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A Genetic and Molecular Analysis of the Control of the Start of the Cell Cycle in *Saccharomyces cerevisiae*

Charles B. Epstein

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**A Genetic and Molecular Analysis
of the Control of
the Start of the Cell Cycle in
*Saccharomyces cerevisiae***

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

by

Charles B. Epstein

2 November 1992
The Rockefeller University
New York, New York

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Dedication

This thesis is dedicated to my parents,
Robert M. Epstein and Lillian C. Epstein,
who taught me to love truth and value hard work,
and encouraged me in my pursuit of an education,
starting at an early age.

*Full many a gem of purest ray serene
The dark unfathom'd caves of ocean bear...*

Thomas Gray, c. 1746

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Abstract

Progression through the eukaryotic cell cycle is controlled by the *CDC28* protein kinase and its homologs. In the budding yeast *Saccharomyces cerevisiae*, this kinase is required for cell cycle START (commitment to cell cycle progression late in G1), and for entry into mitosis. Although constitutively present, *CDC28* is only periodically activated. The activation of *CDC28* involves its physical association with proteins called cyclins.

Budding yeast have three *CLN* genes (*CLNs 1 - 3*), which have limited cyclin homology. At least one of the three is required for cell cycle START. Four B cyclins are known in yeast (*CLBs 1 - 4*); two have been shown to function in mitosis. This thesis reports the discovery of three genes which, when either mutated or overexpressed, relieve the requirement for *CLN* genes for the execution of START.

The first gene was discovered as a mutation which bypasses the requirement for *CLN* genes, and which we have named the *CLN* bypass mutation (CBM). 15 distinct isolates of CBM were obtained; all were dominant, and 12 were shown to be linked or allelic with one another. Two of the isolates caused single division meiosis as an unselected, dominant phenotype.

Two *CEN* plasmid clones were isolated which bypassed the *CLN* requirement. The first of these contained a novel B cyclin, which we have named *CLB5*. *CLB5* transcript abundance peaks in G1, coincident with *CLN2* transcript, but earlier than *CLB2* transcript. *CLB5* deletion does not cause lethality, either alone or in combination with other *CLN* or *CLB* deletions. However, strains deleted for *CLB5* require more time to complete S phase, suggesting that *CLB5* promotes some step in DNA synthesis. *CLB5* is the only yeast cyclin whose

deletion lengthens S phase. *CLB5* may also have some role promoting the G1/S transition, since *cln1 cln2* strains require both *CLN3* and *CLB5* for viability on glycerol media, and *cln1,2,3⁻* strains require *CLB5* for rescue by the *D. melanogaster cdc2* gene.

The other *CEN* plasmid clone rescuing the *cln1,2,3⁻* genotype contained *MPK1*, already known as a gene which could function in this context. We have generated a null allele of *MPK1*, and shown that while *mpk1* strains are only mildly impaired at START execution, *mpk1 cln3* strains are very sick, and *mpk1 cln3* spores are inviable. *MPK1 cln1 cln2* and *mpk1 cln1 cln2* strains are both robust, suggesting that *MPK1* and *CLN1,2* supply substantially the same function in the *cln3* background. *MPK1* appears to be required for the *CDC28* independent phase of pre-START *CLN1* and *CLN2* transcription, which is nearly essential to viability in the *cln3* background.

Chapter 1: Introduction

The cell cycle in budding yeast: logical and biological problems

Introduction. Living organisms are made up of cells, and the propagation of life depends on the replication of cells. Cellular replication is tightly regulated, and generally occurs through the process of cell division, wherein one cell gives rise to two. Since this occurs repetitively (ie. many kinds of cells can divide, then divide again), one conventionally speaks of a "cell division cycle". This thesis describes work in the area of cellular biology concerned with the control of the cell division cycle, in the model organism *Saccharomyces cerevisiae*. The tools of cell physiology, genetics, and molecular biology will be brought to bear.

The cell division cycle encompasses several distinct sub-cellular division cycles: When cells replicate, each component must replicate, prior to the events of cell division. In particular, one thinks of replication of the spindle pole body (Byers, 1981) or centrosome, replication of the genome (ie. all genetic information residing in the nucleus), and replication of all other macromolecules and subcellular organelles as distinct, inter-related processes. These processes are of two, distinct kinds, in the sense that cells replicate their genomes and centrosomes *exactly* once per division, but they replicate all other components an *average* of once per division, while undergoing growth. One of the fundamental questions in our field concerns how growth and division are coordinated, such that after an indeterminate number of cell divisions, the average cell size is unchanged (Pringle and Hartwell, 1981). In other words, we want to understand how cells control how big they are.

The concept of START. The yeast cell cycle, like most eukaryotic cell cycles, is divided into discrete phases. DNA synthesis occurs during "S phase",

and chromosome segregation and nuclear division occur during "M phase", or Mitosis. These two phases are separated by intervening "Gap" phases, called G1 (between mitosis and S phase), and G2 (between S phase and mitosis). It has long been recognized that at least four types of regulation of the yeast cell cycle occur specifically in G1.

First, yeast divide by asymmetrical budding, in which daughter cells are produced that are generally smaller than their mothers. In order for yeast to achieve the coordination of growth and division, daughter cells must refrain from dividing prior to achieving the typical size of mother cells at their first division. It happens that budding yeast postpone the initiation of S phase until they have reached a critical size (Pringle and Hartwell, 1981; Cross et al, 1989a); the coordination of growth and division is achieved in G1. There is no logical necessity to this state of affairs, since in theory cells could complete S phase, and postpone mitosis while waiting to reach the appropriate size. Moreover, the cell size at which S phase begins is not determined by sheer physical constraints, since certain mutants commence S phase at a distinctly smaller size (Carter and Sudbery, 1980; Cross, 1988a; Nash et al, 1988; Hadwiger et al, 1989b).

Second, haploid yeast occur in two distinct mating types (reviewed in Cross et al, 1988b; Herskowitz, 1989). Each mating type secretes a peptide pheromone, and expresses a receptor for the pheromone of the opposite mating type. When exposed to the appropriate mating pheromone, haploid (or mating type homozygous) yeast arrest progression through the cell cycle specifically in G1, and undergo differentiative changes in preparation for mating. Pheromone arrest represents a second instance of G1 specific regulation of cell cycle progression.

Yeast respond to deprivation for a variety of nutrients, including energy, by

arresting progression through the cell cycle, specifically in G1 (Pringle and Hartwell, 1981). Mating type heterozygous yeast respond to deprivation for nitrogen, in the presence of a nonfermentable carbon source, by sporulating; this also commences in G1 (Esposito and Klapholz, 1981).

Hartwell and his colleagues (Pringle and Hartwell, 1981) have defined START as the unique point in the cell cycle when cells become committed to a particular developmental fate. Cells in G1 behave as if choosing among distinct developmental options: dividing, arresting, mating, or sporulating (excepting, of course, that no particular cell must "chose" between mating and sporulating, since these are capabilities of genetically distinct cell types; in addition, although sporulation normally commences in G1, if yeast are transferred back to rich media early in sporulation, they can return to vegetative growth after undergoing meiotic recombination (Esposito and Klapholz, 1981)). G1 yeast that are pre-START are uncommitted to a developmental fate, while post-START, they are committed to completion of an additional vegetative cell cycle, prior to reaching their next decision point. As a corollary, the START concept encompasses the fact that the choice among developmental options is made at a unique point in the cell cycle, rather than as a series of bifurcating choices. A basic objective of our field is to describe, in molecular terms, what happens at START. The three genes described in this thesis are potentially involved in the execution or the regulation of START.

Once yeast have executed START, they have the potential to delay cell cycle progression prior to its completion, due to the existence of "checkpoints" (Hartwell and Weinert, 1989). Checkpoints are inferred to exist when the phenotypes of mutant genes reveal potential negative feedback on cell cycle progression, resulting from the failure to complete a prior cell cycle step.

Checkpoints are believed to ensure the dependence of mitosis on the completion of DNA replication (Weinert and Hartwell, 1988) and microtubule assembly (Li and Murray, 1991), and the dependence of budding on the completion of mitosis (Hoyt et al, 1991).

Genetic analysis of the cell division cycle.

Cell division cycle mutations and CDC28. In budding yeast, cell morphology varies with cell cycle stage: G1 cells are unbudded. Bud emergence and the initiation of S phase are approximately coincident, but independent events. Cells with large buds are usually in G2 or M phase. Hartwell and colleagues (Hartwell et al, 1974) isolated a series of conditional mutations which, when transferred to nonpermissive conditions, arrested cell cycle progression with a homogeneous morphology (ie. unbudded, small budded, large budded). These are called cell division cycle ('cdc') mutations.

Among G1 arresting mutants in this collection, one (*cdc28-1*) was noted to be competent to mate at the arrested stage. Mating competence at the time of arrest was subsequently used to isolate additional alleles of *CDC28*, as well as mutations in three other genes (Reed, 1980). When *cdc28* cells are arrested at their nonpermissive temperature, then exposed to mating pheromone and transferred to permissive temperature, they fail to progress through the cell cycle. Furthermore, when *cdc28* cells are arrested with pheromone, then transferred to fresh media at the nonpermissive temperature, they also fail to progress through the cell cycle. Apparently, cells can't execute the pheromone sensitive step without *CDC28* function, and they can't execute the *CDC28* dependent step while arrested by pheromone. By definition, pheromone arrests cells at START, and by

inference, loss of *CDC28* function does as well. This contributed to the hypothesis that *CDC28* plays a critical role in the execution of START.

CDC28 is a serine/threonine kinase (Reed et al, 1985; Wittenberg and Reed, 1988), and is homologous to *cdc2* from *Schizosaccharomyces pombe*, and to the catalytic subunit of MPF (maturation or mitosis promoting factor, defined as a factor relieving the protein synthesis requirement for the completion of M₁ in amphibian oocytes (Murray and Kirschner, 1989b)). A *CDC28/cdc2* homolog (p34^{cdc2}) occurs in the human genome, and can functionally replace the yeast gene (Wittenberg and Reed, 1989). It is now widely believed that progression through virtually all eukaryotic cell cycles is regulated *via* control over the activation of the *CDC28/cdc2* kinase homologs (Murray and Kirschner, 1989b; Nurse, 1990; Hartwell, 1991). In budding yeast and fission yeast, *CDC28/cdc2* is required for cell cycle START, for mitosis (Hartwell, 1991), and for meiosis (Shuster and Byers, 1989; Niwa and Yanagida, 1988). In higher eukaryotic cells, the involvement of this kinase in mitosis (Hartwell, 1991) and meiosis (Labbe et al, 1988) is established. There are also indications that either *cdc2* or an emerging family of "cyclin dependent kinases" (*cdks*) may have a role at earlier cell cycle stages (Fang and Newport, 1991; Tsai et al, 1991; Pagano et al, 1992; Dulic et al, 1992; Matsushime et al, 1992).

Two of the major unanswered questions about the eukaryotic cell cycle concern the substrates for *CDC28/cdc2* and the *cdks*. In yeast cells, where it appears that a single kinase assumes both G1/S and G2/M functions (Pines and Hunter, 1990b), it remains to be determined how one kinase promotes two distinct transitions. Either one kinase phosphorylates distinct substrates at distinct times, or the phosphorylation of one substrate has different consequences at different times.

In either case, very little is understood about how kinase activation brings about a specific kind of cellular response, appropriate to the transition that eventually occurs. p34^{cdc2} has been reported to phosphorylate the nuclear lamins in *S. pombe* (Enoch et al, 1991).

In higher eukaryotes, where more than one kinase is available, the essential role for each kinase might be confined to a particular cell cycle stage. It remains to be determined, however, what the relevant substrates for these kinases are. Cyclin dependent kinases have been reported to be involved in the phosphorylation of oncogene products (Bischoff et al, 1990; Lin et al, 1991), cytoskeletal elements (Chou et al, 1990), and the nuclear lamins (Dessev et al, 1991; Peter et al, 1990), among other cellular components. Cyclin dependent kinases from both yeast and higher eukaryotes can phosphorylate histone H1, although the significance of this is obscure, since histone H1 is absent in yeast.

The cyclins. In yeast, as in other systems, Cdc28 protein is constitutively present (Mendenhall et al, 1987), but its kinase activity and association with other proteins vary during the cell cycle (Wittenberg and Reed, 1988). *CDC28/cdc2* homologs require physical association with cofactors for activation. The associated proteins which activate *CDC28/cdc2* kinases are called "cyclins", because they cycle in abundance during the cell cycle (Evans et al, 1983). RNAase treated, enucleated, *Xenopus* egg extracts arrest in interphase, but they can be driven into a mitotic state by addition of an RNAase inhibitor plus mRNA encoding cyclin B (Murray and Kirschner, 1989a), demonstrating the sufficiency (but not the necessity) of cyclin synthesis for the activation of MPF. Other means of control, involving both inhibitory (Thr-14, Tyr-15) and stimulatory (Thr-161) phosphorylations of the *Xenopus* p34^{cdc2} kinase also determine the functional state

of *CDC28/cdc2* in mitosis. Such control mechanisms have been particularly well studied in *Xenopus* (Solomon et al, 1990) and in *S. pombe* (Nurse, 1990; Lundgren et al, 1991).

Cyclins of the B-type sequence class are generally associated with the mitotic activation of *cdc2/CDC28*. Four B-type cyclin genes have been reported in *S. cerevisiae* (*CLB1*, *CLB2*, *CLB3*, and *CLB4*; Surana et al, 1991; Ghiara et al, 1991). *CLBs* 1,2, and 4 were discovered as high-copy suppressors of a unique conditional allele of *cdc28* having a G2/M terminal phenotype (Surana et al, 1991). *CLB1* mutations promoting the stability of the protein lead to M phase arrest (Ghiara et al, 1991), while deletion of *CLB2* leads to delayed entry into M phase (Surana et al, 1991). Simultaneous deletion of *CLB2* and either *CLB1* or *CLB3* arrests cells in G2, based on tetrad analysis (Surana et al, 1991; D. Lew and S.Reed, personal communication). In contrast, the *S. pombe cig1⁺* gene is a B cyclin that is required for efficient passage of the G1/S transition (Bueno et al, 1991).

A-type cyclins may also activate *cdc2* in mitosis; however, growing evidence suggests a role for A-type cyclins complexed with Cdc2 or the Cdc2-related kinase Cdk2 earlier in the cell cycle, in particular at DNA replication (Pines and Hunter, 1990b; Girard et al, 1991; Tsai et al, 1991). In higher eukaryotes, it is not clear if cyclin A/kinase complexes are required for S phase throughout its duration, or only at the G1/S transition (Girard et al, 1991). In budding yeast, no cyclin A homologs are known, nor is there any genetic evidence that *CDC28* is involved directly in S phase progression.

In *S. cerevisiae*, START is dependent on the distant cyclin homologs *CLN1*, *CLN2*, and *CLN3* (Richardson et al, 1989), as well as on *CDC28*. *CLN1* and

CLN2 were first identified as high copy suppressors of the G1 arresting temperature sensitive allele *cdc28-4* (Hadwiger et al, 1989b), while *CLN3* was identified as a mutant gene whose phenotypes included small cell size and dominant α -factor resistance (Carter and Sudbery, 1980; Cross, 1988a; Nash et al, 1988). The products of the *CLN* genes bind to (Wittenberg et al, 1990; Tyers et al, 1992; F. Cross and C. Blake, unpublished data) and activate (R. Deshaies, pers. comm.) the *CDC28* protein kinase. The three *CLN* genes are functionally redundant; any one of the three is sufficient for viability (Richardson et al, 1989). Triple *CLN* deficiency causes G1 arrest, at START (Cross, 1990).

The genes for human cyclins C, D, and E (Koff et al, 1991; Lew et al, 1991; Matsushime et al, 1991; Xiong et al, 1991), and *D. melanogaster* cyclin C (Leopold and O'Farrell, 1991; Lahue et al, 1991), were isolated on the basis of their ability to rescue *cln1,2,3⁻* yeast. The normal roles of these cyclins are unclear, but recent work suggests that cyclins D1 and E function in G1 of the mammalian cell cycle (Matsushime et al, 1992; Dulic et al, 1992; Lees et al, 1992). Human cyclin A and cyclins B1 and B2, as well as *S. pombe cdc13⁺*, a mitotic B-type cyclin, also functioned in the *cln1,2,3⁻* rescue assay (Koff et al, 1991; Lew et al, 1991; Xiong et al, 1991). These rescue experiments all employed a strong yeast promoter and high-copy plasmids, presumably promoting gross overexpression of the foreign cyclin.

In this thesis, I report the isolation of a dominant mutation conferring viability on yeast strains deleted for all three *CLN* genes. This mutation, called CBM (*CLN* bypass mutation) was characterized genetically, and shown to be unlinked to several known genes involved in the control of the yeast cell cycle. Among fifteen distinct isolates, two caused single division meiosis as an unselected

phenotype. An attempt was made to clone CBM. While this attempt was unsuccessful *per se*, it led to the isolation of two other yeast genes which could rescue the *cln1,2,3⁻* condition when expressed from their natural promoters, on a centromere containing (low copy number) plasmid.

One of these proved to be a novel B type cyclin, which was named *CLB5* (Epstein and Cross, 1992). Deletion of *CLB5* resulted in a marked slowing of S phase progression, but no detectable delay in the G1/S or G2/M transitions. *CLB5* RNA is expressed early in the cell cycle, in contrast to other B-type cyclins (Surana et al, 1991; Ghiara et al, 1991). These observations suggested that *CLB5* is a B-type cyclin functioning early in the cell cycle, and required for efficient DNA replication.

The other gene found as a dosage suppressor of *cln1,2,3⁻* inviability was *MPK1*. This gene has recently been isolated by others (J.Thorner, B. Futcher, both personal communications). Synthetic lethal analysis revealed that *MPK1* is nearly essential to the function of *CLN1* and *CLN2*. Disruption of *MPK1* caused a detectable delay in the G1/S transition, and caused a dramatic defect in cell cycle progression when combined with a null allele of *CLN3*.

The *CLN* bypass mutation was shown to have the same strain background dependencies as did *CLB5* over-expression for *cln1,2,3⁻* rescue: Both CBM and p*CLB5* rescue of *cln1,2,3⁻* are suppressed by the disruption of either *swi4* or *mpk1*. These results imply a range of possible models integrating roles for *CLB5*, *MPK1*, and CBM in the control of the *cln1,2,3⁻* dependent step. The potential functions of CBM and *MPK1* are discussed at the ends of chapters 3 and 6, respectively.

Chapter 2: Materials and methods

Yeast and bacterial strains

All yeast strains were isogenic with BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*), except as indicated in the text. A nonreverting null allele of *ARG4* was installed in the BF264-15D background using pmlc12Pst*ARG4*BglFill [Bam*URA3*] (Sun et al, 1989). Standard techniques were employed for strain constructions (Sherman et al, 1989). *E. coli* DH5 α was used when blue colonies were desired on X-gal media. YMC10 was used for routine production of plasmid DNA. DK-1 was used for recovery of CEN plasmids from yeast. When necessary for restriction by enzymes sensitive to *dam* methylation, plasmids were prepared in the *dam13::Tn9* strain GM2163 (New England Biolabs).

Synthesis of the genotypes CBM8/cbm⁺/cbm⁺ and cbm⁺/cbm⁺/cbm⁺, for testing dominance of CBM8. Diploid 886-2A, genotype MAT α /MAT α cbm⁺/cbm⁺ *cln1,2,3/cln1,2,3* [*GAL1::CLN3*] (derived from cross 886; see Table 12) was mated to a MAT α *cln1,2,3* CBM8 strain (957-3C), and to a cbm⁺ control. The resultant triploids were tested for growth on YEPD and YEPGal by quantitative plating assay.

Formation and testing of cytoductants with CBM18 (original isolate). To form cytoductants with CBM18, mating partners for CBM18 were prepared which were MAT α *arg4 cln1 CLN2 cln3* ρ° cyh^R and CBM18' or cbm⁺. (The derivation of CBM18' from CBM18 is explained in the text.) These were derived as follows: A spontaneous cyh^R derivative was selected by plating 884-4D (MAT α *cln1::TRP1 CLN2 cln3 Δ arg4*) on YEPD + 10 ug/ml cycloheximide. This was mated (cross 920) to 886-20D (MAT α *cln1::TRP1 cln2::LEU2 cln3 Δ CBM18' ARG4 spo⁺ W16*).

Tetrads were dissected, and four spore clones were retained:

920-4B: *MATa cln1 CLN2 cln3 arg4 cyhR CBM18'*
920-8C: *MATa cln1 CLN2 cln3 arg4 cyhR CBM18'*
920-9A: *MATa cln1 CLN2 cln3 arg4 cyhR cbm⁺*
920-20C: *MATa cln1 CLN2 cln3 arg4 cyhR cbm⁺*

Given the presence of *CLN2*, the genotype at *CBM18'* was assigned based on backcrossing to a *cln1,2,3⁻* W16 strain, dissecting tetrads on YEPD, and examining the segregation of viability in the progeny. Each of the four strains was cultured in the presence of 20 ug/ml ethidium bromide, then plated on YEP + 3% Glycerol + 0.1% Glucose. Slowly growing colonies were picked, and it was confirmed that they grew normally on YEPD, but were inviable on YEPGlycerol. It was also confirmed that they did not revert to viability on YEPGlycerol after culture on YEPD. Each of the four was then mated to *CBM18*, and cytoductants were selected on YEP + 3% Glycerol + cycloheximide. Cytoductants were tested to confirm that they were *MATa arg4 leu2*, all of which ruled out the presence of nuclear genes from *CBM18*. They were then mated to the *MATα* mating type tester PT2, and the diploids were sporulated. All four sporulated to form tetrads.

Derivation and genotyping of cln3 mpk1::ARG4 haploid strains. Although *cln3 mpk1* spores arrest after germinating, without budding, and are consequently inviable, newly germinated *cln3 mpk1* spores are competent to mate, if they germinate in the vicinity of vegetative cells of the opposite mating type. Diploid 1050E (*CLN1/CLN1 CLN2/CLN2 CLN3/cln3::URA3 MPK1/mpk1::ARG4* [CE118/TRP1]) was synthesized by dissecting 1036/A⁺/3 (*CLN1/CLN1 cln2::LEU2/CLN2 cln3::URA3/cln3Δ MPK1::MPK1::TRP1/mpk1::ARG4*) on agar that had been prespread with 1001-1B (*MATα CLN1 CLN2 CLN3 MPK1 arg4* [CE118/TRP1]). Matings between doomed *cln3 mpk1::ARG4* 1036 progeny and

1001-1B were selected via replicas to YcD-arg-trp. Thus it is possible, but unproven, that the terminal phenotype of *cln3 mpk1* spores coincides with the cell cycle stage where they are competent to mate. 1050E was sporulated and dissected to obtain *cln3::URA3 mpk1::ARG4* [CE118/TRP1] spore clones. Only one Ura⁺ Arg⁺ Trp⁺ spore was recovered (1050E-8A), and for unknown reasons it grew very slowly.

As an alternative means of recovering viable *mpk1 cln3* strains, 1036/A⁺/3 (*cln3::URA3/cln3Δ*) was subject to 5-FOA selection (presumably yielding *cln3Δ/cln3Δ* derivatives via mitotic recombination), transformed with [GAL1::CLN3/URA3], sporulated and dissected, yielding *cln3Δ mpk1::ARG4* [GAL1::CLN3] spore clones. Diploid 1052 (*CLN1/cln1::TRP1 CLN2/cln2::LEU2 CLN3/cln3Δ MPK1/mpk1::ARG4 arg4/arg4* [GAL1::CLN3/URA3]) was formed by mating a *cln1,2,3⁻* W16 strain to a *CLN1,2,3⁺ mpk1::ARG4* strain from 1050E. Thus, no auxotrophy was available to score the segregation of *CLN3* among 1052 progeny. However, in *CLN1 cln2 mpk1::ARG4* and *cln1 CLN2 mpk1::ARG4* segregants, genotype assignments at *CLN3* were made based on robust vs. poor (or non-existent) growth on YEPD. The fact that *CLN3* is the only unmarked gene segregating in 1052, combined with the fact that the genotype *mpk1::ARG4 cln3* is spore lethal, make it plausible that the lack of *CLN3* accounts for the poor health of vegetative *mpk1::ARG4* strains.

Null and marked alleles of yeast genes

Alleles from other studies. The *cln1::TRP1* and *cln2::LEU2* alleles were from S. Reed (Hadwiger et al, 1989b). *cln3::URA3* (*daf1::URA3*) was from F. Cross (Richardson et al, 1989). The *cln1Δ*, *cln2Δspe-xho*, *cln3Δ*, and *GAL1::CLN3*

alleles were as described (Cross and Tinkelenberg, 1991; Cross, 1990; Cross, 1988a). *cdc28-13* was from S. Reed (Reed, 1980). Strains bearing null alleles of *CLB1* (Ghiara et al, 1991), *CLB2*, *CLB3*, and *CLB4* (unpublished) were kindly supplied by S. Reed and D. Lew. The *swi4::URA3* allele consisted of a duplication of an internal BamHI fragment of *SWI4*, with the *URA3* between the duplicated sequences. This construct was provided by B. Andrews, and was introduced into the BF264-15D background by J. McKinney. The *hcs26::URA3* allele consisted of the *URA3* gene inserted at the unique *EcoRV* site in *HCS26*. This construct was synthesized by D. Lew, was introduced into the BF264-15D background by D. Lew, and its location was confirmed (by Southern blotting) by C. Epstein (results not shown). *SWI6::TRP1* and *swi6::LEU2* were obtained from B. Andrews, and were introduced into the BF264-15D background by J. Rakonjac. *lys2::URA3* was a gift of J. Thorner.

*Synthesis of *cln2Δspe-sph*.* pJH3-45-2 (containing the yeast *CLN2* gene as a Sau3A fragment cloned into the *Bam*H1 site of yeast ARS plasmid YRP7) was obtained from S. Reed (Hadwiger et al, 1989b). The 5' and amino terminal regions of the *CLN2* gene were subcloned from this source, between unique *Sal*I (5' region) and *Hind*III (coding region) sites, into the integrating vector YIP5 doubly digested with *Sal*I and *Hind*III. This construct was linearized at the unique *Spe*I site lying inside the *CLN2* coding sequence, partially digested with *Sph*I, and size fractionated on a 0.6% agarose gel. A band was recovered, representing the original construct less a deletion of 911 bases between the *Sph*I site 5' of the *CLN2* coding sequence and the *Spe*I site. The DNA was eluted using GENE-CLEAN (Bio 101), blunt-ended with T4 DNA polymerase, recircularized with T4 DNA ligase, and retransformed into *E. coli* strain GM2163. Several transformants were

miniprep and one was demonstrated to have the restriction map predicted for the intended deletion. Plasmid DNA was prepared, linearized inside the *CLN2* coding region with *Bcl*II, and transformed into yeast strain YFC589-1 (relevant genotype *cln1Δ cln2::LEU2 cln3Δ [CLN3/TRP1]*), selecting transformants on YcD-ura. Transformants were patched to 5-FOA to detect *ura3* revertants, which were screened for loss of the Leu⁺ phenotype. One of these was shown by Southern blotting with a *CLN2* probe to have undergone a replacement of the *cln2::LEU2* allele by the newly engineered deletion and truncation (Figure 3). The new *cln2Δspe-sph* allele was shown to be null for *CLN* function by swapping the W16 [*GAL1::CLN3/URA3*] plasmid for the 43-9 [*CLN3/TRP1*] plasmid, and demonstrating that the resulting strain arrested in G1 on transfer to glucose.

Centromere linked markers used to study centromere segregation in single division meiosis. *CEN1::URA3* was plasmid VG90 from D. Kaback, cut with *Eco*RI and transformed into yeast. *LEU2* was pCV13, double digested with *Xho*I/*Sal*II, transformed into yeast, and shown genetically to be linked to *MAT*. *TRP1::TRP1*, *TRP1::CLN3*, and *TRP1::CLN3(x3)* alleles were supplied by F. Cross.

Media

Hydroxyurea (Sigma) was used in solid media at 0.2 M. Nocodazole (Sigma) was used in liquid media at 15 ug/ml, from a DMSO stock at 10 mg/ml. α -factor (Sigma) was used in solid and liquid media at 1 μ M. Galactose, glycerol, and raffinose were used as carbon sources at 3%; glucose was used at 2%.

Construction of CEN plasmid library

Introduction. Since CBM is a dominant mutation, we sought to clone it by making a library in a yeast CEN plasmid, using genomic DNA isolated from strain having CBM. We then screened the library based on function for plasmids having activity at rescue of the genotype *cln1,2,3* [*GAL1::CLN3*] on glucose media. *CLB5* and *MPK1* were cloned fortuitously, while attempting to clone CBM.

Synthesis of pCE101. We made our library in a novel yeast cloning vector, pCE101. pCE101 (Figure 1) was made from pRS314 (Sikorski and Hieter, 1989) by substituting a pBR322 ORI for the existing pUC ORI in pRS314. It was reasoned that libraries should ideally not be made in bacterial vectors having pUC origins, since certain cloned fragments of yeast DNA might be toxic to the bacterial host when expressed at high copy number. The strategy for synthesis of pCE101 was to cut out the ORI region from pBR322 with *BspM2* (pBR322 was produced in *E. coli* strain GM2163) and *ScaI*, and to ligate it to the CEN/*TRP1*/MCS/ β gal region of pRS314, that had been cut out with *Afl*III (Anglian) and *ScaI*. The *ScaI*/*ScaI* junction, in the Ap gene, was to be selected based on recovery of ampicillin resistance. *EcoRI* was included in the digest of pBR322 to allow gel purification of the desired fragment, since *BspM2* and *ScaI* render pBR322 into two nearly equal pieces. Digested pBR322 and pRS314 DNAs were blunt ended with Klenow fragment, and DNAs were ligated together with T4 DNA ligase (New England). Ligated DNA was transformed into DH5 α , and blue colonies were detected on LB/Amp/X-gal agar. Based on restriction mapping, the clones recovered were derived from a pBR322 fragment that had failed to cut at the *ScaI* site, and went all the way from the *BspM2* site to the *EcoRI* site. Hence, there is a small direct duplication of the amino terminal end of the Ap gene in pCE101. This may have resulted from exonuclease contamination of one of the

enzymes used, preventing recovery of an intact Ap gene from a *ScaI/ScaI* junction.

Synthesis of CEN plasmid library. Yeast 933-1C (*MATa cln1::TRP1 CLN2 cln3Δ CBM8*^{4th Backcross}) was the source of the DNA used to make the library. *CLN2* was included in the library as a positive control. The presence of CBM in this strain was confirmed by backcrossing (cross 950) it to 884-39B (*cln1::TRP1 cln2::LEU2 cln3Δ arg4 [GAL1::CLN3]*), and recovering glucose viable Leu⁺ progeny. 933-1C was inoculated into 1 liter YEPD, and cultured to an O.D. of 1.15. Yeast DNA was then isolated essentially as described (Holm et al, 1986). DNA was 1/16 under digested with *Sau3A*, phenol extracted, RNAase treated, and precipitated with PEG to eliminate residual RNA. The pellet was recovered and run over a 10 - 30% glycerol gradient for 5 hours at 40,000 RPM, in an SW40 rotor. The gradient was fractionated and a fraction was retained having a modal DNA fragment length of about 12 KB.

pCE101 was cut with *Bam*HI and treated with calf intestinal phosphatase, then combined, at a concentration of 3 ug/ml, with the glycerol gradient fractionated yeast DNA. The mixture was ligated overnight at 15°C with T4 DNA ligase, and transformed into *E. coli* strain DH5α. Bacterial colonies were rinsed off the LB/amp plates and pooled from 50 independent transformations and platings of the ligated DNA, cultured for 2 hours with an added 100 mls of LB (50 ug/ml ampicillin), and plasmid DNA was prepared by the alkaline lysis method.

Isolation of CLB5 and MPK1 genes from library. Library DNA was transformed into yeast strain 960-3C (*cln1 cln2 cln3 [GAL1::CLN3/URA3]*), and transformants were selected on YcGal-trp. Once colonies had grown up, replicas were taken to YEPD agar. Secondary YEPD replicas were taken from the primary

replicas after 24 hours growth. These were allowed to grow for two to three days, although [p*CLN2/TRP1*] clones formed robust patches after one day's growth. Candidate clones were picked from the secondary YEPD replicas, and colony purified on YcGal-trp-ura. Growth on YEPD was demonstrated to be dependent on inheritance of a *TRP1/CEN* plasmid based on cosegregation, following plasmid loss during nonselective growth. Plasmids were recovered from 1.5 ml overnight YEPD cultures, as described (Ausubel et al, 1987).

Overall, from two independent libraries made from the same glycerol gradient fraction, we recovered eight distinct clones of *CLN2* (a total of 42 times), no clones of *CBM8*, three distinct clones of *CLB5* (one time each), and a single clone bearing *MPK1*, previously found to suppress *cln1,2,3* lethality in a high-copy plasmid (O. Fields and J. Thorner; B. Futcher; both personal communications). These results are summarized and expanded in Table 1. Clones of *CLN2* were identified based on hybridization to a radio-labeled *CLN2* probe.

The *CLB5* gene was subcloned into the integrating vector RS304, yielding pCE105. The *ApaI* fragment was deleted by limit digestion and recircularization. CE105Δ*ApaI* was linearized at the unique *XhoI* site (in the *CLB5* 5' region), and transformed into diploid 957 (*cln1/cln1 cln2/cln2 cln3/cln3 CBM8/cbm*⁺ [p*GAL1::CLN3/URA3*]). Transformants were recovered on YcGal-trp, sporulated, and dissected. Southern blots confirmed that the CE105 derivative had integrated via homologous recombination at the *CLB5* locus, as expected. *CBM8* was not linked to *CLB5*, hence the *CLB5* clone represents wild type DNA.

Plasmids, Subcloning, and Sequencing

CLB5. pCE104 was one of the *CLB5* plasmids isolated from our CEN

Table 1: Plasmids synthesized in this study.

plasmid	gene or origin	library, if applicable	number of times isolated from library
CE101	cloning vector		
CE102	<i>CLN2</i>	[L1]	7
CE103	<i>CLN2</i>	[L1]	2
CE104	<i>CLB5</i>	[L1]	1
CE105	insert from CE104 in RS304		
CE106	<i>CLN2</i>	[L2]	8
CE107	<i>CLN2</i>	[L2]	8
CE108	<i>CLN2</i>	[L2]	9
CE109	<i>CLB5</i>	[L2]	1
CE110	<i>CLB5</i> and <i>CLB2</i>	[L2]	1
CE111	insert from CE104 in RS314		
CE112	<i>SpeI</i> - <i>XhoI</i> fragment from CE104 in RS314		
CE113	<i>CLN2</i>	[L2]	4
CE114	<i>CLN2</i>	[L2]	2
CE115	<i>CLN2</i>	[L2]	2
CE116	<i>MPK1</i>	[L2]	1
CE117	insert from CE116 in RS304		
CE118	insert from CE116 in RS314		
CE119	<i>CLB5</i> subcloned into pBM272 (<i>GAL1::CLB5</i>)		

Distinct <i>CLN2</i> isolates:	8
Total number of times <i>CLN2</i> isolated:	42

Distinct <i>CLB5</i> isolates:	3
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Distinct <i>MPK1</i> isolates:	1
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Each *CLB5* and *MPK1* plasmid was isolated only once.

plasmid library. pCE111 and pCE112 were made by subcloning the pCE104 entire insert and *XhoI*-*ClaI* fragments, respectively, into pRS314. pCE111 Δ *ClaI* and pCE112 Δ *SpeI* were derived from their respective parent plasmids by limit digestion and recircularization. pCE110 was another of the *CLB5* plasmids isolated from the library. Restriction mapping, sequencing, and hybridization experiments revealed that it contained the *CLB2* gene as well as the *CLB5* gene. pCE110 Δ *NsiI*, lacking the *CLB5* gene, was derived from pCE110.

