a**/α diploid was sporulated, and 24 asci were dissected. In all tetrads the temperature-sensitive growth defect of the mutant segregated with *TRP1*. This finding shows that the mutation is closely linked to the centromere; however, it does not prove that the mutation is in the *PTC1* gene. The *PTC1* gene could be a linked low copy supressor of the mutation. To determine whether the mutation is in the *PTC1* gene, a deletion of *PTC1* is necessary. A construct which precisely replaces the complete ORF of *PTC1* with *HIS3* was engineered (pBC29). An **a**/α diploid heterozygous for *TRP1/trp1*Δ was transformed with pBC29 and plated onto -His medium. His^+ single colonies were isolated and sporulated: 29/32 asci dissected showed that His^+ segregated with Trp^- and that His^+ meiotic segregants were viable at 25°C, indicating that the *HIS3* marker had integrated into a non-essential locus linked to *TRP1*. In one tetrad His^+ segregated 3:1, presumably due to gene conversion, and in two tetrads, one meiotic product was His^+ Trp^+, indicating that recombination had occurred. All His^+ segregants had a severe growth defect on YEPD plates at 37°C. Colony PCR using primers outside of the coding sequence of the *PTC1* gene amplified a

fragment of size consistent with *HIS3* integration into the *PTC1* locus. To determine whether the original mutation corresponds to the *PTC1* locus, the *ptc1-1* mutant was mated to the *ptc1*Δ mutant. The **a**/α diploid was temperature-sensitive for growth and had a nucleated/multibudded phenotype indistinguishable from the **a**/α *ptc1-1/ptc1-1* mutant at the non-permissive temperature, proving that the *slc32* mutation was in the *PTC1* gene.

The terminal phenotypes of *ptc1*Δ and *ptc1-1* mutants are similar

*ptc1*Δ mutants (BC128-5B) were grown in rich liquid medium at 25°C to early log phase, and half of the culture was shifted to 37°C. Optical density was used to monitor the growth of the cultures. The cells at 25°C continued to grow exponentially and had normal morphology; the cells at 37°C stopped growing after 6-8 hrs. Approximately 30% of the cells exhibited the multibudded/nucleated phenotype. The doubling time of the mutant in rich medium at 30°C is approximately 2 hrs, somewhat slower than the isogenic wild-type (JO371) and *ptc1-1* (BC93-3C) strains, which have a 1.5 hour doubling time. *ptc1*Δ and *ptc1-1* mutants were streaked onto YEPD plates and grown at various temperatures between 25°C and 37°C. Both mutants form colonies of size similar to wild-type at 25°C-33°C. At 35°C, colony size is noticeably smaller for both *ptc1-1* and *ptc1*Δ mutants, and at 37°C, the *ptc1-1* mutant does not form colonies whereas the *ptc1*Δ mutant forms only very small colonies (data not shown). Thus the *ptc1-1* mutant has a more severe growth defect at high-temperature than the *ptc1*Δ mutant, whereas the

*ptc1*Δ mutant grows slower than the *ptc1-1* mutant at intermediate temperatures; this result is surprising because the *ptc1-1* allele is recessive. One interpretation of the data is that presence of the defective protein product at the non-permissive temperature interferes with some essential process that can occur in its absence. For example Ptc1-1p might bind to a substrate at the non-permissive temperature but cannot dephosphorylate its target. In the absence of Ptc1-1p, another phosphatase less efficient than Ptc1p would be able to bind and dephosphorylate the target.

Cloning of *ptc1-1*

Because the *ptc1-1* allele has a more severe phenotype than the *ptc1*Δ, cloning and sequencing the *ptc1-1* allele might provide some insight into the mutant phenotype. Colony PCR was used to amplify the entire ORF as well as 5' and 3' UTRs from a *ptc1-1* mutant strain. The PCR product yielded a fragment of the expected size, indicating that no large-scale rearrangements had occurred at the *PTC1* locus in the mutant. The PCR products were subcloned (pBC 74, pBC75, pBC76) and the inserts of three independent transformants were sequenced. A single nucleotide change from C to T was found in each mutant clone which results in a threonine to isoleucine change at amino acid residue 216 (Fig. 4a). Sequence comparison between Ptc1p and other 2C phosphatases indicates that this residue is not conserved; however, it borders a potential *CDC28* phosphorylation site that is conserved between yeast and mammals. Various subclones of this mutant allele failed to complement either

*ptc1*Δ or *ptc1-1* strains. Because the *ptc1-1* allele has a stronger phenotype than *ptc1*Δ mutant, overexpression of the *ptc1*$^{216T\text{-}I}$ allele might have a more severe phenotype; however, p*GALptc1*$^{216T\text{-}I}$ (pBC80) and 2μ*ptc1*$^{216T\text{-}I}$ (pBC87) constructs do not enhance the growth defect of the *ptc1*Δ mutant (data not shown).

Additional phenotypes of *ptc1* mutants

ptc1-1 and *ptc1*Δ mutants do not grow on poor carbon sources such as glycerol, acetate, or pyruvate. The temperature-sensitive growth defect is partially complemented by addition of 10% sorbitol to the medium. **a**/α *ptc1*Δ/*ptc1*Δ and **a**/α *ptc1-1*/*ptc1-1* diploids have a more severe growth defect than haploids and do not sporulate on plates or in liquid medium (data not shown).

Antibodies against TrpE-Ptc1p

To examine the expression and activity of Ptc1p, polyclonal antiserum to a TrpE-Ptc1p fusion protein was made (Koerner *et al.*, 1991). TrpE-Ptc1 fusion protein was inducibly expressed and purified from *Escherichia coli* (using pBC27) by inclusion body preparation, SDS-PAGE, and electroelution. This fusion protein was used as antigen in two rabbits, and their sera were screened for production of polyclonal antibodies. Because the sera crossreacted extensively with other proteins in the extract, it was necessary to affinity purify it on a Gst-Ptc1p fusion protein column.

αTrpE-Ptc1p antiserum detects Ptc1p by Western blot

The affinity-purified antiserum recognize a group of at least three bands of approximately 30-34 kd in size in wild-type whole-cell extracts (Fig. 5a). A similar collection of bands is recognized in extracts made from the *ptc1-1* mutant although the uppermost band is of reduced intensity. The bands are not detected in the *ptc1*Δ mutant and are overproduced at least 10-fold in a wild-type strain that harbors a 2μ*PTC1* plasmid (pBC35). Overexpressed Ptc1p could not be detected by Coomassie blue staining in crude lysates, suggesting that Ptc1p is not an abundant protein (data not shown).

To study Ptc1p *in vitro*, Ptc1p was immunoprecipitated from whole-cell extracts using the polyclonal antiserum. Unfortunately, only the lower form immunoprecipitated well, precluding study of the posttranslationally modified forms (data not shown). It is possible that the upper forms of Ptc1p are insoluble and hence cannot be immunoprecipitated by standard protocols. Alternatively, the upper bands could be due to phosphorylation and become dephosphorylated by Ptc1p when immunoprecipitated or cause assembly into complexes which mask the epitopes. Immunoprecipitated Ptc1p was also tested for phosphatase activity against phosphorylated casein, but no significant phosphatase activity could be detected. This negative result could be due to the relatively small and unquantified amounts of Ptc1p in the immunoprecipitates or to loss of enzymatic activity during immunoprecipitation. However, Ptc1p phosphatase activity was

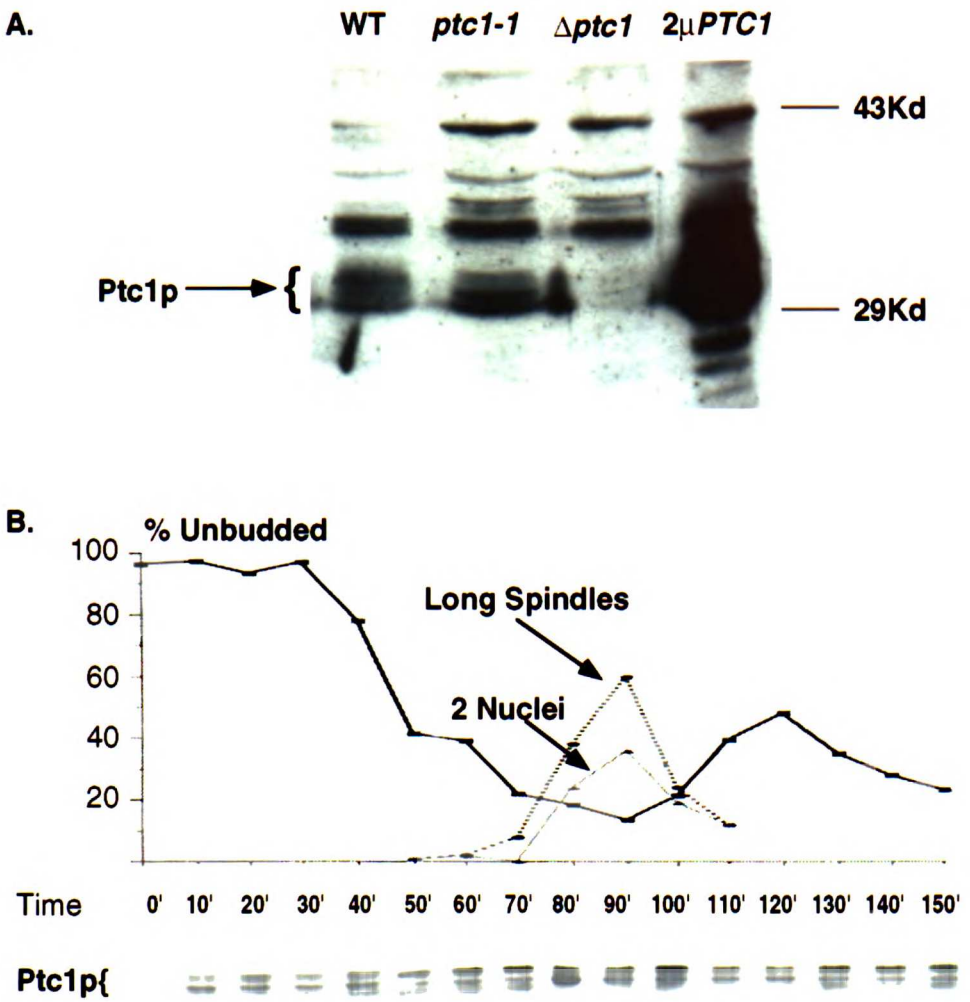


Figure 5 A. Western blot with αTrpE-Ptc1 fusion protein polyclonal antibodies reveals that Ptc1p migrates on SDS-PAGE as a group of bands, the lowest of which corresponds to the predicted molecular weight of Ptc1p. The *ptc1-1* mutant protein is the same size as wild type; however, the uppermost band is of reduced intensity.

B. αTrpE-Ptc1 Western blot of time course following α factor arrest/release. Ptc1p levels are constant over the cell cycle. However, there are changes in its migration pattern, including the appearance of a prominent band at 80' (G2/M). Cell cycle position is monitored by budding index, spindle, and nuclear morphology.

Whole-cell extracts are prepared from liquid cultures growing in rich medium at 30°C.

detected using a Gst-Ptc1p fusion protein (see the next section for details).

To immunolocalize Ptc1p by immunofluorescence, the antiserum was further purified by passage over a yeast protein column prepared from the *ptc1*Δ strain. However, when used in immunofluorescence, the antiserum failed to detect a Ptc1p-specific signal except in cells overexpressing Ptc1p from a 2μ plasmid. In the overexpressing strains, Ptc1p appeared to be in the nucleus and the cytoplasm but was excluded from the vacuole. This staining pattern was specific to the primary antiserum and was dependent on the presence of *PTC1* (data not shown).

Steady-state levels of Ptc1p do not vary over the cell cycle

To examine the expression of Ptc1p over the cell cycle, an α-factor arrest/release experiment was performed. *bar1*$^-$ cells were arrested with α-factor and then released into YEPD. Cells were collected at 10 minute intervals for 150 minutes. Cell cycle position was monitored by assessing the budding index, visualizing the spindles by immunofluorescence, and staining the nuclei with DAPI. The overall levels of Ptc1p were constant over the cell cycle; however, the relative intensities of the bands changed as detected by Western blot. In particular a prominent band appears at 80 minutes, the time corresponding to the G2/M transition (Fig 5b).

Purification of Gst-Ptc1p

To examine the enzymatic activity of Ptc1p *in vitro*, it was necessary to purify Ptc1p from yeast extracts. Gst-Ptc1p fusion protein was expressed (using pBC39) in a protease-deficient strain (FM135) and purified by glutathione-Sepharose 4B affinity chromatography (Smith and Johnson, 1988). 0.25 mg of Gst-Ptc1p can be purified from one liter of log-phase culture induced for six hours. The fusion protein could be visualized in the elution fraction by Coomassie blue staining (Fig 6a). In addition, Western blotting of each of the fractions from the purification revealed that the majority of the fusion protein was in the soluble fraction (Fig 6b). Overexpressed Gst-Ptc1p could be purified using either high or low salt lysis buffers. In contrast, when expressed from the endogenous *PTC1* promoter (using pBC69), the Gst-Ptc1p fusion protein could be purified only from a high-salt (0.5 M) lysis buffer. This observation suggests that Ptc1p might exist in a complex which prevents Gst-Ptc1p from binding to the glutathione-Sepharose resin except in high-salt conditions which dissociate the complex. A portion of the overexpressed fusion protein would presumably not be bound in the complex and, therefore, is free to bind the glutathione-Sepharose resin.

Elution fractions from the Gst-Ptc1p purification were run on SDS-PAGE and silver stained showing that overexpressed Gst-Ptc1p migrates as at least two bands. This result may indicate that even when overexpressed, posttranslational modifications of Ptc1p are preserved during affinity chromatography (Fig 6c).

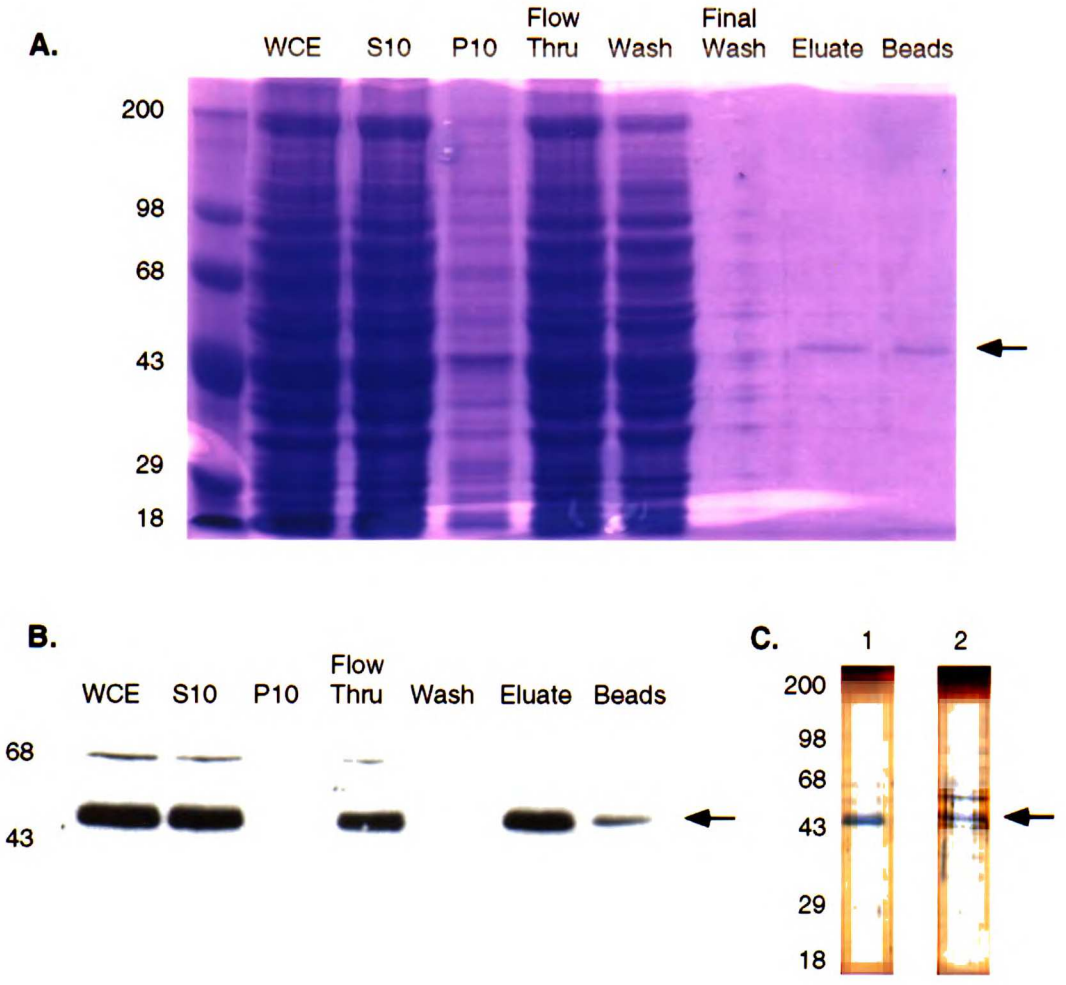


Figure 6 A. Coomassie stained fractions of purification of GstPtc1p by affinity column chromatography from the whole-cell extract (wce) of a protease-deficient yeast strain expressing GstPtc1p from an induced *GAL* promoter.

B. αPtc1p Western blot of fractions from the same purification.

C. Silver-stained elution fractions show that GstPtc1p can be isolated with a high degree of purity when expressed from the *GAL* promoter (pBC39) or its endogenous promoter (pBC69). 1- p*GAL*GstPtc1p, 2- p*PTC1*GstPtc1p

(◄—) indicates GstPtc1p.

Gst-Ptc1p purified from yeast has phosphatase activity

Gst-Ptc1p purified from overexpressing strain was assayed for phosphatase activity using casein (phosphorylated by [γ-^{32}P]ATP and cAMP-dependent protein kinase) as a substrate (Maeda *et al.*, 1993). Gst-Ptc1p could dephosphorylate phosphorylated casein as measured by release of $^{32}P_i$ (Fig. 7). The amount of $^{32}P_i$ released was directly proportional to the amount of Gst-Ptc1p added to the assay. Purified Gstp did not release $^{32}P_i$ from phosphorylated casein. Casein phosphatase activity was dependent on Mn^{++} as expected for a 2C phosphatase (Cohen, 1989; Cohen *et al.*, 1989a; Cohen *et al.*, 1989b). These findings are consistent with previous studies that showed that a Gst-Ptc1p purified from *E. coli* had 2C phosphatase activity using phosphorylated casein as a substrate (Maeda *et al.*, 1993; Robinson *et al.*, 1994).[2]

2μ *CLN2* suppresses the *ptc1-1* mutant

The temperature-sensitive growth defect of *ptc1-1* mutants was tested for suppression by *CLN* gain of function alleles in the S288C strain background (Fig. 8). 2μ*CLN2* (pBC92) partially

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[2] In developing this assay my goal was to be able to purify Gst-Ptc1p from its own promoter and assay its activity over the cell cycle to see if the changes in expression pattern correlated with changes in phosphatase activity. Unfortunately, because of the extremely low level of Ptc1p expression, several liters of culture are required to purify enough Gst-Ptc1 for a single phosphatase assay, making a time course analysis impossible with the available incubation and affinity chromatography equipment.

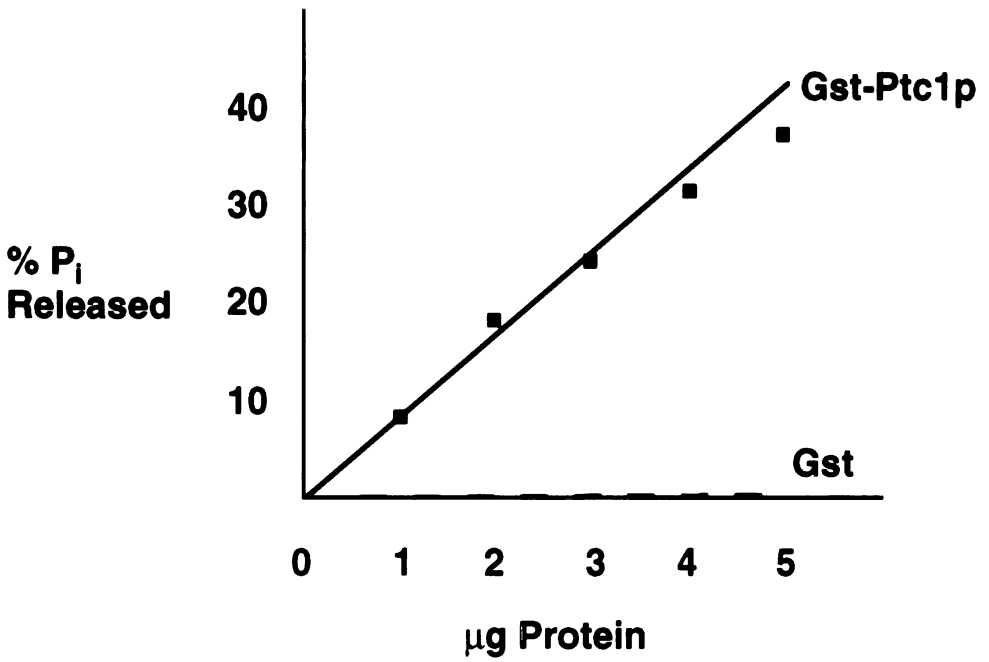

Figure 7. Gst-Ptc1p purified from yeast has phosphatase activity against phosphorylated casein. Gstp expressed from the same strain has no significant phosphatase activity. The enzyme and substrate were incubated for 3 hr at 30°C.

suppresses the *ptc1-1* growth defect, allowing smaller than wild-type colonies to form at 37°C. *ptc1-1* mutant cells harboring 2μ*CLN2* have normal morphology at 25°C but display an enhanced cell-separation defect and abnormal bud morphology when growing at 37°C (Fig. 9). The isogenic wild-type strain harboring 2μ*CLN2* did not manifest the morphological changes associated with overexpression of *CLN2* from a constitutive promoter (data not shown). These observations suggest that overexpression of *CLN2* can compensate for the mutant's presumed G1 block but cannot suppress the cell-separation defect. Indeed this defect appears to be enhanced by continued growth at high temperature. This cyclin-specific interaction is intriguing since it suggests that *CLN2* may have a distinct role with respect to *PTC1* and further supports a function for *PTC1* in the G1 to S transition.