In order to make a null allele of *CLB5*, we partially digested CE111 Δ *ClaI* with *EcoRI*, and ligated it to *EcoRI* digested pMLC28Pst6*ARG4* (Sun et al, 1989). The ligation mixture was transformed into *E. coli*, and transformants were selected on LB, 50 ug/ml ampicillin, and 34 ug/ml chloramphenicol. Several of these were restriction mapped, and one was selected in which the *ARG4* plasmid had been cloned into the *EcoRI* site at residues 290/291 of *CLB5*, with the orientation 5'*CLB5* - camR - *ARG4* - 3'*CLB5*. This was partially digested with *BspEI*, and a fragment lacking the *BspEI* fragment spanning residues 216 to 291 of *CLB5*, plus part of the camR gene, was gel purified and recircularized with T4 DNA ligase. The resulting plasmid, *clb5::ARG4* Δ *BspEI*, was digested with *XhoI* and *SpeI*, and transformed into *arg4* strains. Integration was demonstrated to be at the *CLB5* locus by Southern blotting (data in Figure 2A). *pclb5::ARG4/CEN/TRP1* was also transformed into *cln1,2,3*⁻ yeast as an intact CEN plasmid, and shown to have no activity at *cln1,2,3*⁻ rescue.

Synthesis of MPK1::CE107 Δ *XhoI* (also called *MPK1::MPK1::TRP1*). The yeast chromosome corresponding to the insert in CE116 was marked with *TRP1* as follows: The entire insert from CE116 was subcloned into RS304, yielding CE117. The *XhoI* fragment (left end of insert, Figure 25) was deleted by

linearization and recircularization. The integrating vector was then linearized inside the *MPK1* gene with *HpaI*, and transformed into diploid 957 (*cln1/cln1 cln2/cln2 cln3/cln3* CBM8/cbm⁺ [W16]), selecting on YcGal-trp. Southern blotting revealed that the construct had integrated as expected, since chromosomal DNA hybridizing to the deleted *XhoI* fragment had increased in molecular weight (Figure 2B). 957::CE107Δ*XhoI* was sporulated and dissected; the *TRP1* marker was not detectably linked to CBM8 (Table 6), hence the *MPK1* clone also represents wild type DNA.

Disruption of MPK1 with ARG4. CE118 was linearized with *HpaI* (Figure 25), and ligated to pMLC28*Pst6ARG4* (Sun et al, 1989) that had been linearized with *SmaI* (both enzymes leave blunt ends). This introduced the *ARG4* gene into the coding region of *MPK1*, at least 0.5 KB upstream of the 3' end of *MPK1* (estimated from Figure 25 and sequenced region B). A standard ligase reaction was supplemented with 30 mM KCl, 1 μM hexamine cobalt chloride to enhance the efficiency of blunt end ligation. Bimolecular clones were selected on LB/agar supplemented with 50 μg/ml ampicillin, 34 μg/ml chloramphenicol. A clone was retained having the orientation 5'*MPK1* -- *ARG4* -- cam^R -- 3'*MPK1*. The CE118/pMLC28 fusion clone was doubly digested with *XhoI* and *NotI*, liberating a fragment of yeast DNA containing *MPK1* disrupted with the *ARG4* gene. This was transformed into diploid 1036 (*CLN1/CLN1 CLN2/cln2::LEU2 cln3Δ/cln3::URA3 MPK1::MPK1::TRP1/MPK1*), and five independently transformed colonies were picked, sporulated, and dissected. In none of the 5 was recombination detected between the prototrophies for arginine and tryptophan. Pick #3 was retained (1036/A⁺/3) because it showed linkage (Trp⁺ vs Arg⁺) in *trans*.

Subcloning CLB5 under the control of the GAL1 promoter. *CLB5* DNA

from pCE111 was PCR tailed with unique sites for *SaII* and *BamHI*, using the following primers:

5' primer: CGGGATCCACTGAACAATGG (*BamHI*) (methionine of *CLB5*)

3' primer: TCGCGGTCGACCATTTGAATAAC (*SaII*)

The 3' primer corresponded to *CLB5* DNA 289 nucleotides 3' of the stop codon. PCR was performed using 1' at 95°C, 2' at 55°C, and 2' at 72°C, for 25 cycles. Product DNA was phenol extracted to eliminate residual polymerase activity (which may fill in ends following restriction), precipitated with 2M ammonium acetate and 50% isopropanol, digested with *BamHI* and *SaII*, and purified on an 0.8% LGT agarose gel to eliminate end fragments and oligonucleotide primers. The PCR synthesized band was eluted with β -agarase (New England), phenol extracted and ethanol precipitated. pBM272 (YCP50 plus a 658 nt. *HindIII* - *EcoRI* *GAL1/10* cassette) was cut with *SaII* and *BamHI*, and treated with calf intestinal phosphatase (Boehringer). Plasmid and *CLB5* DNA were ligated together, and transformed into *E. coli* YMC10. Candidate clones were proven by restriction mapping, then transformed into a *cln1,2,3* [*CLN2/TRP1*] yeast strain, and shown to confer galactose dependent viability, upon loss of the *CLN2/TRP1* plasmid.

Dideoxy sequencing. Sequenase (USB) was used according to the manufacturer's instructions. For *CLB5*, double stranded DNA was sequenced on both strands, using ExoIII deletion derivatives of pCE111 and pCE112, restriction enzyme deletion derivatives, and custom synthesized primers. For *MPK1*, sequence was obtained from one strand only, as indicated in the legend to Figure 26.

clb1 clb2 rescue by p[*CEN/CLB2*]

Diploid 997 (*clb1::URA3/CLB1 clb2::LEU2/CLB2*) was transformed with pCE110, pCE112, and pCE110 Δ NsiI (Figure 15). From each transformation, a colony was picked, cultured in YcD-trp liquid, sporulated, and dissected. Replicas were taken from the dissection agar to score *clb1::URA3*, *clb2::LEU2*, and the *TRP1/CEN* plasmid. From 997[pCE110] haploid progeny strains, 5 Ura⁺ Leu⁺ Trp⁺ colonies were found among 22 Trp⁺ spores (23%). From 997[pCE110 Δ NsiI], 2 Ura⁺ Leu⁺ Trp⁺ colonies were found among 7 Trp⁺ spores (29%). From 997[pCE112], no Ura⁺ Leu⁺ Trp⁺ colonies were found among 33 Trp⁺ spores, although 44% of the viable progeny were Trp⁺, 37% were Ura⁺, and 28% were Leu⁺. If pCE112 rescued *clb1 clb2* mutants, then one fourth of Trp⁺ spores (ie. 8.25) should be Ura⁺ Leu⁺, given independent assortment of *CLB1* and *CLB2*. The finding that none of them are Ura⁺ Leu⁺ is significant ($\chi^2 = 11.00$, $p < .005$).

Determination of α -factor and hydroxyurea execution points.

The arrest morphology due to α -factor treatment is a large unbudded cell (Pringle and Hartwell, 1981). Therefore, a cell past the α -factor execution point at the time of plating on α factor-containing solid medium will divide, and each of the new cells will arrest without budding, resulting in an adjacent pair of large unbudded cells. A cell before the α -factor execution point will arrest as a single large unbudded cell.

The arrest morphology due to hydroxyurea treatment is a large-budded cell (Pringle and Hartwell 1981). Therefore, a cell past the hydroxyurea execution point at the time of plating on hydroxyurea-containing solid medium will divide, and each of the new cells will bud and arrest, resulting in an adjacent pair of large-budded cells. A cell before the hydroxyurea execution point will arrest as a

single large-budded cell.

Exponentially growing cultures of 1029-12B (*MATa bar1 CLB5*) or 1029-10B (*MATa bar1 clb5::ARG4*) in YEPD medium were sonicated and plated on YEPD, YEPD + 10^{-8} M α -factor, or YEPD + 0.2 M hydroxyurea (Hartwell 1976). After 4 hrs (3.5 hrs in one experiment) incubation at 30°C, the plates were examined microscopically. By the end of the incubation on the control YEPD plate >90% of the cells had divided and budded again at least once, so we did not consider viability to be a significant concern in the analysis. While ideally all cells on the hydroxyurea plate should be either 2 or 4 cells+buds/microcolony (Hartwell 1976), approximately 10% of the cells were found in the 1, 3, and 5 cells+buds/microcolony categories. The 1's were considered to be before the hydroxyurea execution point; the 3's and 5's were considered to be past the execution point. Similarly for α -factor, ideally all cells should be 1 or 2 cells/microcolony; we observed approximately 10-15% of cells in the 3,4, or 5 categories. These were considered to be past the α -factor execution point in the analysis. The percentage of budded cells was determined at the time of plating. 100 cells each were scored for two independent cultures in three (hydroxyurea execution point) or two (α -factor execution point) separate experiments, and the data were pooled (Table 2). To calculate the proportion of the total cell cycle time at which various events occurred from the percentage of cells in the population past those events, we employed the age-distribution function (Mitchison, 1971) $Ex(F) = 1 - \ln(2 - F)/\ln 2$, where F is the fraction of cells before the execution point, and $Ex(F)$ is the point in the cell cycle at which the execution point occurs (in units of cell-cycle times, from 0 to 1). The time from cell division to the execution point in minutes was calculated by multiplying $Ex(F)$ by the doubling

Table 2. Cell division and budding upon plating wild-type and *clb5* yeast on solid media containing inhibitors.

Strain	Time (Hrs)	Medium	Cells+buds per microcolony (SEM):					#Exp. in mean:
			1	2	3	4	>4	
<i>CLB5 CLB2</i>	0		31(3)	67(3)	2(1)	2(0)	0(0)	6
	4	α F	20(3)	65(4)	6(2)	7(2)	2(2)	4
	4	HU	3(0)	33(2)	4(2)	58(3)	2(1)	6
<i>clb5 CLB2</i>	0		19(2)	76(2)	3(1)	2(1)	0(0)	6
	4	α F	8(0)	79(2)	6(0)	6(2)	1(0)	4
	4	HU	2(1)	56(3)	2(1)	36(2)	5(1)	6
<i>CLB5 clb2</i>	0		12(2)	86(3)	1(1)	1(1)	0(0)	4
	4	HU	0(0)	25(2)	3(1)	70(1)	2(1)	4
<i>clb5 clb2</i>	0		8(3)	89(2)	2(1)	2(2)	0(0)	4
	4	HU	1(2)	35(7)	4(1)	57(6)	3(1)	4

The number of cells+buds formed by each cell plated was determined by microscopic examination of cells plated on appropriate media (see Materials and methods). For each condition in each experiment 100 cells were scored. The means and standard errors (SEM) of the data are shown, and the number of replicate experiments used to calculate the mean is indicated.

time of the strain. The hydroxyurea execution point was determined similarly for a *clb2::LEU2* strain.

Northern blotting

Northern blotting was performed as described in Cross and Tinkelenberg (1991). *cln1,2,3⁻ leu2::LEU2::GAL1::CLN3* cells were synchronized by incubating in YEP 3% raffinose for 150 minutes. Galactose was added to 3% to start the cycle. Nocodazole was used at 15 ug/ml. DNA fragments used as probes were as follows: *CLB5*, the 462 BP *EcoRI* fragment; *CLB2*, the 767 BP *BglII-ClaI* fragment; *CLB4*, the 500 BP *SpeI-SspI* fragment, from a clone supplied by B. Futcher; *CLN2* and *TCM1*, as described (Cross and Tinkelenberg, 1991). *SPO13* DNA was obtained from Dr. Robert Elder.

Determination of Cell Cycle Parameters

Flow cytometric DNA quantitation. Cells were stained with propidium iodide and prepared for FACS analysis using a Becton Dickinson FACScan as described (Lew et al, 1992). In most experiments, 10,000 cells were examined. Events were either live gated to have a DNA fluorescence above background, or gated after acquisition based on forward and side scatter, to exclude cell debris. In the former case (Figures 21, 24, and 28), all 10,000 events are plotted in the DNA content histograms, while in the latter (Figure 20), up to about 20% of the events are discarded. The profiles were affected very little by the gating method chosen. When the latter method was used, the Y axes were adjusted to compensate for the fact that different numbers of events are represented on different histograms. A constant proportion of the cell population corresponds to a constant area in the

histogram, regardless of gating method used. For FACS analysis of synchronized cells, *cln1,2,3*⁻ deficient cells were synchronized exactly as in northern blots, or by treating a *bar1 MATa* strain with 10^{-7} M α -factor (Sigma) for 120 minutes.

Determination of the arrest stage of pheromone treated cells. Exponential cultures of cells were treated with 1 μ molar α -pheromone (or left untreated), then aerated at 30C for an additional 6 hours in YcD-ura media. Cells were sonicated, fixed in 3.7% formaldehyde, and budding indices were determined by microscopic examination.

*Determination of the arrest stage of *cln1,2,3 CBM8 cdc28-13* cells, and controls, at permissive and non-permissive temperatures.* Log phase cultures (OD₆₆₀ about 0.04) of all genotypes were obtained in YcGal-ura media. Cells were pelleted, and resuspended in YcGal or YcD -ura media, and placed at 30° or 38° C. At 0, 2, and 6 hours time, aliquots were taken, fixed in 3.7% formaldehyde, sonicated, and microscopically examined to determine percent cells unbudded.

Doubling times. These were determined from log phase cultures. OD₆₆₀ readings were taken at intervals, log transformed, and regressed against time. Doubling time = $\ln 2$ / slope. Electronic cell volumes were determined as described (Cross, 1988a).

Chapter 3: Isolation and characterization of CBM

Introduction. Isolation of CBM. In order to identify genes involved in the regulation or execution of START, mutations were isolated which enabled *cln1 cln2 cln3* strains to proceed through the cell division cycle and form colonies. It was reasoned that such mutations could be in genes which act at the *CLN* dependent step (ie. a fourth *CLN*-like gene), they could be in genes which act "downstream" of *CLN* products (ie. *CDC28*, its regulatory subunits or its substrates), and they could be in genes which are normally not involved in G1/S regulation, but which can be mutationally recruited to fulfill such a function (ie. S phase, M phase, or meiotic cyclins).

Dr. Cross isolated mutations apparently bypassing the requirement for *CLN1*, *CLN2*, and *CLN3* by creating strains with the genotypes *cln1::TRP1 cln2::LEU2 cln3Δ ura3* plus episomal [*GAL1::CLN3/ URA3*] or [*GAL1::CLN3-2/ URA3*] (Table 3). These were cultured in YEPGal liquid, then plated on YEPD agar, at either room temperature, 23°C, or 37°C, as indicated (Table 3). In some instances, yeast were UV mutagenized prior to plating, as indicated (Table 3). Rare colonies appeared, and replicas were taken to 5-FOA/dextrose solid media (which selects against *Ura*⁺ strains (Boeke et al, 1984)) to identify mutants whose viability was independent of the continued presence of the episomal *GAL1::CLN3* gene. Dr. Cross obtained fifteen independent mutations, to which he gave the provisional name "*CLN*-bypass mutation", abbreviated CBM. He estimated that these mutations occurred at a frequency of one in a hundred million. These isolates were given to me for further characterization when I started work in Dr. Cross' lab.

Table 3: Isolation of *CLN* bypass mutations.

Isolate number	Mutagen	Temp. (°C) at which isolated	Parental Strain
CBM2	---	RT	W11-1A
CBM3	---	RT	D4-1A
CBM4	---	RT	D4-1A
CBM6	---	37	W11-1A
CBM7	---	37	W11-1A
CBM8	---	37	W11-1A
CBM9	---	37	W11-1A
CBM10	---	37	D4-1A
CBM11	---	37	D4-1A
CBM12	---	37	D4-1A
CBM13	---	37	D4-1A
CBM14	---	37	D4-1A
CBM15	UV	23	D4-1A
CBM16	UV	23	D4-1A
CBM18	UV	23	D4-1A
Genotypes:			
W11-1A	<i>MATα cln1::TRP1 cln2::LEU2 cln3Δ [GAL1::CLN3/URA3].</i>		
D4-1A	<i>MATα cln1::TRP1 cln2::LEU2 cln3Δ [GAL1::CLN3-2/URA3].</i>		
(RT)	room temperature.		

The *CLN* bypass mutations are each unlinked to *CLN1* and *CLN2*, and are not rearranged forms of *CLN1*, *CLN2*, or *CLN3*.

The CLN bypass mutations are not reversions of the null alleles of cln1 and cln2 present in the parental strain. CBM was isolated in the strain background *cln1::TRP1 cln2::LEU2 cln3Δ*. In order to exclude the possibility that the residual fragments of *CLN1* or *CLN2*, present in the parental strain, had mutated to restore *CLN* function, each of the 15 original isolates was backcrossed to a strain of genotype *CLN1 CLN2 cln3::URA3*. Diploids were sporulated and, where possible, tetrads were dissected. (CBM15 and CBM18 had dominant mutations causing single division meiosis, hence did not give rise to tetrads; this will be discussed in chapter 4. If CBM were an allele of *CLN1* or *CLN2*, then 100% of spores would inherit either a wild type *CLN* gene or its CBM allele. In no case should germinating spores give rise to the "shmoo" morphology characteristic of cells arrested in G1 due to the lack of *CLN* function. Alternatively, if CBM is unlinked to *CLN1* and *CLN2*, then 1/8 of progeny are predicted to be *cln1::TRP1 cln2::LEU2* CBM, while 1/8 are predicted to be *cln1::TRP1 cln2::LEU2 cbm⁺*. The *cbm⁺* cells are predicted to form shmoos, while the CBM cells are expected to form colonies.

Spores were microscopically examined 24 hours after dissection, to determine morphological phenotypes. For each CBM isolate, "shmoos" were observed on the dissection agar. Pooling results from all CBM isolates, the overall frequency of (unambiguous) shmoos was 9.5% of all spores (including non-germinating spores). The expected frequency would be 12.5% (ie. 1/8) if 100% of spores germinated. However, fewer than 100% germinate, and shmoo

frequency under 12.5% will occur if *cln*⁻ strains preferentially fail to germinate. The occurrence of shmoos in each backcross implied that in no case was CBM allelic with *CLN1* or *CLN2*. This result will be confirmed and extended, below, where it is shown that each isolate of CBM was linked to *CBM9*.

In many instances, the genotypes of shmoos at *CLN1* and *CLN2* could be inferred from the scorable genotypes of *viable* cells in the same tetrad, assuming 2:2 segregation of markers. Overall, out of 35 genotypable shmoos observed in 108 tetrads from the 13 crosses, 32 could be assigned the genotype *cln1::TRP1 cln2::LEU2 cln3*. This lends confirmation to the idea that a cell scored as a shmoo is in fact exhibiting the *cln* deficient terminal morphology.

CLN3 hybridizing sequences are absent in 5-FOA resistant derivatives of CBM strains, as isolated. The strains in which CBM was isolated bore a complete deletion of the *CLN3* gene (Cross, 1990). We were concerned that the *CLN3* gene from the *GAL1::CLN3* plasmid, present in the parental strain, could have re-integrated into the genome under the expression of a new promoter. To exclude this possibility, genomic DNA was prepared from each CBM isolate, restricted, and Southern blotted. The blot was probed with a *CLN3* probe. Each one of the 15 CBM isolates was negative for hybridization to the *CLN3* probe, while a battery of positive controls were all positive (data not shown). Hence CBM is not the result of expressing *CLN3* from an integrated ectopic location.

CBM is neither allelic, nor closely linked, with CLN3. Although it was shown that no DNA hybridizing to a *CLN3* probe was present in CBM strains when originally isolated, one may nevertheless ask whether CBM is genetically linked to the *CLN3* locus. When CBM was first backcrossed, strains of genotype CBM *cln1::TRP1 cln2::LEU2 cln3Δ* were mated to strains of genotype *cbm*⁺ *CLN1*

CLN2 cln3::URA3. In the progeny of these crosses, CBM can be scored indirectly, in two ways. First, viable Trp⁺ Leu⁺ strains are putative CBM strains, since they are inferred to have inherited no intact *CLN* genes, yet are viable. Second, inviable strains which were noted as shmoos on the dissection agar, and which are inferred (from the other spores in the tetrad) to be Trp⁺ Leu⁺, are putative cbm⁺ strains. For both of these classes of strains (ie. CBM and cbm⁺), prototrophy and auxotrophy for uracil were observed with nearly equal frequency. When all data from the backcross of nearly all CBM isolates (ie. 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, and 14) were pooled, among the CBM strains, there were 12 Ura⁺ and 16 Ura⁻ strains among the CBM progeny, and 12 Ura⁺ and 14 Ura⁻ strains among the cbm⁺ progeny. Since uracil prototrophy is linked to *CLN3* in these crosses, we can exclude the possibility that CBM is tightly linked to *CLN3*. The legitimacy of pooling data from these crosses will be established below, when it is demonstrated that all CBM isolates are probably linked to one another.

DNA hybridizing to CLN1 and CLN2 probes is not rearranged in CBM strains. We wished to exclude the possibility that the *CLN* bypass mutations resulted from a chromosomal rearrangement, in which the residual fragments of the *CLN1* or *CLN2* genes (present in the parental strains) had translocated to a novel chromosomal location, and somehow regained function in the process. Such events would yield apparent mutations unlinked to the original *CLN* loci, but would in fact not merit extensive study. In principle, it would be possible to detect such rearrangements by Southern blotting DNA isolated from CBM and cbm⁺ strains, and showing that the pattern of *CLN* probe hybridization was the same in the two strains. However, a potential pitfall in this approach is that if, by chance, the (hypothetical) novel *CLN* locus comigrated on the gel with the bona fide locus, it

would be obscured in the Southern, and consequently overlooked. As a means of avoiding this pitfall, for both *CLN1* and *CLN2*, Southern blots were done using several CBM8 strains each having a different engineered *CLN* null allele, and probed with a *CLN* probe that would detect the particular allele of *CLN* present. Since these each migrate differently on the gel, it is impossible for a hypothetical cryptic band to be hidden in each case. This analysis indicated that there was no material hybridizing to *CLN1* or *CLN2* probes other than the material at the bona fide *CLN1* and *CLN2* loci, respectively. For the sake of illustration, the result for *CLN2* is shown (Figure 3). Consequently, CBM8 does not result from a chromosomal rearrangement leading to the translocation of *CLN1* or *CLN2*. Since all the CBM isolates are linked to one another, it is unlikely that any of them results from such a genetic rearrangement.

*CBM is a true "bypass" suppressor, since its function is not dependent on particular null alleles of *cln1* and *cln2*.* The *CLN* bypass mutations were isolated in the strain background *cln1::TRP1 cln2::LEU2 cln3Δ*. We wished to exclude the possibility that CBM represented a modifier which somehow restored function, in *trans*, to the particular null alleles of *CLN1* and *CLN2* present in the parental strain. A new, null allele of *CLN1* (called *cln1Δ*) was synthesized as described (Cross and Tinkelenberg, 1991). Two new, null alleles of *CLN2* were synthesized, called *cln2Δspe-sph* (Materials and methods) and *cln2Δspe-xho* (Cross and Tinkelenberg, 1991). Strains were constructed with the genotype *cln1Δ cln2(Δspe-sph or Δspe-xho) cln3Δ* W16 CBM8, and shown to retain viability on YEPD media. Hence CBM is a true *CLN* bypass mutation, and not merely a suppressor of the particular disruption alleles present in the parental strains.

Genetic analysis of the CLN bypass mutations

The CLN bypass mutations are dominant. Each of the original CBM isolates were tested for dominance. Since the CBM phenotype can only be detected in the absence of *CLN* genes, the test relied on mating CBM isolates (*cln1::TRP1 cln2::LEU2 cln3Δ* "CBMX" *trp1 leu2 ura3*) to a strain of genotype *cln1::TRP1 cln2::LEU2 cln3Δ* [*GAL1::CLN3/URA3*] *trp1 leu2 ura3*. Diploids were selected by replica plating from the mating plate to YcD-ura. The lack of uracil selected against the CBM strain, while the lack of *GAL1::CLN3* synthesis on dextrose media selected against the *cbm*⁺ strain. However, diploids should only grow if CBM is dominant. For each CBM isolate, growth was apparent, although there were clear differences in the robustness of the diploids, summarized in Table 4.

Diploids were also selected in a fashion that did not rely on CBM function, based on mating a series of six *cln1,2,3*⁻ CBM8^{6x} *leu2::LEU2::GAL1::CLN3* strains to a *cln1,2,3*⁻ *cbm*⁺ [*GAL1::CLN3/URA3*] strain, and selecting the diploids on YcGal-leu-ura (the superscript on CBM refers to the number of generations of backcrossing between the original isolate and the strain in use). These six CBM strains represented all the CBM strains obtained from the sporulation and dissection of diploid 1053. The *CLN* bypass phenotype of all six spore clones was found to be dominant, and no obvious variability was seen in the strength of the dominance (Figure 4). Hence after six backcrosses, CBM8 retained the dominant character observed in the original isolate. This demonstrates that the dominance is not dependent on the action of modifiers which may have been present in the original isolates.

In addition, CBM8 was tested for dominance at high temperature. *cln1,2,3*⁻

Table 4: Phenotypes of *CLN* bypass mutations.

Isolate	Growth on YEPD at 23°C?	Growth on YEPD at 37°C?	Meiosis	Growth of <i>cln1,2,3</i> ⁻ diploid at 30°C
CBM2	+	+	normal	+/-
CBM3	+	+	normal	+
CBM4	+/-	+	normal	+
CBM6	-	+	normal	+
CBM7	-	+	normal	+
CBM8	-	+	normal	++
CBM9	-	+	normal	+
CBM10	-	+	normal	+
CBM11	-	+	normal	++
CBM12	-	+	normal	+
CBM13	-	+	normal	+
CBM14	-	+	normal	+
CBM15	+	+/-	s.d.m.	+/-
CBM16	+	+	normal	+
CBM18	+	+/-	s.d.m.	+

Meiotic phenotype:

normal: upon exposure to sporulation conditions, the majority of sporulated cells give rise to tetrads containing four haploid spores.

s.d.m.: (single division meiosis) upon exposure to sporulation conditions, the majority of sporulated cells give rise to dyads containing two diploid spores.

homozygous diploids were selected in a fashion that did not rely on the dominance of CBM. This was done by mating CBM8^{4X} *cln1Δ cln2::LEU2 cln3Δ arg4* to *cbm⁺ cln1Δ cln2Δ cln3Δ ARG4 [GAL1::CLN3/URA3]*, and selecting for growth (diploid 1000) on YcGal-leu-arg-ura. The diploid so obtained was tested for growth on glucose and galactose complete media at 30° and 38°, based on streaking out. Growth was apparent at both temperatures, although it was less robust on glucose at the higher temperature (data not shown).

Finally, CBM8 was tested for dominance in a triploid. This was done in order to evaluate the possibility that the *CLN* bypass phenotype was the result of a loss-of-function mutation in a negative regulator of the cell cycle. Such a mutation might be dominant if cells were sensitive to the dosage of this gene. A CBM/+ heterozygous diploid has one half the normal gene dosage, while a CBM/+/+ triploid has two thirds the normal gene dosage. Since two thirds approaches unity, a triploid may fail to show a loss-of-function phenotype apparent in a haploid or a diploid. Consequently, if the phenotype persists in the triploid, it renders the possibility that the mutation is due to loss of function all the less plausible, and the alternative (gain of function) all the more plausible. This was of interest both intrinsically, and because we eventually wished to clone CBM by making a library from DNA isolated from a CBM strain, and recovering the clone of interest from the library based on function in a haploid. This strategy could not be expected to work if CBM represented a null allele of some gene, since the addition of a clone of a null allele, unlike the addition of a haploid genome, has no effect on gene dosage.

A CBM8/+/+ yeast strain, and a +/+/+ control, were constructed as described (Materials and methods). Strain construction did not involve selection

for *CLN* bypass activity. Both triploids were nine-fold deleted for *CLN* genes, and had the *GAL1::CLN3/URA3* plasmid. They were tested for growth by streaking on YEPD. Only the CBM8/+/+ strain formed colonies. In addition, plating efficiencies on YcD-ura and YcGal-ura were determined. The CBM8/+/+ triploid plated with an efficiency of about 1/10 on YcD-ura (compared to YcGal-ura), while the +/+ triploid was inviable on YcD-ura. A large range in colony sizes was noted for the CBM8 triploid on YcD-ura, presumably indicative of a common mutational event with an effect on growth rate. Thus, a single dose of the CBM gene is adequate, if barely, for conferring *CLN* bypass activity on a triploid.

At least 10, and probably 12, of the CBM isolates are linked to one another. Fifteen distinct mutations conferring *CLN* independence were isolated. It was of interest to determine how many different genes were represented by these isolates. There is a simple way to determine whether two different mutations in haploid strains are both in the same gene. One simply mates the two isolates to one another, and asks whether the diploid formed thereby retains the phenotype of the mutants (in which case, the mutations are allelic), or is restored to wild type (in which case, the mutations are probably non-allelic). This is conventionally known as a complementation test. Since such tests are based on evaluation of the phenotype of diploid strains, they can only be performed when studying recessive mutations. All of the isolates of the *CLN* bypass mutation were dominant, and therefore, complementation tests could not be employed in their genetic analysis.

The conventional alternative, for dominant mutations, is to determine whether distinct mutant isolates can genetically recombine with one another. The shortcoming of a recombination test is that it provides information which is at best statistical in nature. In other words, if one detects evidence for recombination,

then one can exclude the possibility that two mutant isolates are allelic. But if one fails to find evidence for recombination, this merely proves that two isolates are *linked* to one another. It will usually be impossible to establish that two isolates are in fact *allelic* with one another, because the amount of data required to establish such tight linkage would be prohibitively difficult to collect.

In order to explore the linkage relations of the various CBM isolates, each *cln1,2,3*⁻ CBM isolate was mated to a single, backcrossed, *cln1,2,3*⁻ derivative of CBM9, then sporulated, dissected, and analyzed as described in the following paragraph. The CBM9 strain used carried the 0/9 plasmid (*CLN3/ TRP1/ CEN*). All the CBM mating partners carried the W16 plasmid (*GAL1::CLN3/ URA3/ CEN*). Diploids were selected based on prototrophy for uracil and tryptophan, then maintained on YcGal-ura media to allow loss of the 0/9 plasmid. A *cbm*⁺ strain was included in the study as a negative control, and a CBM9xCBM9 cross was performed (x1076), as a positive control. The absence of the *CLN3/ TRP1* plasmid was confirmed, prior to scoring CBM phenotype, since it provides an alternative means of survival on YEPD. In some cases, the segregation of *MAT* and (where available) *ARG4* were scored, as a means of confirming that the putative spore clones were in fact the haploid products of meiosis, rather than artifacts of tetrad dissection. CBM2 and CBM16 were not included in the study, since previous crosses involving these isolates had been characterized by extremely poor viability of the progeny, and they were deemed too difficult to pursue. CBM15 was not used, since it had a dominant single division meiosis phenotype, preventing tetrad analysis. CBM18 was not used for the same reason, however, a haploid derivative of CBM18, called CBM18', was used. The derivation of CBM18' will be explained in the following chapter.

Diploids were sporulated and dissected on YEPGal media, and the presence of the *GAL1::CLN3* plasmid was determined in the progeny, as was viability on YEPD. Spores inheriting the plasmid should be viable on YEPGal whether or not they have inherited a *CLN* bypass mutation. Viability on YEPD should depend on inheritance of a *CLN* bypass mutation, since both parents were *cln1,2,3*⁻. If a CBM isolate is allelic with CBM9, then 100% of the progeny in the cross to CBM9 should inherit a mutant allele of a gene conferring *CLN* bypass, and all should be alive on YEPD. Alternatively, if a CBM isolate is unlinked to CBM9, then 1/4 of the progeny in the cross to CBM9 should inherit neither mutation, and be inviable on YEPD.

For each cross (summarized in Table 5), at least a dozen tetrads were dissected, and the numbers of glucose inviable and glucose resistant *GAL1::CLN3* spores were determined. In no cross were any glucose inviable *GAL1::CLN3* spores recovered, except in the *cbm*⁺ control, where there were 20 glucose resistant progeny, and 16 glucose inviable progeny, consistent with CBM9 being a single Mendelian gene. Thus, there was no evidence for recombination between CBM9 and any other CBM isolate. How compelling is this *lack* of evidence?

From a statistical point of view, there are two different questions we may ask of this data set. The first question is, what are the odds that the two CBM isolates being tested are really unlinked? Thus, we can form the null hypothesis that "CBMX" (designating any CBM isolate) and CBM9 assort independently, and estimate the odds that we are in error when we reject this null hypothesis. If they are unlinked, we expect 1/4 of the spores to inherit neither *CLN* bypass mutation. Through chance alone, we might fail to detect such a spore, unless we examine a sufficient number. Based on a χ^2 test ($p = 0.05$), I estimate that I should detect

Table 5. Crosses between CBM9 and other CBM isolates.
In no cross were any glucose-inviable *GAL1::CLN3* spores recovered.

Mating partner	Cross #	# glu ^R <i>GAL1::CLN3</i> spores recovered	χ^2	<i>ARG4</i> 2:2?	<i>MAT</i> 2:2?
CBM2	ND				
CBM3	1021	13	4.33	YES	YES
CBM4	1034	16	5.33	YES	YES
CBM6	1046	22	7.33	NA	YES
CBM7	1057	18	6.00	YES	YES
CBM8	1067	15		NA	ND
CBM8	1067 ^{2nd set}	24		NA	YES
CBM8 (overall)		39	13.00		
CBM9	1076	24		NA	ND
CBM9	FC set	25			
CBM9 (overall)		49	16.33		
CBM10	1077	19	6.33	YES	YES
CBM11	1078	14	4.67	YES	YES
CBM12	1079	32	10.67	NA	ND
CBM13	1080	11	3.67	YES	YES
CBM14	1081	10	3.33	YES	YES
CBM15	ND				
CBM16	ND				
CBM18'	1084	47	15.67	YES	YES

χ^2 was calculated based on the null hypothesis that the two CBM isolates are completely unlinked. Under this assumption, one fourth of the *GAL1::CLN3* spores recovered would be predicted to inherit neither CLN bypass mutation, and should therefore be glucose-inviable. The critical value of χ^2 ($p=0.05$) for 1 d.f. is 3.841. Thus, the null hypothesis (non-linkage) can be rejected when zero glucose inviable spores are found among approximately 12 glucose resistant spores. This degree of certainty was achieved for all CBM isolates tested, except CBM13 and CBM14.

(glu^R) glucose resistant, ie. capable of cell division without synthesis of *GAL1::CLN3*

(NA) Data not available, because gene not segregating in this cross.

(ND) Not done or not determined.

approximately 12 glucose resistant *GAL1::CLN3* spores (and no glucose sensitive spores) before rejecting the hypothesis that a CBM isolate is completely unlinked to CBM9. This quantity of data was obtained for each cross except in those to CBM13 and CBM14 (Table 5).

The second question we may ask is, what is the greatest recombination frequency between "CBMX" and CBM9 consistent with the available data. If we completely fail to detect recombinants, then in general, the most probable recombination frequency is zero (allelism). However, non-zero rates of recombination might well be occurring, and we want to know how large those rates might be, without rendering the actual data (in which no recombinants were detected) statistically improbable. In the CBM intercrosses, four possible spore types may be produced: CBM9 CBMX, CBM9 *cbmx*⁺, *cbm9*⁺ CBMX, and *cbm9*⁺ *cbmx*⁺. Of these, the first and last are recombinant, while the middle two are parental. However, only the last would be detected as glucose inviable, hence the detected rate of recovery of recombinant spores is one half their actual rate of production. A χ^2 test may be performed based on the expectation that P% of the spores are recombinant, and it will be seen that to reject the null hypothesis (that P% of the spores are recombinant), *n* non-recombinant (and zero recombinant) spores must be detected, where the smaller P is, the larger *n* must be. Analyzing the data in Table 5 in this way, we conclude that although CBM9 is probably allelic with CBM8 and CBM18', it could be as much as 18 cM from CBM8, and 15 cM from CBM18', without rendering the available data too implausible (*p* = 0.05).

In conclusion, no evidence was obtained that more than one gene can mutate to give the *CLN* bypass phenotype. In general we can at most say that the

various CBM isolates are probably linked to CBM9. If they are all linked to CBM9, then they all maybe allelic with one another. If they are all allelic with one another, then they are all probably the result of mutations in some gene that remain linked to that gene, rather than rearrangements or transpositions involving the duplication and translocation of a section of a chromosome to a novel genomic location. However, even for the crosses which were studied more thoroughly, the possibility can not be excluded that mutations were obtained in several, distinct, linked genes.

CBM is not the result of a chromosomal duplication. Might CBM really be the result of disomy for some chromosome, and not a mutation in a gene? A disome would appear to be a Mendelian gene, since in a heterozygous cross, half the progeny would inherit the disome, while in a homozygous cross, all the progeny would inherit it. In spite of this difficulty, a disome may be detected genetically, based on the fact that in a heterozygous cross, it will segregate (ie. presence vs. absence) at the first meiotic division. In this respect, a disome behaves like any centromere. How do centromeres behave in meiosis? In a cross with two (distinct) tightly marked centromeres segregating, only parental and non-parental ditype tetrads will be produced; tetratype tetrads will be virtually absent. This is because both centromeres are undergoing first division segregation, and tetratypes can only be produced when second division segregation occurs.

In order to evaluate the possibility that CBM behaves like a centromere-linked marker in meiosis, diploid 975 was constructed, segregating both a tightly centromere-linked marker (*TRP1/trp1*, 0.3 cM from *CENIV* (Mortimer et al, 1989)) and *cbm⁺/CBM*. Analysis of tetrads from this diploid suggests that CBM is not detectably centromere-linked (Table 6). The estimated

Table 6: CBM is neither allelic, nor closely linked, to any gene tested.

GENE	Cross #	CBM isolate ¹	Tetrad Types		
			PD	T	NPD
<i>CDC28</i>	846	CBM8 ^{1X}	2	13	2
<i>LYS2</i>	990	CBM8 ^{5X}	0	14	2
<i>CLB1=SCB1</i>	978	CBM8 ^{5X}	3	6	0
<i>CLB2=SCB3</i>	971	CBM8 ^{5X}	1	5	0
<i>CLB2=SCB3</i>	1043	CBM8 ^{5X}	1	6	2
<i>CLB3=SCB2</i>	973	CBM8 ^{5X}	2	6	1
<i>CLB4=SCB4</i>	1031 ²	CBM8 ^{5X}			
<i>CLB5</i>	957 ³	CBM8 ^{4X}	3	22	3
<i>CLB5</i>	1005	CBM8 ^{5X}	3	10	1
<i>HCS26</i>	1042	CBM8 ^{6X}	0	8	4
<i>MPK1</i>	957 ⁴	CBM8 ^{4X}	2	6	1
<i>MPK1</i>	1053	CBM8 ^{5X}	3	3	1
<i>SWI4</i>	1308	CBM8 ^{5X}	2	1	2
<i>SWI6</i>	1307	CBM8 ^{5X}	2	7	1
<i>CENIV(TRP1)</i>	975	CBM8 ^{4X}	3	14	1

The occurrence of recombinant spores (ie. tetratype and non-parental ditype tetrads) establishes that CBM is not an allele of the corresponding marker.

(1) Superscript refers to number of times CBM was backcrossed, from the time it was isolated, to the time it was tested for linkage to the corresponding marker.

(2) Tetrads in cross 1031 were by and large not designated PD,T,NPD. However, at least 8 recombinant spores were recovered, including one clear NPD tetrad with four viable spores. This excludes the possibility of allelism between *CLB4* and CBM.

(3) Diploid 957 (*cln1/cln1 cln2/cln2 cln3/cln3* CBM8/cbm+) was transformed with CE105Δ*ApaI* linearized with *XhoI*. Installation of *TRP1* at *CLB5* was confirmed by Southern (data not shown). Data are from transformed colony #2. Ditype tetrads can not be scored as parental or non-parental, because *CLB5* was not marked until after formation of the diploid. PD refers arbitrarily to +- and -+ spores, while NPD refers to ++ and -- spores.

(4) Diploid 957 was transformed with CE117Δ*XhoI* linearized with *HpaI*. Installation of *TRP1* at *MPK1* was confirmed by Southern (Figure 2). Data are pooled from transformed colonies #1 and #2. See note (3) on ditype tetrads.

frequency of second division segregation (SDS) of CBM was 78%, based on the formula $f(T) = SDS(1) + SDS(2) - 3/2 SDS(1) SDS(2)$ (Suzuki et al, 1981). As a confirmation that *TRP1* is centromere linked, tetrad types between *TRP1* and *ARG4* were evaluated in the same tetrads. *ARG4* is detectably centromere linked, 12.3 cM from *CENVIII* (Mortimer et al, 1989). A preponderance of ditype tetrads were obtained between *ARG4* and *TRP1* (9 ditypes and 5 tetratypes). Assuming that *ARG4* in fact undergoes second division segregation 24.6% of the time (ie. twice the map distance; see Fincham et al, 1979, p. 96 for justification), these data imply that *TRP1* is 8.81 cM from its centromere, which is reasonably consistent with expectation, given the small sample size. In conclusion, since CBM is not detectably centromere linked, we reject the hypothesis that CBM is the result of disomy.

CBM is not an allele of any of several known cell cycle control genes.

Introduction. A logical early step in the study of any new mutation is to determine genetically whether it is an allele of a previously known gene. To this end, allelism tests were conducted between CBM8 and several of the genes known to play a key role in the control of the yeast cell cycle. CBM8 was chosen as the representative CBM isolate, both because it is particularly robust, and because it is linked to CBM9, which in turn was shown to be linked to most of the other CBM isolates (Table 5).

The general procedure for conducting allelism tests was to start by making a strain which was *cln1,2,3⁻ GAL1::CLN3* and had a gene of interest with a tightly linked prototrophic marker (either disrupting or adjacent to the gene of interest). This strain was then crossed to a *cln1,2,3⁻ GAL1::CLN3* CBM8 strain, and the

segregation of CBM and the prototrophy were scored in the progeny. The production of recombinant spores (either CBM prototrophs or *cbm*⁺ auxotrophs) provided evidence against allelism between CBM and the marked gene.

There are two complications involved in this procedure that deserve mention. First, when the *cln1,2,3*⁻ *GAL1::CLN3* strain was made, bearing the marked gene of interest, the *CLN* null alleles were generally unmarked deletions. For this reason, the *cln1,2,3*⁻ genotype was merely inferred from the glucose inviability of the strain. A potential problem with this inference is that the disrupted, null allele of the gene of interest conceivably could affect the viability of strains having less than three, but more than zero, intact *CLN* genes. To confirm that strains were in fact *cln1,2,3*⁻, the putative *cln1,2,3*⁻ strains were mated to a known *cln1,2,3*⁻ strain, sporulated, dissected, and shown to have no progeny viable in the absence of *GAL1::CLN3* synthesis.

A second complication is that the null allele of the gene being tested for allelism to CBM might actually suppress CBM. Suppression of CBM would be manifest as a failure to detect CBM prototrophs in the progeny, which in turn might spuriously suggest allelism between CBM and the gene of interest. Two precautions were taken to avoid this pitfall. First, the *cbm*⁺ progeny were examined, to determine if auxotrophs and prototrophs occurred at equal frequency. Suppression of CBM should not confound the analysis of *cbm*⁺ spores. Second, in cases where suppression was suspected, several prototrophic progeny were obtained and mated to a *cln1,2,3*⁻ *cbm*⁺ auxotrophic tester. These diploids were sporulated, and some were shown to be segregating both *CLN* bypass activity and the gene marked with the prototrophy. In this way, it was possible to confirm the presence of the *CLN* bypass mutation in some progeny where it was apparently absent, but

in fact suppressed by a null mutation in a non-allelic gene having a role in cell cycle control. As will be demonstrated in a subsequent section, CBM is suppressed by *swi4* and *mpk1*.

The fact that CBM is not an allele of *CLN1*, *CLN2*, or *CLN3* was demonstrated earlier, and will not be covered here.

CBM is not allelic with CDC28. *CLN* genes are thought to be cofactors of the *CDC28* protein kinase. This view stems from the limited homology between *CLNs* and cyclins (Hadwiger et al, 1989b; Nash et al, 1988), from the fact that *CLNs* have been demonstrated to physically associate with *CDC28* (Wittenberg et al, 1990; Tyers et al, 1992), and from the genetic association between *CLN1*, *CLN2* and *CDC28* (Hadwiger et al, 1989b). It therefore seemed possible that a mutation in *CDC28* could confer *CLN* independence on cells. CBM was found not to be allelic with *CDC28* (Table 6), based on a cross to the temperature sensitive *cdc28-13* allele of *CDC28*. In addition, as will be described below, CBM was found not to bypass *CDC28*. CBM merely bypasses the *CLN* requirement, in a *CDC28* dependent fashion.

CBM is not linked to LYS2, hence is not a mutation in CKS1. *CKS1* is a subunit of the *CDC28* kinase complex (Hadwiger et al, 1989a). It is the *S. cerevisiae* homolog of the *S. pombe* gene *suc1*⁺ (Hayles et al, 1986), and was discovered as a high copy suppressor of a temperature sensitive allele of *CDC28* (Hadwiger et al, 1989a). *CKS1* was shown (Hadwiger et al, 1989a) to be 24 cM from *LYS2*, based on finding 21 parental ditypes, 19 tetratypes, and 0 non-parental ditypes in a cross segregating both *CKS1* and *LYS2*. We tested for linkage between CBM and *LYS2*, to explore the possibility that CBM was a mutation in *CKS1*. We found no evidence for linkage between CBM and *LYS2* (Table 6). In

addition, based on pooling data from cross 970 as well as other crosses between CBM strains and *lys2::URA3* strains not shown in Table 6, a total of 31 parental spores and 38 non-parental spores were recovered; this is clearly inconsistent with linkage between CBM and *LYS2*. Thus, CBM appears not to be a mutation in *CKS1*.

CBM is not allelic with SWI4 or SWI6. *SWI4* and *SWI6* are involved in the promotion of the transcription of *CLN1* and *CLN2*, as well as the post-START transcription of the HO endonuclease (Andrews and Herskowitz, 1989; Nasmyth and Dirick, 1991). Strains deleted for both *SWI4* and *SWI6* are inviable, for reasons which are not entirely clear, but which involve loss of *CLN* function (Nasmyth and Dirick, 1991). Although CBM can actually bypass all requirement for *CLN* genes, CBM8 was tested for allelism to both *SWI4* and *SWI6* based on the theory that mutant forms of these genes might alter the expression of known (or unknown) genes bearing some functional redundancy to *CLN* genes, such as *HCS26* and *CLB5*. CBM was found not to be allelic with either *SWI4* or *SWI6* (Table 6). However, *swi4::URA3* was found to suppress CBM, as will be described below.

CBM is not allelic with any of the five yeast B cyclins. Five B type cyclins have been described in yeast. The first four were discovered in the Reed (Ghiara et al, 1991) and Nasmyth (Surana et al, 1991) labs, while the fifth resulted from experiments to be described below (Epstein and Cross, 1992). Since there is genetic (Surana et al, 1991) and biochemical (Ghiara et al, 1991) evidence that B cyclins, like *CLNs*, interact with *CDC28*, it was reasonable to test for allelism between CBM and the B type cyclins. Tests were performed using marked alleles of *CLB1*, *CLB2*, *CLB3*, and *CLB4* supplied by the Reed lab, and using the

clb5::ARG4 allele described below (Chapter 5). CBM was found not to be allelic with any of the five B cyclins (Table 6).

CBM is not allelic with HCS26. *HCS26* is a gene which, when overexpressed, can suppress the requirement for *SWI4* in diploids (Ogas et al, 1991). It has extremely limited homology to cyclins. For both of these reasons, it seemed worth checking whether CBM was allelic with *HCS26*. CBM was found not to be allelic with *HCS26* (Table 6), using a null allele (*hcs26::URA3*) kindly supplied by D. Lew.

CBM is not allelic with MPK1. *MPK1* is a gene that was first found as a high copy suppressor of the temperature sensitive phenotype of the genotype *pkc1Δ SKC^d*. (Fields and Thorner, 1991, mentions the cloning of *MPK1*, but says nothing about its properties). This same gene was also found by the present author (Chapter 6) and by B. Futcher (unpublished results) as a dosage suppressor of *cln1,2,3⁻* inviability. If overexpression of a gene can lead to *CLN* bypass, then perhaps mutation can as well. Allelism with CBM was tested using the *MPK1::MPK1::TRP1* and *mpk1::ARG4* alleles of *MPK1* (Materials and methods); CBM was found not to be allelic with *MPK1* (Table 6).

Phenotypic analysis of the *CLN* bypass mutation

CBM cln1,2,3⁻ strains can divide without GAL1::CLN3. *CLN* bypass mutations were selected to confer competence at cell division in the *cln1,2,3⁻* background in the absence of *GAL1::CLN3* synthesis. This is illustrated in Figure 5. *cln1,2,3⁻* CBM cells have a larger mean volume and a longer doubling time than wild type cells; a *cln1,2,3⁻* CBM9^{4x} (1209-21D) strain was found to have an optical density doubling time of approximately 186' in YEPD, compared to a

typical value of about 105' for wild type (*CLN1,2,3*) cells.

It was observed that *MAT α cln1,2,3⁻* CBM strains mate as *MAT α* strains at an elevated frequency (Figure 6), since they form easily detectable diploids with *MAT α* testers. 44 separate diploids formed between *MAT α* CBM strains and a *MAT α* tester were scored for pheromone secretion, and all 44 were α -factor secretors, confirming that their genotype is *MAT α /MAT α* or *MAT α /null* (ie. chromosome III monosomes). In contrast, 22 diploids formed between *cbm⁺ MAT α* strains and a *MAT α* tester were scored for pheromone phenotype; 4 were pheromone non-secretors (and sporulated to form tetrads), while 18 secreted α -factor.

These observations suggest that *MAT α cln1,2,3⁻* CBM strains are partially derepressed for the expression of *MAT α* specific genes. This may be a result of a partial failure of the $\alpha 2$ gene product to repress *MAT α* specific genes (Cross et al, 1988b; Herskowitz, 1989). Consistent with this interpretation is the observation that CBM *MAT α* strains appear to secrete reduced levels of α -factor (Figure 6; this presumably results from the induction of the *MAT α* specific gene *BAR1*, an α -factor protease) and secrete low levels of α -factor. These observations are included here merely for the sake of documentation; their significance, if any, to an understanding of the *CLN* bypass mutation is unclear.

CBM cln1,2,3⁻ MAT α bar1 strains are sensitive to α -factor. α -factor arrests *MAT α* yeast cells prior to bud emergence, in G1 of the cell cycle, at START (Cross et al, 1989a). While the pheromone induced signal transduction pathway has been well studied (reviewed in Cross et al, 1988b), the real effectors of cell cycle arrest remain controversial. The "trident regulation" hypothesis (Chang and Herskowitz, 1990) states that α -factor has an inhibitory effect on *CLN* synthesis

and function, which in turn accounts for the arrest attributable to pheromone. This hypothesis stems from the facts that (a) carboxy-terminal truncation mutations of *CLN2* (Hadwiger et al, 1989b) and *CLN3* (Cross, 1988a; Nash et al, 1988) confer α -factor insensitivity on cells, that (b) α -factor treatment down regulates expression of *CLN1* and *CLN2* transcripts (Wittenberg et al, 1990; Cross and Tinkelenberg, 1991), and that (c) *far1* mutant strains, which fail to arrest in α -factor, are suppressed by mutations at *cln2* (hence *FAR1* is interpreted to function as an α -factor stimulated negative regulator of *CLN2* (Chang and Herskowitz, 1990)).

Since *CLN* bypass mutants are viable in the absence of *CLN* genes, they presented an opportunity to test the idea that *CLN* genes are required for arrest induced by mating pheromone. If this were true, then CBM strains should be insensitive to α -factor. Alternatively, if pheromone arrests yeast via other means, potentially in addition to the effects on *CLN* genes, then CBM strains might be sensitive to α -factor.

A yeast strain was constructed of genotype *MATa bar1 cln1,2,3⁻ CBM8^{6X} W16* (953-7A). *BAR1* encodes a protease that degrades α -factor, hence to determine the intrinsic effect of α -factor on a strain, it is important to work in the *bar1* background; *BAR1* yeast degrade α -factor, hence appear to have some degree of insensitivity, regardless of their true sensitivity (Chan and Otte, 1982). To test the pheromone sensitivity of this strain, it was streaked on YEPGal and YEPD media, with and without added 1 μ M α -factor (Figure 7). The CBM strain failed to divide in the presence of α -factor, consistent with it being sensitive to pheromone in spite of its independence from *CLN* genes. The positive controls for arrest (*cln1,2,3⁻ cbm⁺ W16 MATa bar1* and *CLN1,2,3⁺ MATa bar1*) also arrested (the arrest of the *cln1,2,3⁻* strain is only informative on YEPGal), while the negative

control for arrest (*MAT α cln1,2,3⁻ CBM8*) did not arrest. Thus, this experiment suggests that mating pheromone does not work solely via its effects on *CLN1,2,3* gene synthesis and function.

Pheromone treated cells were examined in liquid culture (Materials and methods) to determine at what stage (budded or unbudded) in the cell cycle they arrested. After six hours pheromone treatment, *MAT α bar1 CBM8 W16* yeast were 92% unbudded in YcD-ura (Table 7). An untreated exponential culture of the same strain was 43% unbudded. A *MAT α* control remained approximately 45% unbudded with or without α -factor. A *cbm⁺ CLN1,2,3⁺ MAT α bar1* control was 91% unbudded in α -factor, while an untreated exponential culture of the same strain was 54% unbudded. Thus, mating pheromone appears to arrest CBM8 *cln1,2,3⁻* yeast in the unbudded stage of the cell cycle, just as it does wild type yeast (Pringle and Hartwell, 1981).

These results imply that α -factor does not arrest cell cycle progression solely as a result of its effects on *CLN1,2,3* gene expression and function. It is not possible to draw a positive conclusion from this experiment without more information about the mode of action of CBM. Pheromone might be a negative regulator of *CDC28* itself, it might be a negative regulator of a gene which is a positive regulator of START, and which is hyperactivated (directly or indirectly) in CBM mutants, or it might be a negative regulator of a gene which is necessary to the function of, but itself unaffected by, CBM.

The CLN bypass mutation does not bypass the CDC28 requirement at START. The *CLN* bypass mutation was selected based on viability in the *CLN* deficient background. The association of *CLN* genes with the product of the *CDC28* gene is necessary to form an active kinase complex (Wittenberg et al,

Table 7. α -factor arrests CBM8 *cln1,2,3⁻* *MATa bar1* strains in the unbudded stage of the cell cycle.

Genotype:		Strain name:		Medium:	Percent unbudded	
					- α F	+ α F
<i>MATa bar1</i>	<i>cln1,2,3⁻</i>	CBM8 W16	953-7A	YcD-ura	43	92
<i>MATa bar1</i>	<i>CLN1,2,3⁺</i>	cbm ⁺	"bar1"	YEPD	54	91
<i>MATα</i>	<i>cln1,2,3⁻</i>	cbm ⁺ W16	884-8D	YcGal-ura	45	46
<i>MATa bar1</i>	<i>cln1,2,3⁻</i>	CBM8 W16	953-7A	YcGal-ura	48	68
<i>MATa bar1</i>	<i>cln1,2,3⁻</i>	cbm ⁺ W16	953-1D	YcGal-ura	49	81

Budding indices were determined after six hours' treatment of liquid cultures with 1 μ M α -factor.

1990; Tyers et al, 1992), which in turn is required to execute START; certain temperature sensitive mutations in *CDC28* cause cell cycle arrest at START (Reed, 1980), as does triple deficiency for *CLN* (Cross, 1990).

It was of interest to determine whether mutations bypassing the *CLN* requirement also bypassed the *CDC28* requirement at START. This might be expected, for example, if CBM were a mutation in a *CDC28* substrate, and if this substrate were the sole substrate whose modification by *CDC28* was necessary for START execution. Alternatively, if more than one substrate must be phosphorylated, or if CBM is a mutation in a kinase activator or a component of the kinase complex, then CBM would not be expected to bypass the *CDC28* requirement at START.

In order to determine whether CBM bypassed the requirement for *CDC28* at START, a *cln1,2,3⁻* strain that was also CBM8^{5X} *cdc28-13* was constructed, and transferred (see Materials and methods) to the non-permissive temperature of *cdc28-13*, which is a temperature sensitive mutation in *CDC28* that causes cell cycle arrest in G1, at START (Reed, 1980). Cells were examined after being held at 38°C for 6 hours, to determine at which stage in the cell cycle arrest had occurred. It was found that cells had arrested 98% unbudded, consistent with arrest occurring in G1. In contrast, the same genotype maintained at 30°C in exponential culture had 39% of cells unbudded, while a CBM8 *CDC28⁺* strain maintained in exponential culture had about 49% of cells unbudded at both 30°C and 38°C (Figure 8). Similar results were obtained under conditions permissive for the synthesis of *GAL1::CLN3* (Figure 8). Therefore, we conclude that *CDC28* is essential to START execution even in the CBM8 *cln1,2,3⁻* background.

Mutations suppressing the CLN bypass mutation. The *CLN* bypass mutation

allows cell division in the *CLN* deficient background. Is bypass activity suppressed by loss of function mutations in other genes involved in the control of the cell division? If some other gene, normally not essential to viability, is essential to bypass activity, it could be informative regarding the mode of action of CBM. In order to determine whether null alleles of other genes suppressed CBM, strains were constructed of genotype *cln1,2,3⁻* CBM8 *sup⁻* [*GAL1::CLN3/URA3*], and maintained on YcGal-ura media; "*sup*" designates the candidate suppressor mutation under test. It was then determined whether such strains were alive on YEPD media. In the case of genes actually suppressing CBM, the presence of the *CLN* bypass mutation in the suppressed strain was confirmed, either by mating to a *cln1,2,3⁻* tester and demonstrating the segregation of *CLN* bypass activity in the progeny, or by the analysis of derivatives that had reverted to *SUP⁺*.

clb2::LEU2, *clb5::ARG4*, and *hcs26::URA3* all fail to suppress CBM. To confirm this, cells were plated on YEPGal and YEPD media, at four different densities (Figure 9). From a comparison of colony numbers on glucose and galactose media, an estimate can be made of the relative plating efficiency in the absence of *GAL1::CLN3* synthesis. In each case, the additional mutation leads to slowed growth on glucose, but does not absolutely prevent cell division. It is indeed possible that the slow down in cell division, resulting from the loss of the additional gene, is a consequence of the partial dependence of the *CLN* bypass phenotype on the presence of that gene. However, it is safer to conclude that the effects these mutations on cell growth rates reflect that rule that sick cells, further insulted by additional mutations, get even sicker, in a way that is not informative about gene product interactions. *CLB2*, in particular, is unlikely to play a role in CBM function, since it is normally not transcribed (Surana et al, 1991) or

synthesized during G1.

swi4::URA3 and *mpk1::ARG4* suppress CBM. In contrast to the genes shown in Figure 9, two genes were identified which are essential or nearly essential for viability in the CBM *cln1,2,3⁻* background, *SWI4* and *MPK1*. Quantitative plating experiments revealed that CBM8 *cln1,2,3⁻ swi4::URA3 GAL1::CLN3* strains plated with an efficiency of about 3.2% (glucose/galactose). However, about 90% of the viable colonies were formed from cells that had reverted to *SWI4* (detected by reversion to auxotrophy for uracil, and resulting from the "popping out" of the insert disrupting the *SWI4* gene), so the true plating efficiency of CBM8 *cln1,2,3⁻ swi4::URA3 GAL1::CLN3* strains was closer to 0.32%. Some fraction of these colonies may result from mutations affecting the glucose repression of the *GAL1* promoter.

Similarly, the *mpk1::ARG4* allele cleanly suppresses rescue of *cln1,2,3⁻* by CBM (Figure 10): *cln1,2,3⁻ CBM8 mpk1::ARG4* strains were completely inviable on YEPD. Furthermore, diploids formed between such strains and *cln1,2,3⁻ cbm⁺ MPK1* strains were also completely inviable on YEPD. Thus, heterozygosity for *MPK1* suppresses the dominance of CBM, previously documented in the *MPK1/MPK1* background. This suggests that yeast are particularly sensitive to *MPK1* gene dosage. The potential significance of this observation will be discussed in Chapter 6.

Discussion.

It remains unclear how CBM functions to allow *CLN* bypass. It has not been possible to identify the mutant gene by allelism studies with other, known mutations. The fact that CBM is not completely suppressed either by deletions of

B cyclins, or by disruption of a gene product (*HCS26*) having limited homology to *CLNs* and cyclins (Ogas et al, 1991), suggests that CBM does not work by affecting the synthesis, stability, or function of other known cyclins or cyclin-like genes. However, it remains possible that CBM is either a mutation in such a gene, or a regulator of such a gene.

The fact that CBM is suppressed by *swi4* and *mpk1* is intriguing. *SWI4* and *MPK1* are both non-essential genes whose mutations cause synthetic lethality in conjunction with particular combinations of *CLN* deletions (results on *MPK1* will be presented in Chapter 6); *swi4 cln3* spores are usually inviable, and *swi4-29 cln3* strains are inviable at 38°C (Nasmyth and Dirick, 1991). *mpk1 cln3* spores are inviable and vegetative cells divide very slowly. *swi4 cln2 cln3* strains are nearly dead (*swi4-29 cln2 cln3* strains are inviable at 35°C) and *mpk1 cln2 cln3* strains arrest as large budded cells. These genetic results are consistent with the interpretation that both *SWI4* and *MPK1* are important to the activity of the *CLN1* and *CLN2* genes. *SWI4* is a component of a transcription factor (CCBF, (Andrews and Herskowitz, 1989)) which binds to a motif called the Cell Cycle Box, and which is involved in promoting cell cycle specific, *CDC28*-dependent transcription of the *HO* gene, and possibly, *CLN1*, and *CLN2* (Ogas et al, 1991; Nasmyth and Dirick, 1991). Preliminary results suggest that *MPK1* may be involved in promoting *CDC28* independent transcription of *CLN1* and *CLN2*. The fact that null alleles of *SWI4* and *MPK1* suppress a mutation which bypasses the *CLN* requirement may indicate that the *CLN* bypass mutation identifies a gene which either is a factor, or modulates a factor, that (like *CLN1* and *CLN2*) promotes START, and depends on *SWI4* and *MPK1* for function.

An attempt was made to clone CBM, relying on the dominance of the

mutation, by making a *CEN* plasmid library from a strain of genotype *cln1 CLN2 cln3* CBM8 (Materials and methods). This library was transformed into *cln1,2,3⁻ GAL1::CLN3* yeast, and plasmids were isolated which conferred viability on the recipient strain on glucose media. Although the library was thoroughly screened (based on recovery of *CLN2* plasmids, Table 1), no clones of CBM were recovered. This might have resulted from several different complications: CBM may be too weakly dominant to confer viability in this fashion; CBM may be a mutation in a very large gene (which would be under-represented in a library); CBM DNA may be toxic to *E. coli*. The possibility that CBM is the result of a duplication of an entire chromosome, and not a simple Mendelian locus, was discounted based on the finding that CBM is not *CEN* linked. The hypothesis that CBM represents a duplication followed by an (unlinked) translocation of a gene, such as *CLB5* or *MPK1*, which was recovered from the library, has not been formally disproved. However, given the linkage of the various CBM isolates, this hypothesis would require that in all the distinct isolates, the duplication had translocated to novel genetic loci which were all linked to one another; there is no basis to expect that such a turn of events is at all likely to have occurred. Until CBM is cloned, its identity, mode of action, and significance will remain enigmatic.

Chapter 4: Single division meiosis and the *CLN* bypass mutation.

Two *CLN* bypass isolates had an unselected, dominant, single division meiosis phenotype.

Two of the isolates of the *CLN* bypass mutation had an unselected trait. Besides having the capacity to divide without *CLN* genes, CBM15 and CBM18 had a dominant single division meiosis phenotype. When the original isolates were mated and sporulated, predominantly two spored ascii (called dyads) were formed (Figure 11). This phenotype will be designated "SPO", while the wild-type phenotype will be designated "spo⁺". Four spored ascii (called tetrads, normal for yeast) were observed at a low frequency; as will be discussed below, these resulted from an obscure, high frequency reversion of SPO to spo⁺; these tetrads were not a result of incomplete penetrance of SPO. For the remainder of this chapter, I will confine my remarks to CBM18. CBM15 was not studied very extensively, due to poor viability of meiotic segregants and certain indications that the *CLN* bypass phenotype in CBM15 was not attributable to a single gene. My belief, based on data not shown here, is that the two mutations are very similar, but that CBM15 has a weaker phenotype and is perhaps dependent on modifiers. The SPO phenotype of CBM18 is strongly dominant, in the sense that when triploid strains were constructed of genotype $a/a/\alpha$ SPO/spo⁺/spo⁺ (by mating *MAT* α CBM18 to diploid *MAT* a/a cbm⁺/cbm⁺ spo⁺ progeny of a cross involving CBM18), they also sporulated to give dyads.

The single division meiosis phenotype is epistatic to the presence of *CLN* genes, and is expressed when the original isolate of CBM18 is crossed outside the BF264-15D strain background (both S288C and 381G were tested). Since SPO is

attributable to a single Mendelian locus (results presented below), this implies that SPO could be linkage mapped using dyad dissection and standard mapping strains.

What is the nature of the single meiotic division in CBM18?

The products of the single meiotic division are diploid. When two spored ascii are produced in meiosis, they can result from normal meiosis followed by the failure to package two of the four haploid nuclei, or they can result from single division meiosis, leading to the production of two diploid spores (Esposito and Klapholz, 1981). There are a few mutations known in *S. cerevisiae* which cause single division meiosis; perhaps the best studied of these is *spo13-1* (Klapholz and Esposito, 1980). The criterion for distinguishing these two means of producing two spored ascii is the ploidy of the resultant spores.

It may be helpful to describe briefly the pattern of chromosome segregation in single division meiosis. Just as tetrad analysis provides information that is unavailable in random spore analysis, it is usually more informative to analyze both viable products from any given dyad. To designate the phenotypes of both spores in a dyad, I will use the notation (&). Consider a heterozygous locus that is not linked to its centromere. Following DNA replication, there are four copies of the locus, two of each allele. These will segregate at random into the two diploid products of single division meiosis. Call these copies *Z1*, *Z2*, *z1*, and *z2*, where *Z1* and *Z2* are sister copies of one allele, and *z1* and *z2* are sister copies of the other. Assume that **Z** is dominant to **z**. If one spore inherits *Z1* and *Z2*, then the other must inherit *z1* and *z2*. The resulting dyad would consist of spores that were genotypically *Z/Z* and *z/z*, hence it would be phenotypically (**Z&z**). Since **Z** and **z** are distinguishable, this is called a ditype dyad.

Alternatively, assume that one spore inherits *Z1*, and the other spore inherits *Z2*. There are two possible ways to complete the picture -- either *z1* goes with *Z1*, or with *Z2*, while *z2* must do the converse. Hence there are two possible (but indistinguishable) ways to make a dyad where each spore is a *Z/z* heterozygote. Given the dominance of *Z*, the dyad will be phenotypically (*Z&Z*), hence this is called a monotype dyad. Since there are twice as many ways to make a monotype dyad as a ditype dyad, *on average* monotypes will be formed in 2/3 of the meioses, while ditypes will be formed in 1/3. Consequently the recessive phenotype *z* will appear in only 1/2 of 1/3 (ie. 1/6) of the diploid spores.

This underscores an important difference between tetrad analysis and dyad analysis. In tetrad analysis, haploid products are recovered and phenotypes can be examined without taking dominance into account. A trait caused by a single gene segregates 2:2 in the progeny of a heterozygous diploid. Finding 2:2 segregation provides a clear criterion that a trait is monogenic. In dyad analysis, in contrast, diploid products are recovered, and recessive phenotypes are only manifest when homozygous. For a trait due to a single gene, this occurs in 1/2 of the spores found in an average of 1/3 of the dyads. The criterion that a trait is monogenic is that the recessive phenotype occurs, on average, in 1/6 of the diploid progeny. Hence it is technically more difficult to demonstrate that a trait is monogenic based on its segregation in dyads.