A *cln2*Δ mutation enhances the growth defect of *ptc1* mutants

To test whether *PTC1* is dependent on *CLN2*, a *cln2*Δ strain was crossed to *ptc1-1* and *ptc1*Δ strains. Double mutants were isolated and tested for growth at a variety of temperatures. *cln2*Δ enhances the growth defects of both *ptc1-1* and *ptc1*Δ mutants (Fig. 10). This finding suggests that *PTC1* functions either upstream of or in parallel to *CLN2*. The terminal phenotype of the *ptc1*Δ *cln2*Δ mutant is consistent with an enhancement of the *ptc1* growth defect. Liquid cultures (BC158-8a) shifted to the non-permissive temperature arrest with a *ptc1*-like multibudded/nucleated phenotype. When maintained at high temperature, the mother cells become very large

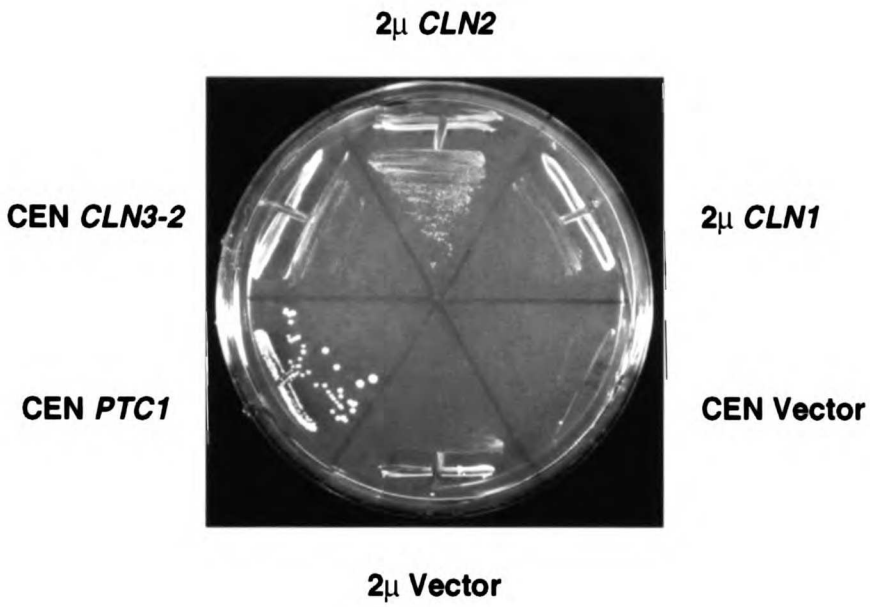

Figure 8. High copy *CLN2* partially suppresses *ptc1-1*^{ts} (BC93-3C). The strains were streaked onto -ura minimal medium and incubated at 37°C for 4 days. Plasmids are: CEN *PTC1*—pBC14; CEN *CLN3-2*—pBC93; 2μ *CLN2*—pBC92; 2μ *CLN1*—pBC91; CEN Vector—pRS316; 2μ Vector—pRS426.

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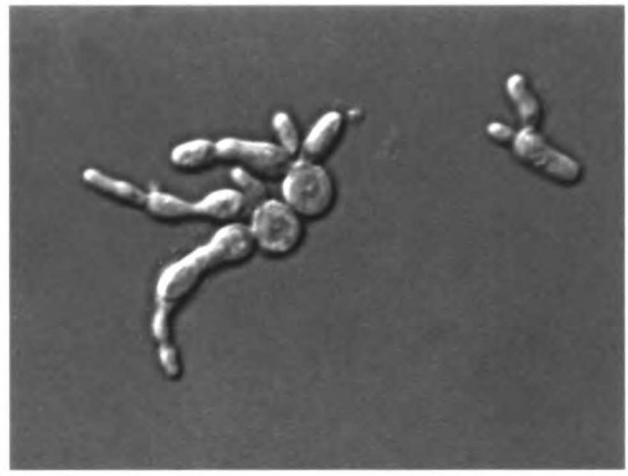

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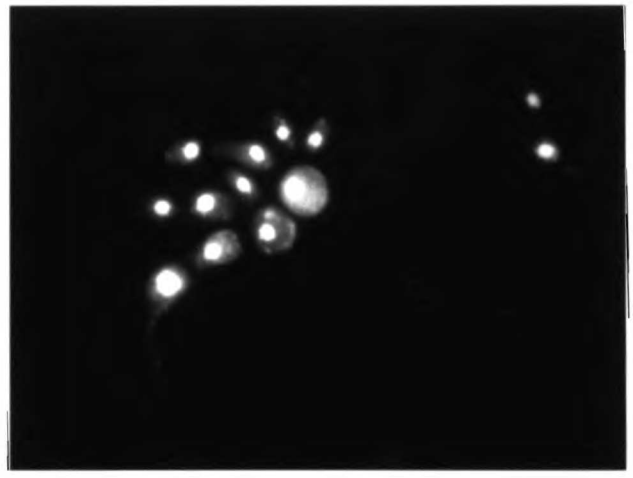

Figure 9. *ptc1-1* cells (BC93-3c) transformed with 2μ *CLN2* (pBC92) grow slowly at 37°C but have abnormal bud morphology and an enhanced septation defect. The culture was grown in -ura, liquid minimal medium at 37°C.

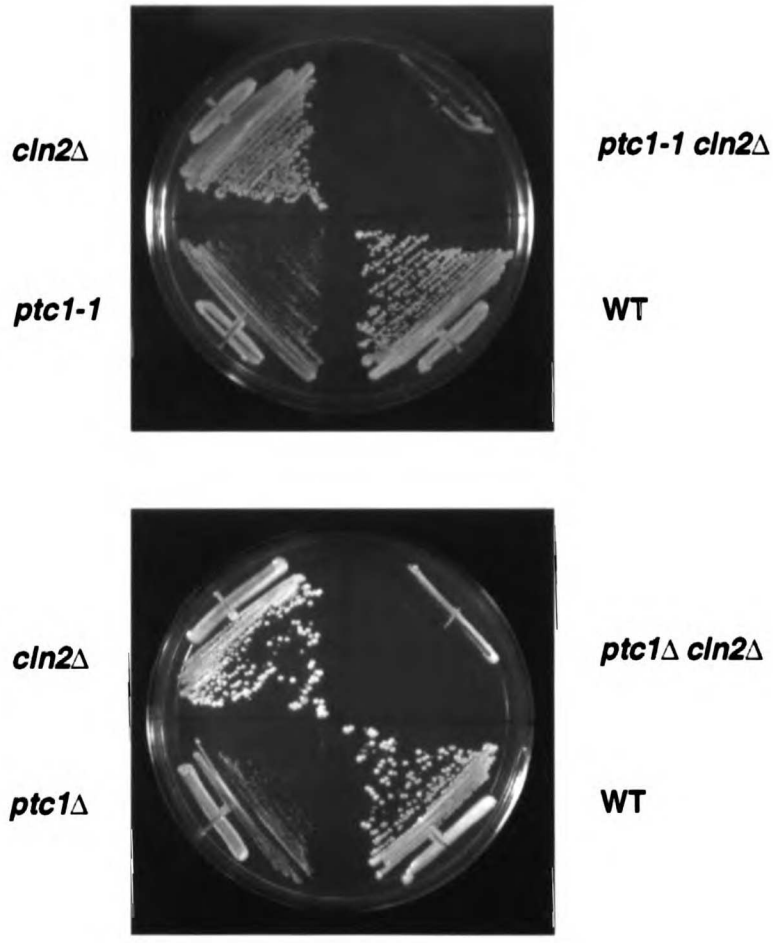

Figure 10. The temperature sensitive growth defects of *ptc1-1* and *ptc1*Δ mutants are enhanced by *cln2* Δ. Tetratype asci from the *ptc1-1* X *cln2*Δ (BC120-14) and the *ptc1*Δ X *cln2*Δ (BC158-8) crosses are shown. Strains were streaked on rich medium and incubated at 33°C for 2 days.

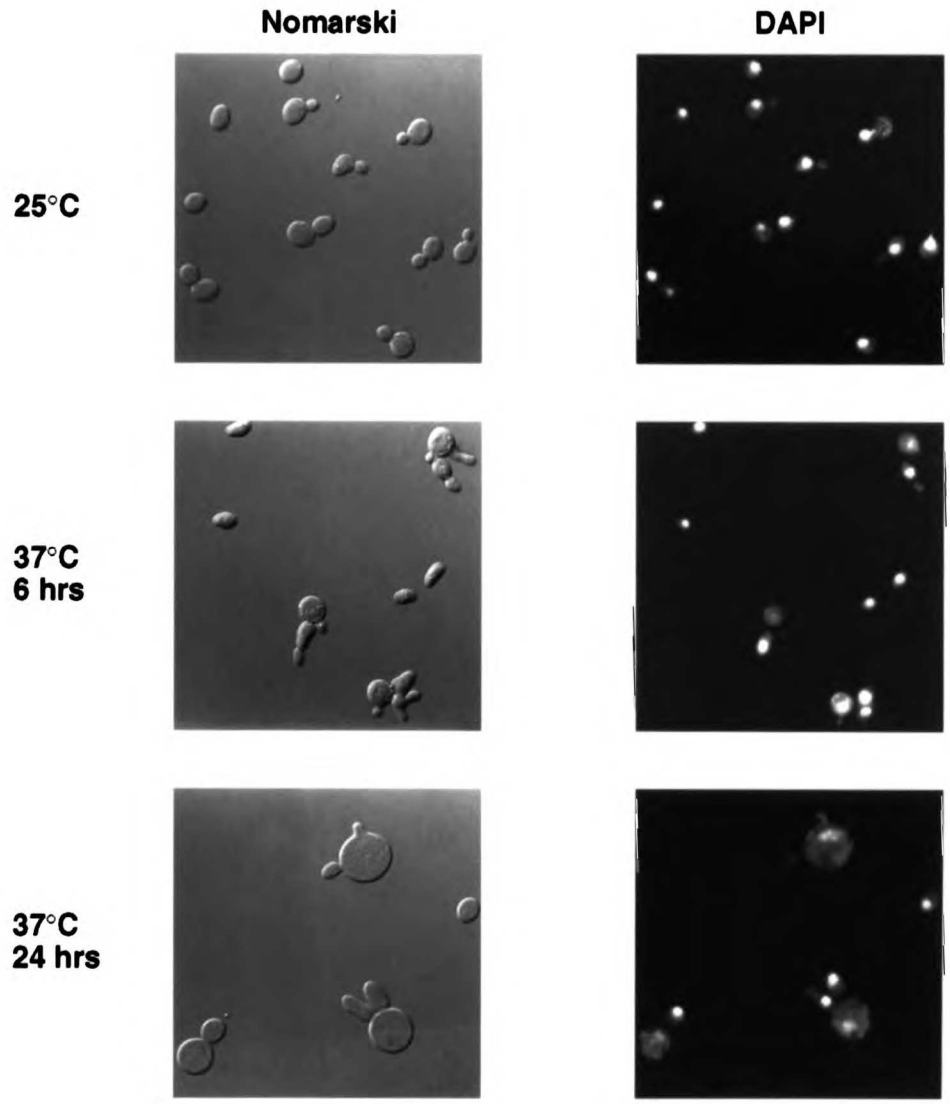

Figure 11. *ptc1*Δ *cln2*Δ cells (BC158-8A) have normal morphology at 25°C. When shifted to 37°C for 6 hours, the cells arrest and many have a pronounced septation defect. The arrested cells continue to grow in size and mother cells have abnormal, lightly staining nuclei. Mid log liquid cultures were grown in liquid rich medium.

(reminiscent of *cdc28* mutants) and have abnormally light staining nuclei (Fig. 11).

ptc1Δ cln1Δ cln2Δ mutants are very sick and *ptc1Δ cln2Δ cln3Δ* mutants are inviable

To investigate further the genetic interactions between *PTC1* and the G1 cyclins, the *ptc1Δ cln2Δ* strain was crossed to an isogenic *cln1Δ cln3Δ* strain and 55 asci were dissected. Double mutants between *ptc1Δ* and each of the *clnΔs* were tested for temperature sensitivity. The *ptc1Δ cln1Δ* mutant had no more severe a phenotype than the *ptc1Δ* mutant, whereas the *ptc1Δ cln3Δ* mutant and the *ptc1Δ cln2Δ* mutant were sicker than the *ptc1Δ* mutant, suggesting that *CLN2* and *CLN3* play greater roles than *CLN1* in the *ptc1Δ* strain. The *ptc1Δ cln1Δ cln3Δ* mutant was comparable to the *ptc1Δ cln3Δ* mutant suggesting that, although impaired for growth, expression of *CLN2* alone was sufficient to drive the cell cycle in a *ptc1Δ* mutant. However, the *ptc1Δ cln1Δ cln2Δ* mutant had a strikingly more severe phenotype than either the *ptc1Δ cln2Δ*, *ptc1Δ cln3Δ*, or *ptc1Δ cln1Δ cln3Δ* mutants. The *ptc1Δ cln1Δ cln2Δ* mutant grew very slowly at room temperature and arrested at 30°C (Fig. 12). These observations show that *CLN3* alone is insufficient to drive G1 progression in a *PTC1* strain and that *CLN1*, *CLN2*, and *PTC1* share a common function. This result is consistent with two interpretations: either Cln3p activity is diminished in a *ptc1Δ* strain or *PTC1* has an essential function parallel to *CLN3*. Since *ptc1Δ cln2Δ cln3Δ* mutants could not be isolated, even when asci were

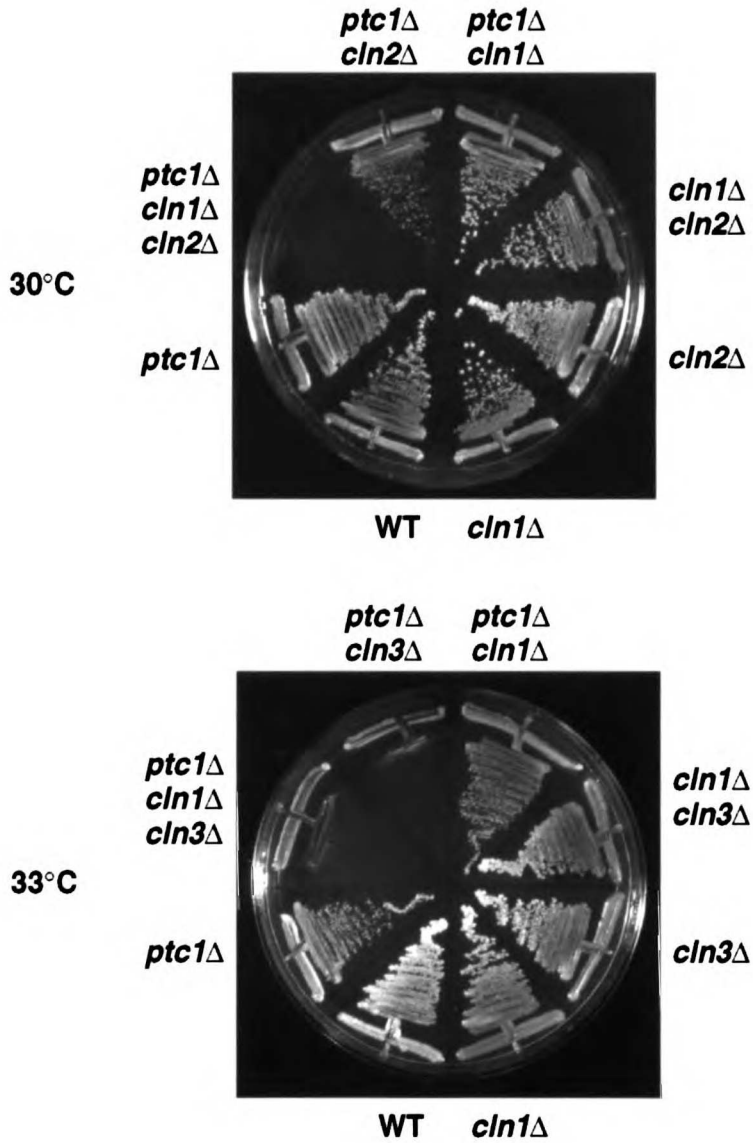

Figure 12. *PTC1* is essential for a *cln1*Δ *cln2*Δ strain to survive at 30°C. Although *ptc1*Δ *cln3*Δ and *ptc1*Δ *cln1*Δ *cln3*Δ strains are viable, they grow very slowly at 33°C. Strains were streaked on rich medium and incubated for 2 days. Strains used are: WT—JO371; *cln1*Δ—BC176-9A; *cln2*Δ—BC176-9B; *cln3*Δ—BC176-5B; *cln1*Δ *cln2*Δ—BC176-3A; *cln1*Δ *cln3*Δ—BC176-2B; *ptc1*Δ—BC176-2A; *ptc1*Δ *cln1*Δ—BC176-6A; *ptc1*Δ *cln2*Δ—BC176-13B; *ptc1*Δ *cln3*Δ—BC176-1D; *ptc1*Δ *cln1*Δ *cln2*Δ—BC176-5D; *ptc1*Δ *cln1*Δ *cln3*Δ—BC176-1A.

dissected on sorbitol slabs (for osmotic stabilization), *CLN1* is incapable of driving the cell cycle without *PTC1*. However, it is important to note that a *cln2*Δ *cln3*Δ mutant grows very slowly in the S288C strain background even after germinating. These results show that *CLN*-deficient strains, like *CDC28* mutants, are sensitive to loss of *PTC1*, strongly suggesting that *PTC1* is required for G1 progression in these mutant strains.

Expression of Cln2p is decreased in *ptc1* mutants

2μ CLN2 can also suppress the *ptc1*Δ suggesting that *CLN2* functions downstream of or in parallel to *PTC1* (data not shown). This observation is consistent with a model in which *PTC1* functions as an upstream positive regulator of G1 cyclin expression. *SIT4*, an essential 2A phosphatase required for progression through G1, is synthetically lethal with *cdc28* and is a positive regulator of G1 cyclin transcription (Arndt *et al.*, 1989; Sutton *et al.*, 1991; Fernandez-Sarabia *et al.*, 1992). Because of the synthetic phenotypes of *PTC1* with various *cln*Δ*s*, and since *CLN2* is a bypass suppressor of *PTC1*, *PTC1* may have a similar function to *SIT4*.

To investigate this hypothesis, isogenic wild-type, *ptc1-1*, and *ptc1*Δ strains were constructed in which the chromosomal copy of *CLN2* was replaced by its HA epitope-tagged counterpart (Tyers *et al.*, 1991). Expression of HA-Cln2p was examined in asynchronous cultures growing in rich medium at 30°C using the 12CA5 monoclonal anti-HA antibody to probe whole-cell extracts by Western blot. Expression of HA-Cln2p is diminished approximately

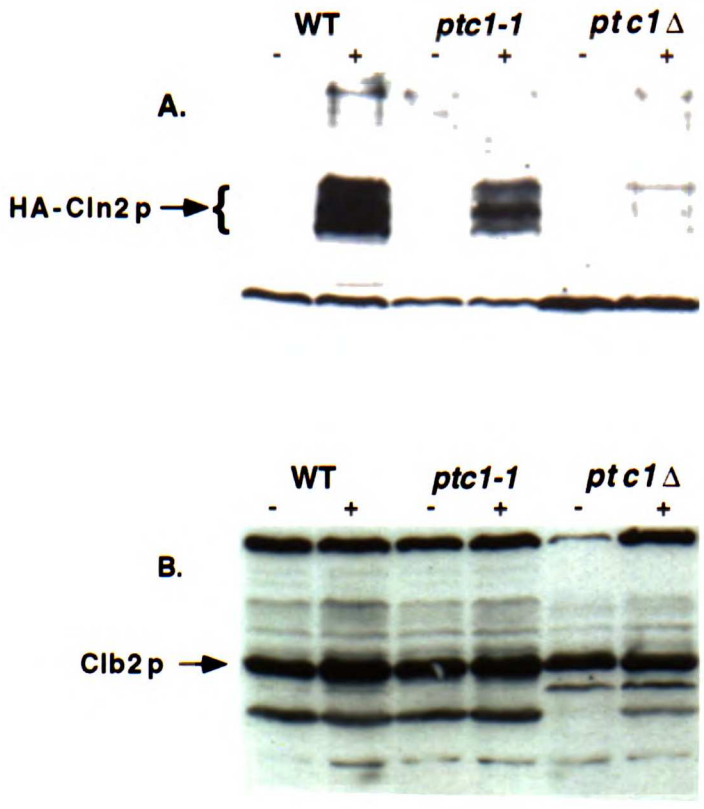

Figure 13 A. Expression of HA-Cln2p is reduced in *ptc1-1* and *ptc1*Δ mutants. Western blot using 12CA5 antisera to probe whole-cell extracts.

B. Expression of Clb2p is normal in *ptc1-1* and *ptc1*Δ mutants. Western blot using Clb2p polyclonal antisera to probe whole-cell extracts.

(+) indicates strains with *HA-CLN2* integrated at the *CLN2* locus.
(-) indicates isogenic strains without the *HA-CLN2* gene replacement.
(⟶) indicates relevant bands.
All extracts were made from asynchronous mid-log liquid cultures growing in rich medium at 30°C. Amount of total protein loaded in each lane is approximately equal as assayed by Ponceau S staining of the transfer membrane. Strains used are: WT—JO14; WT *HA-CLN2*—BC162; *ptc1-1*—BC93-3C; *ptc1-1 HA-CLN2*—BC163; *ptc1*Δ—BC128-5B; *ptc1*Δ *HA-CLN2*—BC164.

five-fold in *ptc1-1* and ten-fold in *ptc1*Δ strains (Fig. 13a). One explanation for this finding is that the *ptc1* cultures are delayed in other parts of the cell cycle; however, the budding index of the *ptc1-1* mutant was comparable to wild-type (% unbudded) and the budding index of the *ptc1*Δ mutant was slightly shifted into G1 (% unbudded). Furthermore, probing the same blot with polyclonal anti-Clb2p antiserum revealed that steady-state Clb2p expression was normal in the *ptc1* mutants (Fig. 13b). This finding suggests that the Cln2p expression defect is specific and is not due to a general problem with cyclin expression. Also if the *ptc1* mutants were delayed in G2/M, then Clb2p levels would be expected to be higher in the mutants relative to wild-type as would the proportion of budded cells.

The effect of Ptc1p on Cln2p protein levels is posttranscriptional

To determine if the diminished Cln2p expression was due to a defect in transcription or mRNA stability, *CLN2* mRNAs were measured. Total RNA was prepared from isogenic wild-type, *cln2*Δ, *ptc1-1*, and *ptc1*Δ asynchronous cultures growing in rich medium at 30°C and probed for steady-state *CLN2* mRNA levels by Northern blot. *CLN2* mRNA levels were comparable to wild-type in the *ptc1* mutants (Fig. 14a). A *TCM1* probe was used as a loading control for the quantity of mRNA since its expression is not subject to cell cycle regulation (Fig. 14b). Therefore the decreased Cln2p expression must be posttranscriptional. This effect could occur either at the level of translation or at the level of protein stability.