To determine the ploidy of the progeny of CBM18/+ heterozygous crosses, genotypes at *MAT* were examined. *MAT* is the ideal locus to consider, since the two alleles are each semi-dominant, and all three genotypes in a diploid can be phenotypically distinguished (ie. *MATa/MATa*, *MATa/MATα*, *MATα/MATα*). The diploid spores produced in single division meiosis may either be *MATa/MATa* and

MAT α /*MAT* α (ditype (a& α) dyads), or both *MATa*/*MAT* α (monotype (N&N) dyads, where "N" designates non-mating). Only rarely will dyads consist of one mating and one non-mating spore, or two mating spores of the *same* apparent mating type. Further, assuming normal levels of recombination occur between *MAT* and *CENIII* (25 cM), then there will be an appreciable frequency of (N&N) monotype dyads consisting of two *MATa*/*MAT* α cells. Hence, the detection of non-mating spores, combined with the failure to find (N&a), (N& α), (a&a), and (α & α) dyads would all be diagnostic of the occurrence of single division meiosis.

CBM18 was mated, dyads were dissected, and it was determined whether spores mated or not. 57% of 187 complete dyads were found to consist of two non-mating spores. These were exposed to sporulation conditions, and generally found to be capable of sporulating. Since sporulation depends on heterozygosity at *MAT* (Herskowitz, 1989), this implied that they were most probably diploid. Further confirmation of the diploidy of these a/ α spores was obtained by sporulating many of them, and then dissecting the products of these sporulations; in general, the spores in the resulting dyads or tetrads showed moderately good viability (data from 930-1 and 930-2 are shown in Table 8), requiring the diploidy or near diploidy of the entire chromosome set in the parental diploids.

Most of the remaining dyads consisted of one phenotypic *MATa* and one phenotypic *MAT* α spore. These were mated to cycloheximide resistant (cyhR) haploid tester strains of the opposite mating type. Since cycloheximide resistance is recessive, the product of mating was cycloheximide sensitive (cyhS). If two haploid strains have been mated, they can generate homozygous cycloheximide resistance following mitotic recombination. Alternatively, if a diploid cyhS strain has been mated to a haploid cyhR tester, producing a triploid, mitotic

Table 8. Genetic data on centromere segregation in single division meiosis.
I. Single centromere linked marker.

centromere:	LOCUS			
	<i>CEN1::URA3</i> I	<i>LEU2</i> III	<i>ARG4</i> VIII	<i>MAT</i> III
cM from <i>CEN</i> :	0	3	12	25
% ditype dyads:	60	55	49	43 ⁽¹⁾
complete dyads examined:	183	47	183	187

(1) 43% ditype is significantly more than the 1/3 ditype expected for CEN unlinked loci ($\chi^2 = 7.863$, $p < 0.005$).

Data are pooled from CBM18/+ dyad dissections of 930, 930-1, 930-2, and 941. 930-1 and 930-2 are the two diploid spore clones derived from the dissection of a single dyad from 930. 930-2 was transformed with *LEU2* DNA prior to sporulation and dissection. CBM18 could not be scored in 930-1 or 930-2, since *CLN* genes are present. However, since both 930-1 and 930-2 sporulated to give dyads, they are both designated SPO heterozygotes. In all of these, the diploid was homozygous null at *CLN3*.

930:	$\frac{a}{\alpha}$	$\frac{CEN1::URA3}{CEN1^+}$	$\frac{CLN1}{cln1::TRP1}$	$\frac{CLN2}{cln2::LEU2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm^+}$	$\frac{ARG4}{arg4}$
930-1:	$\frac{a}{\alpha}$	$\frac{CEN1::URA3}{CEN1^+}$	$\frac{CLN1}{cln1::TRP1}$	$\frac{cln2::LEU2}{cln2::LEU2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{SPO}{spo^+}$	$\frac{ARG4}{arg4}$
930-2:	$\frac{a}{\alpha}$	$\frac{CEN1::URA3}{CEN1^+}$	$\frac{CLN1}{cln1::TRP1}$	$\frac{CLN2}{CLN2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{SPO}{spo^+}$	$\frac{ARG4}{arg4}$ $\frac{LEU2}{leu2}$
941:	same as 930.						

recombination will only very rarely produce phenotypic resistance. Production of *cyhR* mated products was assayed, and from the very low frequency of papillation of the *cyhR* phenotype, it was inferred that the mating progeny of CBM18/+ meiosis were diploids, homozygous at MAT. Taken together, these observations all imply that the two spored dyads produced in CBM18/+ meiosis consist of two diploid cells, which are the products of single division meiosis.

The single meiotic division can be either reductional or equational in character. Normally, meiosis consists of DNA replication, followed by two rounds of chromosome segregation, called M_I and M_{II} . At M_I , homologous chromosomes segregate from one another (reductional division), while at M_{II} , sister chromatids segregate from one another (equational division). In order to determine whether the single meiotic division occurring during CBM18/+ meiosis was equational or reductional in character, CBM18 was mated to strains having marked centromeres. The following centromere linked markers were used (Tables 8 and 9): *CEN1::URA3*, *TRP1*, *LEU2*, *ARG4*, and *MAT*. If a genetic marker recombines infrequently with the centromere of the respective chromosome, then the behavior of the centromere in meiosis may be inferred from an examination of the phenotypes of both of the spores in a complete dyad. A monotype dyad results from equational segregation of a centromere linked marker, while a ditYPE dyad results from reductional segregation (illustrated in Figure 12; see also Figure 7 of Shuster and Byers, 1989). The *URA3* marker linked to *CEN1* is genetically engineered at *CEN1* (Materials and methods); recombination essentially never occurs between *CEN1* and this *URA3* marker. 60% of the dyads were ditYPE for uracil prototrophy, indicating that in 60% of the meioses, *CEN1* segregated reductionally, and in the remaining 40%, *CEN1* segregated equationally (Table 8).

Table 9. Genetic data on centromere segregation in single division meiosis.
II. Multiple centromere linked markers.

Diploid	dyads dis- sected	complete dyads recovered	Number of monotype dyads for the stated marker(s)						
			<i>URA</i> ⁽¹⁾ (+&+)	<i>LEU2</i> (+&+)	<i>ARG4</i> (+&+)	U&A (+&+)	U&L (+&+)	A&L (+&+)	ALL (+&+)
930	131	83	34		38	28			
930-1	31	19	5		9	5			
930-2	99	47	17	21	20	14	12	13	11
941	69	34	18		26	17			
TOTAL	330	183	74	21	93	64	12	13	11
proportion		0.56	0.40	0.45	0.51	0.35	0.26	0.28	0.23
expected proportion						0.20	0.18	0.23	0.09
χ^2						23.90	1.81	0.59	11.40
significance						***	NS	NS	**

(1) ie. *CEN1::URA3*.

Expected proportion of dyads that are doubly or triply monotype is based on the product of the proportions that are singly monotype. χ^2 is calculated based on the null hypothesis that there is no association between being monotype for one marker and being monotype for another marker.

NS not significant
 ** p < 0.005
 *** p < 0.001

Recombination between a marker and its centromere complicates the inference of the behavior of centromeres. In the case where a marker is completely unlinked to its centromere, the behavior of the marker is independent of the behavior of the centromere. In such cases, the expectation is that one third of the dyads will be ditype, and two thirds monotype, as discussed above. At intermediate levels of linkage, the expected frequency of ditypes is intermediate between one third and the rate of reductional division of the centromere.

For the centromere linked markers that recombine at some rate with their centromeres, a somewhat lower rate of production of ditype dyads is seen (Table 8). The greater the recombination frequency between a marker and its centromere, the lower the rate of production of ditypes. This result is consistent with the possibility that the other marked centromeres for which data are available (ie. *CENIII* and *CENVIII*) have the same underlying rate of reductional division as does *CENI*. It will be shown directly in the following section that in any given meiosis, a limited number of centromeres tend to segregate the same way as one another; *CENIII* may be an exception to this rule.

At least three centromeres tend to segregate concordantly in a given meiosis. The previous section established that at least one centromere (*CENI*), and probably two others (*CENIII* and *CENVIII*), can segregate either reductionally or equationally in CBM18/+ single division meiosis. In order to determine whether different centromeres segregated the same way as one another in a given meiosis, CBM18/+ strains were constructed (Table 9) having more than one centromere linked heterozygous marker. Dyads were dissected, and it was determined whether the production of a monotype dyad for one marker was statistically associated with the production of a monotype dyad for other markers. Data are reported in Table

9. Considering *CENI::URA3* and *ARG4* (12 cM from *CENVIII*), there is a significant ($p < 0.001$) statistical association between the occurrence of monotype dyads for the two markers, hence, of equational division for the two centromeres. On the other hand, considering a smaller amount of data for *LEU2* (3 cM from *CENIII*), no association is seen between the behavior of *CENI* and *CENIII*, or *CENVIII* and *CENIII*.

To extend the analysis further, advantage was taken of the fact that *TRP1* lies 0.3 cM from *CENIV*. Diploid 948, heterozygous at both *CENI::URA3* and *TRP1* (Table 10) was found to concordantly segregate *CENI* and *CENIV* in all 23 of 23 dyads. These data contained a surprising result however, in that both *CENI* and *CENIV* appeared to segregate equationally in 96% of the meioses. As already discussed (Table 8), *CENI* had been seen to segregate equationally in 40% of 183 meioses. The finding of 96% equational segregation seemed impossible to reconcile with the larger data set. Since the only obvious difference between the diploids discussed in Table 8 and diploid 948 was the presence of the *CLN3* gene in the latter, it raised the question whether *CLN3* gene dosage affects the propensity to perform equational segregation in CBM18/+ single division meiosis. The limited data presented in Table 10 suggest that when *CLN3* is absent (ie. diploid 954), equational division of *CENIV* is rare, and when it is present (ie. 948, 955, and 956), equational division is more common. These data are confusing, however, since the anomalously high rate of equational division seen in 948 is not reproduced in 955 and 956, which are the other two diploids having *CLN3*.

HOP1 is a gene required for homolog pairing (Hollingsworth and Byers, 1989). As such, it is essential for the execution of reductional, but not equational meiotic chromosome segregation. In normal (tetrad) meiosis, *hop1/hop1* diploids

Table 10. Genetic data on centromere segregation in single division meiosis.
III. Addition of *CLN3*.

Diploid (# of copies of <i>CLN3</i>)	dyads dis- sected	complete dyads recovered	Number of monotype dyads for the stated marker(s)						
			<i>URA</i> ⁽²⁾ (+&+)	<i>TRP1</i> (+&+)	<i>ARG4</i> (+&+)	U&A (+&+)	U&T (+&+)	A&T (+&+)	ALL (+&+)
948 (1)	33	23 ⁽¹⁾	22	22	18	18	22	18	18
954 (0)	33	13		1					
955 (1)	33	20		12					
956 (3)	33	15		9					

(1) One complete dyad, consisting of sick spores of indiscernible phenotype, was omitted from the analysis.

(2) ie. *CEN1::URA3*.

948:	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{CLN1?}$	$\frac{cln2::LEU2}{CLN2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{ARG4}{arg4}$	$\frac{CBM18}{cbm^+}$	$\frac{trp1}{TRP1::CLN3}$
		$\frac{CEN1^+}{CEN1::URA3}$					
954:	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{cln1\Delta}$	$\frac{cln2::LEU2}{CLN2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm+}$	$\frac{trp1}{TRP1::TRP1}$	
955:	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{cln1\Delta}$	$\frac{cln2::LEU2}{CLN2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm+}$	$\frac{trp1}{TRP1::CLN3}$	
956:	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{cln1\Delta}$	$\frac{cln2::LEU2}{CLN2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm+}$	$\frac{trp1}{TRP1::CLN3(x3)}$	

generate almost no viable progeny, while *hop1 spo13 a/α* haploids, which bypass the reductional division, sporulate to produce viable haploid progeny. *a/α hop1/hop1 CBM18/cbm⁺* diploids sporulated to produce virtually no viable progeny, in spite of the fact that individual chromosomes are observed (in other experiments) to segregate equationally much of the time. This suggests that purely equational single division meiosis (in which all 16 chromosomes segregate equationally) does not occur in *CBM18/+* strains.

In summary, it has been demonstrated for *CENI*, *CENIV*, and *CENVIII*, that these centromeres can segregate either equationally or reductionally, but that they tend to segregate the same way as one another in a given meiosis. *CENIII* segregation may be independent of that of *CENI* and *CENVIII*. The rate of equational segregation of *CENI* and *CENIV* may be influenced by the presence of *CLN3*; in its absence, segregation is equational about 40% of the time, and in its presence, for limited data sets, equational segregation was seen in 96% (948) and 60% (955 and 956) of meioses. The significance of perturbation of meiotic phenotype by *CLN3* is difficult to assess, without a better understanding of the underlying phenomenon (ie. single division meiosis) or the potential role of *CLN3* in meiotic regulation.

Meiotic levels of intrachromosomal recombination occur during meiosis in CBM18 heterozygous diploids. An elevated rate of intrachromosomal recombination is one of the hallmarks of meiosis. It was therefore important to establish that intrachromosomal recombination occurred during *CBM18/+* "meiosis". To this end, a *CBM18/+* diploid (930-2, Table 8) was constructed which had a chromosome with two, linked, heterozygous markers: *LEU2* and *MAT*. *LEU2* is 2.9 cM from *CENIII* (left arm), while *MAT* is 25 cM from *CENIII*

(right arm). Since the *LEU2* marker was introduced via transformation of a *leu2* homozygote, it was initially undetermined whether *LEU2* was linked to *MATa* or *MATα*. 930-2 was sporulated and 99 dyads were dissected. 46 complete dyads were obtained having unambiguous phenotypes at *MAT* and *LEU2*. 44 of these (shown in Table 11) were Mendelian, in the sense that they could be explained without assuming the occurrence of gene conversion. In addition, one (N&α) dyad and one (N&a) dyad were recovered; these were excluded from the analysis.

Since 12 *MATa leu2* spores were recovered, and 0 *MATα leu2* spores, the model shown in Table 11 was inferred. This allowed assignment of the each dyad type recovered to one of the four categories shown. Note, since *LEU2* is much closer to *CENIII* than is *MAT*, all (*Leu*⁺&*leu*⁻) ditype dyads are assumed to be reductional. Finally, from the assignments, it was inferred that a single cross over in the interval *LEU2* -- *MAT* occurred in 19/44 meioses, and no cross over occurred in the remaining 25 meioses. The map distance is equal to one half the proportion of meioses having a single cross over (Fincham et al, 1979), or 19/88 = 21.6 cM. This is in very good agreement with the sum of the published map distances for these two loci. Therefore, we may conclude that at least on chromosome III, normal meiotic rates of intrachromosomal recombination occur during meiosis in CBM18/+ diploids.

In CBM18, the mutation causing SPO is linked or allelic with the *CLN* bypass mutation.

SPO was an unselected phenotype of CBM isolate 18. This raised three genetic questions. First, as for all the CBM isolates, it was of interest to establish whether *CLN* bypass activity was a monogenic trait in CBM18. Second, it was of

Table 11. In CBM18/+ single division meiosis, *LEU2* and *MAT* recombine at rates typical of meiotic recombination.

Dyads recovered in each class						
Diploid	LEU ⁺ N	leu ⁻ a	LEU ⁺ N	LEU ⁺ a	LEU ⁺ a	
	$\overline{\text{LEU}^+ \text{ N}}$	$\overline{\text{LEU}^+ \alpha}$	$\overline{\text{leu}^- \text{ N}}$	$\overline{\text{LEU}^+ \alpha}$	$\overline{\text{leu}^- \alpha}$	
930-2	13	12	11	8	0	
interpretation:	equational no rec.	reductional no rec.	reductional s.c.o.	equational s.c.o.	reductional d.c.o.	
<div> <div>Model</div> <div> <div></div> <div>--LEU2--CENIII-----MATα</div> <div>--leu2----CENIII-----MATa</div> </div> </div>						
930-2: $\frac{\text{a}}{\alpha}$	$\frac{\text{CENI}::\text{URA3}}{\text{CENI}^+}$	$\frac{\text{CLN1}}{\text{cln1}::\text{TRP1}}$	$\frac{\text{CLN2}}{\text{CLN2}}$	$\frac{\text{cln3}\Delta}{\text{cln3}\Delta}$	$\frac{\text{SPO}}{\text{spo}^+}$	$\frac{\text{ARG4}}{\text{arg4}} \frac{\text{LEU2}}{\text{leu2}}$

interest to determine whether SPO was a monogenic trait. Finally, it was of interest whether the *same* mutation caused both the *CLN* bypass and SPO phenotypes.

To address all of these questions, CBM18 was mated to *cln1,2,3⁻ GAL1::CLN3* strains. Dyads were dissected on YEPGal media, and the diploid progeny were scored for inheritance of *CLN* bypass activity (ie. viability without *GAL1::CLN3* synthesis), and they were either sporulated (if a/α) or mated and sporulated (if a/a or α/α) to score the inheritance of SPO.

As shown in Table 12, both recessive phenotypes (cbm^+ and spo^+) were recovered in the progeny of these diploids. Further, each recessive phenotype was recovered at a frequency close to (and not statistically different from) 1/6. These results are consistent with each trait being caused by a single dominant mutation. Moreover, all 8 of the 8 cbm^+ diploids recovered were spo^+ . An additional 3 *CLN* bypass progeny also appeared to be spo^+ . These results clearly imply that the mutation causing SPO is linked to the mutation causing *CLN* bypass. This idea is illustrated in the drawing in Figure 13. It is unclear why occasionally strains are CBM but spo^+ . One of the three CBM spo^+ strains was noted to be only marginally viable on YEPD.

One hypothesis for the inheritance of CBM without the inheritance of SPO is that more than one gene mutation is required to cause the SPO trait. One of these might be CBM18, while the other might be unlinked to CBM18. The data are inconsistent with this model. If a trait required two unlinked dominant mutations, whenever either one of them was not inherited, the trait would not appear. In this case, the frequency of non-inheritance (ie. spo^+) would be $1/6 + 1/6 - 1/36$, or $11/36$. As shown in Table 12, spo^+ strains were recovered too rarely

Table 12. CBM18 and SPO are each attributable to single Mendelian loci, which are either linked or allelic with one another.

Diploid	dyads dis- sected	dyads with two viable spores	total viable cells	<i>GAL1::CLN3</i> viable cells	Ura ⁺ cbm ⁺ cells	Ura ⁺ spo ⁺ cells	cbm ⁺ <i>and</i> spo ⁺
886	69	18	66	46	4	6	4
918	36	12	41	34	4	5	4
TOTAL	105	30	107	77	8	11	8
expectation ⁽¹⁾					12.83	12.83	2.14
χ^2					2.18	0.31	16.52
significance					NS	NS	p < 0.005
expectation based on 11/36					23.53	23.53	
χ^2					14.76	9.61	
significance				p <	0.005	0.005	
expectation based on 1/36					2.14	2.14	
χ^2					16.52	37.76	
significance				p <	0.005	0.001	

(1) Expectations are based on 1/6, 1/6, and 1/36, respectively.

886	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{cln1::TRP1}$	$\frac{cln2::LEU2}{cln2::LEU2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm+}$	$\frac{ARG4}{arg4}$	<i>GAL1::CLN3</i>
918	$\frac{a}{\alpha}$	$\frac{cln1::TRP1}{cln1::TRP1}$	$\frac{cln2::LEU2}{cln2::LEU2}$	$\frac{cln3\Delta}{cln3\Delta}$	$\frac{CBM18}{cbm+}$	$\frac{ARG4}{arg4}$	<i>GAL1::CLN3</i>

918 has the same genotype as 886, except the cbm⁺ parent of 918 was derived from dissection of a "rare" tetrad from 886.

to favor this model for the inheritance of SPO.

Another hypothesis for the inheritance of CBM without the inheritance of SPO is that there are two unlinked mutations, either of which confers *CLN* bypass, but only one of which causes SPO. The data are also inconsistent with this model. If a trait could be caused by either one of two unlinked mutations, the frequency of non-inheritance would be the product of the individual frequencies of non-inheritance, or 1/36. As shown in Table 12, *cbm*⁺ strains were recovered too often to favor this model for the inheritance of CBM.

A final hypothesis for the inheritance of CBM without the inheritance of SPO would be if there were two linked mutations, one of which conferred *CLN* bypass, and the other of which caused SPO. If a single crossover occurred between them, it would be possible to generate a dyad that was monotype for CBM and ditype for SPO. This hypothesis predicts that the spore opposing a CBM *spo*⁺ spore is homozygous SPO/SPO, and phenotypic SPO. The phenotypic prediction was realized in two of the three CBM *spo*⁺ spores observed; the third came from an incomplete dyad. Given homozygosity for SPO, sporulation and dissection of the opposing SPO spore should generate no *spo*⁺ progeny; this was never attempted.

In conclusion, while the picture is not crystal clear, the available data suggest that either a single mutation, or two linked mutations, account for both CBM18 and SPO. Whatever the relation between the traits, both appear to be caused by monogenic, Mendelian factors. As will be discussed in the following section, other observations indicate that CBM18 and SPO can become dissociated *without* passing through meiosis, hence we might need ponder no longer why *CLN* bypass-*spo*⁺ progeny were occasionally recovered in dyads.

SPO is a very unstable trait.

SPO is spontaneously lost at a high frequency during the mitotic propagation of CBM18/cbm+ diploids. Examination of sporulations of CBM18 heterozygotes revealed (among sporulated cells) a preponderance of dyads and an occasional tetrad. Typical values (from cross 886, genotype in Table 12) were 50% unsporulated cells, 5% monads, 39% dyads, 3% triads, and 3% tetrads. When tetrads were dissected, they were found to consist either of diploid cells, or of haploid cells, but only rarely of a mixture of the two. For example, from 24 tetrads dissected from cross 886, 16 consisted of haploid cells, 6 of diploid cells, 1 of both, and 1 had no viable spores. The tetrads consisting of diploid cells might simply result from two, attached cells (ie. a mother and a daughter), each of which sporulated to give a dyad. The diploid progeny were found to be mating or non-mating (50% of each), and upon sporulation produced dyads (78%) or tetrads (22%). In all these respects, they are unremarkable.

In contrast, the tetrads consisting of *haploid* cells were quite remarkable: They segregated *MAT*, *CLN* bypass activity, and *ARG4*, all 2:2, but they segregated SPO 0:4 (ie. 0 progeny were SPO, and all viable progeny were *spo*⁺; the sole exception was the one haploid cell from the cross 886-derived tetrad composed of a mix of haploid and diploid cells -- this haploid cell was SPO). 0:4 segregation of SPO came as a surprise, given the linkage of SPO and CBM18, and the 2:2 segregation of *CLN* bypass activity.

The simplest explanation for the existence of these tetrads is incomplete penetrance of SPO. According to such an explanation, however, SPO should segregate 2:2, as do other markers. It does not. The fact that SPO segregated 0:4

suggested an alternative: SPO was somehow lost, prior to sporulation, and this loss accounted for the resulting tetrad.

To test this hypothesis, cross 887 (CBM18 x *CLN1 CLN2 cln3Δ arg4* W16) was colony purified on YcGal-ura, and then restreaked from the single colony. 68 secondary colonies were patched out and replicas were taken to sporulation agar. Patches were examined for sporulation phenotype; 66/68 consisted of dyads, while 2/68 had an abundance of tetrads. Vegetative cells (off the YcGal-ura plate) from one of the two tetrad forming patches were streaked out on YcGal-ura; colonies were patched to YcGal-ura, and replica plated to sporulation agar. 5 patches consisted of dyads, and 3 of tetrads. Thus, SPO was lost at a high frequency during mitotic propagation of CBM18/+ heterozygous diploids. The actual loss rate per mitotic division can be crudely estimated from these data. Assume the loss occurs in 1 colony in 34, when that colony is at the 4 cell stage, such that 3/4 of the colony is SPO, and 1/4 is *spo*⁺. To generate 34 x 4 = 136 cells from 1 cell requires 135 mitoses, hence the per mitosis loss rate is about 0.75%. This figure is about 1/3 (ie. not that different from) the estimated rate of production of tetrads of haploid cells following meiosis. Cross 887-derived tetrads were dissected and marker segregation was scored; Leu and Arg were segregating, and all spores were *Ura*⁻ *Ade*⁻ *His*⁻ *Lys*⁺ *spo*⁺. These observations are consistent with the tetrads being derived from 887, rather than a contaminating strain. In conclusion, it is plausible that the occasional tetrads seen in CBM18/+ sporulation mixes result from the loss of SPO prior to undergoing meiosis.

Spontaneous loss of SPO was never observed during the mitotic propagation of the CBM18 (original isolate) haploid, although in one experiment 192 mated CBM18 patches were examined. It remains unclear whether the spontaneous loss

of SPO depends on the diploid condition. If so, it raises the possibility that mitotic recombination and loss of heterozygosity, or gene conversion, account for the loss of SPO, although the high spontaneous loss rate seems inconsistent with such a mechanism.

When SPO is spontaneously lost, CLN bypass activity is retained as a Mendelian gene linked to CBM9. CLN bypass activity segregated 2:2 in tetrads (consisting of haploid cells) derived from cross 886. This implies that the CLN bypass activity in CBM18 is attributable to a single Mendelian gene. The same conclusion was also reached based on the analysis of dyads (data in Table 12). The CLN bypass activity found in haploids derived from CBM18/+ tetrads was called CBM18'. To determine whether CBM18' was linked to CBM9, CBM18' was backcrossed once to a *cln1,2,3*' strain, and a CBM18' strain derived from that cross was mated to a CBM9 strain (cross 1084, data in Table 5). As was discussed in chapter 3, CBM18' appears to be linked to or allelic with CBM9.

The loss of SPO is not the result of the loss of a cytoplasmic factor, and appears to result from a mutation in a Mendelian gene. The high frequency spontaneous loss of the SPO phenotype, combined with the apparent linkage between SPO and CBM, suggested that SPO might depend jointly on the CBM mutation and a non-Mendelian gene, such as an episome or a mitochondrial gene. Such factors tend to be lost (or to become genetically homogeneous, when initially heterogeneous) at a high frequency during mitotic propagation, since they lack a means of assuring 1:1 segregation between mother and daughter cells. Two experiments were done to test the hypothesis that the *spo*⁺ phenotype resulted when CBM18 lost a non-Mendelian gene. Results from each implied that this was not the correct explanation for the spontaneous loss of SPO. An alternative hypothesis

will be offered below.

A cytoductant is the rare haploid product of a failed mating between two cells, in which mating begins, cytoplasmic fusion occurs, but nuclear fusion does not occur and only one nuclear genome is retained. It is selected by allowing cells to mate, then selecting for the mitochondrial genome of one cell, for the nucleus of the other cell, and against diploidy. It was reasoned that if CBM18' arises when a mitochondrial gene (initially heterogeneous in CBM18) becomes homogeneous, then cytoductants formed between CBM18 mitochondrial and CBM18' nuclear genomes might be SPO. Four distinct cytoductants were selected (Materials and methods), two between CBM18 (original isolate (o.i.)) and *MATa* CBM18' strains, and two between CBM18 and *MATa* *cbm*⁺ strains (negative control). In each case, CBM18 supplied the mitochondrial genome, while the mating partner supplied the nuclear genome. Once derived, cytoductants were mated and sporulated; in each case, tetrads were formed. This result is inconsistent with the hypothesis that the SPO phenotype depends on CBM18' plus a factor linked to the mitochondrial genome in CBM18.

An additional experiment was done to test the hypothesis that the loss of SPO was due to the loss of a non-Mendelian gene. CBM18' strains were backcrossed to CBM18 o.i., and dyads were dissected (Table 13, crosses 916 and 917). As a control, a *cbm*⁺ strain derived from a tetrad segregating CBM18' was also backcrossed to CBM18 o.i. (Table 13, cross 918). When 916 and 917 were sporulated and dyads dissected, 100% of their progeny had *CLN* bypass activity, consistent with allelism between CBM18' and CBM18. Nevertheless, 6 out of 35 dyads (17%) were ditype (SPO&spo⁺). In contrast, cross 918 progeny were also occasionally ditype for SPO, but showed cosegregation of *cbm*⁺ and *spo*⁺, as

Table 13. CBM18' does not support SPO after backcrossing to CBM18.

All numbers refer to complete dyads					
Diploid	number dis- sected	complete dyads recovered	monotype for CBM activity	monotype for SPO	ditype for SPO
916	36	17	17	14	3
917	36	20	20	15 ⁽¹⁾	3
TOTAL	72	37	37	29	6
expectation based on 2/3 χ^2 significance			24.67 18.50 p < 0.005	24.67 3.46 NS	
918	36	12	10	9	3

(1) Sporulation phenotype could not be scored in 2 spore clones. Statistical calculation is based on the assumption that one was in a monotype dyad, and one in a ditype dyad.

916, <u>a</u>	<u><i>cln1::TRP1</i></u>	<u><i>cln2::LEU2</i></u>	<u><i>cln3Δ</i></u>	<u><i>CBM18</i></u>	<u><i>ARG4</i></u>	<u><i>GAL1::CLN3</i></u>
917 <u>α</u>	<u><i>cln1::TRP1</i></u>	<u><i>cln2::LEU2</i></u>	<u><i>cln3Δ</i></u>	<u><i>CBM18'</i></u>	<u><i>arg4</i></u>	

Both 916 and 917 were derived by backcrossing CBM18' haploids to CBM18.

918 <u>a</u>	<u><i>cln1::TRP1</i></u>	<u><i>cln2::LEU2</i></u>	<u><i>cln3Δ</i></u>	<u><i>CBM18</i></u>	<u><i>ARG4</i></u>	<u><i>GAL1::CLN3</i></u>
<u>α</u>	<u><i>cln1::TRP1</i></u>	<u><i>cln2::LEU2</i></u>	<u><i>cln3Δ</i></u>	<u><i>cbm+</i></u>	<u><i>arg4</i></u>	

discussed above.

I interpret the relatively high rate of segregation of *spo*⁺ progeny from 916 and 917 to result, at least in part, from normal meiotic gene segregation (normal, that is, for single division meiosis). This is equivalent to saying that 916 and 917 are heterozygous for a Mendelian dominant which causes SPO, *even though* they are homozygous for *CLN* bypass activity. Since CBM18 is linked to SPO, and CBM18' is not, this could either mean that CBM18' is molecularly different from CBM18, or that something linked to it is different, depending on whether SPO is really a phenotype of CBM18 or of a linked gene. Although these data are limited, they seem inconsistent with the idea that CBM18' results from the spontaneous loss of a non-Mendelian factor. Were that the case, I would expect to find a lower rate of recovery of *spo*⁺ progeny in the crosses described in Table 13, since there should be no contribution from meiotic gene segregation.

It remains uncertain how a phenotype attributable to a nuclear gene could be lost at such a high frequency, in the absence of selection. One possibility consistent with the observed linkage between CBM18 and SPO is that CBM18 o.i. is the result of a UV-induced tandem gene multiplication. If SPO is dependent on elevated copy number of a mutant gene (which causes *CLN* bypass when present in single copy), then loss of SPO might result from loss of some members of this array via looping out (ie. homologous, intrachromatid recombination). Note that two copies (in *trans*) of CBM18' are not enough to cause SPO, since the (presumed) 18'/18' homozygotes generated by sporulating crosses 916 and 917 were *spo*⁺.

SPO is not a consequence of the failure to express *SPO13*.

spo12-1 and *spo13-1* are both recessive mutations that cause single division meiosis (Klapholz and Esposito, 1980). A null allele of *spo13* (*spo13::URA3*) also caused 100% two spored ascii (Wang et al, 1987). This observation suggests that the product of the *SPO13* gene is required for the execution of the first (reductional) meiotic division. CBM18 is not an allele of *SPO13*, since *SPO13* is linked to *ARG4* (Wang et al, 1987), and CBM18 is not (data not shown). However, CBM18 could be a dominant mutation affecting the function of *SPO12* or *SPO13*. An experiment was done to determine if *SPO13* is expressed normally in CBM18/+ diploids. In wild type strains, *SPO13* transcript is only significantly induced in sporulating *MATa/α* cells (Wang et al, 1987). Therefore, CBM18/+ *MATa/α* diploids were constructed, along with *cbm⁺/cbm⁺ MATa/α*, and non-sporulatable *MATa/a* controls. Cells were transferred to sporulation media at 23°C, and after 24 hours, RNA was prepared and analyzed by Northern blotting with a *SPO13* probe. Transcriptional induction of *SPO13* appeared equivalent in *cbm⁺/cbm⁺* and CBM18/+ *MATa/α* diploids (Figure 14), suggesting that the meiotic phenotype of these strains is not a consequence of their failure to express *SPO13*. The *MATa/a* and vegetative controls failed to induce *SPO13* transcript, as expected. It remains possible that CBM18 affects *SPO13* post-transcriptionally, however, the fact that CBM18 is not associated with an exclusively equational phenotype suggests that it does not work via an effect on *SPO12* or *SPO13*.

Discussion.

What is the origin of SPO in CBM18? *CLN* bypass isolates 15, 16, and 18 were selected at 23°C (Table 3). In contrast, other *CLN* bypass isolates were selected at room temperature (2 - 4) or at 37°C (6 - 14). In agreement with this,

isolates 2 - 4 and 15 - 18 grew well at 23°C, while isolates 6 - 14 grew very slowly at 23°C. It is noteworthy that CBM18', which had lost SPO, was cold sensitive, unlike its CBM18 parent. This suggests a hypothetical two step process for the selection of CBM15 and CBM18: First, a mutation was selected which allowed very slow growth on YEPD at 23°C. In addition, a second mutation was selected, either in the same gene or a linked gene, which allowed more rapid growth at 23°C. This mutation was selected for growth at 23°C and, we may suppose, caused SPO. For some reason, this mutation reverted at a high frequency, and when it reverted, SPO was lost along with cold tolerance.

In addition, it is interesting that *CLN* bypass isolates 15, 16, and 18, exclusively, were selected following UV mutagenesis of the parental stock. The relevance of this, if any, to the generation of the SPO trait is unclear. If the two mutation model sketched above is correct, and the use of UV mutagenesis is relevant to SPO, perhaps both the *CLN* bypass and the SPO mutations arose and were selected simultaneously, rather than sequentially.

What could cause single division meiosis? As discussed for *spo12-1* and *spo13-1* (Klapholz and Esposito, 1980), single division meiosis of a predominantly *equational* character might result from one of several different causes, including (1) execution of a single mitotic division instead of meiosis, followed by ascosporeogenesis; (2) execution of an equational division at M_I , due to a failure in homolog pairing, followed by ascosporeogenesis without further segregation; or (3) successful homolog pairing and meiotic recombination, followed either by (i) a defect in movement of the paired homologs to the M_I metaphase plate, (ii) by a defect in the spindle apparatus or centromere - spindle attachment, or (iii) by precocious execution of the M_{II} equational division. Given the propensity of

CBM18 to execute both reductional and equational divisions, in nearly equal numbers, none of these explanations are adequate. Furthermore, as discussed earlier, CBM18 meioses are characterized by normal meiotic levels of recombination in the interval *MAT* -- *LEU2*, ruling out (1) and (2) above. The fact that the CBM18 meiotic phenotype is dominant makes explanation (3)(iii) seem most plausible, although it does not explain why M_{II} fails in those instances where successful reductional segregation has taken place. CBM18 meiosis may best be explained by assuming hyperactivity in some aspect of the control of the timing of the meiotic divisions, associated with a breakdown in the normal control of whether the first division is reductional or equational, such that only one division occurs, of whatever character, and then ascosporeogenesis follows. It has been previously observed (Sharon and Simchen, 1990) that different chromosomes can segregate diversely (ie. some reductionally, some equationally) in a single event of single division meiosis. In that study, strains homozygous for t.s. alleles of *cdc5* and *cdc14* were used, and single division meiosis was induced by shifting cells to the non-permissive temperature 30 minutes after initiating sporulation, then shifting back to permissive temperatures at later times.