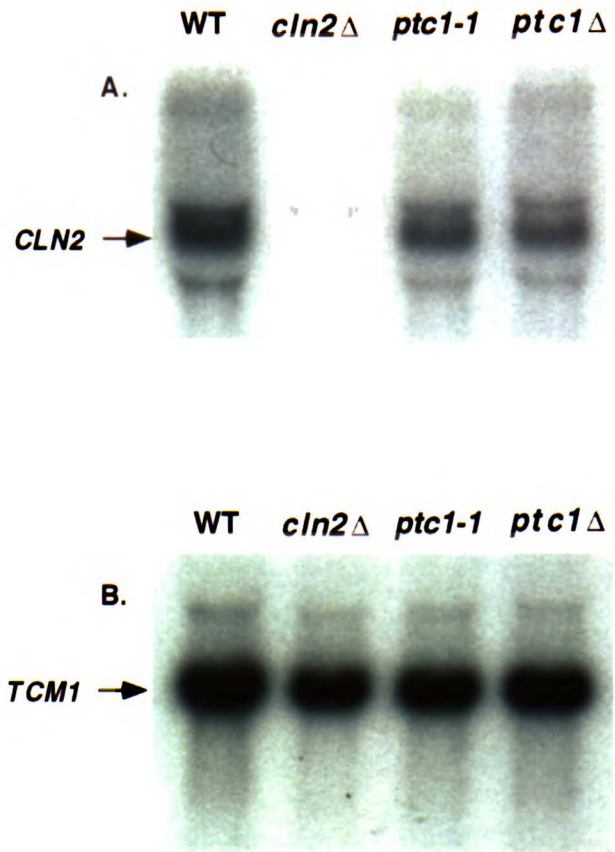

Figure 14 A. *CLN2* RNA levels are normal in *ptc1-1* and *ptc1*Δ mutants. Northern using *CLN2* probe against total RNA.

B. Loading control for "A" using *TCM1* probe against total RNA.

All RNAs were purified from asynchronous mid-log liquid cultures growing in rich medium at 30°C. Strains used are: WT—JO371; *cln2*Δ—BC120-14A; *ptc1-1*—BC93-3C; *ptc1*Δ—BC128-5B. (→) indicates relevant bands.

Cln2p is hyperphosphorylated in *ptc1* strains at non-permissive conditions

Cln2p is a highly unstable protein with a half life of 6-10 minutes (Barral *et al.*, 1995; Salama *et al.*, 1994). Cln2p degradation is triggered by ubiquitination and requires phosphorylation of Cln2p and Cdc28p kinase activity *in vitro* (Deshaies *et al.*, 1995). It is possible that the diminished steady-state levels of Cln2p observed in the *ptc1* mutants is due to decreased stability. Cln2p is observed as a collection of bands on SDS-PAGE, with the slower migrating bands representing phosphorylated states. To determine if the pattern of phosphorylation is dependent on PTC1, isogenic wild-type, *ptc1-1*, and *ptc1*Δ strains containing *HA-CLN2* integrated at the *CLN2* locus were grown at 25°C and then shifted to 37°C. At 25°C, expression of HA-Cln2p in the *ptc1-1* culture was comparable to wild-type; however, at 37°C most of the HA-Cln2p had shifted into the more slowly migrating forms (Fig. 15). Similarly, the levels of HA-Cln2p in the *ptc1*Δ mutant at 25°C were comparable to wild-type but at 37°C only the slowest migrating form of HA-Cln2p was present. Thus at 25°C, *PTC1* is not required for HA-Cln2p expression; however, the HA-Cln2p is hyperphosphorylated in *ptc1* mutants at 37°C. The more rapidly migrating forms of HA-Cln2p, which correspond to the least phosphorylated forms of HA-Cln2p, are dependent on *PTC1*.

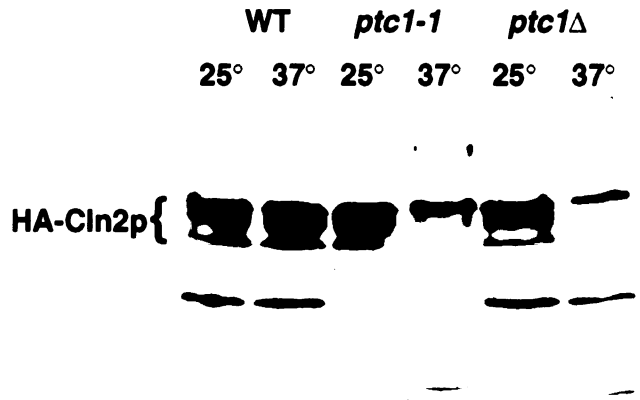

Figure 15. At 25°C expression of HA-Cln2p is normal in *ptc1-1* and *ptc1*Δ cultures. When the cultures are shifted to 37°C for 4 hours, HA-Cln2p is predominantly present in its highest molecular weight form. Approximately equal amounts of total protein are loaded in each lane as assayed by Ponceau S staining of the transfer membrane. Strains used are: WT *HACLN2*—BC162; *ptc1-1 HACLN2*—BC163; and *ptc1*Δ *HACLN2*—BC164.

DISCUSSION

Posttranscriptional expression of *CLN2*

Biosynthesis of G1 cyclins has been thought of as the interplay between an induced transcriptional program which stimulates *CLN* expression (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991) and a proteolytic apparatus which targets Clnps for rapid degradation (Wittenberg *et al.*, 1990; Tyers *et al.*, 1991). From this perspective, cyclin regulation occurs primarily through oscillating transcriptional programs as suggested by the finding that *CLN* expression stimulates *CLB* expression which in turn inhibits *CLN* expression (Amon *et al.*, 1993). The recent finding that Clnps inhibit *CLB*-specific proteolytic machinery demonstrates that both cyclin transcription and degradation are subject to cyclin regulation (Amon *et al.*, 1994).

There are several ways in which Clnps might be positively regulated at the protein level. Clnp protein levels could be subject to translational regulation, which might become important if there were a need to inhibit Clnp function late in G1 when the transcriptional programs operating through SCBs are active. Clnps are not known to be regulated in this manner.

Alternatively, *PTC1* could stimulate Clnp protein levels by affecting Clnp stability. Clnps are highly unstable proteins whose C-termini contain multiple PEST sequences, a motif that is believed to target them for ubiquitination (Rogers *et al.*, 1986; Hadwiger *et al.*, 1989). Truncation of the C-terminus of Cln2p results in a hyper-

stable protein (Hadwiger *et al.*, 1989; Wittenberg *et al.*, 1990). In addition to the PEST sequences, phosphorylation seems to play a part in targeting the Clnps for degradation because *in vitro* Cln2p is phosphorylated by Cdc28p before it is ubiquitinated (Deshaies *et al.*, 1995). Furthermore, mutation of some of the consensus sites for CDKs and MAP kinases in the carboxy terminus of Cln2p increases its half-life ten-fold (C. Wittenberg, personal communication). The observation that Cln2p exists in its slowest molecular weight form in *ptc1* mutants at high temperature suggests that Cln2p is hyperphosphorylated in the absence of Ptc1p and therefore should be less stable (Fig. 16). Cln2p instability would not only reduce its steady state level but may also make the CDK complex unstable as well (unless the formation of the complex somehow masks the PEST sites). Perhaps by keeping Cln2p dephosphorylated, Ptc1p makes not only Cln2p more stable but the CDK complex as well and thereby stimulates CDK activity (Fig. 16). It will be interesting to test whether Cln2p is a substrate for Gst-Ptc1p *in vitro*. If so, then Ptc1p may render Cln2p resistant to destruction through direct dephosphorylation.

Roles of PTC1 in the G1 to S transition

The network of genetic interactions between *PTC1* and the *CLNs* strongly implicates a role for Ptc1p in G1. That Ptc1p is required for normal Cln2p protein levels helps explain some of the genetic interactions but cannot account for the severity of the *ptc1*

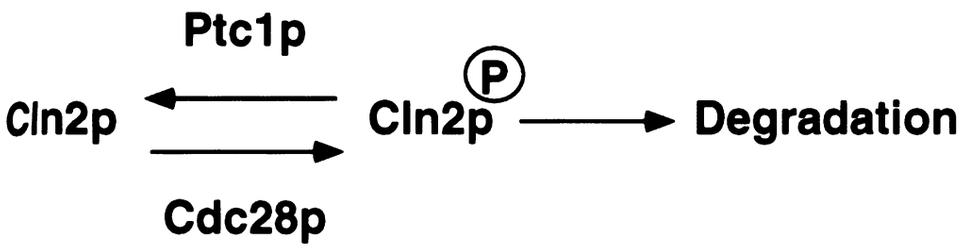

Figure 16. A model for interactions of Ptc1p with Cln2p. Ptc1p increases the steady-state level of Cln2p by stabilizing it through dephosphorylation. This increases the level of active Cdc28p/Cln2p and thereby promotes the G1 to S transition.

mutant phenotype since a *cln2*Δ strain is viable at all temperatures (Fig. 11). Therefore, *PTC1* must have other functions. One interpretation of the synthetic phenotypes of *ptc1*Δ with *cln*Δs is that *PTC1* functions to activate all three *CLNs*. Perhaps Cln1p and Cln3p protein levels are also dependent on Ptc1p. If Ptc1p does not regulate the protein levels of the Cln1p and Cln3p, then *PTC1* must be involved in a process parallel to or downstream of *CLN1* and*CLN2* to explain the strong synthetic phenotypes of the *ptc1*Δ *cln1*Δ *cln2*Δ mutant since the *cln1*Δ *cln2*Δ mutant is viable at all temperatures (Fig. 13).

Interpretation of the *ptc1-1* phenotype

The complex mutant phenotype suggests that *PTC1* may function at two points in the cell cycle: at G1 in daughter cells and at cell-separation in mother cells (Fig. 3). An alternative interpretation is that *PTC1* functions only at cell separation but that this event is required for progression through G1 by daughter cells. Thus there may be a checkpoint at cell separation which must be passed in order for daughter cells to progress through G1. The checkpoint may control passage through START by negatively regulating Clnp protein levels. This model requires that the cell separation checkpoint becomes active only after cytokinesis is complete. Because some mother cells only give rise to one daughter cell, at least some mother cells may also respond to the proposed checkpoint and cease nuclear division. It is important to note that multinucleated cells are never seen in the *ptc1-1* mutant in liquid

culture; thus DNA synthesis is not uncoupled from nuclear segregation.

A role for PTC1 in cell separation

Although cell separation immediately precedes G1, events in G1 could have ramifications much later in the cell cycle. For example, septin proteins are some of the first proteins to be located at the site of bud emergence in G1 (Kim *et al.*, 1991); yet these proteins are necessary for cytokinesis (Byers and Goetsch, 1976). A second connection between cytokinesis and bud emergence is the observation that mutation in one of the septins, *cdc10-10*, is synthetically lethal with *spa2*Δ, a non-essential protein that is localized to the site of bud emergence in G1 (Flescher *et al.*, 1993). Indeed, it has been proposed that polarity establishment and bud emergence are actually the first stages of cytokinesis (Nasmyth, 1993).

The G1 cyclins are also involved in polarity establishment since their absence results in a delay in cortical actin polarization (Lew and Reed, 1993). In addition, the ability to bud in a *cln1*Δ *cln2*Δ strain is dependent on the bud site selection gene *BUD2* since mutations in *BUD2* result in a lethal failure of bud emergence in *cln1*Δ *cln2*Δ strains (Benton et al., 1993; Cvrckova and Nasmyth, 1993; H.-O. Park, personal communication). As a positive regulator of the G1 to S transition, *PTC1* could assist *CLNs* in establishing cytokinetic machinery during G1. A rationalization for the *ptc1* phenotype is suggested: in *PTC1* mutants, failure to establish the

cytokinesis machinery results in a cell-separation defect. Daughter cells do not progress through G1 in *PTC1* mutants because the Clnp protein levels are limited. Some mother cells continue to bud, resulting in the multibudded/multinucleate phenotype.

PTC1 may be involved in polarity establishment in G1 through interactions with the septins. In fact, similar to a *spa2*Δ, *ptc1* mutants enhance the temperature-sensitive growth defects of septin mutants *cdc10-1* and *cdc3-1*. The double mutants not only have a lower restrictive temperature but also have an enhanced morphological septation defect (see Appendix II). It will be interesting to ascertain whether *ptc1* mutations effect cortical actin polarization and whether they are synthetically lethal with mutations in bud emergence genes such as *SPA2*.

Interpretation of the *ptc1*Δ phenotype

PTC1 is required for growth at 37°C but is not essential at lower temperatures. An explanation of why *PTC1* is essential only at high temperature is that *PTC1* has a thermolabile redundant partner which can complement the *ptc1*Δ mutation at low temperature but not at 37°C. Alternatively, *PTC1* might be required only at high temperature and be an essential component of the heat adaptive response. If the first model were correct, one might expect to find a mutation affecting a second phosphatase that is synthetically lethal with *ptc1*⁻. A second phosphatase is also predicted by the increased severity of the *ptc1-1* allele. If the second model were correct, high temperature might induce either

expression or activity of *PTC1*. These models are not mutually exclusive, and *PTC1* could have multiple partners as well as play roles at low and high temperature.

In fact, *PTC1* may have a partner: *PTP2*, which encodes a protein tyrosine phosphatase. *PTP2* was previously identified by degenerate oligo PCR (Guan *et al.*, 1992), but *ptp2*Δ did not have an obvious phenotype, even in combination with *ptp1*Δ (Guan *et al.*, 1991; Guan *et al.*, 1992; James *et al.*, 1992; Ota and Varshavsky, 1992). A synthetic lethal screen searching for the redundant partners of these phosphatases identified *PTC1* (Maeda *et al.*, 1993). In addition to this synthetic phenotype, *PTP2* can complement a *ptc1*Δ mutant when overexpressed from the *GAL* promoter.

Independently, I identified *PTP2* as a dose dependent suppressor of the *ptc1-1* mutant; *PTP2* was the only strong suppressor other than *PTC1* isolated in a 2μ suppressor screen (data not shown). These genetic interactions strongly suggest that *PTP2* and *PTC1* have overlapping functions. What is intriguing about these results is that *PTC1* and *PTP2* belong to different classes of phosphatases with different substrate specificities. It is therefore likely that the targets of *PTC1* and *PTP2* are subject to at least two levels of regulation since it is doubtful that these phosphatases dephosphorylate the same residue. Examples of systems where proteins are phosphorylated at ser, thr, and tyr residues include the CDK/cyclins and the MAP kinases (Anderson *et al.*, 1990; Krek and Nigg, 1991a; Krek and Nigg, 1991b; Norbury *et al.*, 1991). Thus it is possible that *PTC1* regulates either CDKs or MAP kinases through dephosphorylation.

Bud emergence checkpoints: a G2/M role for *PTC1*?

Quite unexpectedly, the diploid formed from the cross between *ptc1*Δ and *cln2*Δ mutants formed eight-spore asci when sporulated (data not shown). These "octads" occur in 1% of all asci in a diploid heterozygous for both *ptc1*Δ and *cln2*Δ. Each octad spore receives a nucleus and most spores are viable, inheriting markers as expected from a 4N parent. Octads have previously been reported in mutants with nuclear migration defects (Kormanec *et al.*, 1991). Interestingly, one class of nuclear migration mutants may be involved in checkpoint regulation during G2/M that ensures that bud emergence has properly occurred (Lew and Reed, 1995; M. von Lohuizen, personal communication). One of these mutants, *gas1*Δ, has a multibudded/multinuclear phenotype similar to *ptc1* mutants (Ram *et al.*, 1995). It will be interesting to determine if *ptc1*Δ *cln2*Δ diploids have additional phenotypes similar to this class of mutants and, if so, whether they are defective in feedback inhibition of nuclear segregation.

Other potential targets

At present, it seems likely that *PTC1* exerts a positive influence in promoting G1 progression through regulation of cyclin expression, but another possibility is that it inhibits a negative growth regulator such as *SWE1* (Booher *et al.*, 1993) or *SIC1* (Nugroho and Mendenhall, 1994). Cdc28p is subject to the same

pattern of phosphorylation that controls activity of the *Schizosaccharomyces pombe* homolog *Cdc2+*. However, the physiological function of the phosphorylation on $CDC28^{Y19}$ is unclear because mutation of this site or its positive and negative regulators, *MIH1* and *SWE1* respectively, are not essential. That *PTC1* might negatively regulate a CDK inhibitor is an exciting and as yet unexplored possibility. It is thought that one function of Cln1p and Cln2p in driving the G1 to S transition is to inhibit Sic1p, thereby allowing Cdc28p to associate with Clb5p and Clb6p (Dirick and Nasmyth, 1995; Schwob *et al.*, 1994). The synthetic phenotype of the *ptc1Δ cln1Δ cln2Δ* mutant could be explained by a failure to inactivate Sic1p. This hypothesis could be directly tested by looking for prolonged association of Cdc28p with Sic1p in *ptc1* mutants. Overexpression of *PTC1* in a *cln1Δ cln2Δ* mutant might also promote START at smaller cell volumes by facilitating association of Cdc28p with Clb5p and Clb6p.

PTC1 could also function to regulate a MAP kinase. PTC1 could negatively regulate a MAP kinase that has an inhibitory effect on progression through START or act in concert with a MAP kinase that stimulates the G1 to S transition. Of the MAP kinases known in yeast, one participates in pheromone response (Marsh *et al.*, 1991), one is induced by heat shock (Kamada *et al.*, 1995), and one responds to high osmolarity (Brewster *et al.*, 1993). At first glance it seems unlikely that *PTC1* would be involved in negative regulation of any of these processes. Investigations of *PTC1* as a MAP kinase regulator are presented later in this dissertation.

Type 2C phosphatastases

Mammalian type 2C phosphatases were first isolated by biochemical fractionation and are distinguished from other phosphatases by their primary amino acid structure and by their biochemical profile (Cohen, 1989; Cohen *et al.*, 1989a; Cohen *et al.*, 1989b). Type 2C phosphatases have monomeric ser/thr protein phosphatase activity which is Mg^{++} dependent and okadaic acid resistant. They share no sequence similarity to other phosphatases, and their closest relative is yeast adenylate cyclase, with which they share 32% identity and 60% homology over a 200 amino acid stretch that lies between the RAS response domain and catalytic domain (Colicelli *et al.*, 1990, Suzuki *et al.*, 1990). It is likely that 2C phosphatases function to regulate the activity of other proteins by dephosphorylation although the physiological substrates for 2C phosphatases are as yet unknown.

PTC1 was also identified in a screen for mutants defective in tRNA processing (Robinson *et al.*, 1994). However, the basis for this defect is obscure since *ptc1* mutants are not defective in the specific biochemical activites required for tRNA processing (Robinson *et al.*, 1994). *PTC1* may regulate tRNA processing indirectly, perhaps by regulating the activity of other genes involved in tRNA processing. Indeed, there are five known genes (*LOS1*, *PTA1*, *RNA1*, *SEN1*, and *STP1*) that affect tRNA splicing without being directly involved in the catalytic events (Robinson *et al.*, 1994).

Thus *PTC1's* role in tRNA splicing could be indirect, possibly through regulation of one of these other genes. These authors also noted the multinucleated/multibudded phenotype of *PTC1* mutants, the inability of *PTC1* mutants to grow on poor carbon sources, and sporulation defects in both heterozygous and homozygous *PTC1* diploid mutants.

S. cerevisiae has at least three 2C phosphatases: *PTC1*, *PTC2*, and *PTC3* (Maeda *et al.*, 1993). *PTC1* was identifed in a synthetic lethal screen with *PTP2* and *PTC2* and *PTC3* were identified by degenerate oligonucleotide PCR and low stringency hybridization (Maeda *et al.*, 1993). *PTC1* and *PTC3* may function as negative regulators of a pathway involved in accommodation to high osmolarity medium (Maeda *et al.*, 1994). Deletion of a presumed osmosensing receptor (*SLN1*) results in constitutive activation of a MAP kinase signal transduction pathway. Constitutive activation of this pathway is lethal to the cell but can be suppressed by overexpression of *PTP2*, *PTC1*, and *PTC3*. Thus *PTP2*, *PTC1*, and *PTC3* may function as negative regulators of the MAP kinase cascade (the *HOG1* pathway) that responds to medium of high osmolarity.

In *Schizosaccharomyces pombe*, a gene encoding a type 2C phosphatase, *ptc1+*, was identified as a dosage suppressor of the temperature-sensitive mutant *swo1-26* (Shiozaki *et al.*, 1994). *swo1+* encodes the heat shock protein hsp90p and was isolated as a second site suppressor of overproduction of Wee1p (Aligue *et al.*, 1994). Wee1p is a kinase that phosphorylates Cdc2p on an inhibitory tyrosine residue; overproduction of Wee1p results in a block in G2 (reviewed in Atherton-Fessler *et al.*, 1993). Although it is not

known why *ptc1*$^+$ can suppress *swo1*$^+$, *ptc1*$^+$ is induced by heat shock, and *ptc1*$^-$ mutant cells are hypersensitive to heat shock. These observations led to the proposal that *ptc1*$^+$ has an important role for survival in response to heat stress. Two additional type 2C phosphatases have been identified in *Schizosaccharomyces pombe* (Shiozaki and Russell, 1995). The interactions of *ptc2*$^+$ and *ptc3*$^+$ with *ptc1*$^+$, their role in osmoregulation, and their interactions with a MAP kinase kinase homolog are discussed in Chapter 2.

CHAPTER 2

PTC1 Negatively Regulates the *HOG1* Pathway, which Functions in Opposition to the *PKC1* Pathway

INTRODUCTION

Life is dangerous. Organisms are subjected to a constantly varying environment. The capacity to respond to changes in external conditions by altering internal biochemical pathways is a major evolutionary advantage. In *S. cerevisiae* there are several examples of adaptive responses to external stimuli. The best studied example is the response of haploids to mating pheromone, resulting in arrest of the cell cycle in G1 and preparation for mating (Marsh *et al.*, 1991). Yeast also responds to fluctuations in temperature, osmolarity, and nutrient conditions through activation of specific signal transduction pathways (Roberts and Fink, 1994; Kamada *et al.*, 1995; Maeda *et al.*, 1995).

The cell senses the environment, e.g. presence of mating pheromone, using surface receptors that transmit this information to the cell interior to effect the appropriate response. Although the external signals can be different, with specialized surface receptors to sense each signal, similar mechanisms are used by different response pathways to propagate the signal within the cell. A common feature of several signal transduction pathways in *S. cerevisiae* is a mitogen activated protein (MAP) kinase cascade consisting of three sequentially ordered protein kinases. MAP kinase cascades are used in signal transduction pathways that sense and respond to mating pheromone, thermal stress, and high osmolarity.

The most extensively studied MAP kinase cascade is found in the pheromone response pathway (Fig. 17). Activation of this pathway is initiated by pheromone binding to a seven-membrane-

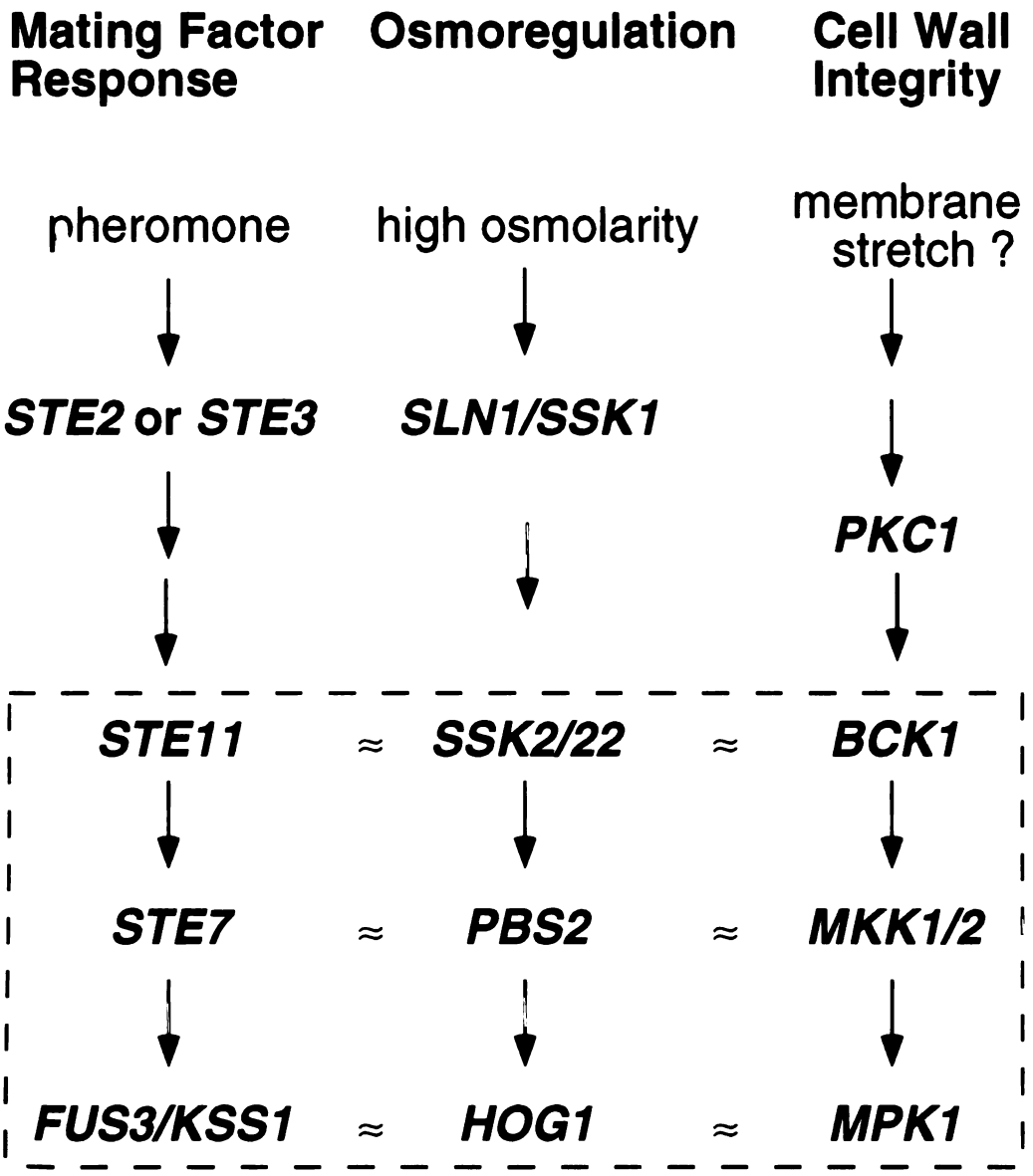

Figure 17. The signal transduction pathways which respond to the presence of mating pheromone, high extracellular osmolarity, and heat stress (possibly changes in membrane stretch) all employ cascades of homologous kinases.

spanning cell surface receptor which causes dissociation of the βγ subunit from the α subunit of a heterotrimeric G protein (Marsh *et al.*, 1991). $G_{\beta\gamma}$ somehow stimulates Ste11p, the first member of the MAP kinase cascade (the MAP kinase kinase kinase or MEKK). Ste11p then phosphorylates and activates Ste7p (the MAP kinase kinase or MEK), which in turn phosphorylates and activates the MAP kinases Fus3p (Neiman, 1993; Errede *et al.*, 1993) and Kss1p (Zhou *et al.*, 1993). The outputs of Fus3p include phosphorylation of Far1p, a CDK inhibitor, and phosphorylation of Ste12p, a transcription factor (Peter *et al.*, 1993; Elion *et al.*, 1993). Kss1p does not phosphorylate Far1p but can activate Ste12p (Peter *et al.*, 1993; Elion *et al.*, 1993). Activation of Far1p and Ste12p through phosphorylation by these kinases results in inhibition of the cell cycle at START and induction of many genes required for mating (Fields and Herskowitz, 1985; Song *et al.*, 1991; Elion *et al.*, 1993; Peter *et al.*, 1993).

This system illustrates two common features of MAP kinase cascades: 1) passage of the signal through three levels of protein kinases and 2) transcription of genes that allow specific adaptation to the stimulus. Two other signal transduction pathways in *S. cerevisiae* reiterate these themes.

The response to high extracellular osmolarity employs a MAP kinase cascade composed of Ssk2p and Ssk22p (two MEKKs), which activate Pbs2p (the MEK), which in turn activates Hog1p (the MAPK, Brewster *et al.*, 1993; Maeda *et al.* 1995) (Fig. 17). The targets of Hog1p are not known; however, transcription of *GPD1*, which encodes the gene for glycerol-6-phosphate dehydrogenase, is induced, resulting in accumulation of intracellular glycerol stores which

helps bring internal osmolarity into balance with the external conditions (Albertyn *et al.*, 1994). In contrast to the pheromone response pathway, a two-component receptor composed of Sln1p and Ssk1p activates this MAP kinase cascade (Maeda *et al.*, 1994; Maeda *et al.*, 1995).

A third pathway may respond to deformation in the cell membrane as a consequence of high temperature by activation of protein kinase C (Pkc1p) (Levin *et al.*, 1990; Kamada *et al.*, 1995), which in turn stimulates a MAP kinase cascade consisting of Bck1p —the MEKK (Lee and Levin, 1992), Mkk1p and Mkk2p—two MEKs (Irie *et al.*, 1993), and Mpk1—the MAPK (Lee *et al.*, 1993a) (Fig. 17). Neither the identity of the cell surface receptor(s) nor the targets of Mpk1p are known. However, recent work indicates that transcription of some genes expressed in late G1 is induced by this pathway (D. Levin, personal communication).

Several observations prompted the exploration of possible interactions between *PTC1* and the *PKC1* pathway. First, the temperature-sensitive growth defect of *ptc1* mutants is partially rescued by addition of 10% sorbitol to the medium (Fig. 23); mutations in the *PKC1* pathway are also rescued by sorbitol (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). Second, overexpression of *CLN2* can partially suppress mutations in the *PKC1* pathway as well as *PTC1* (J. Gray, personal communication). Third, activation of the *PKC1* pathway can stimulate the G1 to S transition (J. Gray, personal communication); *PTC1* also appears to be necessary for progression though G1 (Chapter one of this thesis). Fourth, two phosphatases, *PPZ1* and *PPZ2* (protein phosphatase Z)

when overexpressed can suppress *pkc1*Δ mutations (Lee *et al.*, 1993b). Like mutants in *PPZ1* and *PPZ2* (Lee *et al.*, 1993b), **a**/α *ptc1/ptc1* diploids are sicker than their haploid counterparts (data not shown). Because mutations in *PTC1* and *PKC1* pathway components share these four phenotypes, it is possible that *PTC1* could have a positive role in activation of the *PKC1* pathway or function in parallel to it, as *PPZ1* and *PPZ2* are thought to (Lee *et al.*, 1993b).

The experiments that I will describe were originally designed to test the hypothesis that *PTC1* functions in concert with the *PKC1* pathway. However, the initial results suggested that *PTC1* might negatively regulate the *PKC1* pathway. Further experiments were incompatible with this second hypothesis and suggested that *PTC1* inhibits a pathway that functionally opposes the *PKC1* pathway. Experiments described below indicate that the pathway negatively regulated by *PTC1* is the *SLN1/HOG1* pathway; and hyperactivity of this pathway is incompatible with mutations in the *PKC1* pathway. I propose that the functional opposition of the *PKC1* and *SLN1/HOG1* pathways occurs through activation of incompatible transcriptional programs that regulate cell wall remodeling. In this model, a function of the *PKC1* pathway is to stimulate transcription of *FKS2*, a β–glucan synthase (D. Levin, personal communication). The *SLN1/HOG1* pathway regulates transcription of *EXG1*, a β–glucanase (Jiang *et al.*, 1995). Simultaneous activation of the *PKC1* and *SLN1/HOG1* pathways is incompatible because genes that function to assemble and modify the cell wall are inapropriately expressed.

RESULTS

Overexpression of *PKC1* pathway components enhances the *ptc1* growth defect.

Overexpression of PKC1 pathway components *MPK1*, *BCK1*, or *MKK1* partially rescues the growth defect of *ppz1Δ and ppz2Δ* mutants (Lee *et al.*, 1993b). To test whether the growth defect of *ptc1* mutants is affected by the *PKC1* pathway, gain-of-function alleles of the *PKC1* pathway were transformed into *ptc1* mutant strains. Activation of the *PKC1* pathway not only failed to complement *ptc1* mutants but made the cells sicker (Figs. 18 and 19). The dominant gain-of-function allele, *BCK1-20* (Lee and Levin, 1992), very poorly transformed *ptc1* mutant strains, whereas the isogenic wild-type strain transformed well (Fig. 18). The few *BCK1-20* transformed colonies that grew up failed to restreak or accumulated plasmid-independent suppressors since they were no longer temperature-sensitive after the plasmid was lost by counter selection. *ptc1* mutant strains could be transformed with 2μ*PKC1*, 2μ*MPK1* and 2μ*HCS77*, another potential activator of the PKC1 pathway (Ogas, 1992; J. Gray, personal communication). When streaked for single colonies, *ptc1* strains transformed with these plasmids grew less well than strains carrying vector controls (lower restrictive temperature and smaller colony size) (Fig. 19). 2μ*MPK1* was the strongest enhancer of the *ptc1* temperature-

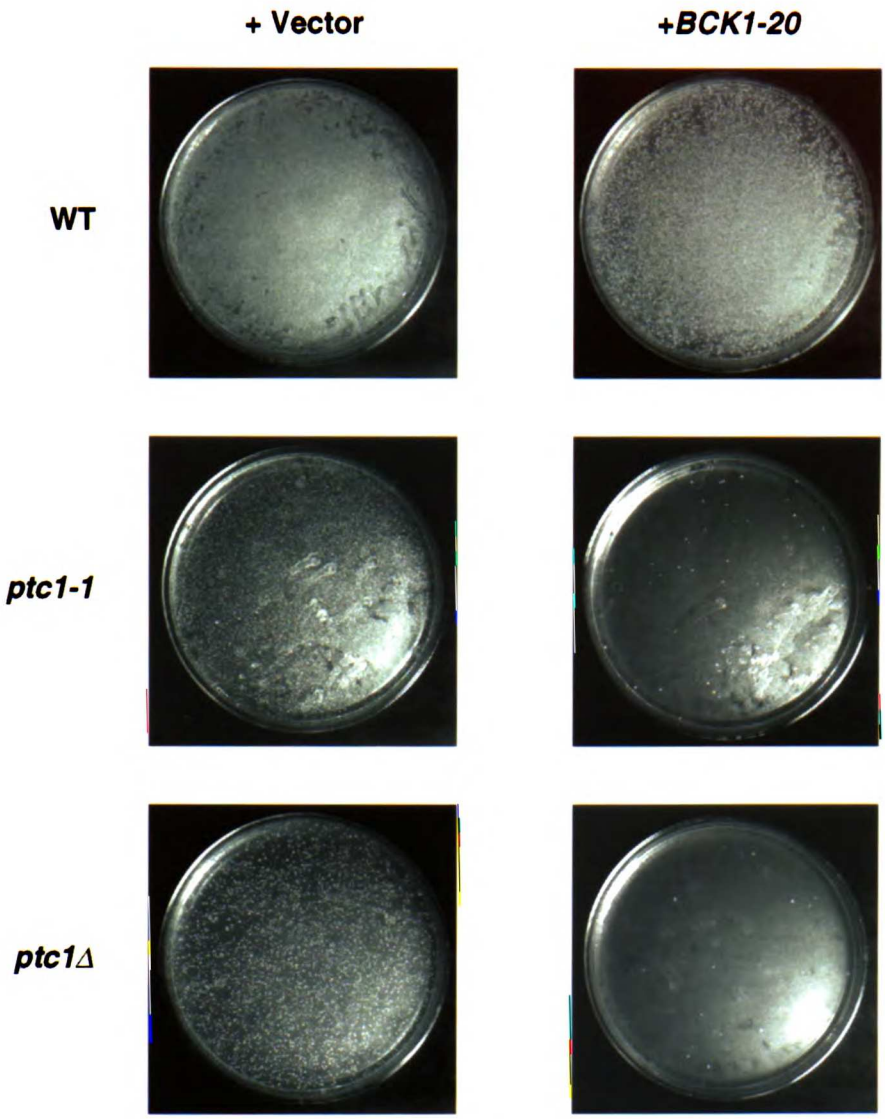

Figure 18. *BCK1-20* poorly transforms *ptc1* mutant strains. Strains are plated on -ura minimal media and incubated at 25°C for 3 days. Strains used are: WT—JO371; *ptc1-1*—BC93-3C; *ptc1Δ*—BC128-5B.

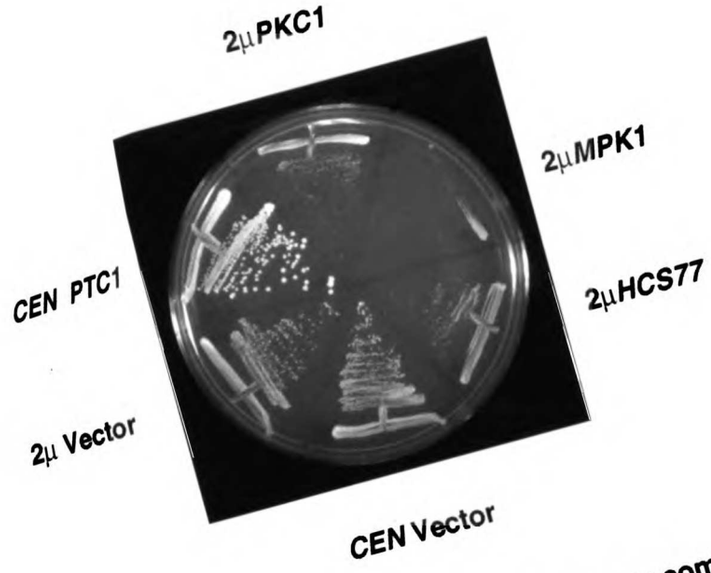

Figure 19. Overexpression of *PKC1* pathway components enhances the temperature-sensitive growth defect of a *ptc1*Δ (BC151) mutant. The transformed strain was streaked on -ura minimal medium and incubated at 30°C for 3 days. Plasmids used are *CEN PTC1*—BC14; 2μ*PKC1*—pBC88; 2μ*MPK1*—pBC90; 2μ*HCS77*—pBC94; CEN Vector—pRS316; 2μ Vector—pRS426.

sensitive growth defect, reducing the restrictive temperature from 37°C to 30°C in a *ptc1*Δ mutant. Wild-type isogenic strains harboring these plasmids grew equally well as strains harboring vector controls at all temperatures and had normal nuclear and budding morphologies. Because gain-of-function alleles of the *PKC1* pathway exacerbate the growth defects of *ptc1* mutants, it seems unlikely that *PTC1* acts like *PPZ1* and *PPZ2*, since mutations in *PPZ1* and *PPZ2* are suppressed by activation of the *PKC1* pathway. Furthermore, in contrast to *PPZ2*, which was isolated as a dosage suppressor of an *mpk1*Δ strain (Lee *et al.*, 1993b), 2μ*PTC1* does not complement the growth defect of *mpk1*Δ mutants (data not shown). These results contrast with the observations that *PTC1* shares some phenotypes in common with the *PKC1* pathway (suppresion by osmotic stabilizers or overexpression of *CLN2*, enhancement of the growth defect of the diploid, and stimulation of the G1 to S transition). Thus the phenotypes shared by *PTC1* and *PKC1* pathway components must have different origins.

Overexpression of *PTC1* suppresses the high-temperature slow growth defect of *BCK1-20*.

One interpretation of the observation that activation of the *PKC1* pathway enhances the growth defects of *ptc1* mutants is that *PTC1* has a role in negatively regulating the *PKC1* pathway. Thus in the absence of *PTC1*, activated alleles of the *PKC1* pathway have a more severe growth defect due to enhanced hyperactivation of the pathway. Since MAP kinase cascades are activated by sequential

phosphorylation, it is likely that phosphatases participate in their negative regulation. *BCK1-20*, a dominant gain-of-function allele, has a slow growth phenotype at 37°C, suggesting that hyperactivation of the *PKC1* pathway may cause temperature sensitivity (Lee and Levin, 1992). Hyperactivation of the pathway through loss of a negative regulator might also cause temperature-sensitivity. Thus the growth defect of *ptc1* mutants may be, in part, due to hyperactivation of the *PKC1* pathway. If Ptc1p functions to down-regulate activation of Mpk1p, then overexpression of Ptc1p might counteract hyperactivation of Mpk1p by Bck1-20p. In support of this hypothesis, 2μ*PTC1* suppresses the slow growth defect at high temperature of *BCK1-20* mutants (data not shown).

*ptc1*Δ strongly enhances the temperature induced lyis of *mpk1*Δ

If the temperature-sensitive growth defect of *ptc1* is due to hyperactivity of the *PKC1* pathway, then loss-of-function alleles in the *PKC1* pathway may partially suppress the *ptc1* phenotype. Because most of the analysis of the *PKC1* pathway has been done in the EG123 strain background, it was necessary to construct a *ptc1*Δ mutant in the isogenic strain background. Since EG123 is *his4*$^-$ the *ptc1*Δ::*HIS3* deletion construct was not usable. A deletion construct was engineered to replace the entire ORF of *PTC1* with *TRP1*. **a** and α EG123 strains were mutagenized with this construct and deletions were confirmed by their temperature-sensitive phenotype, colony PCR, and rescue with various *PTC1* plasmids. In all of the following experiments in this chapter, the *ptc1*Δ mutant referred to is

*ptc1*Δ::*TRP1* in EG123. In addition, the strain used in Fig. 19 is also EG123 *ptc1*Δ::*TRP1* . Deletion of *PTC1* in the EG123 strain background has a more severe phenotype than in the S288C strain background as evidenced by a restrictive temperature of 35°C compared to 37°C for S288C. When the EG123 *ptc1*Δ strain was transformed with *pBCK1-20*, no transformants could be isolated from multiple transformation attempts (data not shown).

The *ptc1*Δ mutant was crossed to a *mpk1*Δ mutant, the heterozygous diploid was sporulated, and 48 asci were dissected. Because *mpk1*Δ is also marked with *TRP1*, non-parental ditypes were used to identify double mutants. Surprisingly, the double mutant had a severe synthetic phenotype, forming very small colonies at 25°C and was inviable at 33°C (Fig. 20). To test if this synthetic interaction was true for other members of the *PKC1* pathway, the *ptc1*Δ strain was crossed to a *bck1*Δ::*URA3* strain. The heterozygous diploid was sporulated and 48 asci were dissected; Ura^+ Trp^+ double mutants were readily isolated. The *ptc1*Δ *bck1*Δ mutant also has a severe growth defect. Because synthetic interactions between two temperature-sensitive mutants can be additive, it was important to assess the terminal phenotype of the double mutant. Liquid cultures of *ptc1*Δ *mpk1*Δ and *ptc1*Δ *bck1*Δ strains were grown at 25°C and shifted to 33°C. Within one hour at the restrictive temperature, *ptc1*Δ *mpk1*Δ and *ptc1*Δ *bck1*Δ cells began to lyse either as unbudded or small-budded cells. By four hours, over 70% of the cells stained with methylene blue, indicating cell death (data not shown). Blue-stained intracellular contents were observed spilling forth from the

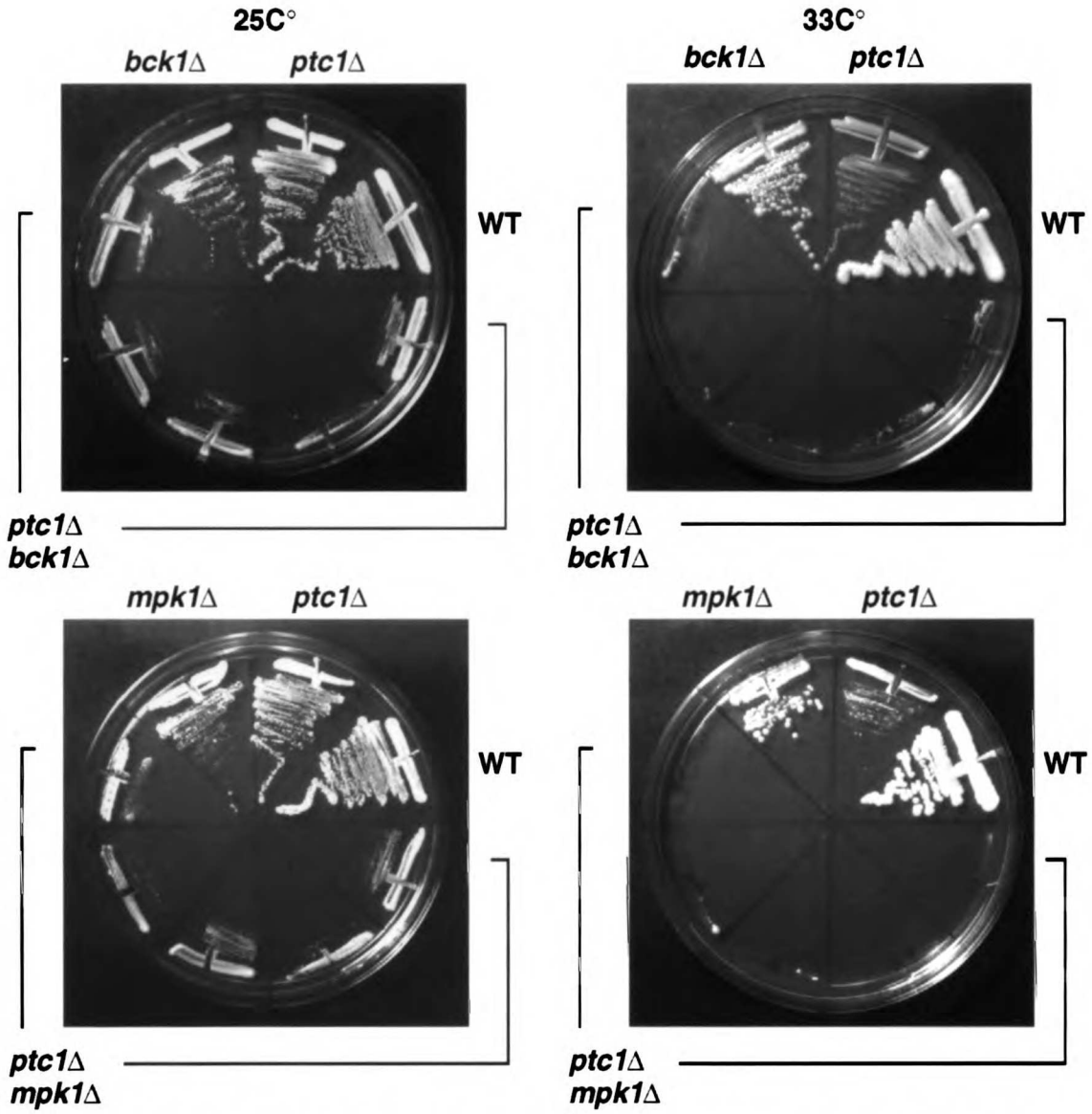

Figure 20. *ptc1* Δ is synthetically sick with *bck1*Δ and *mpk1*Δ. *ptc1*Δ *bck1*Δ and *ptc1*Δ *mpk1*Δ double mutants form small colonies at 25°C and are inviable at 33°C. Five independent isolates of the double mutants are shown. Strains were streaked on rich medium and incubated for 3 days. Strains used are: WT—IH1783; *ptc1* Δ—BC151; *mpk1*Δ—JG217; *bck1*Δ—JG206; *ptc1*Δ *mpk1*Δ—BC155-4B, 4C, 10A, 10D, 15C; *ptc1*Δ *bck1*Δ—BC157-9B, 11A, 13A, 14A, 14B.

bud or projections on the unbudded cells that may represent sites of attempted new growth. Thus deletion of *PTC1* enhances the temperature-sensitive lytic phenotype of *mpk1*Δ and *bck1*Δ mutants, implying that *PTC1* is essential in *PKC1* pathway mutants.