Why should a CLN bypass mutation cause single division meiosis? There are precedents which indicate that genes controlling the mitotic cell cycle are also involved in the control of the meiotic divisions (Simchen, 1974; Shuster and Byers, 1989). *CDC28*, in particular, is required during both M_I and M_{II} divisions (Shuster and Byers, 1989). Furthermore, the yeast B cyclins *CLB1* (N. Grandin and S.I. Reed, unpublished results) and *CLB5* (results presented below) are both essential to the execution of meiosis. Since *CLNs*, like B cyclins, are probably *CDC28* cofactors, dominant mutations relieving the *CLN* requirement might well have a

direct or an indirect effect on *CDC28* activity. Taken together, these points suggest a framework for contemplating the meiotic phenotype of a *CLN* bypass mutation. Unfortunately, without a better understanding of CBM, and of the relationship between CBM18 and SPO, the meiotic behavior of CBM18 sheds light neither on the nature of CBM nor on the shared meiotic and mitotic functions of cell cycle regulatory components.

Chapter 5. Cloning and Characterization of *CLB5*

Isolation of a novel B cyclin rescuing the *cln1,2,3*⁻ genotype.

While attempting to clone CBM, we screened our library for plasmids that could rescue a strain with the genotype *cln1 cln2 cln3* [p*GAL1::CLN3/URA3*], under conditions (ie. glucose media) where *GAL1::CLN3* was not being synthesized. We recovered eight distinct clones of *CLN2*, and three distinct clones of a novel gene. Subcloning experiments identified the minimal region of these plasmids required for *cln1,2,3*⁻ rescue (Figure 15). This region was sequenced in its entirety (Figures 16,17), and found to contain a single open reading frame of 435 amino acids. The translated sequence was aligned using the FASTA homology search program (Pearson and Lipman, 1988) to the contents of GenBank (Bilofsky and Burks, 1988), and found to represent a new B cyclin. We named this gene *CLB5*, since four B cyclins (*CLB1*, *CLB2*, *CLB3*, and *CLB4*) were already known in budding yeast (Surana et al, 1991). *CLB5* can be further identified as a B cyclin since it contains the conserved FLRR_SK motif (residues 301 - 307), diagnostic for distinguishing B and A type cyclins (O'Farrell and Leopold, 1991).

CLB5 is adjacent to one of the other B cyclins, *CLB2* (Figure 15). We tested various plasmids containing *CLB5* only, or *CLB2* only, for *cln1,2,3*⁻ rescue activity and for rescue of a *clb1,2*⁻ double mutant (see Materials and methods; the double mutant is lethal [Surana et al, 1991]). We found that the *CLB2*-containing plasmid rescued the *clb1,2*⁻ lethality but not the *cln1,2,3*⁻ lethality; the *CLB5*-containing plasmid rescued the *cln1,2,3*⁻ lethality, but not the *clb1,2*⁻ lethality (Figure 15). Thus, *CLB5* is qualitatively different from *CLB2*. The failure of

CLB2 to rescue *cln1,2,3⁻* lethality, together with the fact that no other B cyclin genes were recovered from our library, suggest that *CLB5* is unique among yeast B cyclins in its ability to rescue *cln1,2,3⁻* lethality when cloned on a CEN plasmid. Lew et al. (1991) were unable to rescue a *cln1,2,3⁻* strain by overexpressing integrated *CLB1* from the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, again supporting the inference that *cln1,2,3⁻* rescue may be unique to *CLB5*. Although addition of *CLB5* on a CEN plasmid was adequate at *cln* rescue, but not at *clb* rescue, this may simply reflect the time of expression of *CLB5* from its natural promoter, rather than the intrinsic potential of the protein. Overexpression of *CLB5*, or expression from a deregulated promoter, might suffice to rescue *clb1 clb2* mutants. These experiments are in progress.

Northern analysis of *CLB5*

Four yeast cyclins are known to be subject to cell cycle periodic transcriptional control: *CLN1* and *CLN2* are maximally expressed just prior to cell cycle START (Wittenberg et al, 1990; Cross and Tinkelenberg, 1991), while *CLB1* and *CLB2* are maximally expressed prior to mitosis (Surana et al, 1991; Ghiara et al, 1991). For these genes, the time of expression is consistent with the time of function; *CLN1* and *CLN2* function to promote START (Richardson et al, 1989), while *CLB1* and *CLB2* regulate entry into mitosis (Surana et al, 1991; Ghiara et al, 1991). Since the time in the cell cycle when a transcript is abundant may provide some insight into the gene's function (McKinney and Heintz, 1991), we examined *CLB5* expression in synchronized cultures. G1 arrest was induced in a *cln1 cln2 cln3 GAL1::CLN3* strain by incubation in raffinose medium, and cycling was induced by galactose addition, as described (Cross and Tinkelenberg, 1991). The

defective *cln2* gene in this strain produces a properly regulated mRNA (Cross and Tinkelenberg, 1991), serving as a control for a gene turned on at START. We found that *CLB5* RNA is expressed exactly in parallel with *cln2* RNA in this protocol (Figure 18, *upper* panel). In contrast, *CLB2* RNA comes on later, and peaks when *CLB5* and *cln2* transcripts are near their trough, just preceding nuclear division. This pattern is detectable for two cell cycles in this experiment. *CLB4* transcript is also cell cycle periodic, and peaks at a time intermediate between *CLB5* and *CLB2* transcripts (data not shown). We have also observed that *CLB5* RNA fluctuates exactly in phase with *CLN2* RNA in an α -factor block-release synchronization protocol using a *CLN1 CLN2 CLN3* strain (data not shown). The fact that *CLB5* expression peaks earlier in the cell cycle than *CLB2* may indicate that *CLB5* principally functions at an earlier cell cycle stage.

To further examine the regulation of the *CLB5* transcript, we repeated the block-release protocol described above, but released in the presence of nocodazole, a microtubule depolymerizing agent that prevents mitosis (Jacobs et al, 1988). In this protocol, the first bud emergence occurs with normal timing, but mitosis never occurs, and cells arrest in G2 with a single large bud (Jacobs et al, 1988). We found that *cln2* and *CLB5* RNAs peak with timing equivalent to the nocodazole free treatment, then decline (Figure 18, *bottom* panel). The second peak of these RNAs is not observed. *CLB2* RNA also comes up on schedule, but in contrast, remains on at the nocodazole block. These results suggest that the fall in *CLB2* RNA levels requires nuclear division, and that the time in the cell cycle when these gene products function is early for *CLN2* and *CLB5*, and late for *CLB2*.

The initial rise in *cln2* RNA levels in this protocol has been ascribed to *CLN*-dependent positive regulation of *cln2* RNA (Cross and Tinkelenberg 1991;

Dirick and Nasmyth 1991). The Swi4/Swi6 transcriptional regulators have been proposed as mediators of this regulation, acting through the "cell cycle box" (CCB) target sequences ACGAAA and C₂CGAAA (Nasmyth and Dirick, 1991; Ogas et al, 1991). While *CLN1* and *CLN2* have such upstream sites, *CLB5* has none (except in the coding sequence; Figure 16, legend). *CLB5* transcript had a similar pattern of cell cycle regulation in both *cln1,2,3⁻ GAL1::CLN3 swi4::URA3* and *SWI4⁺* strains (data not shown). The *swi4::URA3* allele abolishes CACGAAAA binding activity and HO transcription (B.J. Andrews, personal communication). Thus, Swi4 is not essential for periodic transcription of *CLB5*.

Yeast have a second class of genes whose transcripts fluctuate in a cell cycle periodic fashion and peak late in G1. These genes are all involved in DNA replication, and include *CDC21* (thymidylate synthase), *CDC9* (DNA ligase), *POL1* (DNA polymerase I), and others (reviewed by McKinney and Heintz, 1991; Andrews and Herskowitz, 1990). All have a sequence motif called the MCB (ACGCGT, *MluI*-containing cell cycle box) in their 5' untranscribed region. The MCB is necessary and sufficient for the periodic, late G1 pattern of expression of these genes (McIntosh et al, 1991; Lowndes et al, 1991; Gordon and Campbell, 1991; Marini and Reed, 1992). We examined the sequence of *CLB5* for the occurrence of this element, and found five ACGCG sites in the 5' untranslated region of the gene. Four are clustered (three on the sense strand, one on the anti-sense strand) in the region 407 to 339 nucleotides upstream of the presumed initiator ATG (Figure 16), while a fifth was found at -47. No complete *MluI* sites are present in *CLB5*; however, the sixth nucleotide of the *MluI* site may not be critical for MCB activity (McIntosh et al, 1991). The sixth nucleotide of each ACGCG site in *CLB5* is a C. While we have not demonstrated the role of

ACGCGC elements in *CLB5* transcription, it seems possible that MCB activity contributes to the cell-cycle regulation of *CLB5* expression.

***CLB5* is required for efficient progression through S phase.**

Phenotypes of the clb5::ARG4 allele. To determine the role of *CLB5* in cell cycle progression, we disrupted the gene, substituting the region between residues Y²¹⁵ and E²⁹⁰ with the yeast *ARG4* gene (Figure 15). The substitution removes most of the cyclin box. Strains carrying the deletion have a 20% increase in cell volume and a 10% increase in doubling time, compared to isogenic wild type strains. In addition, the proportion of unbudded cells in an exponential culture was significantly lower in strains lacking *CLB5* (Table 2). These observations suggested that strains lacking *CLB5* are delayed at one or more stages during the budded portion of the cell cycle.

Hydroxyurea (HU) interferes with DNA replication, and imposes a delay on cell cycle progression in cells that have not yet completed most of S phase (Hartwell, 1976). We observed that *clb5* null strains form colonies extremely slowly on 0.2 M HU supplemented YEPD agar (Figure 19, plating assay). In contrast, wild type and *clb2* strains were relatively mildly delayed by HU. *CLB5* deletion and HU have a synergistic effect on cell cycle duration. This suggested the possibility that the delay in cell cycle progression associated with loss of *clb5* is specific to progression through S phase. Although *clb5* strains grow very slowly on HU media, they eventually form tiny colonies with high plating efficiency in the presence of 0.2M HU.

DNA flow cytometry of asynchronous cultures. To assess the effect of *clb5* deletion on cell cycle distribution, we performed flow cytometric DNA analysis on

exponentially growing cultures of isogenic wild type and *clb5* strains (Figure 20, upper panels). Wild type yeast display a characteristic pattern in which over half of the cells are in G2, a smaller portion are in G1, and only a small fraction are in S phase. In contrast, *clb5* strains exhibit a significant increase in the proportion of cells in S phase, and a diminished fraction of cells in G1. The increase in the S phase fraction suggests that *clb5* strains progress more slowly through S phase than *CLB5*⁺ strains. The reduced G1 proportion could also be due to a delay during progression through S phase, because delay in S phase leads to larger sized daughter cells at the time of cell separation, reducing the requirement for growth (and time) in G1 in the subsequent cycle (Singer and Johnston, 1981; Johnston and Singer, 1983; reviewed in Cross et al, 1989a). The decrease in the G1 population caused by *clb5* deletion is likely to work by this indirect mechanism, rather than by directly accelerating the G1/S transition, since *clb5* cells are larger than wild-type. Mutations (such as the *DAF1-1* allele of *CLN3*; Cross, 1988a) that reduce the length of G1 by accelerating the G1/S transition are associated with a smaller cell size.

DNA flow cytometry of synchronous cultures. To directly test whether the loss of *CLB5* affects the duration of S phase, we examined the kinetics of S phase transit in *CLB5* and *clb5* cultures synchronized by *CLN*-block-release (as in Figure 18). We obtained flow cytometric DNA profiles at intervals after releasing cells from the G1 block. *CLB5* and *clb5* strains both budded and entered S phase simultaneously, approximately 36 minutes after release from the G1 block (Figure 21A,B). We detected no indication that *clb5* strains entered S phase behind wild type controls. This suggested that the interval between START and the beginning of S phase was the same in *CLB5* and *clb5* strains. In *CLB5* strains, S phase

lasted about 24 minutes, while in *clb5* strains, S phase lasted about 48 minutes. Synchronized cultures therefore confirm that loss of *CLB5* slows progression through S phase. We can distinguish between a slowed progression through S phase and delayed beginning of S phase, since the *clb5* mutant culture showed DNA content between 1N and 2N for a protracted period compared to the *CLB5* control. In addition, a similar result was obtained using α -factor to synchronize *CLB5* and *clb5 CLN1,2,3⁺* strains; hence the *clb5* S phase delay is not dependent on the *cln* deficient, *GAL1::CLN3* background (data not shown).

Execution point analysis. The FACS data indicate that *clb5* strains are delayed during passage through S phase. We wished to confirm this finding using an alternative method. We used α -factor and hydroxyurea to estimate the proportion of cells in a log phase population that lie between the α -factor and hydroxyurea execution points (Materials and methods). An execution point is defined as the point in the cell cycle when cells have completed the steps which make them sensitive to an agent, such as α -factor or hydroxyurea (Hartwell, 1976). Once a cell has passed an execution point, it is insensitive to the corresponding agent for the remainder of the current cell cycle. Since S phase starts shortly after the α -factor execution point (Hereford and Hartwell, 1974), and ends at about the hydroxyurea execution point (Hartwell, 1976), the interval between these points approximates S phase.

In the *CLB5* wild type strain, 20% of the cells in an exponential culture were between the α -factor and hydroxyurea execution points (Table 14). In contrast, in the *clb5* deleted strain, 50% of the cells were in this interval. Given the doubling times of *CLB5* and *clb5* strains, these percentages allow estimation that S phase requires 17 minutes in a *CLB5* strain, and 48 minutes in a *clb5* strain

Table 14. The *clb5* mutation increases the amount of time between the alpha factor (α F) execution point and the hydroxyurea (HU) execution point.

Percentage of cells before event (minutes after cell division that event occurs)						
	Bud emergence	α F ex. pt.	S phase duration	HU ex. pt.	G2+M duration	Doubling time
<i>CLB5 CLB2</i>	31%(24)	20%(15)	17'	40%(32)	68'	100'
<i>clb5 CLB2</i>	19%(16)	9%(7)	48'	59%(55)	55'	110'
<i>CLB5 clb2</i>	12%(9)	ND	ND	28%(22)	80'	102'
<i>clb5 clb2</i>	8%(7)	ND	ND	40%(36)	76'	112'

The data in Table 2 were analyzed as described in Materials and methods to yield an estimate of the percentage of cells in an asynchronous population that are before a cell cycle event, and the time after cell division that the indicated event occurs. S phase duration was estimated as HU execution point (minutes) minus α F execution point (minutes). G2 + M duration was estimated as doubling-time (minutes) minus HU execution point (minutes).

(Table 14). These results are in reasonable agreement with the length of S phase estimated from the FACS analysis of synchronous cell cycles (ie. 24 and 48 minutes for *CLB5* and *clb5*, respectively; Figure 21).

Deletion of *CLB5* does not prolong G2.

DNA flow cytometry of asynchronous cultures. Since *CLB5* is a B cyclin, and typically B cyclins promote G2 to M phase progression, we determined whether *CLB5* deletion prolonged G2. Based on the FACS analysis of synchronized cells, we estimate that the *clb5* strain entered nuclear division about 12 minutes after completion of S phase, compared to about 18 minutes for *CLB5*. (Completion of S phase was inferred at the earliest time when all cells were in a well defined G2 peak [Figure 21A], while onset of nuclear division was inferred from the rapid increase in percent binucleate cells [Figure 21C]; see Figure 21 legend). Hence there is no indication from these data that *clb5* deletion caused any delay during G2.

Execution point analysis. A similar conclusion was reached from the determination of hydroxyurea execution points in exponential cultures. Assuming that G2 starts at the hydroxyurea execution point, the length of G2 + M (including cytokinesis) can be estimated from the data in Table 14 to be 68 minutes for *CLB5* and 55 minutes for *clb5* (see Table 14 legend). Here again, G2 is possibly shorter, and certainly no longer, in strains lacking *CLB5*. Note that the G2 + M estimate is substantially greater than the FACS based G2 estimate, because it includes the interval between nuclear division and cell separation. In contrast, a *clb2* strain was estimated to require 80 minutes for the completion of G2 + M (Table 14). This increase in the proportion of the cell cycle after the hydroxyurea

sensitive step is presumably due to G2 delay (Surana et al, 1991). This is consistent with the view that *CLB2* has an important role in G2, rather than in S phase; the major role of *CLB5* appears to be in S phase and not in G2.

Interactions between *clb5* and other B cyclin mutations.

We reasoned that if other B cyclins acted in concert with *CLB5* to promote S phase transit, then strains bearing combinations of B cyclin mutations might either have a more pronounced S phase delay than *clb5* single mutants, or be altogether unable to transit S phase, and consequently inviable. We used tetrad analysis to generate all viable combinations of B cyclin null mutations. The simultaneous deletions *clb1 clb2* and *clb2 clb3* are inviable, while genotypes *clb1 clb3 clb4* and *clb2 clb4* are viable (S. Reed and D. Lew, personal communication). We found that deletion of *CLB5* did not cause lethality in conjunction with any viable set of B cyclin deletions.

DNA flow cytometry of asynchronous cultures. We therefore determined the cell cycle distributions of viable combinations of B cyclin null mutations. We found that exponential cultures of *clb1,3,4* strains have a similar FACS profile to wild type strains (Figure 20, compare *left-top* and *left-central* panels), however their G1 peak is slightly depressed. The deletion of these B cyclins does not cause a detectable S phase delay; some delay in G2 may be inferred from the diminution of the G1 peak. In contrast, *clb1,3,4,5* strains display a FACS profile suggestive of a combination of S phase and G2 delays (Figure 20, *right-central* panels). These data do not suggest that quadruply deleted strains are significantly more delayed in S phase than *clb5* single mutants, although some effect of *clb5* on G2 delay, or of *clb1,3,4* on S phase delay, cannot be ruled out. Exponential cultures

of *clb3,4,5⁻* strains appear similar by FACS to *clb1,3,4,5⁻* strains, indicating that the presence of *CLB1* does little to overcome the delays attributable to the simultaneous deletion of *CLBs* 3, 4, and 5 (data not shown).

Deletion of *CLB2* alone (Surana et al, 1991), or *CLB2* and *CLB4* leads to accumulation of cells in G2 in a log phase culture (Figure 20, *lower-left* panels). The FACS profile of *clb2,4,5⁻* strains is also consistent with accumulation of cells in G2, but some S phase delay attributable to loss of *CLB5* function may still be apparent, since the G2 peak in the latter genotype is shorter and broader (Figure 20, *lower-right* panels).

Execution point analysis. To further examine the possible interaction of *CLB5* and *CLB2*, we determined where in the cell cycle the HU execution point occurs in double mutant strains (Table 14, Line 4). We found that in a *clb2* background, the loss of *CLB5* increases the interval between bud emergence and the HU execution point from 13 to 29 minutes, while the interval between the HU execution point and cell division remains virtually unchanged (80 vs. 76 minutes). Using bud emergence as a rough morphological marker of START (Pringle and Hartwell, 1981), this result is consistent with our conclusion from DNA flow cytometry (Figure 20, bottom panels) that loss of *CLB5* prolongs S phase in a *clb2* background, while having no incremental effect on the G2 delay attributable to the loss of *CLB2*.

Is *CLB5* functionally redundant with the *CLN* genes?

Synthetic lethal analysis. Since we discovered *CLB5* based on its ability to rescue *cln1,2,3⁻* yeast when overexpressed, and then found that its transcript is present at times in the cell cycle coinciding with those of *CLN2* (and *CLN1*), we

were curious to determine whether it could be demonstrated to have *CLN*-like functions. We tested all combinations of *cln* deletions with the *CLB5* deletion, and found that *CLB5* was not essential in strains bearing any viable combination of *cln* deletions (Figure 22, *left* panels). However, when cells were tested on YEPGlycerol media, *cln1 cln2 clb5* strains were nearly inviable, while *cln1 cln2 CLB5* strains were fully viable (Figure 22, *right* panels). This observation is suggestive of partial functional redundancy between *CLB5* and *CLN* genes: *cln1,2* strains rely on both *CLN3* and *CLB5* for growth on glycerol media. Liquid cultures were used to assess the terminal phenotype of inviable *cln1 cln2 clb5* strains in glycerol (Figure 23). The terminal phenotype (lower right panel) is not reminiscent of true START arrest, since cells appear to bud several times prior to death. It was not determined whether the intervening stages of the cell cycle occurred between consecutive budding cycles. Deletion of *CLB2* in the *cln1,2* background did not impair viability on glycerol (Figure 22, *bottom* panels). Diploids made by mating *cln1 cln2 clb5* strains to a ρ° (mitochondrial deficient) *cln3* strain were viable on glycerol media, hence the glycerol lethality of *cln1 cln2 clb5* strains is not due to a defect in their mtDNA.

DNA flow cytometry of asynchronous cultures. We further examined the potential functional overlap between *CLB5* and the *CLNs* by performing DNA flow cytometry on exponential cultures of all genotypes having a single *CLN* gene, with and without *CLB5* (Figure 24). We were curious to determine whether the deletion of *CLB5*, in conjunction with *CLN* deletions, would cause a delay in G1, or alternatively, whether the deletion of *CLN* genes would exacerbate the S phase delay already seen in the *clb5* background. While a number of subtle effects make the data in Figure 24 difficult to interpret, the major effect of deleting *CLN* genes

in a *clb5* background is to restore the G1 subpopulation to normal proportions from its diminished status in the *clb5* single mutant. In fact, if one compares the height of G1 and G2 peaks in a given population, it appears that in the *CLN⁺* background, *clb5* deletion depresses the G1 peak, while in the doubly deleted *cln* background, *clb5* deletion either enhances the G1 peak at the expense of the G2 peak (*cln1 CLN2 cln3* and *cln1 cln2 CLN3* strains), or leaves the G1/G2 ratio unaffected (*CLN1 cln2 cln3* strains). This result obtains in spite of the fact that *clb5* deletion continues to cause an S phase delay, at least in the *CLN1 cln2 cln3* and *cln1 CLN2 cln3* backgrounds (compare the heights of the S phase troughs between *CLB5* and *clb5* portions of Figure 24). How could *clb5* deletion enhance the proportion of cells in G1? Normally, one expects that delay during S phase will compensate for defects in G1 functions, so that these defects become less consequential (Singer and Johnston, 1981; Johnston and Singer, 1983). The fact that the triple mutants *cln1 CLN2 cln3 clb5* and *cln1 cln2 CLN3 clb5* manifest larger G1/G2 ratios than seen in isogenic *CLB5* strains implies that in these strains, compensation fails to occur. This may imply that *CLB5* promotes both START execution and S phase progression, and that *clb5* deletion hinders these strains in G1 (ie. increases the G1/G2 ratio) by enough to prevent the S phase delay from restoring G1 timing to normal.

Leopold and O'Farrell (1991) reported that overexpression of the *D. melanogaster cdc2* gene could rescue *cln1,2,3⁻* yeast strains. They speculated that the *Drosophila cdc2* protein was interacting with an unidentified cyclin. We have found that rescue of *cln1,2,3⁻* yeast by *Drosophila cdc2* is dependent on *CLB5*. *cln1,2,3⁻ clb5 GAL1::CLN3* yeast transformed with the *Drosophila cdc2* plasmid are inviable on glucose, while isogenic *CLB5* transformants are viable (data not

shown). This result further supports the idea that *CLB5* function may overlap with *CLN* gene functions.

***CLB5* is essential for the normal execution of meiosis.**

Many gene products are involved in the control of both the mitotic and meiotic cell cycles (Niwa and Yanagida, 1988; Westendorf et al, 1989; Simchen, 1974). In yeast, mutations affecting the mitotic cell cycle are sometimes found to have meiotic phenotypes as well (Esposito and Klapholz, 1981; Shuster and Byers, 1989). We therefore tested diploids bearing homozygous *clb5* deletions for their ability to undergo meiosis. We found that the loss of *clb5* in an otherwise wild type background was sufficient to dramatically impair the performance of meiosis (Table 15). Under growth conditions normally used to induce meiosis, homozygous *clb5* strains produced a very low frequency of asci, virtually all of which were morphologically abnormal and contained fewer than four spores. Hence while they are capable of initiating and performing ascosporeogenesis, they have an uncharacterized defect in the execution of meiosis. This defect was partially suppressed by transformation with a CEN plasmid bearing intact *CLB5* (CE112, Table 15).

Discussion.

CLB5 functions to promote *S* phase. We report the discovery of a fifth B cyclin in budding yeast. While this gene is not essential, its deletion significantly depresses the rate at which cells replicate chromosomal DNA. DNA flow cytometry of both exponential and synchronized cultures shows that *clb5* strains are slowed during progression through *S* phase. Also, we have shown that the

Table 15. *CLB5* is essential for normal meiosis.

Strain	Genotype	Spores/ascus: (percentages)		
		0	1,2,3	4
1014 A1,A2	<i>clb1/+ clb3/+ clb5/clb5</i>	94.0	6.0	0.0
A3	" " <i>clb5/CLB5</i>	69.0	15.5	15.5
1014 B1,B2	<i>clb1/+ clb4/+ clb5/clb5</i>	92.0	8.0	0.0
B3	" " <i>clb5/CLB5</i>	66.0	26.0	8.0
1027- A,C,E,G	<i>clb5/clb5</i> [pRS314]	96.3	3.1	0.6
B,D,F,H	" [pCE112]	89.9	3.3	6.9
1027- I,J,K,L	<i>CLB5/clb5</i>	51.4	9.8	38.9
1027- M,N	<i>CLB5/CLB5</i>	60.5	7.3	32.3

[CE112] is the *CLB5* gene in the RS314 vector.

proportion of the cell cycle falling between the α -factor and hydroxyurea execution points expands in *clb5* strains. It is plausible to infer that the α -factor execution point approximates the start of S phase (Hereford and Hartwell, 1974), and the hydroxyurea execution point marks the completion of S phase, in both *clb5* and wild type strains (Hartwell, 1976). Hence both experimental approaches indicate that *CLB5* functions to promote S phase transit. *CLB5* is the only yeast cyclin whose deletion causes prolonged S phase. Deletion of other B cyclins either prolongs G2 (*CLB2*) or has little effect on cell cycle distribution (*CLB1*, *CLB3*, and *CLB4*; Figure 20), while deletion of any two *CLN* genes does not cause an S phase delay detectable by DNA flow cytometry (Figure 24). Deletion of the B cyclin *cig1⁺* in *S. pombe* causes a delay during G1 or in the G1/S transition, but does not affect the rate of S phase progression (Bueno et al, 1991).

The loss of *CLB5* seems to affect the overall rate of progression through S phase. In contrast, other known cyclins promote transitions between discrete phases of the cell cycle, such as the START event (*CLNs*, Richardson et al, 1989), the G2/M transition (Murray and Kirschner, 1989; Nurse, 1990; Hartwell, 1991), and meiotic induction (Westendorf et al, 1989). However, *CLB5* may not act throughout S phase. An effect on the rate of S phase progression could be caused by a defect in initiation (for example, due to use of a smaller number of origins of replication). Moreover, we note the possibility that *CLB5* affects the efficiency of S phase transit by acting at a time other than S phase, eg. during G1, or G2 of the preceding cycle.

We considered that slow S phase transit in *clb5* strains might result from defective regulation of the genes involved in DNA replication, and expressed under control of the DSC1 binding activity and the MluI-containing cell cycle box

(MCB; Lowndes et al, 1991; reviewed by McKinney and Heintz, 1991). In the synchronous time course shown in Figure 18, we examined the regulation of *CDC21* (an MCB gene). *CDC21* RNA accumulation peaked with similar levels and kinetics in *CLB5* and *clb5* strains (data not shown). We also did not detect a significant defect in histone H2A transcription in *clb5* strains. H2A is an S-phase-transcribed gene not under MCB control (reviewed by Osley, 1991). In light of these results, it seems unlikely that the basis for the prolonged S phase of *clb5* strains is a defect in the transcriptional control of gene products involved in DNA synthesis. Consistent with this conclusion is the observation that *clb5* deletion fails to affect DSC1 DNA binding activity (L. Johnston, personal communication).

In human cells, there is some evidence for the involvement of a cyclin dependent kinase in DNA synthesis (D'Urso et al, 1990; Blow and Nurse, 1990; Furakawa et al, 1990; Fang and Newport, 1991). In higher eukaryotes, it appears that *cdc2* has multiple homologs (called 'cdk's). Distinct kinases may be involved in S phase and M phase promotion (Fang and Newport, 1991). Multiple cyclin/cdk complexes are present during S phase, and at least three of them (cyclin A/cdk2, cyclin A/cdk2, and cyclin E/cdk2) are catalytically active at this time (Pines and Hunter, 1990b; J. Roberts, pers. comm). No A-type or E-type cyclins are known in budding yeast. Perhaps *CLB5* plays a role in yeast similar to the role played by cyclin A or cyclin E in human cells. In addition, it remains to be tested whether *CLB5* activates Cdc28 kinase.

Does CLB5 function elsewhere in the cell cycle? Although *CLB5* was discovered based on *cln1,2,3*⁻ rescue, we can not be sure if *CLB5* functions in concert with *CLNs* in their role in START. The fact that the *CLB5* transcript appears and disappears in synchrony with the *CLN2* transcript, together with the

fact that extra copies of the *CLB5* gene under control of the natural promoter rescue *cln1,2,3⁻* strains, suggest that the Clb5 protein may be present early enough to have a role in START. The fact that strains lacking *CLN1* and *CLN2* require *CLB5* for viability on glycerol suggests that *CLB5*, like *CLN3*, can promote *CLN* dependent events. A similar conclusion is implied by the fact that *cln1,2,3⁻* strains require *CLB5* for rescue by the *D. melanogaster cdc2* gene. Does *CLB5* have an additional role in promoting the G2 to M phase transition? The data presented (Figures 20 and 21, and Table 14) do not suggest any delay during G2 in *clb5* mutants. However, the presence of a potential mitotic destruction box (Glotzer et al, 1991) in Clb5 suggests the protein may persist until M-phase, consistent with a possible role in G2.

Chapter 6: Cloning and preliminary characterization of *MPK1*

***MPK1* rescues *cln1,2,3*⁻ strains when expressed from a *CEN* plasmid.**

A second gene was recovered while attempting to clone CBM8. It was described earlier how a library was constructed and screened in an attempt to clone CBM8. During the screening of this library, plasmid CE116 was recovered. Restriction mapping indicated (Figure 25) that the insert in CE116 was distinct from *CLB5*. A probe made from the *EcoRI* fragment internal to *CLB5* failed to hybridize to the CE116 insert. Both criteria implied that CE116 was not a clone of *CLB5*. Similar criteria ruled out *CLN2*. Was this CBM8?

The gene on CE116 is not CBM8. The entire insert from CE116 was recloned in a yeast integrating vector (RS304), to yield CE117. The sequence to the left of the *XhoI* site (Figure 25) was deleted from CE117 to yield CE117 Δ *XhoI*, which was integrated into diploid 957 (CBM8/+ heterozygote; *cln1,2,3*⁻ homozygote) as described (Materials and methods). Southern blotting confirmed that the integration of CE117 Δ *XhoI* was associated with a change in the restriction map of the DNA hybridizing to the deleted *XhoI* fragment (Figure 2), hence integration had occurred at the genomic site of the insert in CE117. 957::CE117 Δ *XhoI* was sporulated and dissected; prototrophy for tryptophan was not detectably linked to *CLN* bypass activity, hence CE117 appears not to represent a clone of CBM8.

The gene on CE116 is *MPK1*. The region of the insert in CE116 necessary for function at *cln1,2,3*⁻ rescue was mapped to the vicinity of the *SpeI* and *EcoRI* sites, based on tests of the deletion constructs shown in Figure 25. The regions designated A and B were sequenced, and the sequence data reported in Figure 26

were obtained. Both A and B consist of uninterrupted open reading frames, which read from right to left in Figure 25. These sequences were not present in Genbank. However, sequence B (Figure 26B) contained an exact match of a 15 nucleotide long sequence obtained in the lab of Dr. Bruce Futcher (Cold Spring Harbor) from a clone of a high copy suppressor of *cln1,2,3⁻* inviability. Dr. Futcher communicated this sequence to me in November, 1991, in order to inquire whether a clone obtained in his lab might be *CLB5*, whose sequence we had not published at that time. I contacted Dr. Futcher early in 1992, and informed him that I had obtained a clone of the same gene; he informed me that he suspected that we both had cloned a gene called *MPK1*, already discovered and sequenced in the lab of Dr. Jeremy Thorner (UC Berkeley). I consulted my notes on Dr. Thorner's seminar at the April, 1991 Cold Spring Harbor Symposium on "The Cell Cycle", and found that Dr. Thorner had described the cloning of *MPK1* (a high copy suppressor of the temperature sensitive phenotype of a dominant mutation suppressing the inviability caused by the deletion of *PKC1*; screen described in Fields and Thorner, 1991). During his seminar, Dr. Thorner supplied the predicted amino acid sequence data underlined in Figure 26A, remarking that it contained a tandem pair of consensus *CDC28* phosphorylation sites; no physical data on phosphorylation were presented. Dr. Thorner's studies of *MPK1* remain unpublished (October 12, 1992).

Rescue of cln1,2,3⁻ by pMPK1 is suppressed by clb5, mpk1, and swi4, but not swi6. It was determined whether CE118 (ie. p*MPK1*) could rescue *cln1,2,3⁻* strains that had additional mutations in a few other genes involved in cell cycle regulation. Rescue of *cln1,2,3⁻* inviability by p*MPK1* is marginal to begin with. Null alleles of *CLB5*, *MPK1*, and *SWI4* all suppressed rescue by p*MPK1*, but a

null allele of *SWI6* apparently did not prevent rescue (Figure 27). Given the weakness of rescue by CE118, and the fact that loss of the endogenous copy of *MPK1* suppresses rescue by p*MPK1*, it is unclear what to make of these results. Additional mutations, that make a strain sick, may prevent rescue whether or not the lost gene is specifically involved in the function of the plasmid gene conferring rescue activity.

*Rescue of *cln1,2,3*⁻ by pCLB5 is suppressed by *mpk1* and *swi4*, but not by *clb5* or *swi6*.* In contrast to the results just presented, it was found that the rescue of *cln1,2,3*⁻ by CE111 (ie. p*CLB5*) was relatively robust (Figure 27). Moreover, null alleles of *CLB5* (and of *SWI6*) did not prevent (or apparently, affect) p*CLB5* rescue. However, null alleles of *SWI4* and *MPK1* suppressed rescue. Given the more robust status of the p*CLB5* rescue, the suppression of p*CLB5* by *swi4* and *mpk1* is very intriguing. As was presented in Chapter 3, *swi4* and *mpk1* are also the two mutations that suppress *cln1,2,3*⁻ rescue by the *CLN* bypass mutation. These observations suggest that there may be some similarity in function or mode-of-action between *MPK1* and *SWI4*, on the one hand, and between CBM and p*CLB5* on the other. This theme will be developed more fully in the discussion section at the end of this chapter.

***MPK1* is an essential gene in *cln3* spores, and contributes to the efficient execution of the G1/S transition in *CLN*⁺ vegetative cells.**

A null allele of *MPK1* was made and installed, as described (Materials and methods). When a *cln3*Δ/*cln3*::*URA3* diploid (1036/A⁺/3) segregating *MPK1*::*MPK1*::*TRP1*/*mpk1*::*ARG4* was sporulated and 16 tetrads were dissected, it was found that all viable cells were Trp⁺, hence *MPK1*::*MPK1*::*TRP1*, and no viable

cells were *Arg*⁺ (ie. *mpk1::ARG4*). No tetrad had more than two viable spores. Thus, the disruption of *MPK1* is usually or always associated with inviability, in *cln3* spores. The parental diploid was *CLN1/CLN1*, hence triple deficiency for *CLN* was not a potential source of inviability among these progeny. The dissection agar was examined microscopically, and in each case, inviable spores were seen to have remained unbudded, expanded substantially in volume, and assumed an ovoid (usually not shmoo like) shape. The *mpk1* heterozygous diploid was transformed with a [*GAL1::CLN3*] plasmid, sporulated, and dissected on YEPGal media, and *Arg*⁺ spores were recovered, confirming the presence of the *mpk1::ARG4* allele in this strain, and indicating that the requirement for *MPK1* in *cln3*⁻ spores is relieved by *GAL1::CLN3*.

Strains lacking *mpk1* had a 3% longer doubling time than wild type (109') and a 1/3 increase in modal cell volume; both of these observations suggest that loss of *MPK1* prolongs some stage in the cell cycle. Exponential cultures of strains lacking *mpk1* showed a slight elevation in the percentage of cells that were in the unbudded stage of the cell cycle (29% unbudded vs. 24.5% unbudded for wild type). Asynchronous cultures of *MPK1* and *mpk1* strains were examined by DNA flow cytometry (Figure 28). Genotype at *MPK1* affected the distribution of cells throughout the cell cycle: the lack of *MPK1* is associated with a slight contraction in the G2 peak, and a slight expansion in the G1 peak. This suggests that G1 is slightly prolonged in *mpk1* mutants. Overall, these results imply that *MPK1* contributes to the efficient execution of the G1 to S transition in vegetative cells. In the absence of *MPK1*, cells are larger, doubling times are slightly longer, unbudded percentages are slightly higher, and the FACS profile is detectably shifted from G2 to G1.

mpk1 cln3 vegetative cells are sick or dead, depending on their genotype at *CLN1* and *CLN2*.

The initial results with *mpk1::ARG4* established that *mpk1* was essential in *cln3* spores, but that *MPK1* was not required in the presence of *GAL1::CLN3*. It remained to be determined whether *MPK1* was also essential in *cln3* vegetative cells. To further investigate the *mpk1* requirement, the strains shown in Figure 29 were constructed, as described (Materials and methods). Two noteworthy observations were made. First, it was found that strains of genotype *cln1 CLN2 cln3 mpk1* and *CLN1 CLN2 cln3 mpk1* can survive as vegetative cells without *GAL1::CLN3*, but their growth is significantly slower than *GAL1::CLN3* synthesizing controls. Thus, the joint deletion of *cln3* and *mpk1* causes sickness in vegetative cells.

Second, it was seen that vegetative cells of genotype *CLN1 cln2 cln3 mpk1* are inviable without *GAL1::CLN3*, just as are *cln3 mpk1* spores. To determine the terminal phenotype of *CLN1 cln2 cln3 mpk1 W16* cells upon transfer to YEPD media, photomicrographs of cells on solid media were obtained, approximately 18 hours after plating (Figure 30). Cells apparently arrest during the first cycle after transfer to glucose media, since they arrest with either one or two cell bodies. The unbudded cells probably represent cells that were unbudded at the time of plating, while the two bodied cells probably represent cells that were in the budded phase of the cell cycle. To confirm that cells arrest in G1 upon removal of *GAL1::CLN3*, YEPGal liquid cultures of 1052-2D and 1052-7D (*CLN1 cln2::LEU2 cln3Δ mpk1::ARG4 [W16]*) were transferred to YEPD media, and DNA flow cytometry was performed at two hour intervals for six hours after transfer. Most

cells had an apparent G1 DNA content after two hours in YEPD, consistent with first cycle arrest in G1 (data not shown). However, interpretation is complicated by the fact that cell volume, and fluorescence, increased substantially between 2 and 6 hours after transfer to YEPD.

Discussion.

MPK1 was discovered in the present study as a gene whose overexpression could rescue *cln1,2,3*⁻ lethality. Its null phenotypes (slightly increased cell volume, shift of DNA flow cytometric profile towards G1) imply that the normal function of this gene quite plausibly involves a positive regulatory role in the execution of START. Hence, the overexpression and loss-of-function phenotypes of this gene go in opposite directions, and both imply an involvement in the regulation of START.

Synthetic lethality analysis of *mpk1* strongly suggests that *MPK1* works in concert with *CLN1* and *CLN2*. The reasoning behind this conclusion is as follows: *mpk1 cln3* spores are apparently inviable, and *mpk1 cln3* vegetative cells are very sick. The simplest interpretation of these observations is that *CLN3* and *MPK1* supply a redundant essential function. However, *CLN1* and *CLN2* are two other genes that also supply a redundant essential function with *CLN3* (Richardson et al, 1989). Hence a critical test is to determine the phenotype of *cln1 cln2 mpk1* strains. These are apparently quite robust (Figure 29). The finding that there is little or no synthetic phenotype due to the simultaneous deletion of *cln1*, *cln2*, and *mpk1* is consistent with the view that *MPK1* is directly involved in the function (ie. synthesis or utilization) of *CLN1* and *CLN2*, or vice versa. Therefore, it is likely that the *CLN3*-redundant essential functions supplied by *CLN1* and *CLN2*, on the

one hand, and by *MPK1* on the other hand, overlap significantly.

Preliminary results (Epstein, Hoek, and Cross) indicate that the loss of *MPK1* negatively affects (by undetermined means) the level of "basal" transcription of *CLN1* and *CLN2*. Basal transcription is the cell-volume dependent (Lew et al, 1992) (but *CDC28*-independent (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991)) transcription that is seen in *cln1,2,3⁻* arrested cultures after 2.5 hours' block in G1 (see Figure 18). This tantalizing observation suggests a specific means of involvement of *MPK1* in *CLN1* and *CLN2* function. If *MPK1* is required for basal transcription of *CLN1* and *CLN2*, and if, in the absence of *CLN3*, this basal transcription is required or nearly required to initiate the *CDC28*-dependent (*MPK1* independent) phase of the transcription of *CLN1* and *CLN2* (and presumably, *CLB5*), then loss of *MPK1* in the *cln3* background would be lethal, or nearly so, essentially due to the loss of *CLN1* and *CLN2* function.

Alternatively, *MPK1* might be involved in the interaction of *CDC28* with *CLN1* and *CLN2*, rather than in their synthesis. *CLN1 cln2 cln3 mpk1* strains are inviable, suggesting that *CLN1* (if synthesized) can not activate *CDC28* in the absence of *MPK1*. Suppose that *MPK1* is a factor which is required for *CDC28* activation by limiting amounts of cyclin-like proteins. Even at a *cln1,2,3⁻* block, there might be some trace level of *CDC28* activity, due to the presence of eg. *CLB5* and *HCS26*. If this trace activity were *MPK1* dependent, and if basal *CLN1* and *CLN2* transcription was in turn dependent on *CDC28*, then the loss of *MPK1* would have a negative effect on basal transcription of *CLN1* and *CLN2* via a negative effect on *CDC28* activity. Arguing against this scenario are the data which show that the basal transcription of *CLN2* is *CDC28* independent (Cross and Tinkelenberg, 1991, Figure 5; Dirick and Nasmyth, 1991, Figure 3).

Whatever the true mode of action of *MPK1*, it seems certain that *MPK1* does not affect only *CLN1* and *CLN2*: Given that *MPK1* overexpression can rescue *cln1,2,3⁻* strains, there must be some other component(s) besides *CLN1* and *CLN2* which are sensitive to and dependent on *MPK1* gene dosage. Furthermore, *mpk1* suppresses both CBM and p*CLB5* rescue of *cln1,2,3⁻* inviability. This suggests that both of these forms of rescue or bypass rely upon genes that are also dependent on *MPK1* for their synthesis or utilization, and there are likely to be at least two other genes which somehow require *MPK1*: one accounting for *mpk1* suppression of p*CLB5* (this is potentially *CLB5* itself), and one accounting for *mpk1* suppression of CBM. Note that *clb5* fails to suppress CBM (Figure 9), so CBM can't work purely through *CLB5*.

p*MPK1* rescue of *cln1,2,3⁻* was suppressed by *clb5* and *swi4* (Figure 27). As mentioned in the results section, these facts may tell us nothing of real interest. If meaningful, however, they tend to suggest that p*MPK1* rescue works by affecting either the expression or utilization of *CLB5*, and potentially, of additional components (besides *CLN1* and *CLN2*) which are also dependent on *SWI4*.

It remains unclear how cells monitor their size (Pringle and Hartwell, 1981; Cross et al, 1989a), but a contemporary view (Lew et al, 1992) is that a "putative size sensor" detects that a critical size has been reached, and directly or indirectly causes basal *CLN1* and *CLN2* transcription to begin. This eventually leads to *CDC28* activation, *CDC28*-dependent *CLN1* and *CLN2* transcription (Cross and Tinkelenberg, 1991), and the execution of START. In such a system, a signal transduction pathway must somehow connect the attainment of a critical size to the initiation of basal transcription.

If *MPK1* is really necessary for cell size dependent, *CDC28* independent,

"basal" transcription, then perhaps it is a component of such a signal transduction pathway. Genes which are part of the size control pathway have been expected to show dosage effects on cell volume (Cross, 1988a; Cross, 1989b). Does *MPK1* show dosage effects on cell volume? The effect of overexpressing *MPK1* on cell volume was not determined in the current study, although *MPK1* was discovered based on its overexpression rescuing *cln1,2,3* lethality. Deletion of *MPK1* did lead to an increase in cell volume; this increase was similar to the increase caused by the simultaneous deletion of *CLN1* and *CLN2*. In this context, it is interesting to recall that CBM8 dominance is sensitive to *MPK1* dosage in diploids (Figure 10). While this is not a dosage effect on cell size *per se*, it does argue that certain genotypes are uncommonly sensitive to *MPK1* gene dosage.

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Figure 1. Restriction map of CE101.

pCE101 was synthesized from pRS314 (Sikorski and Hieter, 1989) and pBR322 for the purpose of cloning CBM. The sites shown in parenthesis are no longer present, but indicate the sites used to form the junctions between pBR322 and pRS314. The *CEN/ARS* and *TRP1* inserts are present in pRS314. The numbers given refer to the standard coordinate systems of the parental vectors: pBR322 (from the New England Biolabs catalog), pBS+/-, and pBSKS+/- (both from the Stratagene catalog). This map is not to scale.

The restriction maps of *CLB5* and *MPK1* presented later in this thesis are based on *Sau3A* fragments cloned into the *Bam*HI site of pCE101, and read (from left to right) from the *Kpn*I site toward the *Sac*I site of the multiple cloning site. Thus, the *Kpn*I, *Apa*I, *Xho*I, *Sal*I, *Cla*I, *Hind*III, *Eco*RV, *Eco*RI, *Pst*I, and *Sma*I sites occur to the immediate left of the insert, and the *Spe*I, *Xba*I, *Not*I, *Eag*I, *Bst*XI, *Sac*II, and *Sac*I sites occur to the immediate right of the insert.

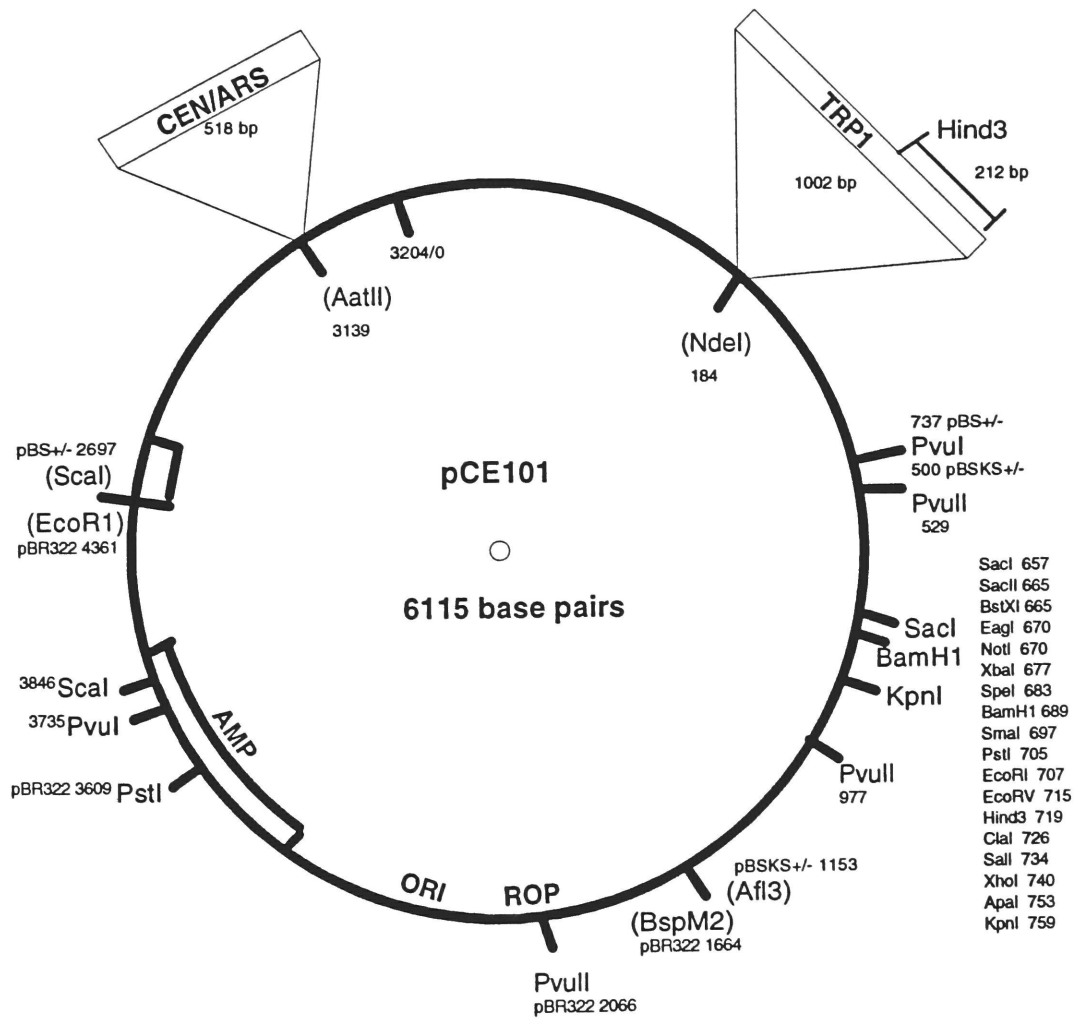


Figure 2. Installation of the *clb5::ARG4ΔBspEI* and *MPK1::CE117ΔXhoI* alleles into yeast.

(A) Installation of *clb5::ARG4ΔBspEI*.

Lane	Genotype	Strain
1	<i>CLB5/CLB5</i>	994
2	<i>CLB5/clb5::ARG4</i>	994/A ⁺ /1
3	<i>CLB5/clb5::ARG4</i>	994/A ⁺ /2
4	<i>CLB5/clb5::ARG4</i>	994/A ⁺ /3
5	<i>CLB5/clb5::ARG4</i>	994/A ⁺ /3
6	<i>CLB5/CLB5</i>	998
7	<i>CLB5/clb5::ARG4</i>	998/A ⁺ /1
8	<i>CLB5/clb5::ARG4</i>	998/A ⁺ /2
9	<i>CLB5/clb5::ARG4</i>	998/A ⁺ /3
10	<i>CLB5/clb5::ARG4</i>	998/A ⁺ /4

Yeast genomic DNA was digested with *XhoI*, fractionated on agarose/TAE, and transferred to GeneScreen *Plus*[™] (Dupont). The screen was probed with the radio-labelled *SalI* - *BglII* fragment of CE110, corresponding to the *CLB2* gene. *XhoI* does not cut inside the *clb5::ARG4* construct.

994: $\frac{MATa}{MAT\alpha} \frac{CLN1}{cln1\Delta} \frac{cln2::LEU2}{CLN2} \frac{cln3\Delta}{cln3\Delta} \frac{arg4}{arg4}$ W16

998: $\frac{MATa}{MAT\alpha} \frac{cln1\Delta}{cln1\Delta} \frac{cln2\Delta}{cln2\Delta} \frac{CLN3}{cln3\Delta} \frac{arg4}{arg4} \frac{leu2::LEU2::GAL1::CLN3}{leu2} \frac{bar1}{BAR1}$ W16

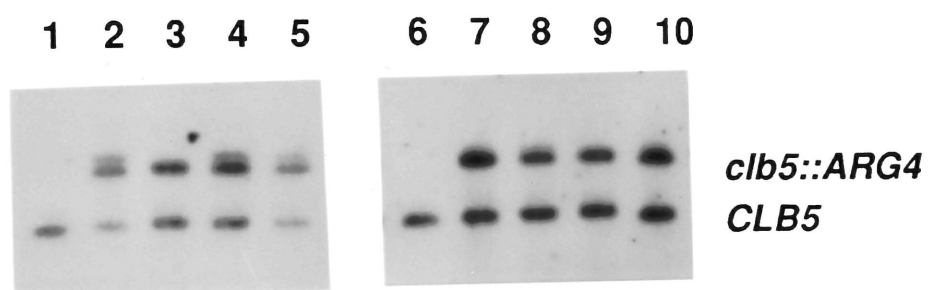
(B) Installation of *MPK1::CE117ΔXhoI*.

Lane	Genotype	Strain
11	<i>MPK1/MPK1::CE117ΔXhoI</i>	957/T ⁺ /4
12	<i>MPK1/MPK1::CE117ΔXhoI</i>	957/T ⁺ /3
13	<i>MPK1/MPK1::CE117ΔXhoI</i>	957/T ⁺ /2
14	<i>MPK1/MPK1::CE117ΔXhoI</i>	957/T ⁺ /1
15	<i>MPK1/MPK1</i>	957

Yeast genomic DNA was digested with *ClaI*, fractionated on agarose/TAE, and transferred to GeneScreen *Plus*[™] (Dupont). The screen was probed with the radio-labelled *XhoI* fragment from the left end of CE117, which had been deleted from CE117ΔXhoI. *ClaI* does not cut inside the insert in CE117.

957: $\frac{MATa}{MAT\alpha} \frac{cln1\Delta}{cln1\Delta} \frac{cln2::LEU2}{cln2\Delta} \frac{cln3\Delta}{cln3\Delta} \frac{trp1}{trp1} \frac{CBM8}{cbm^+}$ W16

A



B

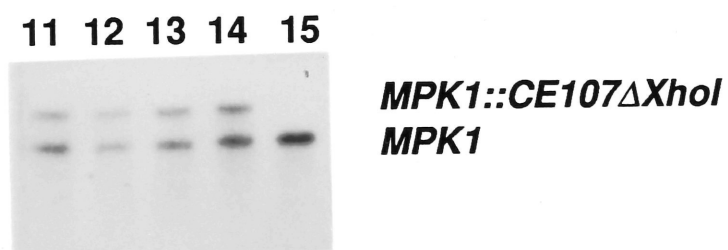


Figure 3: CBM8 is not the result of a rearrangement of the *CLN2* locus.

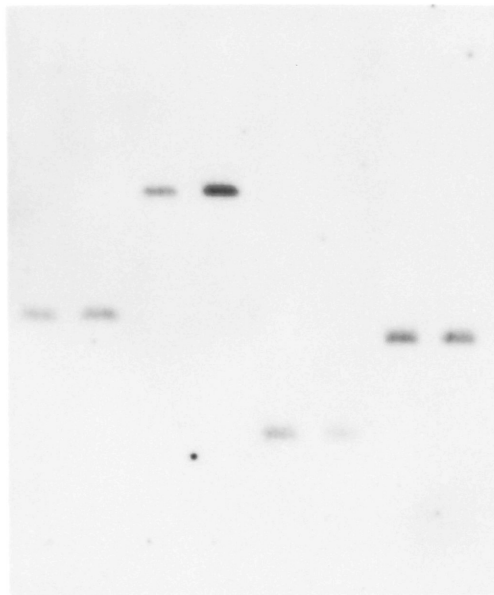
Lane	Genotype		Strain
1	CBM8 ^{4X}	<i>CLN2</i>	933-1C (*)
2	CBM8 ^{4X}	<i>CLN2</i>	933-3D
3	CBM8 ^{5X}	<i>cln2::LEU2</i>	957-5B
4	CBM8 ^{5X}	<i>cln2::LEU2</i>	957-9A
5	CBM8 ^{5X}	<i>cln2Δ (spe-sph)</i>	957-4B
6	CBM8 ^{5X}	<i>cln2Δ (spe-sph)</i>	957-8C
7	CBM8 ^{5X}	<i>cln2Δ (spe-xho)</i>	952-1B
8	CBM8 ^{5X}	<i>cln2Δ (spe-xho)</i>	952-4A

Yeast genomic DNA was digested with *Hind*III and *Nru*I, fractionated on 0.7% agarose/TAE, and transferred to GeneScreen *Plus*[™] (Dupont). The screen was probed with the radio-labelled *Hind*III - *Xho*I fragment of *CLN2*.

All strains bore null alleles at *CLN1* and *CLN3*. CBM8 was scored in strains 3 - 8 based on growth on YEPD, and in strains 1 and 2 based on backcrossing to a *cln1 cln2::LEU2 cln3* strain and detecting glucose viable Leu⁺ progeny. Strains 3 - 8 had an episomal source of *GAL1::CLN3* and were maintained on galactose media.

(*) CEN plasmid library was made from DNA isolated from this strain.

1 2 3 4 5 6 7 8



— 1.6 KB

Figure 4. CBM8 retains a homogeneous, dominant phenotype after extensive backcrossing.

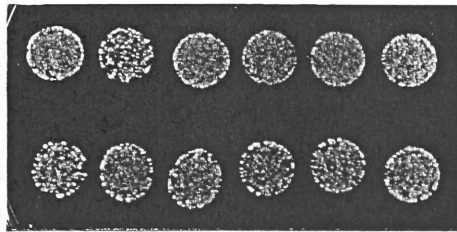
Genotype		Strain
<i>MATα</i>	CBM8 ^{6X}	1053-21B
<i>MATa</i>	CBM8 ^{6X}	1053-19D
<i>MATa</i>	CBM8 ^{6X}	1053-11D
<i>MATa</i>	CBM8 ^{6X}	1053-8B
<i>MATa</i>	CBM8 ^{6X}	1053-5A
<i>MATa</i>	CBM8 ^{6X}	1053-3B

All strains are *cln1 Δ cln2 Δ cln3 Δ CBM8 arg4 MPK1 leu2::LEU2::GAL1::CLN3*, and all are derived from cross 1053, which was a sixth backcross of CBM8. Diploids were formed by mating haploids to 960-48C (*MAT α*) or 960-5A (*MATa*) *cln1 Δ cln2 Δ cln3 Δ cbm⁺ ARG4 MPK1 [GAL1::CLN3/URA3]*, and selecting on YcGal-leu-arg agar. All strains were cultured to saturation in YEPGal liquid, prior to plating at 1:1000 dilution on YEPGal and YEPD. YEPGal photos are after 2 days, and YEPD photos are after 2 and 3 days.

YEPGal

2 DAYS

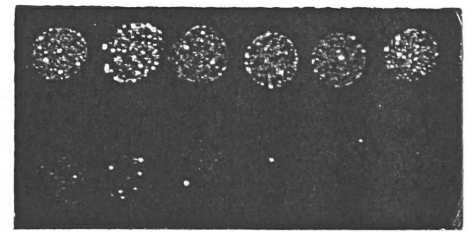
Haploids



Diploids

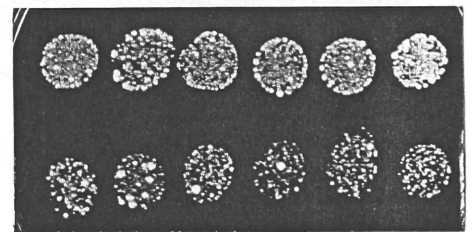
YEPD

2 DAYS



3 DAYS

Haploids



Diploids

Figure 5. *CLN* bypass mutants can divide without *GAL1::CLN3* synthesis.

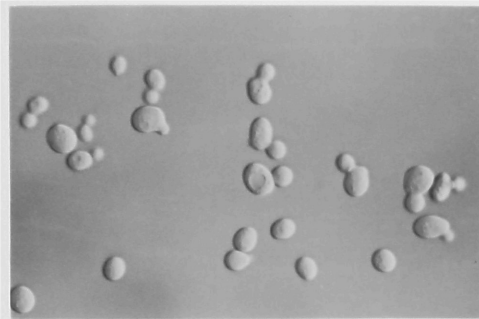
Strains of the indicated genotypes were cultured in the indicated media. All strains are *cln1,2,3*⁻ and have a source of *GAL1::CLN3*.

cln1,2,3 strains growing with or without *GAL1::CLN3* expression

YEPGal

YEPD

cbm⁺



CBM9

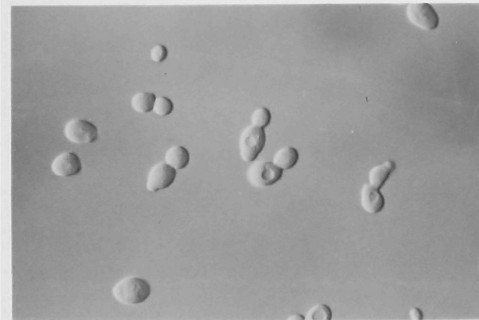


Figure 6. *MAT α* CBM strains behave as *MATa* strains at an abnormally high frequency.

Segregants from the dissection of 8 tetrads from diploid 934 were patched out on YEPGal, and replicas were taken to YEPD, YcGal-ura, mating type and pheromone testers.

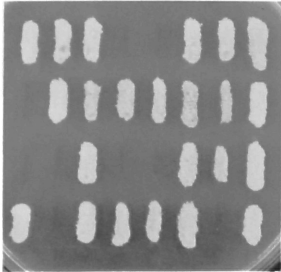
Key	Genotype	Phenotype
a	cbm ⁺ <i>MATa</i> W16	normal mating and pheromone secretion
b	cbm ⁺ <i>MATα</i> W16	normal mating and pheromone secretion (*)
c	CBM8 <i>MATa</i> W16+/-	normal mating and pheromone secretion
d	CBM8 <i>MATα</i> W16	elevated a mating, weak α pheromone (**)
e	CBM8 <i>MATα</i>	elevated a mating, little or no α pheromone

W16 is the [*GAL1::CLN3/URA3*] CEN plasmid. All strains are *cln1,2,3*⁻.

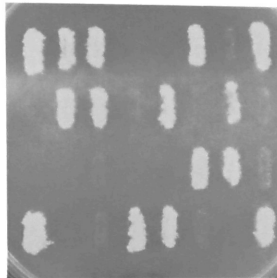
(*) except 6D: very occasional **a** mating, even though cbm⁺.

(**) except 6C: normal α pheromone secretion, even though CBM8 *MAT α* W16.

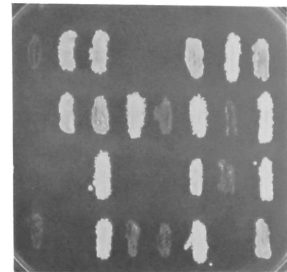
YEPGal



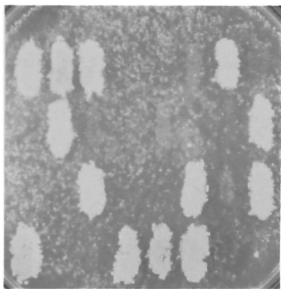
YEPD



YcGal-ura

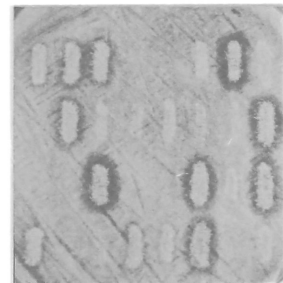


MAT alpha

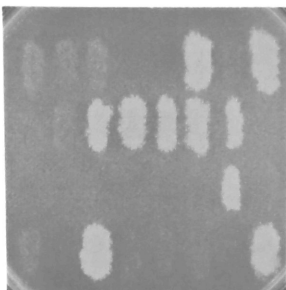


	1	2	3	4	5	6	7	8
A	e	d	d			c	b	c
B		d	c	a	c	a	c	b
C			b			d	c	b
D	e		a	e	e	b		c

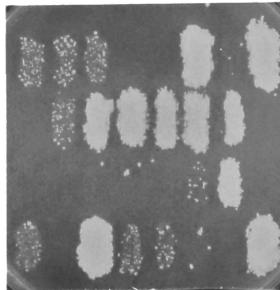
alpha pheremone



MAT a



MAT a, extra time



a pheremone

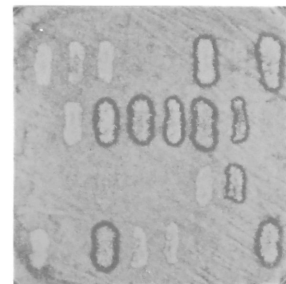
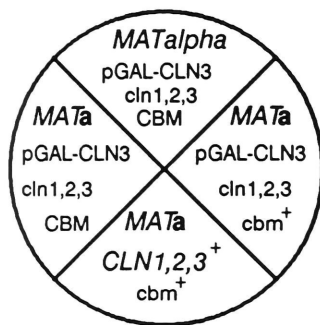


Figure 7. *MATa bar1* CBM8 strains are sensitive to α -factor.

Genotype				Strain
<i>MATa cln1,2,3</i>	CBM8	W16	<i>bar1</i>	953-7A
<i>MATa cln1,2,3</i>	cbm+	W16	<i>bar1</i>	953-1D
<i>MATa CLN1,2,3</i>	cbm+		<i>bar1</i>	"bar1"
<i>MATα cln1,2,3</i>	CBM8	W16		953-4A

Strains of the indicated genotypes were streaked on YEPD or YEPGal, with or without added α -factor at 1 μ M (final concentration in the agar).



All MAT α strains are *bar1*

- alpha
factor

+ alpha
factor

YEPD

YEPGal

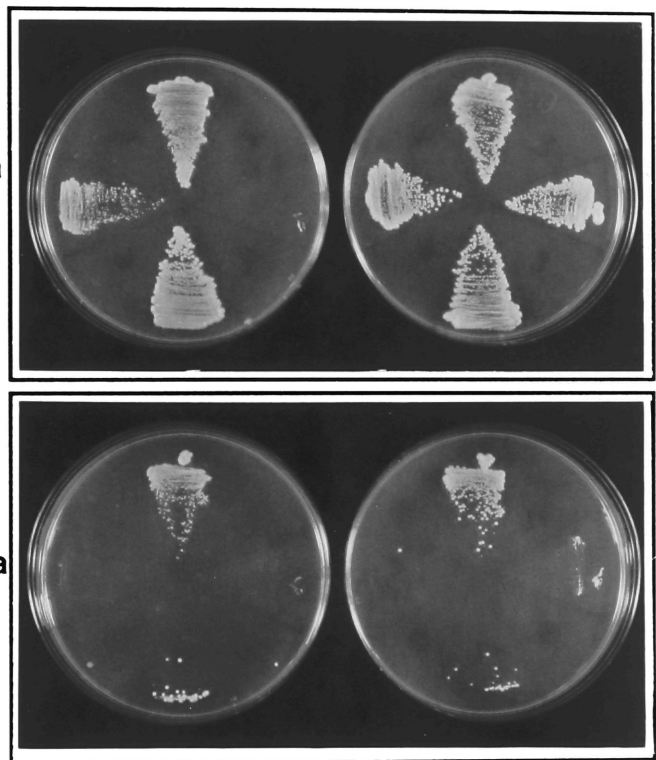


Figure 8: CBM does not bypass the requirement for *CDC28* at START.

Genotype		Strain
cbm ⁺	<i>CDC28</i>	884-17B
cbm ⁺	<i>cdc28-13</i>	939-4B
CBM8	<i>CDC28</i>	939-6C
CBM8	<i>cdc28-13</i>	939-6D

All strains are *cln1,2,3*⁻ [*GAL1::CLN3/URA3*]. Budding indices were determined after 6 hours at the indicated temperature.

Percent Unbudded Cells

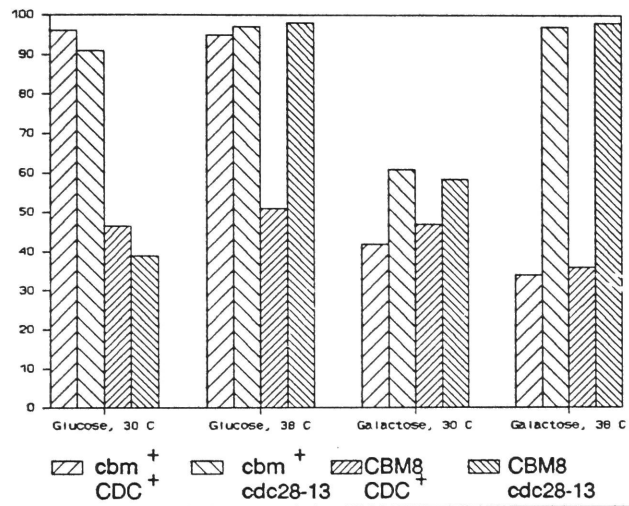


Figure 9: CBM is not suppressed by mutations in *CLB5*, *CLB2*, or *HCS26*.