The severe growth defects of *ptc1*Δ *mpk1*Δ and *ptc1*Δ *bck1*Δ double mutants are suppressed by an osmotic stabilizer

Mutations in the *PKC1* pathway are partially suppressed by the addition of 10% sorbitol, which acts as an osmotic stabilizer, to the medium (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). Since *PKC1* pathway mutants lyse at sites of new growth, such as the bud tip in mitotically growing culture and the shmoo tip in pheromone arrested cultures, and because their cell wall is morphologically abnormal, it is thought that sorbitol acts as an osmotic stabilizer, preventing the weakened cell wall from bursting under the cell's intracellular pressure (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). To test if the growth defects of the *ptc1*Δ *mpk1*Δ and *ptc1*Δ *bck1*Δ double mutants were suppressed by the presence of an osmotic stabilizer, the double mutant was streaked onto YEPD+10% sorbitol plates (Fig. 21). The presence of sorbitol greatly reduced the growth defect of both *ptc1*Δ *mpk1*Δ and *ptc1*Δ *bck1*Δ double mutants. Less than 10% of *ptc1*Δ *mpk1*Δ cells stained with methylene blue in sorbitol-supplemented medium growing at 33°C indicating that sorbitol suppresses the lytic defect (data not shown). Control cultures lysed at 33°C as previously described. That the lytic phenotype can be suppressed by an osmotic

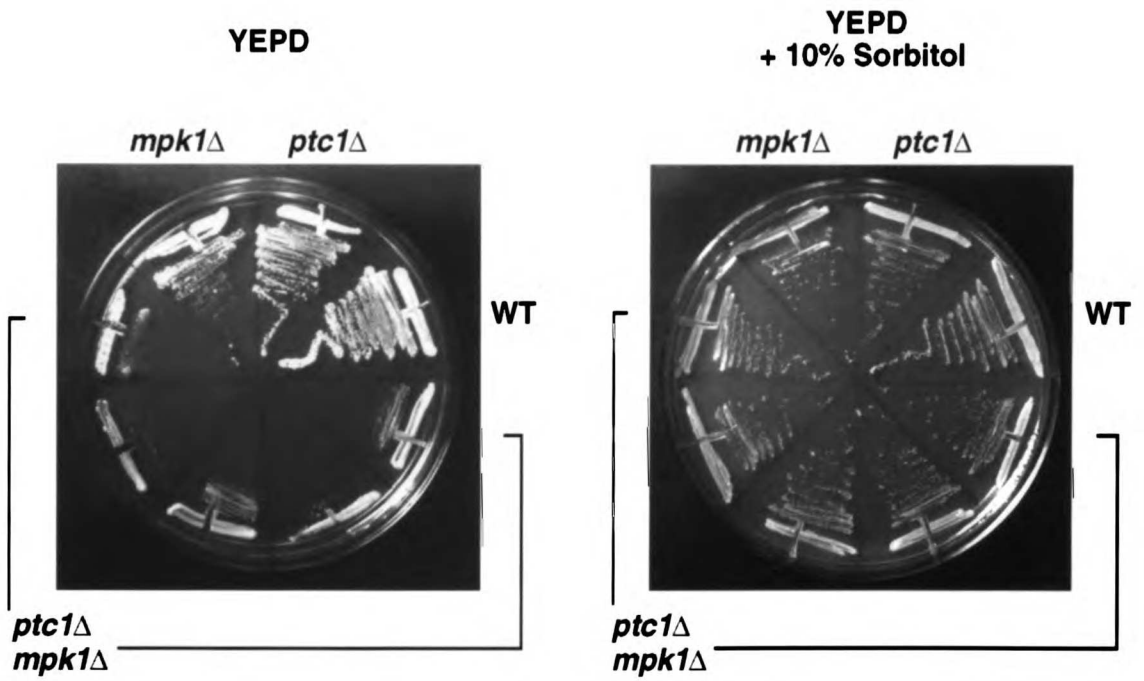

Figure 21. The addition of 10% sorbitol partially rescues the synthetic growth defect of the *ptc1*Δ *mpk1*Δ mutant. Five independent isolates of the double mutant are shown. Strains were streaked on rich medium and incubated at 25°C for 3 days. Strains used are: WT—IH1783; *ptc1*Δ—BC151; *mpk1*Δ—JG217; *ptc1*Δ *mpk1*Δ—BC155-4B, 4C, 10A, 10D, and 15C.

stabilizer suggests that the poor viablity of the double mutants is due to additive effects of *ptc1*Δ and *mpk1*Δ on cell wall integrity.

The synthetic interactions between *PTC1* and *PKC1* pathway deletion mutants, taken together with the findings that gain-of-function alleles of the *PKC1* pathway enhances the growth defect of *PTC1*, indicates that *PTC1* does not negatively regulate the *PKC1* pathway. The synthetic lethal interactions between *ptc1*Δ and *mpk1*Δ might be interpreted as *PTC1* positively regulating a pathway that is redundant with *PKC1*. However, this model is not easily reconciled with the observation that increased expression of *PKC1* pathway components exacerbates the *ptc1* growth defect. I speculated that *PTC1* could negatively regulate another pathway that, when hyperactive, enhances the lytic defect of *mpk1*Δ mutants. In addition, increased activity in the *PKC1* pathway would be incompatible with simultaneous hyperactivity (due to *ptc1*) in the proposed pathway, thereby enhancing the *ptc1* growth defect. Thus it is possible that *PTC1* negatively regulates a pathway whose action opposes that of the *PKC1* pathway.

The severe growth defect of *ptc1*Δ *ptp2*Δ is suppressed by sorbitol.

A hint to the identity of this other pathway came from studying the *ptc1*Δ *ptp2*Δ double mutant, which has a severe temperature-sensitive growth defect. *PTC1* was previously identified in a synthetic lethal screen with *PTP2*, a protein tyrosine phosphatase (Maeda *et al.*, 1993). *ptp2*Δ strains do not have a discernible phenotype but in combination with mutations in *PTC1*

display a severe growth defect. The *ptc1*Δ *ptp2*Δ double mutant was generated by mating the *ptc1*Δ mutant to an isogenic *ptp2*Δ::*LEU2* strain, sporulating the diploid, and dissecting 25 asci to identify 13 Leu^+ Trp^+ segregants. The double mutant forms very small colonies at 25°C and is inviable at 30°C (Fig. 22). Remarkably, this mutant is rescued very well by sorbitol and grows to form small colonies even at 35°C (Fig. 23). The double mutant was examined for lytic defects; however, arrested cultures did not stain with methylene blue and were viable upon return to permissive conditions even after 24 hrs at high temperature. Indeed, the double mutant has a similar morphology to arrested *ptc1* mutants (data not shown). Because the double mutant has a growth arrest phenotype rather than a cell lysis phenotype, it seems that sorbitol was suppressing the mutant by doing something other than supporting a fragile cell wall.

The growth defects of *ptc1*Δ and *ptc1*Δ *ptp2*Δ mutants are partially suppressed by deletion of *HOG1*

Since *HOG1* (high osmolarity glycerol response) is involved in the response to high osmolarity, I reasoned that, if it were hyperactive, cells would overproduce glycerol which might inhibit growth (Brewster *et al.*, 1993; Albertyn *et al.*, 1994). If Ptc1p and Ptp2p negatively regulate the *HOG1* pathway, then Hog1p hyperactivity could contribute to the *ptc1*Δ *ptp2*Δ mutant phenotype. The growth defect of such mutants might be suppressed by sorbitol, which would bring the cells into better osmotic balance with their medium. This hypothesis could also explain why deletion of *PTC1* is

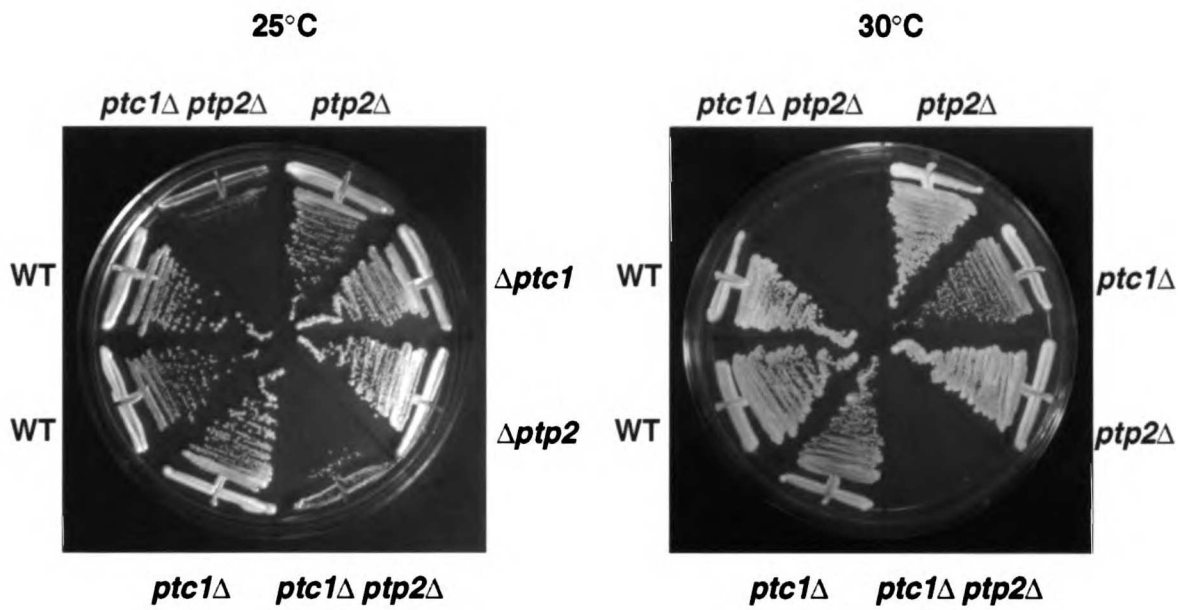

Figure 22. *ptp2*Δ strongly enhances the temperature-sensitive growth defect of a *ptc1*Δ mutant. Double mutants form small colonies at 25°C and are inviable at 30°C. Two tetratype tetrads from a *ptc1*Δ X *ptp2*Δ cross are shown: one on the top half, and one on the bottom half of each plate. Strains were streaked on rich medium and incubated for 3 days.

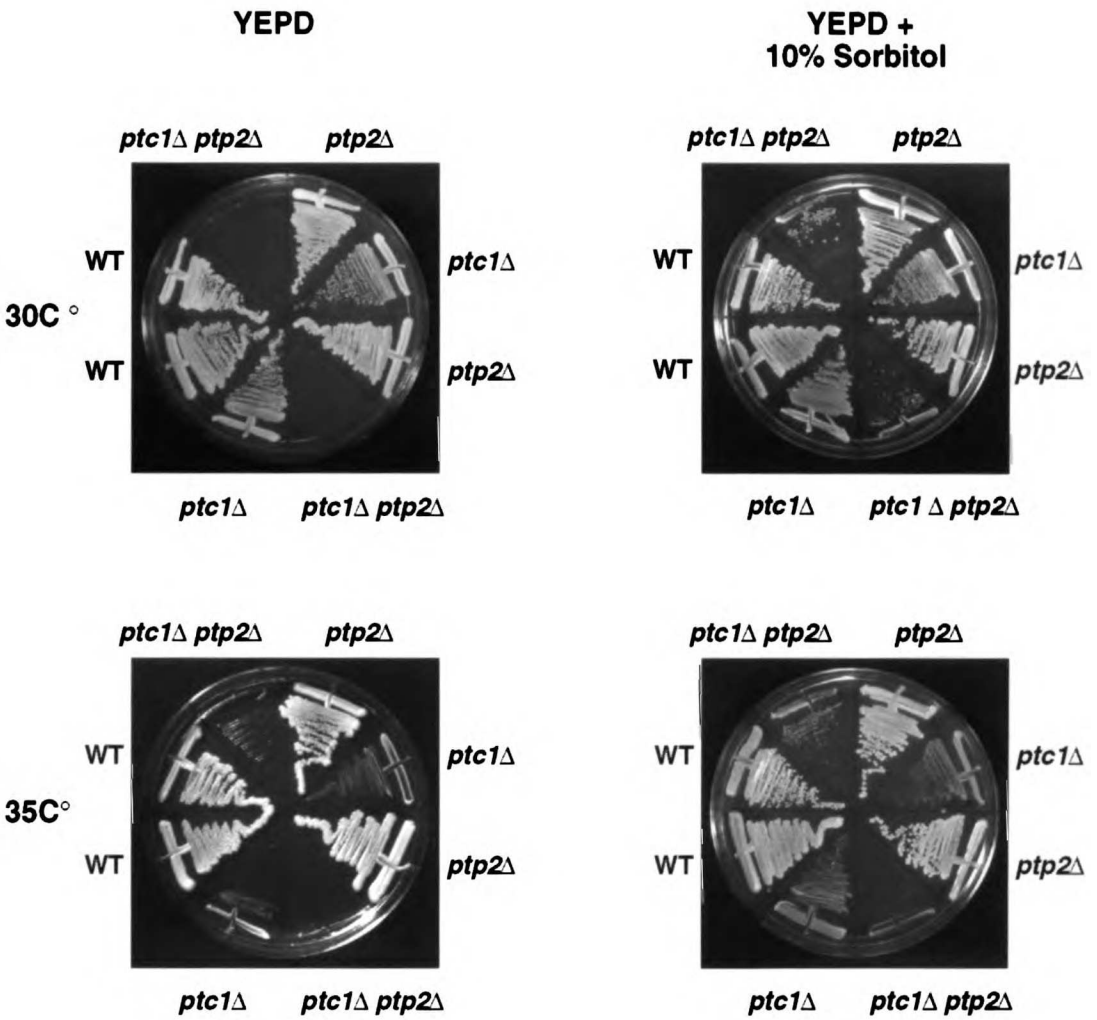

Figure 23. The addition of 10% sorbitol partially rescues the temperature sensitive growth defect of *ptc1*Δ and *ptc1*Δ *ptp2*Δ. Two tetratype tetrads of a *ptc1*Δ X *ptp2*Δ cross are shown: one on the top half (BC156-2), and one on the bottom half (BC156-3), of each plate. Strains were streaked on rich medium and incubated for 3 days.

synthetically lethal with deletion of *MPK1* since the cells would be hyperosmotic and make the fragile cell wall even more susceptible to lysis. A prediction of this model is that the temperature-sensitive phenotypes of *ptc1*Δ and *ptc1*Δ *ptp2*Δ and *ptc1*Δ *mpk1*Δ might be *HOG1* dependent.

To test this hypothesis, a construct replacing 95% of the coding sequence of *HOG1* with the *HisG-URA3-HisG* disruption sequence was made (Alani *et al.*, 1987). Because the *URA3* gene in this sequence is flanked by repeated elements, loss of the *URA3* gene due to mitotic recombination can be selected for by growth on 5-FOA, allowing the deletion strain to be made *ura3*$^-$. It has been demonstrated that *hog1*Δ strains grow as wild-type under most conditions but grow poorly in medium of high osmolarity (Brewster *et al.*, 1993; M. Gustin, personal communication). Haploid wild-type and *ptp2*Δ strains were transformed with the *HOG1* deletion plasmid and Ura$^+$, salt-sensitive mutants were isolated. Deletion of *HOG1* was confirmed by colony PCR.

The *hog1*Δ *ptp2*Δ strain was then mated to the *ptc1*Δ strain, diploids were sporulated, and 32 asci were dissected. 17 *ptc1*Δ *hog1*Δ (Trp$^+$ Ura$^+$) double mutants and 10 *ptc1*Δ *ptp2*Δ *hog1*Δ (Trp$^+$ Leu$^+$ Ura$^+$) triple mutants were identified. Remarkably, the temperature-sensitive growth defect of *ptc1*Δ and the severe growth defect of *ptc1*Δ *ptp2*Δ were partially suppressed by *hog1*Δ, strongly suggesting that the growth defects of *ptc1*Δ and *ptc1*Δ *ptp2*Δ mutants are, in part, due to hyperactivation of *HOG1* (Fig. 24).

The *hog1*::*HisG-URA3-HisG* disruption contruct was also transformed directly into the *ptc1*Δ strain. 12 Ura$^+$ transformants

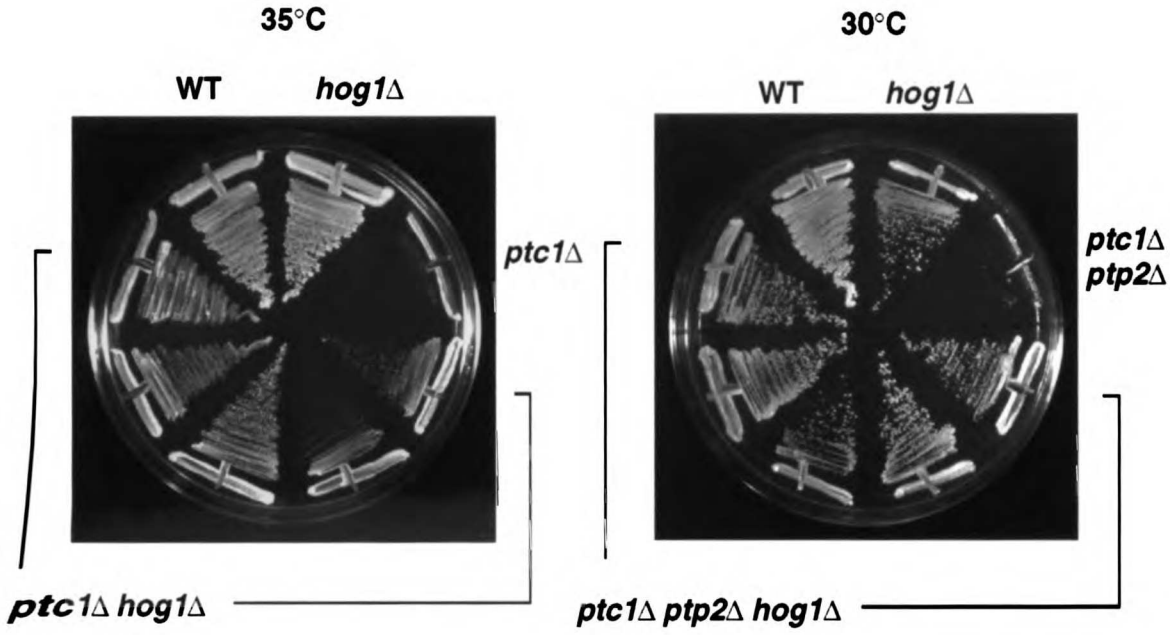

Figure 24. *hog1*Δ partially suppresses the temperature-sensitive growth defect of the *ptc1*Δ mutant and the severe growth defect of the *ptc1*Δ *ptp2*Δ mutant. Five independent isolates of the *ptc1*Δ *hog1*Δ and *ptc1*Δ *ptp2*Δ *hog1*Δ strains are shown. Strains were streaked onto rich medium and incubated for 2 days. Strains used are: WT—IH1783; *hog1*Δ—BC166; *ptc1*Δ—BC151; *ptc1*Δ *hog1*Δ—BC174-2D, 5D, 6C, 10B, 12D; *ptc1*Δ *ptp2*Δ—BC174-7A; *ptc1*Δ *ptp2*Δ *hog1*Δ—BC174-4B, 5C, 8C, 11D, 14D.

were isolated. Four Ura+ transformants are osmo-sensitive and grow at 37°C (data not shown). In contrast, the eight Ura+ transformants that are not osmo-sensitive are inviable at 37°C. Colony PCR confirmed deletion of *HOG1* in the osmo-sensitive transformants. The eight transformants that are not osmo-sensitive have intact chromosomal copies of *HOG1* (confirmed by colony PCR). Therefore, directed deletion of *HOG1* in *ptc1*Δ mutants results in suppression of the *ptc1*- temperature-sensitive phenotype.

The *ptc1*Δ *ptp2*Δ mutant was also transformed directly with the *hog1*::*HisG-URA3-HisG* disruption contruct. Ura+ transformants were isolated and colony PCR was used to assess if *HOG1* had been deleted. Five Ura+, osmo-sensitive, *hog1*- transformants grow well at 30°C (data not shown). 19 Ura+, *HOG1*+ transformants are not osmo-sensitive and grow very poorly. These results confirm that deletion of *HOG1* partially suppresses the severe growth defect of the *ptc1*Δ *ptp2*Δ strain.

Spontaneous second-site suppressors of *ptc1*Δ *ptp2*Δ are osmo-sensitive

As a second test of the hypothesis that the growth defect of *ptc1*Δ *ptp2*Δ is due to increased *SLN1/HOG1* pathway activity, 12 spontaneous revertants of the *ptc1*Δ *ptp2*Δ mutant were isolated. These strains, capable of forming single colonies at the non-permissive temperature (33°C), were tested for salt sensitivity. 12 revertants grew poorly on plates supplemented with either 10% sorbitol or 0.9 M NaCl. In contrast, the growth defect of the isogenic

parent strain was suppressed by these hyperosmolar conditions (data not shown). It is likely that these revertants contain suppressor mutations affecting components of the *SLN1/HOG1* osmosensing pathway since the majority of mutants that are sensitive to high osmolarity thus far isolated correspond to genes in this pathway (Brewster *et al.*, 1993; Maeda *et al.*, 1995; M. Gustin, personal communication). These suppressors are currently being tested for complementation by *SLN1/HOG1* pathway components.