Genotype		Strain
CBM8 ^{6X}	<i>CLB5</i>	1005-17B
CBM8 ^{6X}	<i>CLB5</i>	1005-21B
CBM8 ^{6X}	<i>clb5::ARG4</i>	1005-15A
CBM8 ^{6X}	<i>clb5::ARG4</i>	1005-23A
CBM8 ^{6X}	<i>CLB2</i>	1043-3B
CBM8 ^{6X}	<i>clb2::LEU2</i>	1043-1B
CBM8 ^{7X}	<i>HCS26</i>	1042-7D
CBM8 ^{7X}	<i>hcs26::URA3</i>	1042-8D

Cells were diluted 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} from saturated cultures, sonicated, and 10 μ l were applied to the named media. Photographs were taken after 3 days' incubation at 30°C. All strains are *cln1,2,3*⁻ and have a chromosomally integrated source of *GAL1::CLN3*.

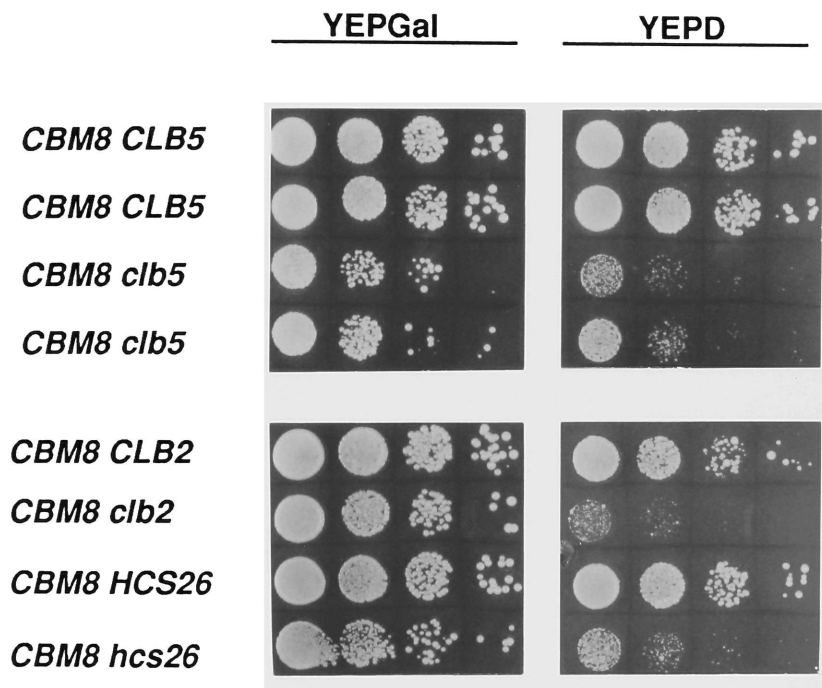


Figure 10. *mpk1::ARG4* suppresses CBM8 rescue of *cln1,2,3*⁻ inviability.

Genotype			Strain
<i>MATa MPK1</i>	CBM8 ^{6X}	<i>GAL1::CLN3</i>	1053-8B
<i>MATa mpk1::ARG4</i>	CBM8 ^{6X}	<i>GAL1::CLN3</i>	1053-23A
a/α <i>MPK1/MPK1</i>	CBM8 ^{6X} /cbm ⁺	<i>GAL1::CLN3</i>	1055(8B)
a/α <i>mpk1::ARG4/MPK1</i>	CBM8 ^{6X} /cbm ⁺	<i>GAL1::CLN3</i>	1055(23A)

All strains are *cln1Δ cln2Δ cln3Δ*. Diploids were formed by mating haploids to 960-45A (*MATα cln1Δ cln2Δ cln3Δ MPK1 cbm⁺ [GAL1::CLN3/URA3]*), and selecting on YcGal-leu-ura agar. All strains were cultured in YEPGal liquid, prior to plating at four densities on YEPGal and YEPD. YEPGal photos are after 2 days, while YEPD photos are after 3 days.

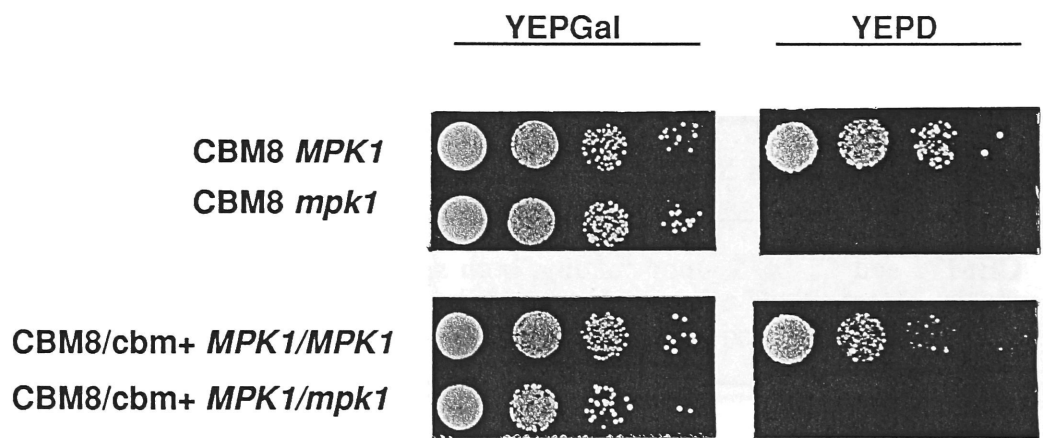
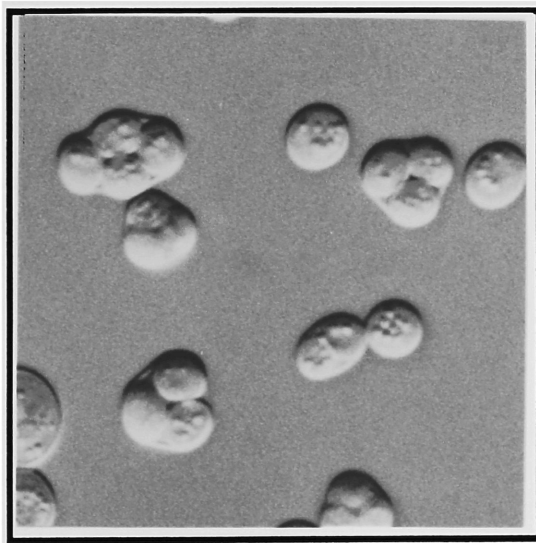
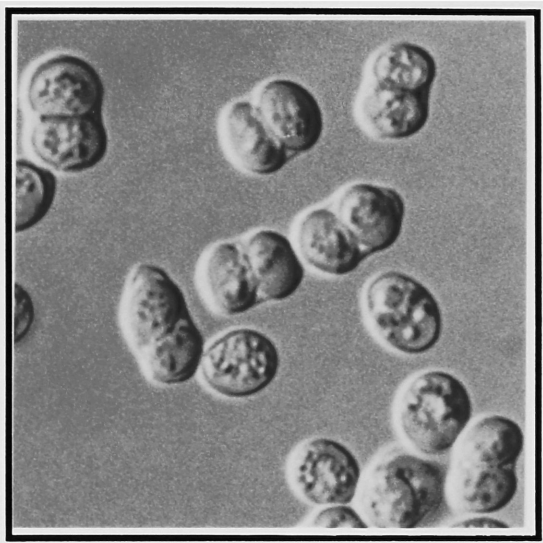


Figure 11. CBM18 has a dominant single division meiosis phenotype.

CBM18 and CBM15, upon mating, both sporulated to yield a high proportion of dyads. Most of the apparent triads on the left are in fact tetrads with the fourth spore out of the focal plane.



Tetrads

$$\frac{cbm^{+}}{cbm^{+}}$$


Dyads

$$\frac{CBM18}{cbm^{+}}$$

Figure 12. Equational vs. reductional segregation in single division meiosis.

In single division meiosis, one diploid cell gives rise to a dyad of two diploid spores. Each chromosome can segregate equationally or reductionally.

Assume that no recombination occurs between a genetic marker (heterozygous in the parental diploid) and its centromere. Following reductional centromere segregation, a dyad will be formed in which each spore is homozygous for a different allele of the initially heterozygous marker. Following equational centromere segregation, a dyad will be formed in which each spore preserves the parental heterozygosity.

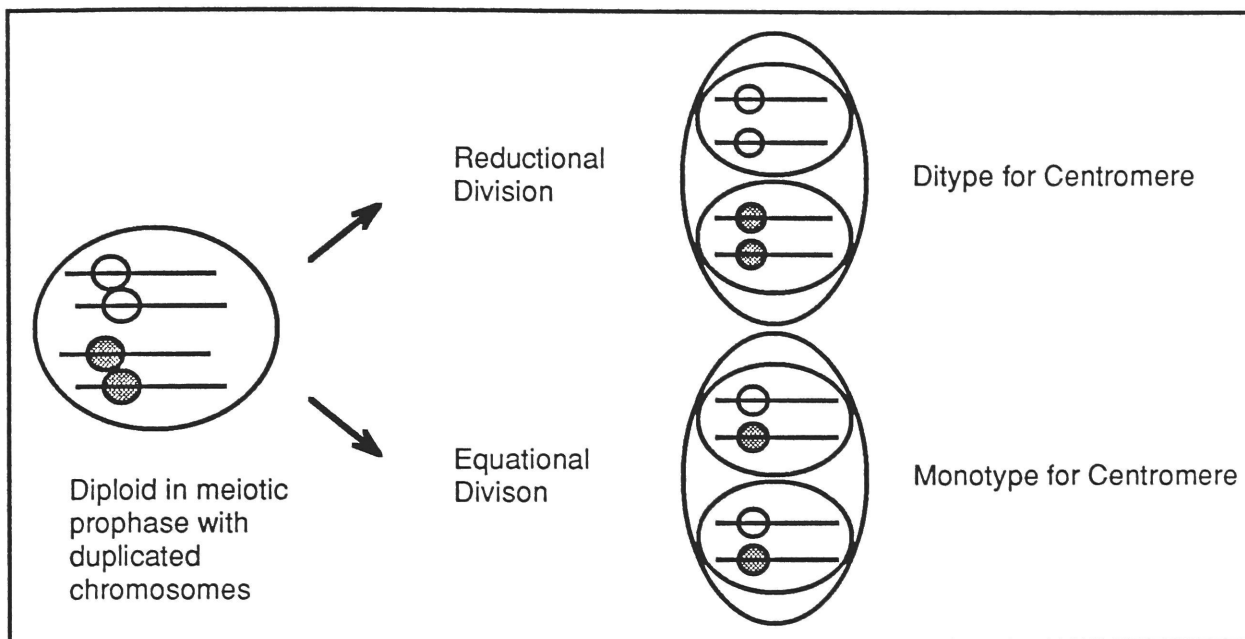


Figure 13. Method for the detection of linkage between CBM18 and SPO.

If CBM18 and SPO are linked, then upon sporulation of a heterozygous CBM18/+ diploid, it is predicted that in dyads ditype for *CLN* bypass activity (as shown), the CBM18/CBM18 spore will be phenotypically SPO, and the cbm⁺/cbm⁺ spore will be spo⁺.

Given the dominance of SPO, it is further predicted that dyads that are monotype for *CLN* bypass activity will also be monotype for SPO.

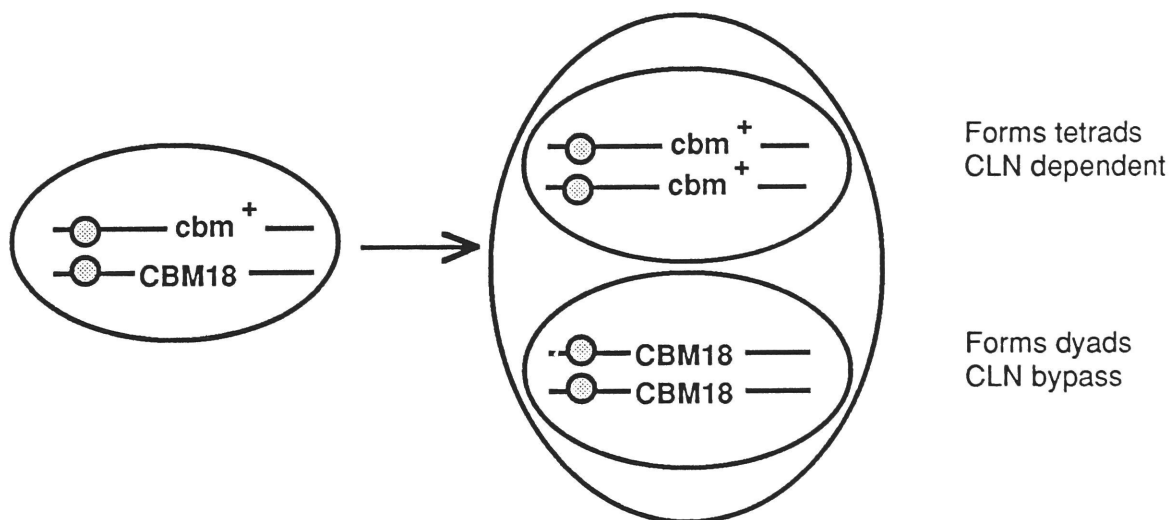


Figure 14. *SPO13* mRNA induction is normal in CBM18/+ strains during sporulation.

Lane	Genotype	Condition	Strain		
1	CBM18/+	a/α vegetative	818		
2	cbm ⁺ /+	a/α vegetative	885		
3	CBM18/+	a/α 24 hours spm.	818		
4	cbm ⁺ /+	a/α 24 hours spm.	885		
5	cbm ⁺ /+	a/a 24 hours spm.	818-35B		
818:	<u>a</u> α	<u>CLN1</u> <i>cln1::TRP1</i>	<u>CLN2</u> <i>cln2::LEU2</i>	<u><i>cln3::URA3</i></u> <i>cln3Δ</i>	<u><i>CBM18</i></u> <i>cbm⁺</i>
818-35B:					
	<u>a</u> a	<u>CLN1</u> <i>CLN1</i>	<u>CLN2</u> <i>CLN2</i>	<u><i>cln3::URA3</i></u> <i>cln3</i>	<u><i>spo⁺</i></u> <i>spo⁺</i>
(818-35B is a <i>MATa spo⁺</i> diploid obtained by sporulating and dissecting 818.)					
885:	<u>a</u> α	<u>CLN1</u> <i>cln1::TRP1</i>	<u>CLN2</u> <i>cln2::LEU2</i>	<u><i>CLN3</i></u> <i>cln3Δ</i>	

SPO13 probe was a gift of Dr. Robert Elder. PC4 is a control probe apparently exhibiting non-specific background hybridization.

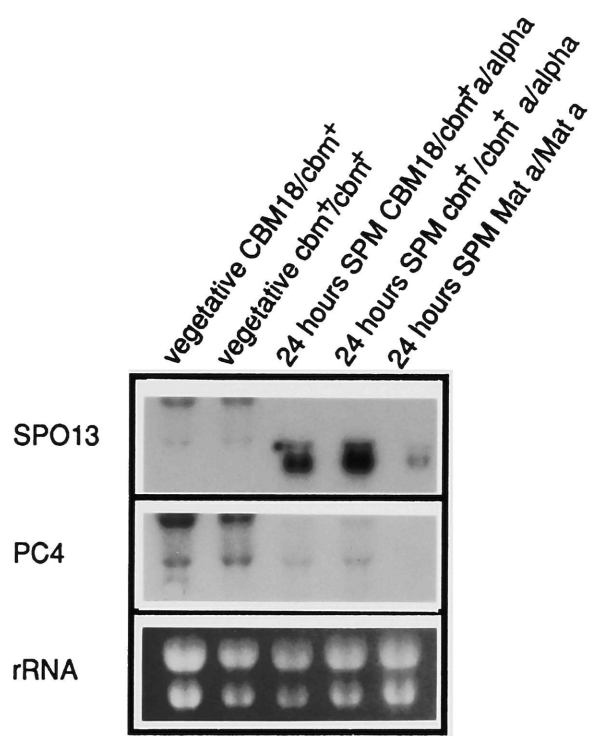


Figure 15. Restriction map of *CLB5*.

CE104 and CE110 were among three overlapping CEN plasmid clones recovered which rescued *cln1,2,3* inviability. CE110 contained intact copies of the *CLB2* and *CLB5* genes, but *cln* rescue activity was unique to *CLB5*, while *clb1 clb2* rescue was unique to *CLB2*. The region of *CLB5* between the designated *BspEI* and *EcoRI* sites was replaced with the yeast *ARG4* gene to generate the *clb5::ARG4* allele.

ND=Not Determined.

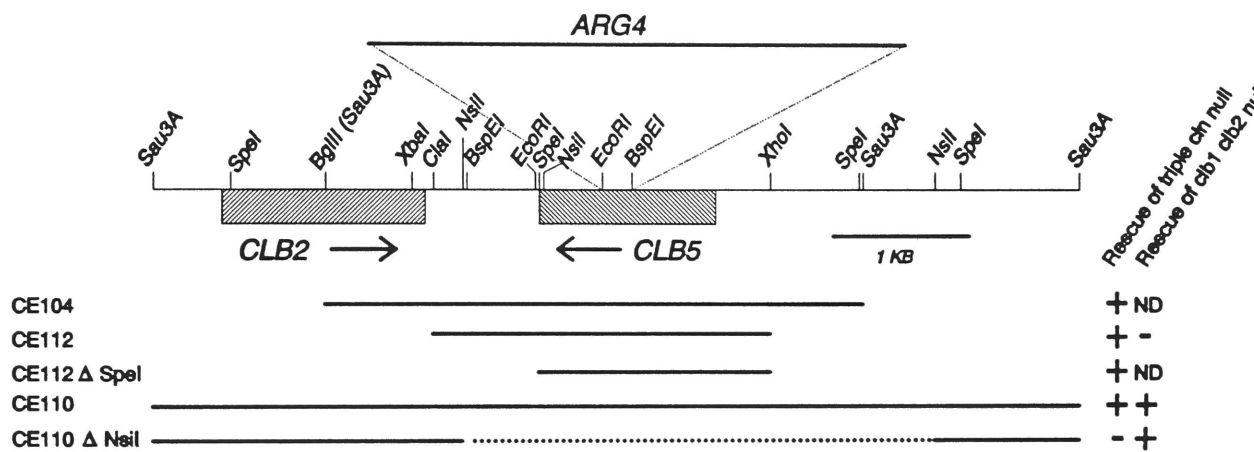


Figure 16. *CLB5* is a B cyclin.

DNA: The minimal fragment having activity on a CEN plasmid at *cln1,2,3*⁺ rescue (CE112 Δ*Spe*, Figure 15) is the region between the indicated *Xho*I and *Spe*I sites. The DNA between the indicated *Bsp*EI and *Eco*RI sites, which was replaced by the yeast *ARG4* gene, falls near the start of the region of cyclin homology, hence nearly the entire cyclin box is absent in the *clb5::ARG4* allele. Sequence was determined for both strands, except in the region -438 to -386.

Potential Transcriptional Control Elements: Five occurrences of a sequence (ACGCGC and its inverse complement) resembling the "MluI-containing cell cycle box" (MCB) are underlined in the 5' region of the gene. No "cell cycle box" (CCB) motifs (CACGAAA, NACGAAA, CNCGAAA and their inverse complements) are found, with the exception of a single CACGAAAA (underlined), which occurs in the coding region, spanning residues 209 to 211 of the protein. Previously described CACGAAAA sequences have all been in the 5'-nontranscribed regions (Nasmyth 1985; Nasmyth and Dirick 1991; Ogas et al 1991). Sequence from -1037 to -438 (data in GenBank, accession number M91209) showed no further occurrences of CCB or MCB motifs.

Protein: In the cyclin homology region (residues 197 to 370), we consulted O'Farrell and Leopold (1991) to determine amino acids conserved in at least 90% of 14 B cyclins compared. For those residues, we placed an asterisk under the Clb5 sequence where it matches other B cyclins, and place the B cyclin consensus amino acid where Clb5 differs. Clb5 matches the consensus at 35/46 positions, and matches fungal B cyclins at many additional residues (see following figure). A potential "destruction box" (Glotzer et al, 1991) is underlined, with the consensus given below the line. Consensus destruction boxes begin at residue 42, while the Clb5 destruction box begins as residue 41 or 56, depending on the true initiator methionine.

XhoI

-438 GTGCCCTCAGTGAAAAGAACCAGCAAAAGAACGCGCATCTCGAGTGAAGACGCGCCTTG
-378 ATGGTACAAAATTAAACGGGAAGGCGCGTCTGATGTTTCACGCGCTTTGCCACATTGGG
-318 ATAGCGCCACAGCATATCTGTGCTAAACTCACTTTCTAGTGACTGCCGATAGCTACT
-258 GCCATCTACCGCGAAGGGAACCTTCATTTGCGTTCATCGGTTTATTAGAAGCTACTTGGAA
-198 CTAATTCCTTAAGCTTCTCAAGAAAAGTTTTTTCTGCTATCTATTGAAGCTTTTTGT
-138 CTTTGTACTTCAAGAGACTCAATCACCTAAAGCTTTTCACGCCAATTAGTTGCTCACA
-78 CAAAGCAAAATAAGCTTAATAATTAGCAGTAACGCGCTTTCCCTGTTATTTAAAGCCGCT
-18 GAACACCTTTACTGAACAATGGGAGAGAACCACGACCATGAGCAGAGATTATAAAGAAAT
M G E N H D H E Q S I K R N 14

43 TCTATGATTTATAATGAAATGAGAGGCGAGTTGTGCAATTCAAACCTAAAGATTCTTCAA
S M I Y N E N E R Q L C N S N L K I L Q 34

103 AATAAAGGGCCCTTTCAAAAATGACAGCTCTAGTAAGCAGCAGTTTCAGGATTCTAAA
N K R A L S K N D S S S K Q Q V Q D S K 54

163 CCAAGAAGGGCTTTAACAGATGTACCAGTGAACAATAATCCTTTAAGCCAGAACAGAGA
P R R A L T D V P V N N N P L S Q N K R 74
R X A L G D I X N <--Destruction box consensus

223 ATAGTAGCAGGGAGCAAGGCGGCCAAAGTACGAAGAGAAGAAAACATTAGACCTATTGTT
I V A G S K A A K V R R E E N I R P I V 94

283 AGCGCCGTTCAAAAAGACAGATATATAACGATCGAACGGCAGCAGCAAGAAGAAGAA
S A V Q K R Q I Y N D R T A A E Q E E E 114

343 GAAGAAGAAGAAGGAGAAGATGATGATGCTGCTTCGATAGTGAACAAAAACGCAGAATA
E E E E G E D D D A A S I V N K K R R I 134

403 GACGCTGAAGGAGTGAAGTAAATAGTAGGCTGGCAGGACCTAGATTATGTTGAAAAAGAT
D A E G V S E I V G W Q D L D Y V E K D 154

463 GATACTGCAATGGTAGCAGAATATTCTGCTGAAATTTTGCATTTTATATAGAAGAGAA
D T A M V A E Y S A E I F A F L Y R R E 174

523 TTAGAAACGTTACCATCGCACAACTATTTACTCGACAAAACGTCCAAGTATTATTGAGG
L E T L P S H N Y L L D K T S K Y Y L R 194

CellCycleBox

583 CCTTCCATGAGAACAATATTAGTGGATTGGCTGGTAGAGGTGCACGAAAAATTTCAATGC
P S M R T I L V D W L V E V H E K F Q C 214
* * * * * L CONS B

BspEI

643 TATCCGGAACGTTATTCTATCCATAAACTTAATGGATAGATTTTTAGCTAAAAATAAA
Y P E T L F L S I N L M D R F L A K N K 234
L * * * * * CONS B

703 GTTACGATGAACAAGTTACAATTTATGGCAGTTACCTCACTTTTCATCGCGGCAAAATTT
V T M N K L Q L L A V T S L F I A A K F 254
* * V G * * * Y CONS B

763 GAAGAGGTAAATTTGCCCAAAGTACGCTGAATACGCTTATATCACTGACGGCGCGGCTTCT
E E V N L P K L A E Y A Y I T D G A A S 274
* * F CONS B

EcoRI

823 AAAAACGACATAAAAAATGCGGAAATGTTTCATGCTCACCTTCTAGAAATTCACATTTGGT
K N D I K N A E M F M L T S L E F N I G 294
* * * * * CONS B

883 TGGCCCAACCCACTCAATTTCTAAGGAGGATCTCCAAGGCAGATGATTACGATCCGGTT
W P N P L N F L R R I S K A D D Y D P V 314
* * * * * CONS B

943 AATAGAAATATTGGTAAGTTTATTTTAGAGTATGCCTACTGCTGCCACCAATTCATTCAT
N R N I G K F I L E Y A Y C C H Q F I H 334
A * L * CONS B

1003 TTACCTCCATCTACCGTAAGCGCAATGGCAATGTATATAGCGAGAAGAAATGACCAACAGA
L P P S T V S A M A M Y I A R R M T N R 354
* * A * L CONS B

1063 AACAGAACGAGCTATGGAATGGAACACTACAGCATTACAGTGGTGGTATCGATCCAATA
N K N E L W N G T L Q H Y S G G I D P I 374
* * Y CONS B

1123 CACGATGAAGCGTTTCAGTCTCTCTGATTGATCTAGTCAAAGACATCGCTAGTTCCAAA
H D E A F Q S L C I D L V K D I A S S K 394

1183 ACTCATTTAGATTCAATTGATTTTGAAGTACAAGAAACCAAGGTATGGCTCTGTTTATTTTC
T H L D S L I L K Y K K P R Y G S V Y F 414

1243 CAAACTTTCAAGTGGTGTACATCCGAAATGCATAGCAACTTTCAAAATCTATTTAATCTT
Q T F K W C T S E M H S N F Q N L F 434

SpeI

1303 AAGTAGATTAGTACTAGTAATGCTCATGAATTGCGCATACTCTTTACATTTTCTTTCTTT
K AMB 435

1363 TTGCTGAACATAAAGGAAAAATTTTTTGCATCTATCTATCTATCTATATATATGTATAT
1423 ATAGTTTATAGTGATATGCGGTGTTCACTTCAACATATATAATACCACGATATTACTACT
1483 ATTATCATCTAATATAGAAATTTTTTGTGTTTAAATTTATCTTTTATTTATTTA
1543 TTATTATTATCATTTACTATATAAAAAATATTATTATTAAATGTTATTCCAATGTT 1599

Figure 17. Alignment of Clb5 with other yeast B cyclins.

The Clb5 sequence was aligned to all B cyclins known from the yeasts *S. cerevisiae* and *S. pombe*, using the CLUSTAL program within PC/GENE (Higgins and Sharp, 1988). CLUSTAL places a '*' under absolutely conserved residues, and a '.' under well conserved residues. The Clb5 sequence is underlined at residues where it fails to conform to sequence conserved in most B cyclins (as determined in the legend to the previous figure).

CLB5	WQDL DYVEKDDTAMVAEYSAEIFAFLYRRELETLP SHNYLLDKTSKY YLR	194
CLB1	WDDLDEEDCDDPLMVSEEVNDIFDYLH HLEIITLPNKANLYKHKN---IK	238
CLB2	WEDLDAEDVNDPFMVSEYVNDIFEYLHQLEVITLPKKEDLYQH RN---IH	258
CDC13	WDDLDAEDWADPLMVSEYVVDIFEY LNELEIETMPSPTYMDRQKE---LA	232
CIG1	MVPDYDPEIFHYMASLERKLAPPPNYMSVQQE---ID	192
	* * * * *	
CLB5	PSMRTILVDWLVEVHEKFQCY PETLFLSINLMDRFLAKNKVTMNKLQ LLA	244
CLB1	-QNRDILVNWI IKIHNKFGLLPETLYLAINIMDRFLCEEVVLNRLQLVG	287
CLB2	-QNRDILVNWLVIHNKFGLLPETLYLAINIMDRFLGKELVQLDKLQLVG	307
CDC13	WKMRGILTDWLVIEVHSRFRLLPETLFLAVNIIDRFLSLRVCSLNKLQLVG	282
CIG1	WVTRHMLVDWIVQVQIHFRLLPETLFLAVNLIDRFLSIKVVSLQKVQLVG	242
CLB3	--FRSTLIDWIVQVHEKFQLLPETLYLCINIIDRYLCKEVVPVNKFQLVG	
CLB4	--FRRTMIDWLVLQHFRLLPETLYLTINIVDRFLSKKTVTNLNRFQLVG	
	* . . . * * * *	
CLB5	VTSLFIAAKFEEVNLPKLA EYAYITDGAASKNDIKNAEMFMLTSLEFNIG	294
CLB1	TSCLFIASKYEEIYSPSIKH FAYETDGACSVEDIKEGERGILEKLDFQIS	337
CLB2	TSCLFIASKYEEVYSPSIKH FASETGDCTEDEIKEGEKFIKTLKFNLN	357
CDC13	IAALFIASKYEEVMCP SVQNFVYMDGGYDEEEILQAERYILRVLEFNLA	332
CIG1	LSALLIACKYEEIHPPSIY NFAHVVGIFTVDEIIRAERYMLMLLDFDIS	292
CLB3	AASLFIAAKYEEINCPTIKDFVYMSENCYSRNDLLDAERTILNGLEFELG	
CLB4	VSALFIAAKFEEINCPTLDDL VYMLENTYTRSDIIRAEOQYMTDTLEFEIG	
	. * . * . * . * . * . * * *	
CLB5	WPNPLNFLRRISKADDYDPVNRNIGKFILEYAYCCHQFIHLPPSTVSAMA	344
CLB1	FANPMNFLRRISKADDYDIQSR TLAKFLMEISIVDFKFIGILPSLCASAA	387
CLB2	YPNPMNFLRRISKADDYDIQSR TLAKFLLEISLVDFRFIGILPSLCAAAA	407
CDC13	YPNPMNFLRRISKADFYDIQTR TVAKYLVEIGLLDHKLLPYPPSQQCAAA	382
CIG1	WPGPMSFLRRISRAHSYDHDIR MLAKYLQEVTLMDEIFIGAHISFIAATA	342
CLB3	WPGPMSFLRRISKADDYEHDTRTLAKYLLESTIMDHRLVSAQPSWLAAGA	
CLB4	WPGPMPFLRRISKADDYDFEPRTLAKYLLETTIVEPKLVAAAPSWLAAGA	
	. . . * . * . * . * . * . * . * . . . * . . . *	
CLB5	MYIARRMTNRNKNELWNGTLQHYSGGIDPIHDEAFQSLCIDLVKDIASS	393
CLB1	MFLSRKMLGKGT---WDGNLIHYSGGYT KAKLYPVCQLLMDYLVGSTIH	433
CLB2	MFMSRKMLGKGK---WDGNLIHYSGGYT KEELAPVCHMIMDYLVSPIVH	453
CDC13	MYLAREMLGRGP---WNRNLVHYSGYEYQLISVVKKMINYL-QKPVQH	427
CIG1	YYLSMQMLGHLDD---WTPCHVYYSGYTARQLKPCANIIWECLVDAPNH	388
CLB3	YFLSKIILGQNG---WSLAHVYYSNYTQE QILPLATIIL	
CLB4	YFLSRTILGSND---WSLKHVFYSGYTSSQIIPLASLIL	
 * . * .	

Figure 18. Northern analysis of *CLB5* transcription through the cell cycle.

cln1 cln2 cln3 leu2::LEU2::GAL1::CLN3 strains were grown to log phase in YEP 3% galactose at 30°C, and arrested by 150 minutes growth in YEP 3% raffinose at 30°C. Cultures were released from cell cycle arrest by addition of galactose to 3%. At 12 minute intervals thereafter, samples were taken and RNA was isolated. Replicate blots were hybridized with *CLB5*, *CLN2*, *CLB2*, and *TCM1* (used as a loading control) probes, as indicated. Timing of bud emergence (BE) and nuclear division (ND) was determined by microscopic examination of fixed, sonicated cells, in the latter case stained with DAPI and UV illuminated. In the bottom set of panels, nocodazole was added (15 ug/ml final concentration) at the time of galactose addition. Lane 1: asynchronous cultures. Lane 2: cells arrested 150' at the *cln* block. Lane 3: 12 minutes after galactose addition. Subsequent lanes, additional 12 minute intervals.

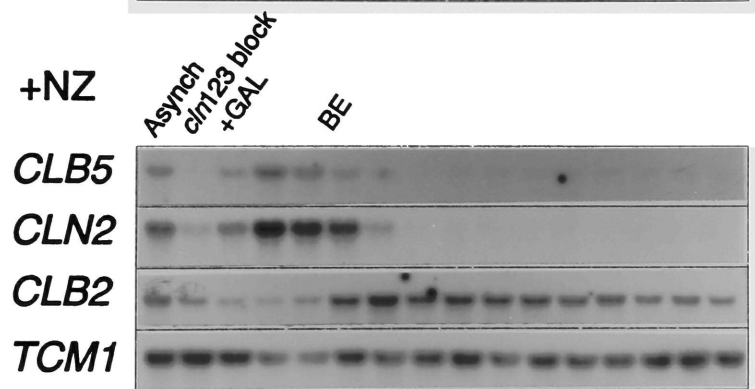
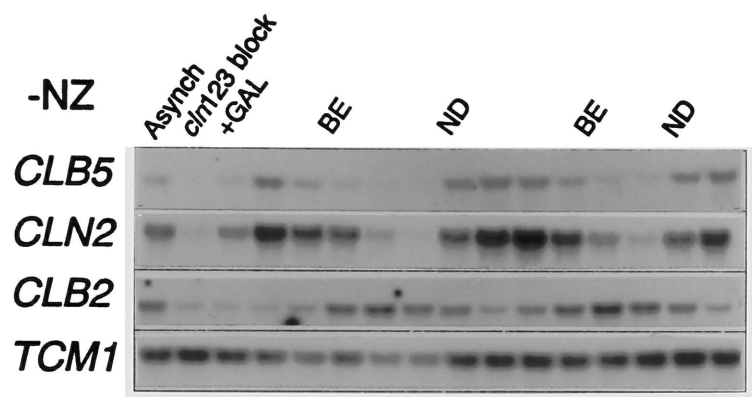


Figure 19. Loss of *CLB5* makes cells sensitive to hydroxyurea.

Genotype	Strain	plating efficiency (*)
<i>CLB2 CLB5</i>	1024-29C	1.19
<i>CLB2 CLB5</i>	1024-31A	1.00
<i>CLB2 clb5</i>	1011-5D	0.88
<i>CLB2 clb5</i>	1011-10A	0.88
<i>clb2 CLB5</i>	1003-8C	0.83
<i>clb2 CLB5</i>	1003-5A	0.82

YEPD agar was prepared with or without supplemental 0.2 M hydroxyurea. All strains are *CLN1 CLN2 CLN3*. Plates were photographed after 3 days

(*) Plates were incubated a total of 7 days to determine that *clb5* strains eventually formed tiny colonies with wild type efficiency in the presence of HU.