*hog1*Δ partially rescues the lytic defect of *ptc1*Δ *mpk1*Δ

To test if hyperactivation of Hog1p enhances the lytic defect of the *ptc1*Δ *mpk1*Δ double mutant, the *hog1*Δ strain was mated to the *ptc1*Δ *mpk1*Δ strain, the diploid was sporulated, and 28 asci were dissected. Because both *PTC1* and *MPK1* deletions are marked with *TRP1*, Ura$^+$ non-parental ditypes with respect to Trp$^+$ were identified. The six *ptc1*Δ *mpk1*Δ *hog1*Δ triple mutants that were isolated grow much better than the *ptc1*Δ *mpk1*Δ mutant, indicating that the severe growth defect of the *ptc1*Δ *mpk1*Δ mutant is partially *HOG1* dependent (Fig. 25). This result suggests that the proposed Hog1p hyperactivity associated with deletion of *PTC1* accounts for the severe growth defect of *ptc1*Δ *mpk1*Δ mutants.

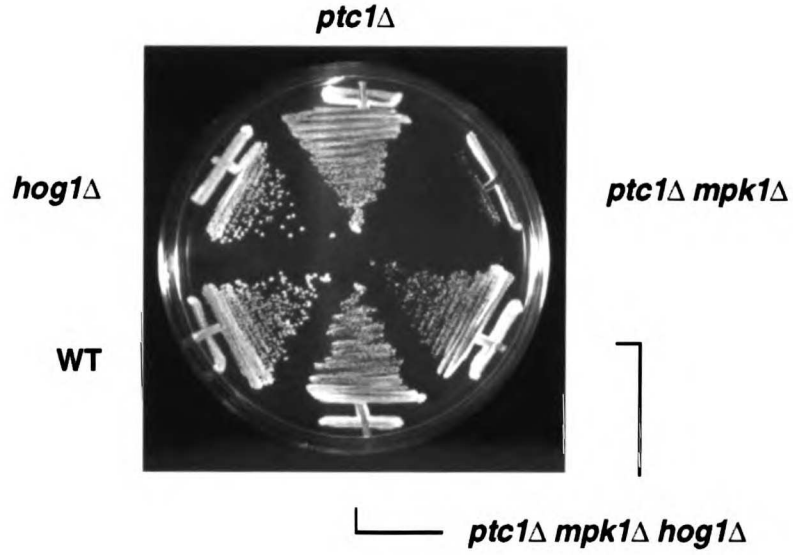

Figure 25. *hog1*Δ partially suppresses the severe growth defect of *ptc1*Δ *mpk1*Δ. Two independent isolates of the triple mutant are shown. The strains were streaked onto rich medium and incubated at 30°C for two days. Strains used are: WT—IH1783; Δ*hog1*—BC166; *ptc1*Δ—BC151; *ptc1*Δ *mpk1*Δ—BC155-4B; *ptc1*Δ *mpk1*Δ *hog1*Δ—BC175-12D, 16A.

Overexpression of *HOG1* enhances the temperature-sensitive growth defect of *mpk1*Δ

One interpretation of the result that deletion of *HOG1* partially suppresses the growth defects of *ptc1*Δ *mpk1*Δ mutants is that hyperactivation of *HOG1* in an *mpk1*Δ strain exacerbates the lytic phenotype. This hypothesis was further tested by transforming *mpk1*Δ mutants with 2μ*HOG1*. The *mpk1*Δ strain transformed with 2μ*HOG1* grows poorly at room temperature relative to the *mpk1*Δ mutant transformed with the vector control, which grows normally at room temperature (data not shown). This observation indicates that high copy *HOG1* exacerbates the growth defect associated with deletion of *MPK1*. In contrast, the isogenic wild type strain transformed with 2μ*HOG1* grows normally at all temperatures.

These results demonstrate that increased Hog1p activity, either due to overexpression or to relieving negative regulation by deletion of phosphatases *PTC1* and *PTP2*, exacerbates the lysis caused by mutations in the *PKC1* pathway. Furthermore, activation of the *PKC1* pathway in strains where Hog1p is also hyperactive results in growth inhibition. Taken together, these results suggest that the *PKC1* pathway and *HOG1* pathway functionally oppose each other.

DISCUSSION

PTC1 and *PTP2* negatively regulate the *HOG1* pathway

Because deletion of *HOG1* partially suppresses the temperature-sensitive phenotype of *ptc1*Δ mutants and the severe growth defect of *ptc1*Δ *ptp2*Δ mutants, it is likely that the growth defects of the mutants are due to hyperactivation of Hog1p. Overexpression of *PTC1* and *PTP2* from the strong *ADH1* promoter rescues the lethality associated with constitutive activation of the *HOG1* pathway as a consequence of deletion of *SLN1* (Maeda *et al.*, 1994). Together, these results strongly demonstrate that a role of *PTC1* and *PTP2* is to negatively regulate the *HOG1* pathway. However, it is not known at what level this negative regulation occurs, and it is important to determine if these phosphatases function by directly dephoshorylating the MAP kinases or by inhibiting other components of the *HOG1* pathway.

In *Schizosaccharomyces pombe* there are three 2C phosphatases thus far identified: *ptc1*$^+$, *ptp2*$^+$, and *ptc3*$^+$ (Shiozaki and Russell, 1995). Δ*ptc1* Δ*ptc2* Δ*ptc3* strains are swollen at 30°C and lyse at 36°C. Δ*ptc1* Δ*ptc3* mutants have abnormal morphologies at 30°C, grow poorly at 36°C, and are Ca^{++} sensitive. The morphological and growth defects of Δ*ptc1* Δ*ptc3* mutants can be suppressed by addition of osmotic stabilizers to the medium. In addition, mutations in a MAP kinase homolog, *wis1*$^+$, suppress the morphological and Ca^{++} sensitive phenotypes of Δ*ptc1* Δ*ptc3*

mutants. *wis1*+ was isolated as dosage dependent inducer of mitosis and is similar to *PBS2* in primary amino acid sequence (Ogden and Fantes, 1986; Warbrick and Fantes, 1991). Δ*wis1* mutants are osmosensitive and temperature-sensitive (Shiozaki and Russell, 1995). One interpretation of these observations is that *Ptc1*+ and *Ptc3*+ counteract the role of *Wis1*+ in the signal transduction response to medium of high osmolarity. In addition to these type 2C phosphatases, two protein tyrosine phosphatases, *pyp1*+ and *pyp2*+ also negatively regulate the osmosensing signal transduction pathway in *Schizosaccharomyces pombe* (Millar *et al.*, 1995). *pyp1*+ and *pyp2*+ were isolated as dosage dependent inhibitors of mitosis (Millar *et al.*, 1992). Conversely, disruption of *pyp1*+ or *pyp2*+ causes premature entry into mitosis and simultaneous disruption of both genes is lethal. Mutations in *wis1*+ and *sty1*+, the *Schizosaccharomyces pombe* homolog of *HOG1*, suppress the *pyp1*- *pyp2*- growth defect (Millar *et al.*, 1995).

Thus *Schizosaccharomyces Pombe* has elements of an osmosensing pathway that are similar to those of the *HOG1* pathway in *S. cerevisiae*. However, one difference between *ptc* mutants in *Schizosaccharomyces pombe* and *S. cerevisiae* is in their terminal phenotypes. Mutation of all three *ptc* + genes in *Schizosaccharomyces pombe* causes a swollen, lytic phenotype, presumably due to excessive accumulation of intracellular glycerol as a result of hyperactivation of Wis1p. In contrast, *ptc1* and *ptc1 ptp2* mutants in *S. cerevisiae* have a multibudded/multinucleated phenotype. This observation suggests that the hyperactivity of Hog1p caused by elimination of the negative regulators Ptc1p and

Ptp2p may affect other processes, possibly cell wall remodeling necessary for cell separation, in addition to glycerol biosynthesis. Furthermore, mutations in *pyp1*$^+$ or *pyp2* cause premature entry into mitosis, and mutations in *wis1*$^+$ and *sty1*$^+$ cause a delay in mitotic initiation. Analogous mitotic roles for *PTC1*, *PTP2*, and *HOG1* have not been demonstrated.

Activation of the *PKC1* pathway is incompatible with simultaneous hyperactivation of Hog1p

The results presented in this chapter indicate a need for balance between the *HOG1* and *PKC1* pathways. Simultaneous activation of both pathways appears to inhibit growth. It seems that when the *PKC1* pathway is activated, the *HOG1* pathway must be inhibited. Perhaps *PKC1* inhibits *HOG1* by positively regulating *PTC1*, which then attenuates the *HOG1* pathway, preventing simultaneous activation of both pathways (Fig. 26). *HOG1* may also be inhibited by induction of *PTP2*. Transcription of *PTP2* is induced by heat shock (Ota and Varshavsky, 1992). It is possible that activation of *PKC1* by heat shock (Kamada *et al.*, 1995) results in induction of *PTP2* transcription which in turn attenuates the *HOG1* pathway. It will be interesting to determine if the heat activated induction of *PTP2* is *PKC1* dependent.

If the enhanced growth defect from overexpression of *PKC1* pathway components in the *ptc1* mutants is due to simultaneous activation of both *PKC1* and *HOG1* pathways, then this phenotype

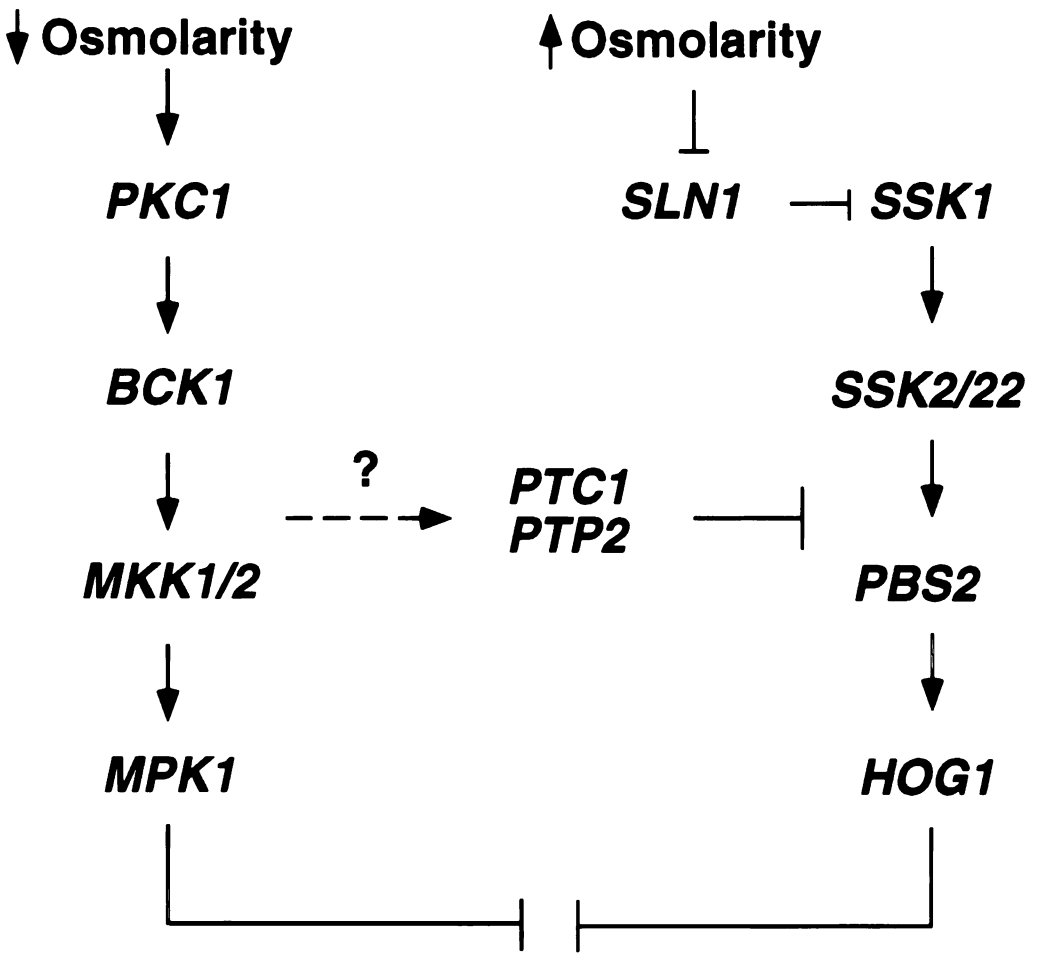

Figure 26. The network of genetic interactions between *PTC1/PTP2* and the *PKC1* and *HOG1* pathways suggests a model wherein *PTC1* functions as a negative regulator of the *HOG1* pathway. The *PKC1* pathway acts in opposition to the *HOG1* pathway such that simultaneous activation of both pathways is incompatible. In addition, activation of the *HOG1* pathway in the absence of the *PKC1* pathway is deleterious, suggesting a need for balance between the pathways. It is possible that the *PKC1* pathway stimulates *PTC1/PTP2* to inhibit the *HOG1* pathway.

should be *HOG1* dependent. The *HOG1* deletion construct was engineered with this in mind: the *URA3* gene used to delete *HOG1* is flanked by repeated elements to allow selection for excision of *URA3* by homologous recombination. The *ptc1*Δ *hog1*Δ strain can then be transformed with *URA3*-marked activated alleles of the *PKC1* pathway. These experiments are in progress.

Opposition suggests cross regulation

That the *HOG1* and *PKC1* pathways have opposing roles is not surprising since the *HOG1* pathway responds to hyperosmotic conditions, and recent evidence indicates that the *PKC1* pathway is activated in response to hypotonic shock (M. Gustin, personal communication). Also some evidence suggests a physiological interplay between these pathways. When cells are subjected to hypertonic shock, the *PKC1* pathway is induced transiently. It is hypothesized that the *PKC1* pathway responds to membrane stretch (Kamada *et al.*, 1995); in hyperosmotic conditions cells decrease their volume by half (Klis, 1994) thereby activating *PKC1*. However, this activation is transient—consistent with the proposed need for the *PKC1* pathway to be attenuated when the *HOG1* pathway is induced. Also consistent with the idea that the *PKC1* pathway is turned down when *HOG1* is active is the observation that growth in high osmolarity inhibits heat shock induction of Mpk1p kinase activity (D. Levin, personal communication). This inhibition occurs independently of *HOG1*, suggesting that some signal in response to

high osmolarity inhibits the *PKC1* pathway but that this signal is not transmitted through *HOG1*.

Recently, a potential negative regulator of the *PKC1* pathway was identified as a dosage suppressor of $Mkk1^{P386}$, a temperature-sensitive gain-of-function mutation (K. Irie, personal communication). The suppressor is *MSG5*, a previously identifed tyrosine phosphatase that is involved in adaptation to pheromone (Doi *et al.*, 1994). When yeast cells are exposed to mating pheromone, they arrest the cell cycle at START in preparation for mating. If mating does not occur, cells exposed to phermone eventually resume the cell cycle (Marsh *et al.*, 1991). Deletion of *MSG5* diminishes the ability to recover from pheromone arrest, and overexpression of *MSG5* accelerates the recovery response. Overexpresion of Msg5p can also inhibit hyperactivation of the mating pheromone response pathway, presumably by dephosphorylating Fus3p (Doi *et al.*, 1994). Because of its positive role in adaptation to pheromone, and since it can suppress gain-of-function mutations in some members of the *PKC1* pathway, *MSG5* might assist in attenuating the *PKC1* pathway during hypertonic conditions. As negative regulators of MAP kinases, phosphatases are excellent candidates for coordinating activity between different signal transduction pathways. Thus phosphatases could be regulated by one pathway to, in turn, coregulate another pathway.

Hyperactivation of Hog1p enhances the lytic defects of mutations in *PKC1* pathway components

Hyperactivation of the *HOG1* pathway in the absence of *PKC1* pathway components results in cell lysis, demonstrating that the basal level of *PKC1* activity is essential when *HOG1* is active. Thus although the *HOG1* pathway may down-regulate *the* PKC1 pathway it must not completely inhibit it. The enhanced lysis observed in the *ptc1*Δ *mpk1*Δ mutants may be a consequence of increased glycerol production due to hyperactive Hog1p. The increased level of glycerol would increase internal osmolarity and render the cell, which already has a fragile cell wall due to deletion of *MPK1*, more susceptible to lysis.

The observation that simultaneous activation of both *PKC1* and *HOG1* pathways inhibits growth is more difficult to rationalize. Why should activation of a pathway that presumably helps strengthen the cell wall in combination with activation of the hyperosmotic response pathway make a cell sicker? I believe the answer lies within the complex nature of transcriptional programs activated by both pathways. Recent work suggests that the *PKC1* and *HOG1* pathways regulate cell wall remodeling.

The cell wall is essential for yeast to survive in a varied environment; however, it is by no means a static structure. All cell cycle events from bud emergence to isotropic growth to cell separation involve radically changing the cell wall, a delicate process since improper execution results in lysis. Thus, the cell

wall must simultaneously be resilient and plastic, responding to precise cell cycle regulation.

Although, as discussed in the introduction to Chapter One, the budding, nuclear, and spindle pole body cycles can be disjoined by specific mutations, during physiological growth these cycles are precisely coordinated. This regulation ensures that the cell wall modifications that result in bud growth are coordinated with the nuclear cycle. I have presented evidence that Ptc1p is a positive regulator of the G1 to S transition, possibly by stabilizing Cln2p (Chapter One of this thesis). I have also shown that *PTC1* in conjunction with *PTP2* negatively regulates the *HOG1* pathway and have demonstrated a need for coordination of *PKC1* and *HOG1* pathway activity. I believe that these two functions of *PTC1*, stimulation of the G1 to S transition and negative regulation of the *HOG1* pathway, help coordinate cell cycle progression with cell wall biosynthesis. I will present several lines of evidence that suggest connections between the *PKC1* and *HOG1* pathways and cell wall remodeling; and propose that the function of *PTC1* is to coordinate multiple biochemical pathways that are essential for cell division.

Connections between the PKC1 pathway and cell wall biogenesis

Mutations in the *PKC1* pathway cause cells to lyse at sites of new growth at high temperature—small buds in mitotic growth (Torres *et al.*, 1991; Lee and Levin, 1992; Paravicini *et al.*, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993a; Shimuzu *et al.*, 1994) and shmoo tips in the presence of pheromone (J. Gray, personal communication).

Electron microscopic analysis of a *pkc1*Δ strain growing in osmotically stabilized media shows that the cell walls of *pkc1*Δ mutants are abnormally dark compared to those of wild-type cells, with the darkest areas corresponding to the small bud tip (Paravicini *et al.*, 1992). The role of *PKC1* in cell wall integrity is inferred by the correlation of the lytic phenotype with the morphological abnormalities of the cell wall. The *PKC1* pathway is also involved in the G1 to S transition and appears to activate late G1 transcripts (J. Gray, personal communication). That the *PKC1* pathway is involved in both cell wall integrity and progression through G1 suggests that its function may be to coordinate the G1 to S transition with cell wall modification. A prediction of this model is that the *PKC1* pathway postively regulates transcripts necessary for both cell cycle regulation and cell wall biogenesis. The *PKC1* pathway would also be expected to interact genetically with genes involved in progression through G1 and cell wall biosynthesis.

Cell wall composition and identification of cell wall biosynthetic genes

The cell wall is a bilayered structure consisting of a fibrillar outer layer and an amorphous inner layer (reviewed in Klis, 1994). Mannoproteins make up much of the outer layer and are thought to limit the permeability of the cell wall whereas β-glucans, which make up the inner layer, provide structural support. The inner layer is composed of 80% (1→3) *β*–glucans, glucose homopolymers of roughly 1500 residues, and 20% (1→6) *β*–glucans, which are 140-200

residues long and composed primarily of (1→6) but also some (1→6), (1→3) triple linkages. Some of the (1→3) β–glucans are cross-linked to chitin, which may play a part in enhancing rigidity (Hartland *et al.*, 1994).

Several lines of evidence indicate that a function of the *PKC1* pathway is to regulate β-glucan synthesis. The total glucan content of the cell wall in *PKC1* pathway mutants is reduced by approximately 30%, suggesting that a role for the *PKC1* pathway in cell wall integrity is to promote β-glucan synthesis (Roemer *et al.*, 1994; Shimizu *et al.*, 1994). In addition, a general screen for cell wall biosynthetic components identified mutants that are calcoflour white hypersensitive (Ram *et al.*, 1994); some of these mutations interact with the *PKC1* pathway (Garrett-Engle *et al.*, 1995). Calcoflour white is a general inhibitor of cell wall assembly; therefore, mutants defective in cell wall biosynthesis can be hypersensitive to the drug. Two of the *CWH* mutants are specifically defective for (1→3) β–glucan synthesis. *CWH52* corresponds to *GAS1* (Nuoffler *et al.*, 1991; Vai *et al.*, 1991), and *CWH53* is a novel 16 membrane spanning protein (Ram *et al.*, 1995). Transcription of both genes is induced late in G1; certain alleles of each gene, when exposed to lethal concentrations of calcoflour, arrest with a small bud in contrast to wild type cells which arrest heterogeneously. These observations suggest that (1→3) β–glucan has a role during bud growth and may be regulated by the *PKC1* pathway, which arrests at the same point in the cell cycle (Ram *et al.*, 1995).

CWH53 was also identified in a synthetic lethal screen with calcineurin (*cna1* *cna2* or *cnb1*) and in a screen for mutants

hypersensitive to the calcineurin inhibitors FK506 and CsA (Eng et al., 1994; Douglas *et al.*, 1994; Garrett-Engle *et al.*, 1995). Additional mutants that are colethal with calcineurin include *PKC1* pathway components (Garrett-Engle *et al.*, 1995). Furthermore, mutations in the *PKC1* pathway are synthetically lethal with mutations in *CWH53*. *FKS2* is a 16 membrane spanning protein that was identified by low stringency hybridization with *CWH53*; its transcription is calcineurin dependent and mutations in *FKS2* are synthetically lethal with mutations in *CWH53* (Douglas *et al.*, 1994).

FKS2 transcription is *PKC1* dependent (D. Levin, personal communication) suggesting the following explanation for these disparate observations. *CWH53* and *FKS2* are subunits of (1→3) β–glucan synthase; transcription of *FKS2* is regulated by *PKC1* and by calcineurin. Mutations in calcineurin and *PKC1* are synthetically lethal with mutations in *CWH53* since at least one of the two β-glucan synthases are required for viability. Thus one function of the *PKC1* pathway is to produce (1→3) β–glucans through transcription of *FKS2*.

Et tu *HOG1*?

β-glucan synthesis alone cannot account for the dramatic changes that occur during budding. β-glucans are extensively cross-linked to each other and to chitin; therefore, enzymatic machinery to modify this interwoven structure must exist. One group of candidates to accomplish this task is the β-glucanases. *EXG1* encodes a cell wall exoglucanase/transglycosylase thought to be

involved in (1→6) β–glucan integrity (Vazquez de Aldana *et al.*, 1991; Larriba et al., 1993). Deletion of *EXG1* results in increased levels of (1→6) β–glucans; in contrast, overexpression of *EXG1* results in decreased levels of (1→6) β–glucans (Jiang *et al.*, 1995). Interestingly, overexpression of *PBS2* also results in decreased levels of (1→6) β–glucans and increased transcription of *EXG1*. Moreover, overexpression of *PTC1* has the opposite effect, causing decreased transcription of *EXG1* and increased levels of (1→6) β–glucans. Thus one target of the *HOG1* pathway's transcriptional program is *EXG1*, an enzyme that helps regulate levels of (1→6) β–glucan in the cell wall.

These observations prompt the hypothesis that the synthetic interaction between *PKC1* pathway overexpression and *ptc1* mutants is due to an imbalance in cell wall biosynthesis. Overexpressed *PKC1* results in increased expression of *FKS2* and presumably elevated levels of (1→3) β–glucan, whereas mutations in *PTC1* activate *HOG1*, causing increased *EXG1* transcription and decreased levels of (1→6) β–glucans (Figure 27). In this model, increased expression of (1→3) β–glucans is incompatible with decreased levels of (1→6) β–glucan, thereby inhibiting cell growth. If true, then the synthetic interaction between *PKC1* pathway overexpession and *ptc1* mutants may be *EXG1* dependent. However, *EXG1* is likely to have redundant partners: six β–glucanases have been purified, and it is not clear if *EXG1* corresponds to any of them (Hien and Fleet, 1983). Thus, it will be important to determine if other glucanases are also regulated by *HOG1* and *PTC1*.

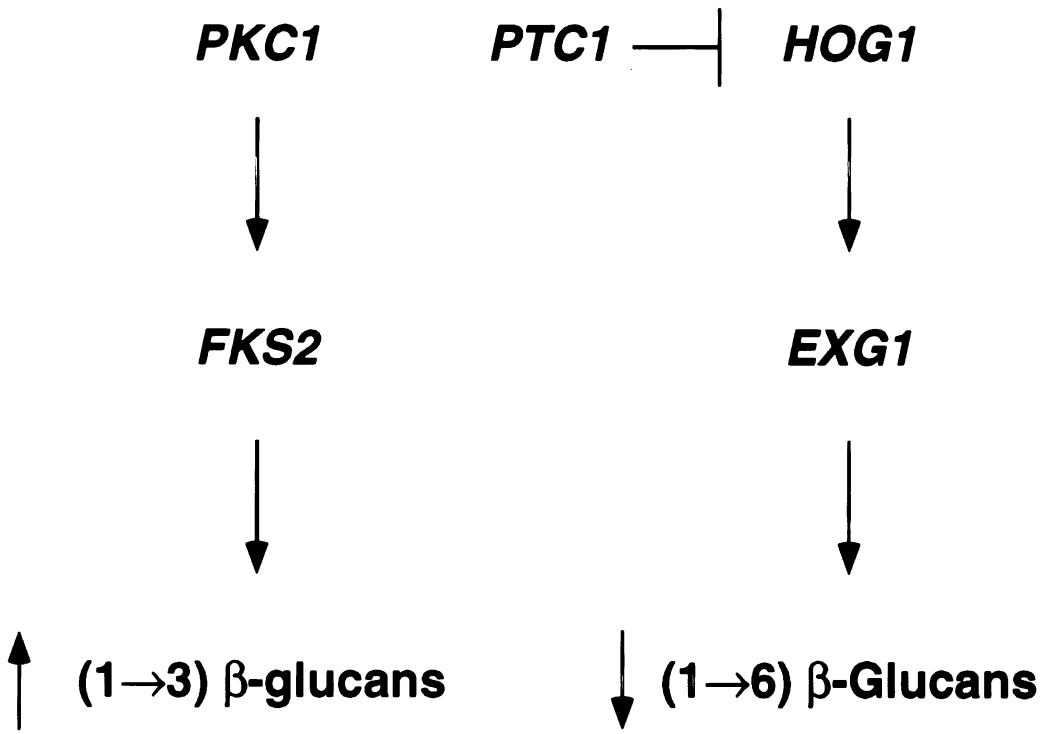

Figure 27. The *PKC1* and *HOG1* pathways regulate transcription of genes that are involved in cell wall remodeling. The inhibition of cell growth that results from simultaneous activation of both pathways might be caused by an imbalance in the levels of cell wall structural polysaccharides.

Bigger picture

The network of interactions between the *PKC1* and *HOG1* pathways can be rationalized if the pathways govern opposing aspects of cell wall biosynthesis. It is likely that these pathways have been adapted to function both during vegetative growth and periods of osmotic stress. These pathways must also be interlinked with the cell cycle machinery to allow proper synchronization of cell wall biosynthesis with the nuclear and mitotic cycles. These pathways may impinge upon the cell cycle during times of osmotic stress. For example, when subjected to hypertonic shock, yeast cells transiently arrest in G1 (M. Gustin, personal communication). Perhaps cell wall modification (budding) during hyperosmotic conditions is harmful. It would thus be beneficial for the cell to arrest in G1 and build up glycerol stores before commencing the next cycle. Examination of *CLNs* during hypertonic treatment shows that their protein levels rapidly decline (M. Peter, personal communication). Taken together, this observation with the result that Ptc1p is required for Cln2p protein levels prompts the hypothesis that Hog1p may negatively regulate the Clnps by decreasing their stability through phosphorylation. Indeed, the Clnps have MAP kinase consensus sites that may be important for targetting them for degradation. Thus, the decreased levels in Cln2p in *ptc1* mutants may be *HOG1* dependent. Experiments to test this hypothesis are underway.

PTC1 may function to synchronize the activity of the *PKC1*, *HOG1*, and *CDC28* pathways (Fig. 28). In this model, Ptc1p increases

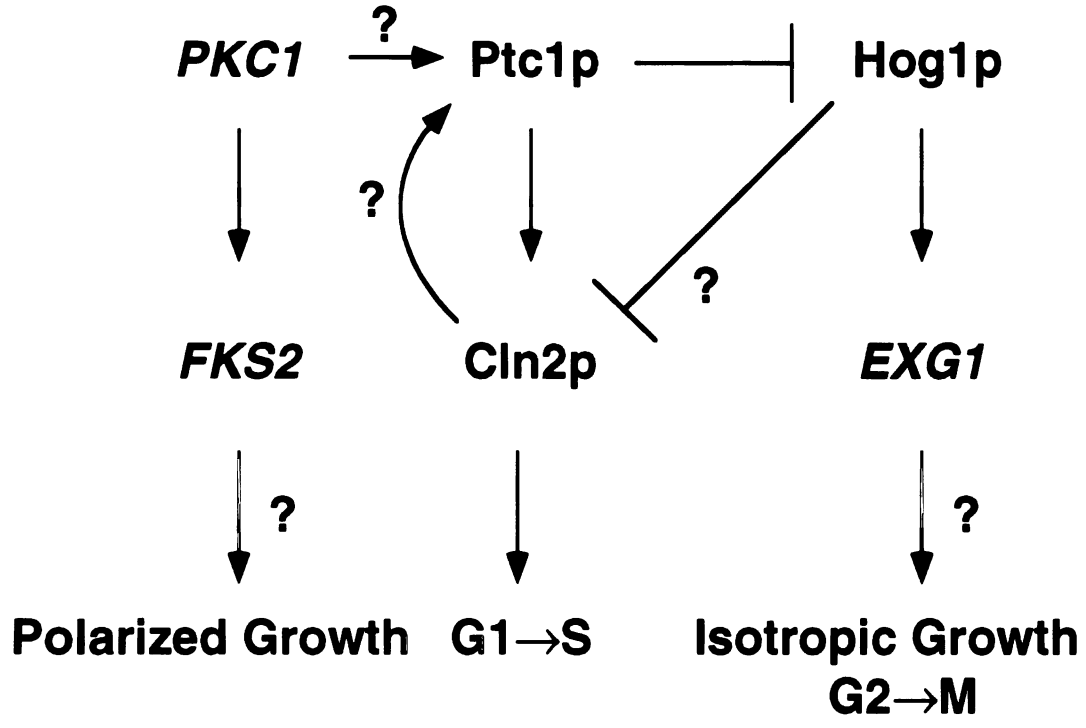

Figure 28. ***PTC1*** **may function to coordinate the activity of the** ***PKC1***, ***HOG1***, **and** ***CDC28*** **pathways. In this model the** ***PKC1*** **pathway functions to activate late G1 genes necessary for polarized growth such as** ***FKS2***, **a gene involved in cell wall biosynthesis. The** ***PKC1*** **pathway also activates Ptc1p which increases Cln2p protein levels thereby stimulating the G1 to S transition. CDC28p/Cln2p might also stimulate Ptc1p in a feed forward pathway. Thus, the cell wall biosynthesis associated with bud emergence is synchronised with progression through G1. Ptc1p also functions to inhibit Hog1p which may destabilize Cln2p through phosphorylation.** ***HOG1*** **is a homolog of** ***WIS1***, **a mitotic inducer in** ***Sz. pombe***, **and may stimulate G2/M. During G2/M, Hog1p is active to ensure that G1 cyclins are not present. Hog1p also stimulates transcription of genes involved in cell wall remodeling such as** ***EXG1*** **which may have a role in isotropic growth.**

Clnp protein levels, either by directly dephosphorylating the Clnps, or indirectly by inhibiting the *HOG1* pathway. The *PKC1* pathway positively regulates *PTC1* to ensure that the *HOG1* pathway is attenuated when the *PKC1* pathway is activated. Ptc1p might also be activated by Cdc28p/Clnps, which would enhance their own stability, thereby stimulating progression through G1. Thus the *PKC1* transcriptional program in G1 necessary for the cell wall modifications associated with bud emergence is coordinated with progression through G1. Hog1p may also function to destabilize Clnps during mitosis thereby preventing inappropriate Cdc28p/Clnp kinase activity. The *HOG1* pathway also may be involved in promoting mitosis since the *Schizosaccharomyces pombe* homologs of *HOG1* and *PBS2, sty1*$^+$ and *wis1*$^+$, are mitotic inducers (Ogden and Fantes, 1986; Millar *et al.* 1995). Perhaps *HOG1* has a role in mitotic induction by activating transcripts required for the cell wall remodeling associated with isotropic growth.

A phosphatase for all seasons?

The complex phenotypes of many phosphatases has led to their being dubbed *pleiotropic*—a damning word in yeast cell biology and genetics often interpreted as undecipherable or not worth studying. It is possible that the same phosphatase is arbitrarily used in many different contexts, as the term pleiotropic implies. I think that this is unlikely given nature's penchant for elegant and sophisticated systems. Rather, it seems that the confusion associated with "pleiotropism" stems from a lack of understanding and imagination.

For example, if we did not know so much about cell cycle progression surely *cdc28* mutants might seem unfathomably pleiotropic given that *cdc28* mutations affect the bud, the nucleus, and microtubules. I think that it is likely that phosphatases such as *PTC1* serve very specific functions to coordinate multiple biochemical pathways. If so, the study of phosphatases will be important in attaining an understanding of the cell as an integrated system displaying the emergent and resilient property we call life.

APPENDIX 1: A SYNTHETIC LETHAL SCREEN WITH *CDC28-1*

The strain PS11 and PS12 (*cdc28-1* in the S288C strain background, harboring *CDC28* on a *URA3* marked centromere-based plasmid) were mutagenized in water by UV radiation. Radiation dosage was tittered so that 10-15% of mutagenized colony forming units were viable. Mutagenized cells were plated onto -Ura minimal medium and allowed to form colonies in darkness. Since the initial screening was by replica plating, the colony density was 100-200 colonies per plate. Approximately 30,000 colonies were screened for 5-FOA sensitivity. 5-FOA selects against Ura^+ cells, therefore colonies that are unable to lose the *URA3* marked *CDC28* plasmid are inviable on 5-FOA (Boeke *et al.*, 1984; Frank *et al.*, 1992). 51 recessive mutants that are inviable on 5-FOA were identified and designated *slc* for synthetic lethal with *cdc28-1*. 23 of these mutants have temperature-sensitive growth defects with phenotypes at the non-permissive temperature consistent with CDC arrest or with abnormal bud morphology. The temperature-sensitive phenotypes of ten of these mutants segregated as single gene traits. These mutants were placed in nine complementation groups. Complementation tests indicated that one mutant was allelic to *CDC37* and two were allelic to *CDC28*. The remaining seven mutants were not allelic to *CDC28*, *CDC36*, *CDC37*, *CDC39*, *SIT4*, or *POLII* (other genes that have G1 arrest alleles). These seven mutants were crossed to the *cdc28-1* strain. One of these mutations, *slc2*, is synthetically lethal with *cdc28-1* at germination. The remaining six mutations, *slc1*, *slc15*, *slc23*, *slc32*, *slc45*, and *slc67*, are not.

These six mutations are "synthetically sick" with the *cdc28-1* allele; the double mutants exhibit growth defects that are more severe than either single mutant.

The *slc2* mutant arrests with 65% of cells exhibiting a large unbudded phenotype and 10% of cells exhibiting a multibudded morphology. The *slc2* mutant is inviable at 33°C. The *slc2* temperature-sensitive growth defect is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, *SSD1-v1* and *SGV1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1*(*HCS26*), *HCS77*, *ORC6*, and several high copy suppressors of *cdc37-1*. Seven low copy clones that complement the growth defect of the *slc2* mutant were isolated. Restriction mapping reveals that the seven clones correspond to two overlapping inserts.

The *slc1* mutant arrests with 70-77% of cells exhibiting an unbudded, uninucleate morphology. The mutant is inviable at 33°. The temperature-sensitive growth defect of *slc1* is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, and *SGV1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1* (*HCS26*), *ORC6*, and several high copy suppressors of *cdc37-1*. *slc1* is suppressed by 2μ*HCS77* and by low copy *SSD1-v1*suggesting that it may have a role in cyclin expression or in the *PKC1* pathway. As with *hcs77*Δ strains, expression of β-galactosidase from the *PCL1* upstream activating sequence is reduced in the *slc1* mutant. Two high copy suppressors of the temperature sensitive growth defect of **a**/α *hcs77*Δ/*hcs77*Δ and

sit4-1, pMP4 and pMP24, also suppress *slc1*. Complementing clones of *slc1* have not been isolated.

The *slc15* mutant arrests in G1 at the non-permissive temperature. 75% of *slc15* cells are unbudded and all cells are uninucleate. The FACS profile of the *slc15* mutant arrested for four hours at the non-permissive temperature is consistent with a 1N nuclear content. The *slc15* mutant is inviable at 33°. The temperature-sensitive growth defect of *slc15* is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, *SSD1-v1* and *SGV1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1* (*HCS26*), *HCS77*, *ORC6*, and several high copy suppressors of *cdc37-1*. Interestingly, the *slc15* mutant is also an **a**-specific sterile. The *slc15* mutant responds to α-factor by arresting and shmooing implying that the mating pheromone response signal transduction pathway is intact in the *slc15* mutant. Two complementing clones of the *slc15* mutant were isolated. A four kb fragment from one of the clones was subclones and shown to complement both growth and mating defects. LukTn10 mutagenesis (Huisman *et al.*, 1987) was used to localize the complementing region and sequencing from the ends of the transposon hops revealed that the complementing clone corresponded to *SUP45* (Himmelfarb *et al.* 1985; Breining and Piepersberg, 1986). *sup45* mutants are omnipotent suppressors and are capable of suppressing more than one nonsense codon (UAA and UAG). Sup45p is a 49 kd protein and is not part of the ribosomal complex. It has sequence similarity to aminoacyl tRNA synthetase and ATPase motifs.

The *slc23* mutant arrests in G1. 85-96% of arrested cells are unbudded and uninulceate. FACS analysis indicates that after 2 hours at 37°, the nuclear content of *slc23* mutants shifts to 1N. The FACS profile broadens at later time points. DAPI staining reveals that the nuclei of *slc23* mutants grow large and break down after prolonged arrest (greater than eight hours at the non-permissive temperature). *slc23* mutants are inviable at 35°. The temperature-sensitive growth defect of *slc23* is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, *SSD1-v1* and *SGV1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1* (*HCS26*), *HCS77*, *ORC6*, and several high copy suppressors of *cdc37-1*. A low copy complementing clone of the *slc23* mutant has been identified.

The *slc32* mutant arrests with approximately 30% of cells having more than one bud and is the subject of this thesis.

The *slc45* mutant arrests in G1. 80-95% of arrested cells are unbudded and uninucleate. The FACS profile indicates that by eight hours at the non-permissive temperature, *slc45* mutant cells have a 1N nuclear content. The *slc45* mutant is inviable at 33°. The temperature-sensitive growth defect of *slc45* is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, and *SSD1-v1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1* (*HCS26*), *HCS77*, *ORC6*, and several high copy suppressors of *cdc37-1*. The *slc45* mutant is partially suppressed by low copy *SGV1* at 35°. Complementing clones of *slc45* have not been identified.

The *slc67* mutant arrests in G1. 80-85% of cells are unbudded and uninucleate. FACS analysis indicates a 1N nuclear content. The *slc67* mutant is inviable at 37°. The temperature-sensitive growth defect of *slc67* is not suppressed by low copy plasmids of *CDC37*, *SIT4*, *POLII*, *SWI4*, *ORC6*, and *SSD1-v1* or by high copy plasmids of *CDC36*, *CDC39*, *CDC37*, *CDC28*, *CLN1*, *CLN2*, *CLN3*, *HCS24*, *PCL1* (*HCS26*), *HCS77*, and *ORC6*. The *slc67* mutant is suppressed by low copy *SGV1* and by two high copy suppressors of *cdc37-1*, pMG11 and pMG50. A complementing clone of *slc67* was isolated and subcloned. Sequence analysis revealed that it corresponds to *PEP3*, a protein with Zinc finger motifs thought to be involved in vacuolar biogenesis (Preston *et al.*, 1991; Robinson *et al.*, 1991). pMG50 also corresponds to *PEP3*.

Table 1: List of strains used in this screen

The strain background is S288C and all strains are *ura3 his3 leu2 lys2 ade2* unless otherwise indicated.

PS11: mat**a** *cdc28-1 tyr1 ADE2*$^+$

PS12: matα *cdc28-1 tyr1 HIS3*$^+$

BC89-1C: *mat**a** slc1*

BC89-4A: *matα slc1*

BC100-1D: *mat**a** slc2*

BC100-2C: *matα slc2*

BC91-1C: *mat**a** slc15*

BC91-1A: *matα slc15*

BC94-9C: *mat**a** slc23*

BC94-2C: *matα slc23*

BC93-3C: *mat**a** slc32*

BC93-2B: *matα slc32*

BC99-6C: *mat**a** slc45*

BC99-7C: *matα slc45*

BC101-3C: *mat**a** slc67*

BC101-12C: *matα slc67*

Table 2: List of plasmids used in the screen

All plasmids are marked with *URA3*.

pCEN *CDC28*, from J. Ogas

pCEN *CDC37*, from M. Gerber

pCEN *SIT4*, from K. Arndt

pCEN *POLII*, from C. Peterson

pCEN *SWI4*, from J. Ogas

pCEN *ORC6*, from J. Li

pCEN *ssdv-1*, from K. Arndt

pCEN *SGV1*, from K. Matsumoto

p2μ*CDC36*, from A. Neiman

p2μ*CDC39*, from A. Neiman

p2μ*CDC37*, from M. Gerber

p2μ*CDC28*, from J. Ogas

p2μ*CLN1*, from J. Ogas

p2μ*CLN2*, from J. Ogas

p2μ*CLN3*, from J. Ogas

p2μ*HCS24* ,from J. Ogas

p2μ*PCL1*(*HCS26*), from J. Ogas

p2μ*HCS77*, from J. Ogas

p2μ*ORC6* , from J. Li

pMG11: high copy suppressor of *cdc37-1*, from M. Gerber

pMG50: high copy suppressor of *cdc37-1*, from M. Gerber

pMP4: high copy suppressor of **a**/α *hcs77*Δ/*hcs77*Δ, from M. Peter

pMP24: high copy suppressor of **a**/α *hcs77*Δ/*hcs77*Δ, from M. Peter

pBC4 : complementing clone of *slc2*

pBC2: complementing clone of *slc15*

APPENDIX 2: MUTATIONS IN *PTC1* ENHANCE THE GROWTH DEFECTS OF SEPTIN MUTANTS

To examine possible interactions between *PTC1* and the septins, double mutants between *ptc1* mutants and septin mutants were made. The available septin alleles were in a strain background without auxotrophic markers so the septin alleles *cdc3-1*, *cdc10-1*, *cdc11-1*, and *cdc12-1* were first crossed into the wild-type S288C strain background three times before being crossed to *ptc1-1* (BC93-3C) and *ptc1*Δ (BC128-5B). Thus the septin mutant strains are not strictly isogenic with the *ptc1* mutant strains. The double mutants were tested for growth at a variety of temperatures. In all cases the double mutants grow more slowly than the single mutant counterparts. However, double mutants with the *ptc1*Δ allele have a noticeably stronger phenotype than with the *ptc1-1* allele. *ptc1*Δ *cdc10*Δ double mutants (BC136-3B) had the most pronounced phenotype compared to other *ptc1* septin double mutants. Double mutants shifted from room temperature to the non-permissive temperature exhibited enhanced morphological defects.

To determine if the *ptc1* mutant phenotype was effected by overexpression of the septins, 2μ *CDC3*, *CDC10*, *CDC11*, *CDC12* were transformed into *ptc1-1* and *ptc1*Δ and wild-type strains. No substantial effects on growth were observed in any of the transformants.

Table 3: List of strains used in Appendix 2

BC93-3C: *mat***a** *ptc1-1*

BC128-5B: *mat***a** *ptc1*Δ

BC129-2C: *mat*α *cdc3-1*

BC130-3A: *mat*α *cdc10-1*

BC131-5A: *mat*α *cdc 11-1*

BC132-4B: *mat*α *cdc12-1*

BC142-9D: *mat***a** *ptc1-1 cdc3-1*

BC141-2D: *mat***a** *ptc1*Δ *cdc3-1*

BC139-15A: *mat***a** *ptc1-1 cdc10-1*

BC136-3B: *mat***a** *ptc1*Δ *cdc10-1*

BC140-7A: *mat***a** *ptc1-1 cdc11-1*

BC134-6A: *mat***a** *ptc1*Δ *cdc11-1*

BC143-12A: *mat***a** *ptc1-1 cdc12-1*

Table 4: List of plasmids used in Appendix 2

pBC59: 2μ*CDC3 URA3*, from M. Longtine and J. Pringle

pBC60: 2μ*CDC10 URA3*, from M. Longtine and J. Pringle

pBC61: 2μ*CDC11 URA3*, from M. Longtine and J. Pringle

pBC62: 2μ*CDC12 URA3*, from M. Longtine and J. Pringle

APPENDIX 3: LIST OF PLASMIDS

pBC7: complementing clone of *ptc1* mutants from genomic fragment library (YCP50) (Rose *et al.*, 1987)

pBC14: *PTC1* subcloned in pRS316

pBC16: *PTC1* with BamHI site after first ATG

pBC36: *PTC1* in pBluescript KS with modified polylinker

pBC42: *ptc1*::*HIS3* deletion construct

pBC27: TrpE-Ptc1p bacterial expression fusion protein construct (pATH11)

pBC21: Gst-Ptc1p bacterial expression fusion protein construct (pGEX2T)

pBC35: 2µ*PTC1* (pRS426)

pRD56: p*GAL1-10GST* yeast expression vector (pRS316)

pBC39: p*GAL1-10GST-PTC1* inducible yeast exression vector (pRS316)

pBC69: p*PTC1GST-PTC1* (pRS316)

pBC74, pBC75, pBC76: *ptc1-1* in TA cloning vector

pBC80: p*GAL1-10GST-ptc1-1* (pRS316)

pBC87: 2µ*ptc1-1* (pRS426)

pBC91: 2µ*CLN1* (YEP24) also pJO1; Ogas, 1992

pBC92: 2µ*CLN2* (YEP24) also pJO21; Ogas, 1992

pBC93: *CLN3-2* (YCP50), from F. Cross

pMT184: *cln2*::*HACLN2/LEU2* gene replacement, from M. Tyers

pBC94 : 2µ*HCS77* (YEP24), from J. Ogas

pBC88: 2µ*PKC1* (YEP352, *URA3*), from D. Levin

pBC90: 2µ*MPK1* (YEP352, *URA3*), from D. Levin

pBC89: *BCK1-20* (pRS316), from D. Levin

pBC78: *ptc1*Δ::*TRP1* deletion construct

pAS135: *HisG-URA3-HisG* in pRS56, from Anita Sil

pJB30: 2μ*HOG1* (*URA3*), from M. Gustin (pRS426)

pBC99: *hog1*Δ::*HisG-URA3-HisG* deletion construct

APPENDIX 4: LIST OF STRAINS

The S288C background.

All strains carry the mutations *ura3 his3 leu2 lys2 ade2 gal2.*

JO20: *mat**a***

JO14: *matα*

JO371: *mat**a** trp1 GAL2*$^{+}$

JO314-3C: *matα cln1*Δ *cln3*Δ

BC93-3C: *mat**a** ptc1-1*^{ts}

BC128-5B: *mat**a** ptc1*Δ

BC178-9A: *mat**a***

BC178-9B: *mat**a***

BC178-9C: *matα ptc1-1 cdc28-1*

BC178-9D: *matα ptc1-1 cdc28-1*

BC178-10A: *matα cdc28-1*

BC178-10B: *mat**a** cdc28-1*

BC178-10C: *mat**a** ptc1-1*

BC178-10D: *matα ptc1-1*

BC120-14A: *mat**a** ptc1-1 cln2*Δ

BC120-14B: *matα cln2*Δ

BC120-14C: *mat**a** ptc1-1*

BC120-14D: *matα*

BC158-8A: *mat**a** ptc1*Δ *cln2*Δ

BC158-8B: *matα cln2*Δ

BC158-8C: *matα ptc1*Δ

BC158-8D: *mat**a***

BC176-2A: *mat**a** ptc1*Δ

BC176-9A: *mat**a** cln1*Δ

BC176-9B: *mat**a** cln2*Δ

BC176-5B: *mat**a** cln3*Δ

BC176-3A: *mat**a** cln1*Δ *cln2*Δ

BC176-2B: *mat**a** cln1*Δ *cln3*Δ

BC176-8A: *matα ptc1*Δ *cln1*Δ

BC176-13B: *matα ptc1*Δ *cln2*Δ

BC176-1D: *mat**a** ptc1*Δ *cln3*Δ

BC176-5D: *matα ptc1*Δ *cln1*Δ *cln2*Δ

BC176-1A: *mat**a** ptc1*Δ *cln1*Δ *cln3*Δ

BC162: *matα cln2*Δ::*HACLN2*; *LEU2*

BC163: *mat**a** ptc1-1 cln2*Δ::*HACLN2*; *LEU2*

BC164: *mat**a** ptc1*Δ *cln2*Δ::*HACLN2*; *LEU2*

The EG123 strain background.

All strains carry the mutations *ura3 his4 leu2 trp1 can1*.

IH1783: *mat***a**

BC151: *mat***a** *ptc1*Δ

JG217: *matα mpk1*Δ

JG206: *matα bck1*Δ

BC150: *matα ptp2*Δ (also DL975)

BC155-4B: *matα ptc1*Δ *mpk1*Δ

BC155-4C: *mat***a** *ptc1*Δ *mpk1*Δ

BC155-10A: *mat***a** *ptc1*Δ *mpk1*Δ

BC155-10D: *matα ptc1*Δ *mpk1*Δ

BC155-15C: *mat***a** *ptc1*Δ *mpk1*Δ

BC157-9B: *mat***a** *ptc1*Δ *bck1*Δ

BC157-11A: *mat***a** *ptc1*Δ *bck1*Δ

BC157-13A: *mat***a** *ptc1*Δ *bck1*Δ

BC157-14A: *mat***a** *ptc1*Δ *bck1*Δ

BC157-14B: *matα ptc1*Δ *bck1*Δ

BC156-2A: *matα*

BC156-2B: *mat***a** *ptc1*Δ *ptp2*Δ

BC156-2C: *mat***a** *ptp2*Δ

BC156-2D: *matα ptc1*Δ

BC156-3A: *matα*

BC156-3B: *mat***a** *ptc1*Δ *ptp2*Δ

BC156-3C: *mat***a** *ptp2*Δ

BC156-3D: *matα ptc1*Δ

BC174-2D: *mat***a** *ptc1*Δ *hog1*Δ

BC174-5D: *matα ptc1Δ hog1Δ*

BC174-6C: *mat***a** *ptc1Δ hog1Δ*

BC174-10B: *mat***a** *ptc1Δ hog1Δ*

BC174-12D: *matα ptc1Δ hog1Δ*

BC174-7D: *matα ptc1Δ ptp2Δ*

BC174-4B: *mat***a** *ptc1Δ ptp2Δ hog1Δ*

BC174-5C: *mat***a** *ptc1Δ ptp2Δ hog1Δ*

BC174-8C: *matα ptc1Δ ptp2Δ hog1Δ*

BC174-11D: *mat***a** *ptc1Δ ptp2Δ hog1Δ*

BC174-14D: *mat***a** *ptc1Δ ptp2Δ hog1Δ*

BC175-12D: *mat***a** *ptc1Δ mpk1Δ hog1Δ*

BC175-16A: *matα ptc1Δ mpk1Δ hog1Δ*

APPENDIX 5: MATERIALS AND METHODS

Media and yeast genetic manipulations

Standard media and yeast genetic techniques were used (Mortimer and Hawthorne, 1969; Hicks and Herskowitz ,1976; Sherman *et al.*, 1982; Rose *et al.*, 1990). 5-FOA plates were prepared as described in Boeke *et al.* (1987). Transformations were performed as described in Ito *et al.* (1983).

DNA and Protein Manipulations

Standard DNA and protein manipulations were performed according to Ausubel *et al.* (1989). DNA fragments were eluted from agarose gels using Qiaex (Qiagen). Polymerase chain reaction (PCR) primers were synthesized using a Millipore oligonucleotide synthesizer.

Colony PCR

A "toothpick head full" of yeast is scraped from a colony and resuspended in 100λ PCR reaction mix (10mM Tris HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, 0.2mM dGTP, 0.2mM dATP, 0.2mM dTTP, 0.2mM dCTP, 1μM primer 1, and 1μM primer 2). The sample is boiled for 5 minutes (min) and briefly spun, and then placed on ice. 2.5 Units of Taq polymerase is added and the sample is overlayed with oil. The sample is amplified for 30 cycles as follows: 94°C for 1 min/ 42°C for 2 min/ 65°C for 4 min. The final cycle is extended at 65°C for 5 min.

Strains

E. coli Strains

The *E. coli* strain used for propagation of plasmids is DH5α. The *E. coli* strain used for TrpE-Ptc1p and Gst-Ptc1p induction is NB42. The *E. coli* strain GM33 (dam$^-$) was used to propagate plasmids that required digestion with dam-methyl sensitive enzymes.

S. Cerevisiae strains are listed in Appendix 3.

Strain construction

For single step gene replacement of *PTC1* with *HIS3*, plasmid pBC42 was cut with SalI/NotI and transformed into strains with selection on -His minimal medium. Transformants were screened for temperature sensitivity, complementation by plasmid pBC14, and colony PCR using the primers:
5'-GTCATGTGAGAGATGCATGC and 5'-TAGTTTAAGTAAGTGAACGC.

For single step gene replacement (Rothstein, 1983) of *PTC1* with *TRP1*, plasmid pBC78 was cut with SalI/NotI and transformed into strains with selection on -Trp minimal medium. Transformants were screened for temperature sensitivity, complementation by plasmid pBC14, and colony PCR using the following pairs of primers:
5'-GTCATGTGAGAGATGCATGC and 5'-TAGTTTAAGTAAGTGAACGC;
5'-GTCATGTGAGAGATGCATGC and 5'-GTAATCAACCTAAGGAGGATG;
and 5'-TAGTTTAAGTAAGTGAACGC and 5'-CATCCTCCTTAGGTTGATTAC.

For single step gene replacement of *HOG1* with *HisG-URA3-HisG*, plasmid pBC99 was cut with XhoI/BamHI and transformed into strains with selection on -Ura minimal medium. Transformants

were screened for sensitivity to medium of high osmolarity, and PCR using the following pairs of primers: 5'-CGCAAGTTGTTAGGAAAGCG and 5'-AATGTCATCATCACGCAGGC; 5'-GAAAGGCCGACAATTCTGCC and 5'-GAAGTTTCTGCCACAGTCCGT.

Plasmid Construction

Subcloning *PTC1*. pBC7 is the YCP50 based genomic complementing clone of *ptc1-1*. pBC14 (CEN *PTC1 URA3*) was made by subcloning a 1.5 kb SalI/HindIII fragment, derived from pBC7, into pRS316. pBC14 contains the entire ORF of *PTC1*, the promoter of *PTC1*, and 300 bp of 3'UTR and fully complements the *ptc1-1* and *ptc1*Δ mutants. pBC35 (2μ*PTC1 URA3*) was made by subcloning the 1.5 kb SalI/HindIII fragment of pBC14 into pRS426.

pBC16 was generated by a trimeric ligation of: 1) a synthetic linker made from primers 5'-GATCCATGAGTAATCATTCTGAAATCTTA GA AAGGCCAGAAACACCA and 5'-TATGGTGTTTCTGGCCTTTCTAAGATT TCAGAA TGATTACTCATG, 2) the 1.1 kb NdeI/SalI fragment of pBC14, and 3) BamHI/SalI digested pBluescript KS.

pBC21 (p*GST-PTC1*) was generated by ligating the 855 bp BamHI fragment of pBC16 into pGEX2T (Pharmacia). This plasmid is used to inducibly express Gst-Ptc1p in *E. coli*.

pBC27 (pTrpE-PTC1) was made by ligating the 855 bp BamHI fragment of pBC16 containing the entire ORF of *PTC1* into the BamHI site of pATH11 (Koerner *et al.*, 1991). This plasmid is used to inducibly express TrpE-Ptc1p in *E.coli*.

pBC31 (pGAL1-10*GST-PTC1 URA3 CEN*) was made by first ligating the 855 bp BamHI fragment of pBC16 into BamHI digested

pRD56. The 7.0 kb fragment of a SalI / BamHI-partial digest of this plasmid was then ligated to the 302 bp SalI/BamHI fragment of pBC14 to make pBC31. This plasmid is used to express Gst-Ptc1p from the strongly inducible *GAL1-10* promoter from a centromeric plasmid in yeast.

pBC36 (PTC1 in modified Bluescript KS) was made by first subcloning the 1.5 kb SalI/NotI fragment from pBC14 into pBluescript KS. This plasmid was then digested with EcoRI and XbaI, filled in with Klenow, and religated to remove EcoRI and BamHI sites from the polylinker, to make pBC36.

pBC42 (*ptc1*::*HIS3*) was made as follows. pBC36 was amplified by PCR using primers 5'-CAGAATGATTACTCATGAATTCTATAA and 5'-5'-GGCTGCAGGAATTCGATATCAAGC. The PCR product was digested with HindIII and NdeI and cloned back into the same sites in pBC36. The 3.2 kb EcoRI/BamHI fragment of this plasmid was ligated to a 1.1 Kb EcoRI/BamHI fragment containing the *HIS3* gene (gift of J. Li), to yield pBC42 (*ptc1*::*HIS3*). This plasmid is used to precisely replace the entire ORF of *PTC1* with *HIS3* by single step gene replacement.

pBC69 (p^{PTC1}*GST-PTC1 URA3 CEN*) was made in a trimeric ligation using these fragments: 1) the 6.0 kb fragment from a completely XbaI and BamHI partial digest of pBC31, 2) the XbaI/NdeI digested PCR product of pBC14 amplified by primers T7 and 5'-GCCTTCTAAGATTTCAGAATGATTACTCATATGTATAATGATTTTT AAAAGATAAATGC, and 3) the 660 bp BamHI/NdeI fragment of pRD56. This plasmid is used to express Gst-Ptc1p from the PTC1 promotor on a centromeric plasmid.

Isolation and subcloning of *ptc1-1*. pBC74, pBC75, and pBC76 were made by colony PCR of strain BC93-3C (*ptc1-1*) using primers 5'-GTCATGTGAGAGATGCATGC and 5'-TAGTTTAAGTAAGTGAACGC, and subcloning the PCR product into the TA cloning kit (Stratagene). The *ptc1-1* mutation was subcloned by ligating the 306 bp BamH**I**/BspM**I** fragment from pBC74 into the 4.1 kb BamH**I**/BspM**I** fragment of pBC36 to make pBC77. pBC87 (2µ*ptc1-1 URA3*) was made by ligating the 1.5 kb Not**I**/Sal**I** fragment of pBC77 into Not**I**/Sal**I** digested pRS426. pBC80 (p$_{GAL1-10}$*GST-ptc1-1 URA3 CEN*) was made by ligating the 1.0 kb Snab**I**/Sal**I** fragment of pBC77 to the 6.3 kb Snab**I**/Sal**I** fragment of pBC31.

pBC78 (*ptc1*::*TRP1*) was made by digesting pBC42 with EcoR**I** and BamH**I**, filling in the ends with klenow, and ligating a blunt ended 1.0 kb fragment containing the *TRP1* gene. This plasmid is used to precisely replace the entire ORF of *PTC1* with *TRP1* by single step gene replacement.

pBC99 (*hog1*::*HisG-URA3-HisG*) was made by ligating the 7.1 kb EcoR**I**/Bcl**I** fragment of pJB30 to the 3.9 kb EcoR**I**/BamH**I** fragment of pAS135. This construct is used to replace 95% of the *HOG1* ORF with *HisG-URA3-HisG* by single step gene replacement.

Antibodies

TrpE-Ptc1p was inducibly expressed and purified from *E. Coli* essentially as described in Koerner *et al.* (1991) except that the strain NB42 was used. The purified TrpE-Ptc1p was used as antigen for two rabbits. Antigen injection and serum collection was performed by Northview Pacific Laboratories. The sera was

screened for production of anti-TrpE-Ptc1p antibodies by Western blot of yeast whole cell extracts.

The antiserum was affinity purified by column chromatography over a Gst-Ptc1p column and a column made from the whole cell extract of a *ptc*Δ strain. The Gst-Ptc1p column was made by inducibly expressing the Gst-Ptc1p fusion protein in *E. coli*, purifying it by glutathione-Sepharose 4B affinity chromatography (Smith and Johnson, 1988), and coupling it to Affigel-10 beads (BioRad). The "*ptc*Δ column" was made by coupling an extract from the *ptc*Δ strain, BC128-5B, to a mixture of Affigel-10 and Affigel-15 beads (BioRad). The extract was made by pelleting 1.0 L of BC128-5B at $O.D._{660}$ of 0.5, resuspending the pellet in lysis buffer (50 mM HEPES pH 7.6, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, 1mM PMSF, and 1.0% SDS, 4°C) and grinding the pellet with glass beads. The extract was then heated to 100°C for 10', spun at 4°C for 5' @ 4000rpm, and transferred to a fresh centrifuge tube. The extract was then spun for 30' @ 10,000rpm, 4°C. The supernatant (S-10) was then coupled to a mixture of Affigel-10 and Affigel-15 (Biorad). To affinity purify the antisera the antisera was cycled over the Gst-Ptc1p column by use of a peristaltic pump. The column was then washed with 25 column volumes of buffer (0.1 M Tris pH7.5, 0.5 M NaCl, 1mM EDTA). The bound antibodies were eluted by gravity filtration using 100 mM glycine pH2.5. 1 ml fractions were collected and neutralized with 17λ Tris (unpHed). Peak fractions were determined by O.D.260. For immunoflourescence, this affinity purified antisera was further purified by passage over the "*ptc*Δ column" using the peristaltic pump.

Antibodies against the HA epitope (12CA5) were obtained from Berkeley Antibody Company.

Purification of Gst-Ptc1p and Gstp from yeast

The strain FM135 transformed with pBC31 was grown to an $O.D._{660}$ of 0.5 at 30°C in -Ura minimal medium and then induced for 5 hours to express Gst-Ptc1p from the *GAL1-10* promoter by addition of galactose to the medium to a final concentration of 10%. All further manipulations take place at 4°C. All centrifugations used a Sorvall SS34 rotor. The strain was pelleted, resuspended in low salt lysis buffer (50 mM HEPES pH 7.6, 50 mM KCl, 5mM EDTA pH 8.0, 5mM EGTA pH 8.0, 10% glycerol, 0.1% NP40, 1mM DTT, 1mM benzamidine hydrochloride, 1X Aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1μg/ml pepstatin, 10 mM NaF, 80 mM β-glycerol phosphate), and ground with glass beads. The extract was spun for 5' @ 2000rpm and the supernatant (the whole-cell-exract) was transferred to a fresh centrifuge tube. The whole cell extract was then spun for 30' @ 10,000 rpm, PMSF was added (to 1mM), and the extract was spun again for 30' @10,000 rpm. The S-10 fraction, the supernatant, was separated from the P-10 fraction, the pellet, and was pressed through a 0.45μ filter. PMSF (to 1mM), DNAse (to 10 μg/ml), and RNAse (to 10 μg/ml) were added. The S-10 fraction was then loaded onto a lysis buffer equilibrated Sepharose 4B column (Pharmacia) and allowed to flow through by gravity filtration. The Gst-Ptc1p bound column was washed with 15 column volumes of lysis buffer, 5 column volumes of final wash buffer (50 mM HEPES pH 7.6, 50 mM KCl, 5mM EDTA pH 8.0, 5mM EGTA pH 8.0, 10% glycerol, 1mM DTT, 10

mM NaF, 80 mM β-glycerol phosphate). The column was then eluted with 8 column volumes of elution buffer (50 mM HEPES pH 7.6, 20 mM reduced glutathione, 10% glycerol, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.1% Octyl-β-D-glucopyranoside). Fractions were collected and assayed for protein by micro Bradford (Biorad); the peak fractions were pooled together. The eluate was then concentrated and exchanged into storage buffer (1X PBS, 10% glycerol, 1 mM DTT, 1 mM EDTA) using a centricon-30 microconcentrator (Amicon). Protein concentration was determined by Bradford (Biorad).

Gstp was expressed from the inducible *Gal1-10* promoter from FM135 transformed with pRD56. Purification was carried out exactly as described above except that a Centricon 10 microconcentrator was used (Amicon).

Gst-Ptc1p expressed from the *PTC1* promoter was purified from strain Y57 transformed with pBC69 essentially as described above except that a high salt lysis buffer was used (50 mM HEPES pH 7.6, 0.5 M KCl, 5mM EDTA pH 8.0, 5mM EGTA pH 8.0, 10% glycerol, 0.1% NP40, 1mM DTT, 1mM benzamidine hydrochloride, 1X Aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1μg/ml pepstatin, 10 mM NaF, 80 mM β-glycerol phosphate) and that the strain was harvested from -Ura minimal medium at an $O.D._{660}$ of 1.0 without the addition of galactose to the medium.

For large scale preparations, I would grow up six, 1.5 L cultures, and make extracts from 3.O L of culture to be run over a 2 ml (4 mls of slurry) glutathione Sepharose 4B column (Pharmacia).

Phosphatase Assays

Casein (Sigma C4765) was phosphorylated by bovine heart muscle kinase (Sigma P2645) in the presence of $^{32P}\gamma$ATP and kinase buffer (50 mM Tris pH 6.5, 10 mM $MgCl_2$, 0.05 mM ATP). A typical reaction would use 100λ of casein (5.6mg), 10λ of $^{32P}\gamma$ATP (100 μCi), and 100λ of kinase (100units) in a 500λ reaction at room temperature for 1 hour. The reaction was stopped by the addition of an equal volume of 40% TCA and placed on ice for 30'. The casein was pelleted by centrifugation for 10' @ 14,000 rpm (eppendorf 5415C centrifuge) and washed with 1 ml of 20% TCA X 2, and 1 ml of acetone X 2. The pellet was then dried and vigorously vortexed into 2X phosphatase buffer (100mM Tris pH 7.5, 0.2% β-mercaptoethanol, 20 mM $MnCl_2$) and counted using a Beckman scintillation counter. The concentration of casein was adjusted to1 X 10^4 cpm/λ. 100λ phosphatase reactions consisted of the desired amount of purified Gst-Ptc1p or Gstp and 5 X 10^5 cpm of resupended casein, taking up the remaining volume with H_20 to a buffer concentration of 1X. Reaction were incubated at 30°C for 3 hours. The reactions were stopped by addition of 0.75mls of 5% charcoal in 20mM phosphoric acid, vortexed briefly and spun. The supernatant was transferred to a fresh tube and counted. The percentage of P_i released was calculated by dividing the cpm of the reaction supernatant by the cpm from the casein prior to addition of enzyme.

Western and Northern blots

Immunoblots were performed as described in Peter *et al.* (1993). Northern blots were performed as described in Cross and Tinkelenberg (1991).

Microscopy and FACS analysis

Photo microscopy and FACS analysis were performed as described in Ogas *et al.* (1992). DAPI staining and immunoflouresence were performed as described by Pringle *et al.* (1989).

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