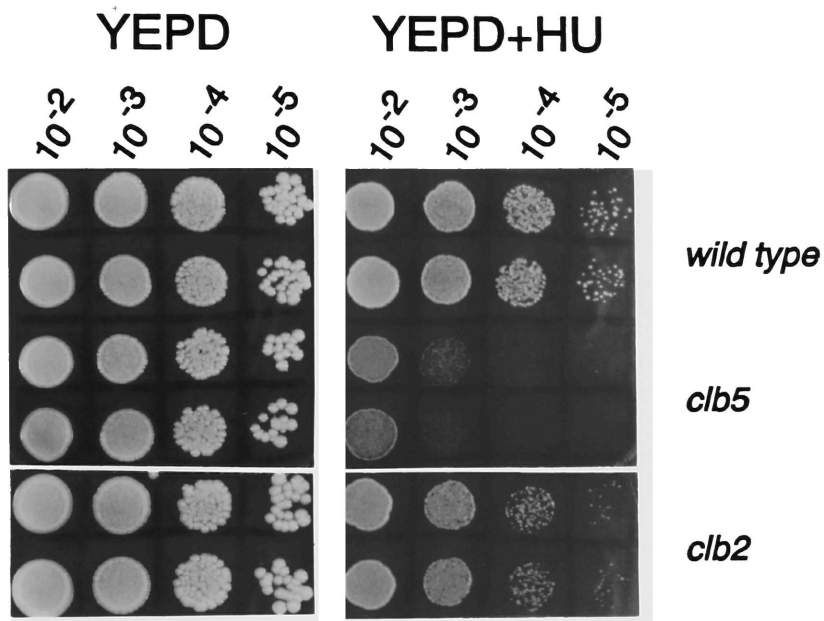


Figure 20. DNA flow cytometry of asynchronous cultures, with and without *CLB5*.

Genotype	Strain
wild type	1001-1C
wild type	1001-2A
<i>clb5</i>	1011-5D
<i>clb5</i>	1011-10A
<i>clb1,3,4</i>	1018-8A
<i>clb1,3,4</i>	1018-23B
<i>clb1,3,4,5</i>	1018-10D
<i>clb1,3,4,5</i>	1018-22D
<i>clb2,4</i>	1008A-1B
<i>clb2,4</i>	1008A-5A
<i>clb2,4,5</i>	1019-4B
<i>clb2,4,5</i>	1019-7A

Log phase YEPD cultures of the indicated genotypes were sonicated, fixed, and stained with propidium iodide for flow cytometric analysis. For each genotype, two distinct spore clones were used, to confirm that phenotypes observed are characteristic of the genotype. Histograms depict relative DNA content (X) vs. cell number (Y).

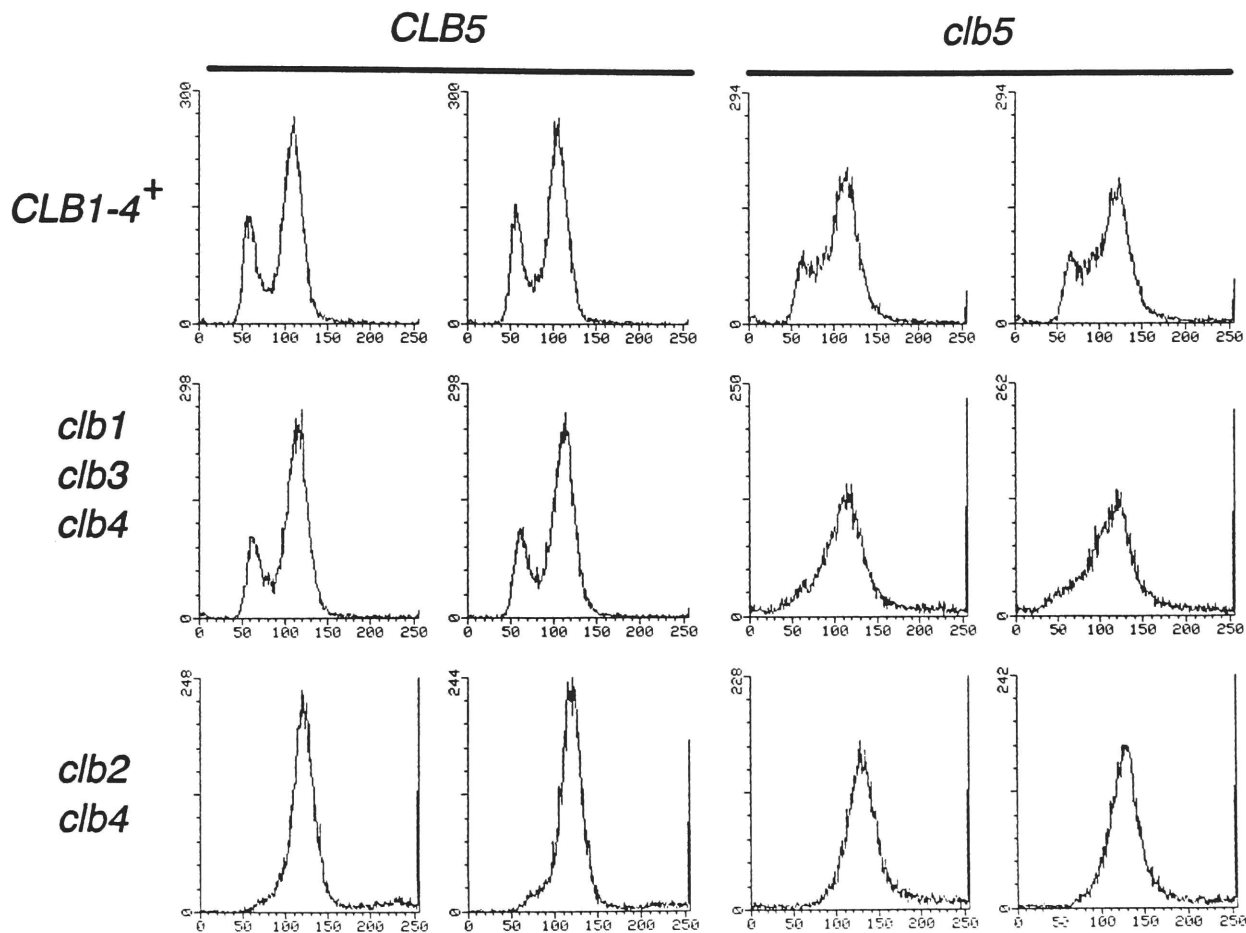


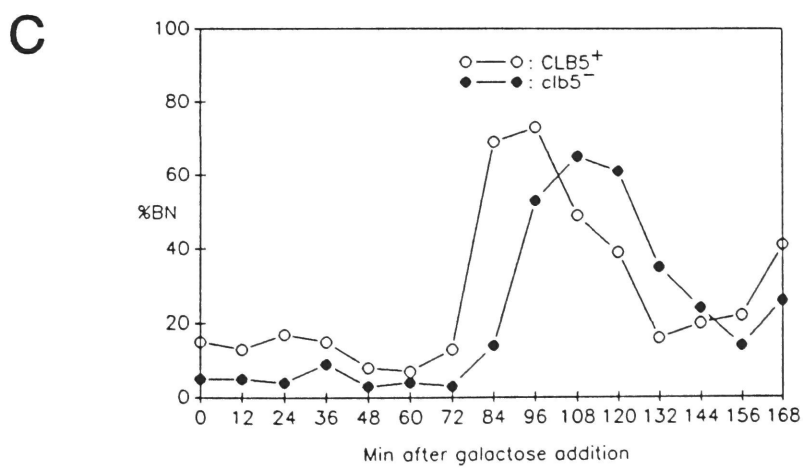
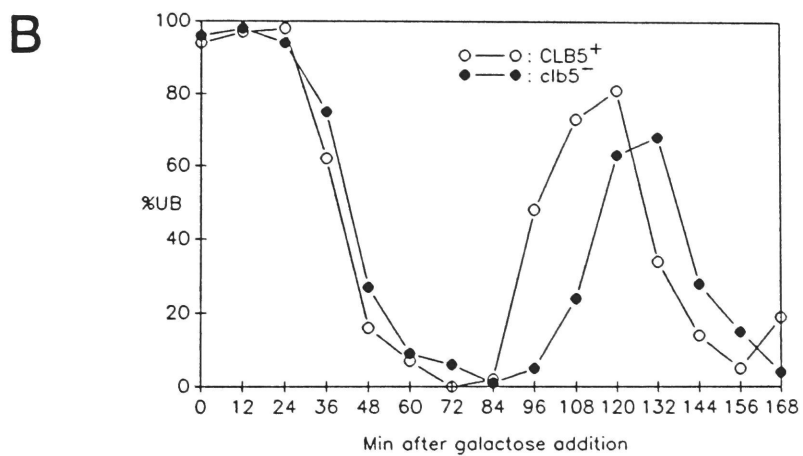
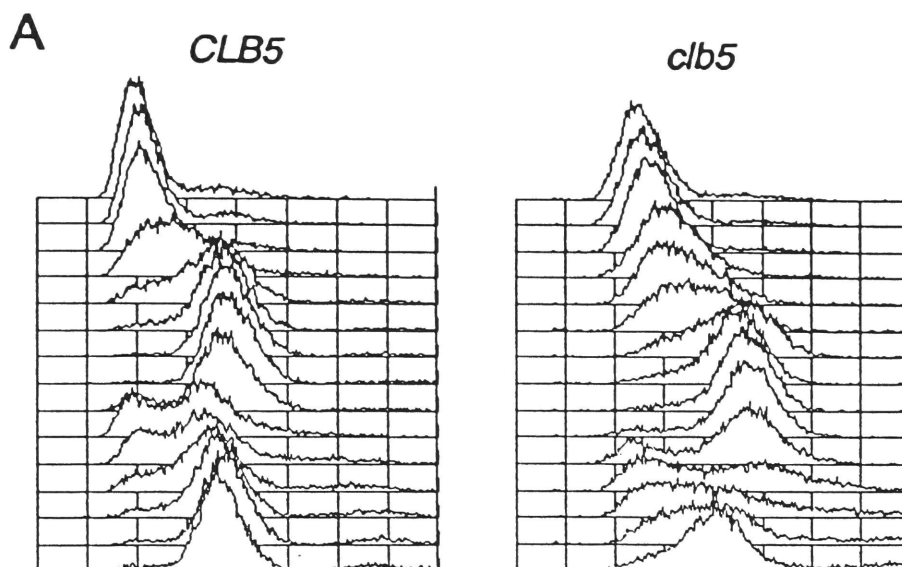
Figure 21. DNA flow cytometry of synchronized cultures following release from arrest in G1.

cln1,2,3⁻ deficient strains were synchronized as in Figure 18. Strains were either *CLB5* or *clb5::ARG4*, as indicated. Initial samples were taken after 150 minutes' arrest, and subsequent samples were taken at 12 minute intervals following addition of galactose.

(A) DNA content histograms. Top curve represents the arrested culture; subsequent curves are arranged below. S phase is complete 60 minutes after release in *CLB5* cells, but not until 84 minutes after release in *clb5* cells.

(B) Percent unbudded cells.

(C) Percent binucleate cells. Nuclear division occurs about 78 minutes after release in *CLB5* cells, and 96 minutes after release in *clb5* cells.



DATE: 12-SEP-92

TIME: 12:09:29

LYS CMD HIST HSTATS DOT-PLOT CONTOUR/3D 2D-STATS GATES WINDOWS

CLB5

clb5::ARG4

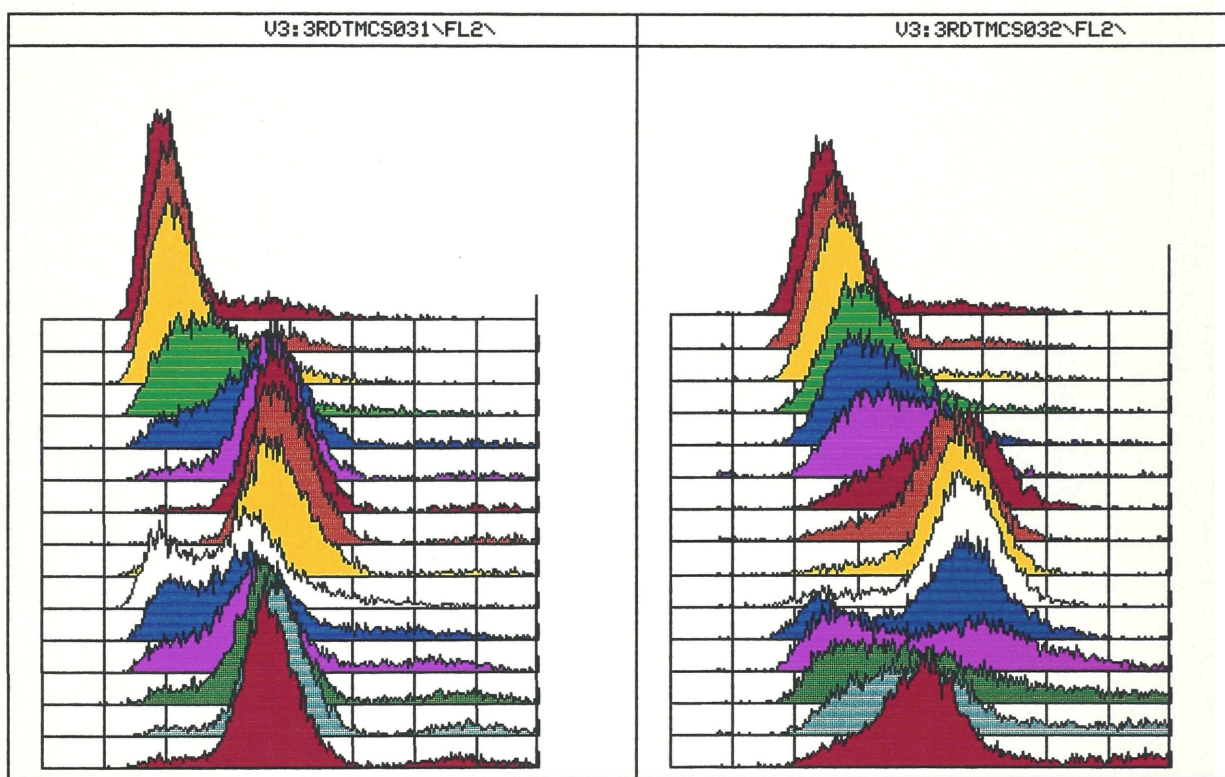


Figure 22. *cln1 cln2 clb5* strains are nearly inviable on glycerol media.

Strains of the indicated genotypes were serially diluted, and plated at two densities on YEP media supplemented with either 2% glucose (YEPD) or 3% glycerol. Photographs were taken after growth for the indicated number of days. *cln1 cln2 clb5* strains formed detectable colonies on YEPGlycerol after approximately one weeks' growth, with a plating efficiency of about 5% (glycerol/glucose).

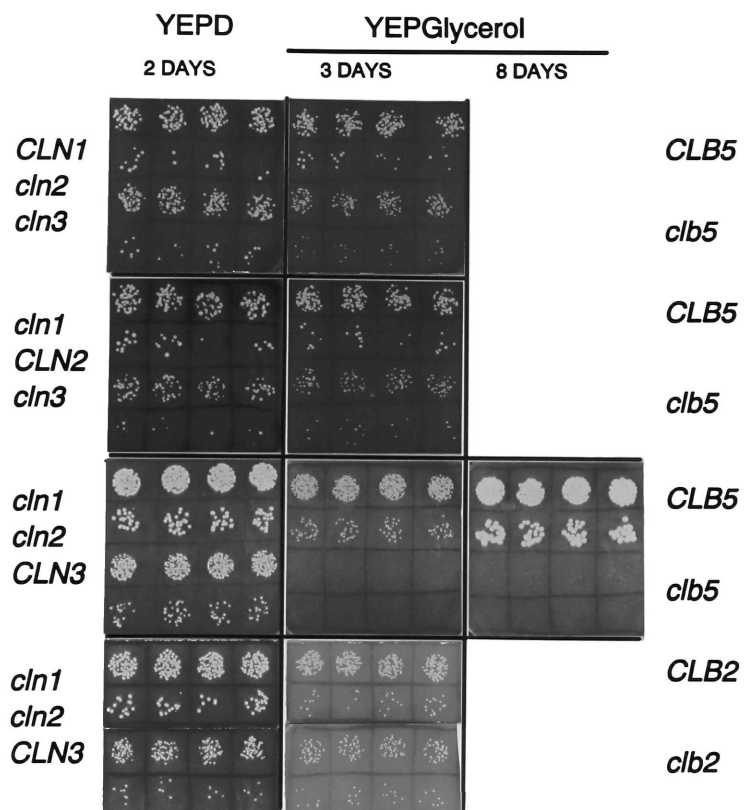


Figure 23. *cln1 cln2 clb5* yeast develop an aberrant morphology upon culture in YEPGlycerol.

Genotype					Strain
<i>CLN1</i>	<i>cln2</i>	<i>cln3</i>	<i>CLB5</i>	[<i>GAL1::CLN3</i>]	994/A ⁺ /2-2A
<i>CLN1</i>	<i>cln2</i>	<i>cln3</i>	<i>clb5</i>	[<i>GAL1::CLN3</i>]	994/A ⁺ /2-3A
<i>cln1</i>	<i>CLN2</i>	<i>cln3</i>	<i>CLB5</i>	[<i>GAL1::CLN3</i>]	994/A ⁺ /2-3D
<i>cln1</i>	<i>CLN2</i>	<i>cln3</i>	<i>clb5</i>	[<i>GAL1::CLN3</i>]	994/A ⁺ /2-8A
<i>cln1</i>	<i>cln2</i>	<i>CLN3</i>	<i>CLB5</i>	<i>leu2::LEU2::GAL1::CLN3</i>	998/A ⁺ /2-3A
<i>cln1</i>	<i>cln2</i>	<i>CLN3</i>	<i>clb5</i>	<i>leu2::LEU2::GAL1::CLN3</i>	998/A ⁺ /2-4B

Yeast were precultured in YcGal-ura (first four) or YEPGal (last two), then inoculated into YEPGlycerol (3%), and further cultured overnight. Cells were sonicated prior to photography.

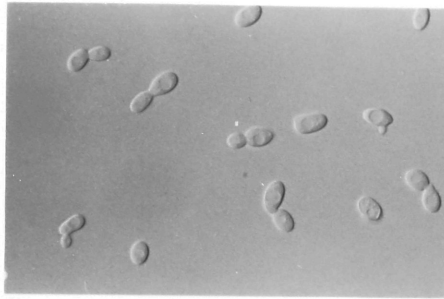
CLB5

clb5

CLN1

cln2

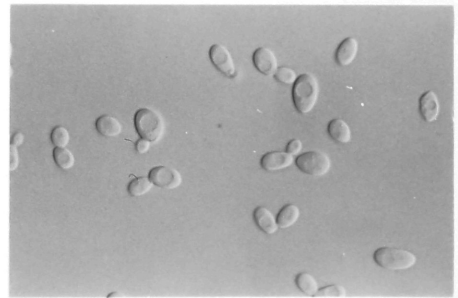
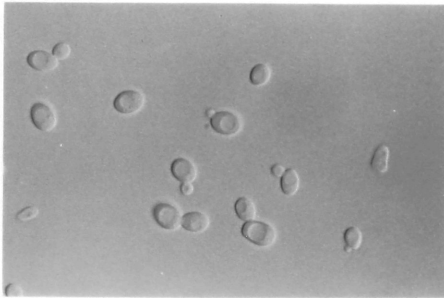
cln3



cln1

CLN2

cln3



cln1

cln2

CLN3

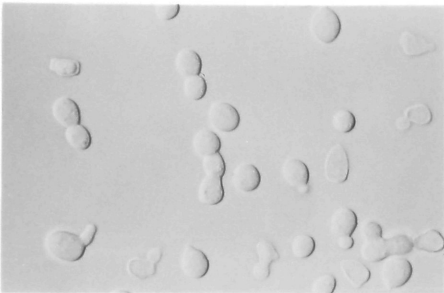


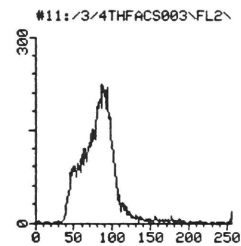
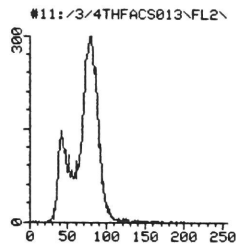
Figure 24. DNA flow cytometry of *CLB5* and *clb5::ARG4* strains having only a single *CLN* gene.

Genotype	Strain
<i>CLN1 CLN2 CLN3 CLB5</i>	1024-29C
<i>CLN1 CLN2 CLN3 clb5</i>	1011-5D
<i>CLN1 cln2 cln3 CLB5</i>	994/A ⁺ /4-4A
<i>CLN1 cln2 cln3 CLB5</i>	994/A ⁺ /4-10B
<i>CLN1 cln2 cln3 clb5</i>	994/A ⁺ /4-13B
<i>CLN1 cln2 cln3 clb5</i>	994/A ⁺ /4-14A
<i>cln1 CLN2 cln3 CLB5</i>	994/A ⁺ /4-1A
<i>cln1 CLN2 cln3 CLB5</i>	994/A ⁺ /4-9C
<i>cln1 CLN2 cln3 clb5</i>	994/A ⁺ /4-9B
<i>cln1 CLN2 cln3 clb5</i>	994/A ⁺ /4-18B
<i>cln1 cln2 CLN3 CLB5</i>	998/A ⁺ /2-4B
<i>cln1 cln2 CLN3 CLB5</i>	998/A ⁺ /2-5B
<i>cln1 cln2 CLN3 clb5</i>	998/A ⁺ /2-3A
<i>cln1 cln2 CLN3 clb5</i>	998/A ⁺ /2-6C

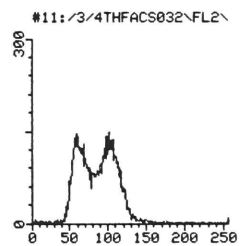
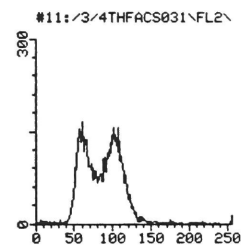
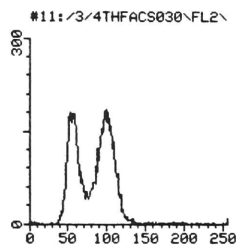
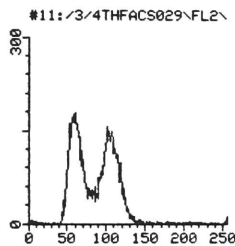
CLB5

clb5

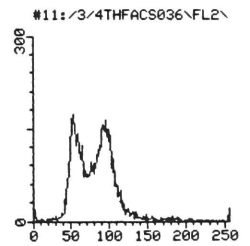
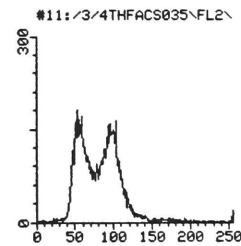
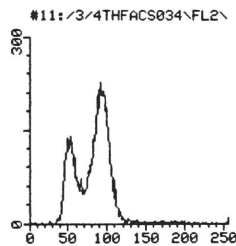
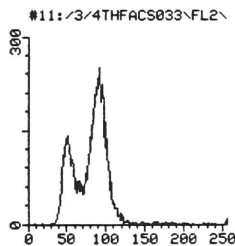
CLN1
CLN2
CLN3



CLN1
cln2
cln3



cln1
CLN2
cln3



cln1
cln2
CLN3

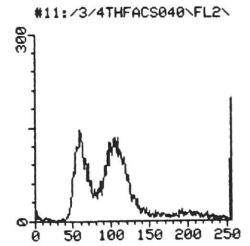
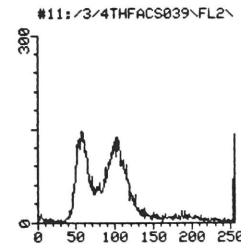
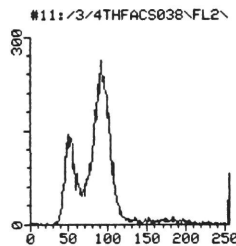
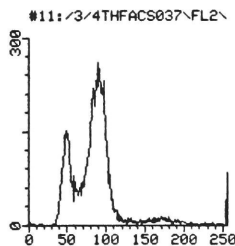
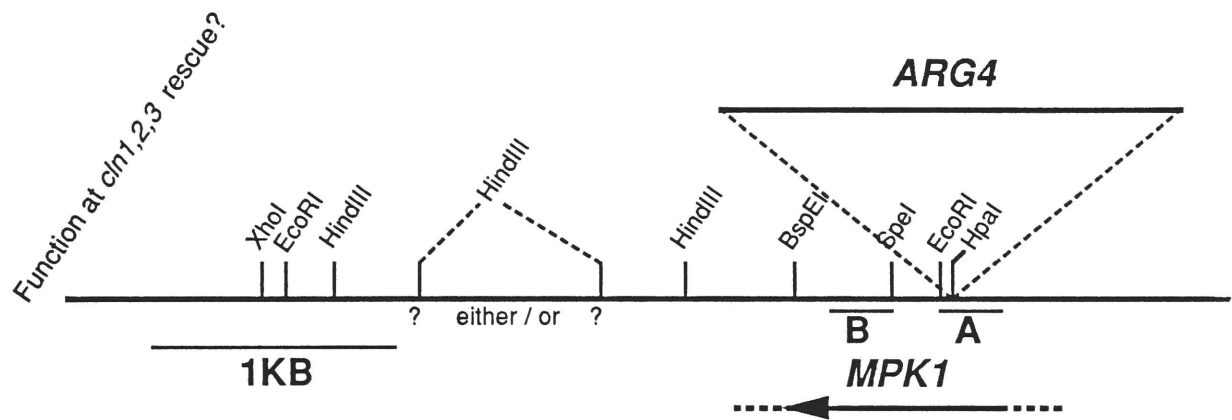


Figure 25. Restriction map of CE116.

CE116 is not cut by *Apa*I, *Bam*HI, *Cla*I, *Kpn*I, *Sac*I, *Sal*I, or *Sma*I. The DNA sequence of regions A and B is reported in the following figure.



NO _____ *CE116ΔSpe I*

YES *CE116ΔXho I* _____

NO _____ *CE116ΔEco RI* _____

Figure 26. Partial sequence of *MPK1*.

(A) Translation of the inverse complement of the sequence of CE116Δ*EcoRI*, obtained using the T3 primer. Sequence reads from right to left on the restriction map in Figure 25, and terminates upstream of, but near, the *EcoRI* site. Noted as region A on that map.

(B) Translation of the sequence of CE116Δ*SpeI*, obtained using the T7 primer. Sequence reads from right to left on the restriction map in Figure 25, and originates downstream of, but near, the *SpeI* site. Noted as region B on that map.

A

CCACTTCAGTTGGCTCATTATCATCCATGTCAAATCGTTATTCACCAATAAGAGTTGCAT
T S V G S L S S M S N R Y S P I R V A

lation site

CGCCAGGAAGAGCAAGATCCGCAACTCGTGGGTCTTCCCTTTATAGATTATCCAGAGACC
S P G R A R S A T R G S S L Y R L S R D

TTAATTCTTTACCAAGCGTCACTGATCTACCAGAAATGGATAGTACAACCCAGTTAACG
L N S L P S V T D L P E M D S T T P V N

AAATATTCTTGGATGGCCAACCAACAGCATAAAAGTGGCAGTGTCAAAGGAGGGCATAG
E I F L D G Q P Q H K S G S V K G G H

B

ACTACAACAAGCAACGGACCGGAAACTACTTTCAAGATCAAGATAAGTATACATTGGTAA
Y N K Q R T G N Y F Q D Q D K Y T L V

ATACGGGATTGGGATTGAGTGATGCAAACCTCGATCATTTTATTAGATCTCAATGGAAAC
N T G L G L S D A N L D H F I R S Q W K

ACGCTTCTCGATCAGAATCCAATAATAATACCGGAAATCGCGTTTCTTACAGTGGCTCAA
H A S R S E S N N N T G N R V S Y S G S

CACCAAACAATGTTGATACAACAAAGACTAATTTGCAAGTGCATACCGAGTTCGATTTTG
T P N N V D T T K T N L Q V H T E F D F

AAA
E

Figure 27. *mpk1* and *swi4* suppress p*CLB5* rescue of *cln1,2,3*⁻ inviability.

Genotype	Strain
<i>CLB5 MPK1 leu2::LEU2::GAL1::CLN3</i>	YFC1317-13D
<i>clb5 MPK1 leu2::LEU2::GAL1::CLN3</i>	YFC1317-3D
<i>CLB5 mpk1 leu2::LEU2::GAL1::CLN3</i>	YFC1371-2B
<i>SWI4 SWI6 [GAL1::CLN3/URA3]</i>	YFC1242-4B
<i>swi4 SWI6 [GAL1::CLN3/URA3]</i>	YFC1370-2B
<i>SWI4 swi6 [GAL1::CLN3/URA3]</i>	YAT1927-17A

All strains are *cln1Δ cln2Δ cln3Δ*. Yeast were transformed with p*CLB5* (CE111), p*MPK1* (CE118), or left untransformed. They were precultured in YcGal-trp-ura or YcGal-trp media (depending on whether integrated or episomal *GAL1::CLN3* was present), and plated at 4 densities on YEPGal and YEPD media. Photographs were taken after 5 days growth.

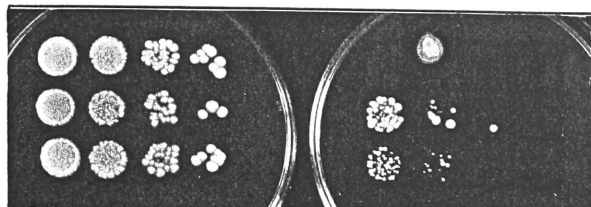
YEPGal

YEPD

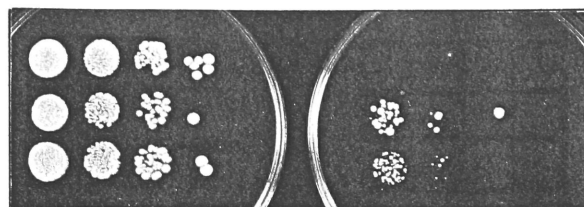
YEPGal

YEPD

1
2
3

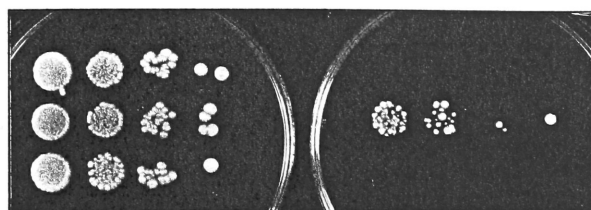


cln1 cln2 cln3 GAL1::CLN3

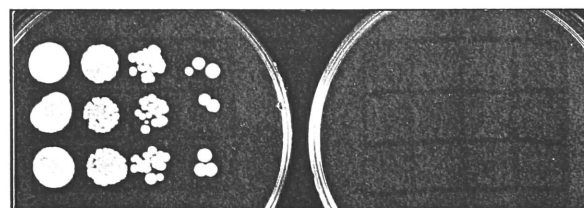


cln1 cln2 cln3 GAL1::CLN3

1
2
3



cln1 cln2 cln3 clb5 GAL1::CLN3

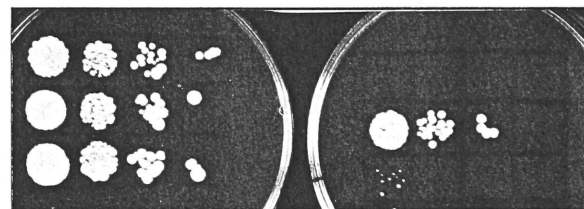


cln1 cln2 cln3 swi4 GAL1::CLN3

1
2
3



cln1 cln2 cln3 mpk1 GAL1::CLN3



cln1 cln2 cln3 swi6 GAL1::CLN3

1 no plasmid
2 pCLB5
3 pMPK1

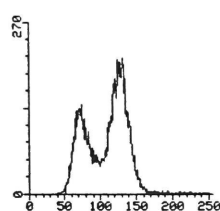
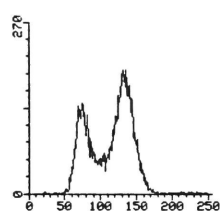
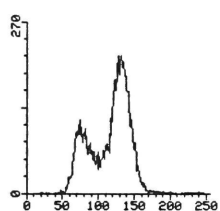
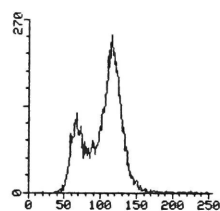
Figure 28. DNA flow cytometry of *MPK1* and *mpk1::ARG4* strains.

Genotype	Strain
<i>CLN1 CLN2 CLN3 MPK1</i>	1050E-1B
<i>CLN1 CLN2 CLN3 MPK1</i>	1050E-12A
<i>CLN1 CLN2 CLN3 mpk1</i>	1050E-1D
<i>CLN1 CLN2 CLN3 mpk1</i>	1050E-3A
<i>cln1 cln2 CLN3 MPK1</i>	1052-5A
<i>cln1 cln2 CLN3 MPK1</i>	1052-12C
<i>cln1 cln2 CLN3 mpk1</i>	1052-7C
<i>cln1 cln2 CLN3 mpk1</i>	1052-5D

MPK1

mpk1::ARG4

CLN1 CLN2 CLN3



cln1 cln2 CLN3

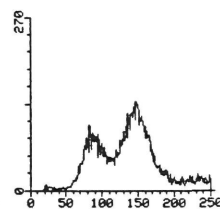
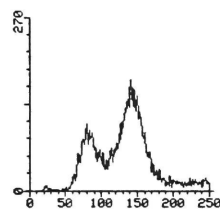
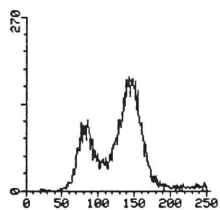
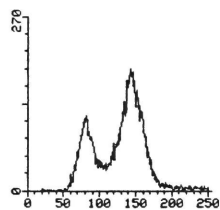


Figure 29. *mpk1::ARG4* strains are sensitive to the absence of *CLN3*.

Genotype	Strain
<i>CLN1 CLN2 cln3 mpk1</i> W16	1036/A ⁺ /3/FOA ^R /W16-5C
<i>CLN1 CLN2 cln3 mpk1</i> W16	1036/A ⁺ /3/FOA ^R /W16-2C
<i>CLN1 cln2 cln3 mpk1</i> W16	1052-2D
<i>CLN1 cln2 cln3 mpk1</i> W16	1052-7D
<i>cln1 CLN2 cln3 mpk1</i> W16	1052-3D
<i>cln1 CLN2 cln3 mpk1</i> W16	1052-20D
<i>cln1 cln2 CLN3 mpk1</i>	1052-7C
<i>cln1 cln2 CLN3 mpk1</i>	1052-5D

The derivation and genotype assignments of these strains is described in Materials and methods.

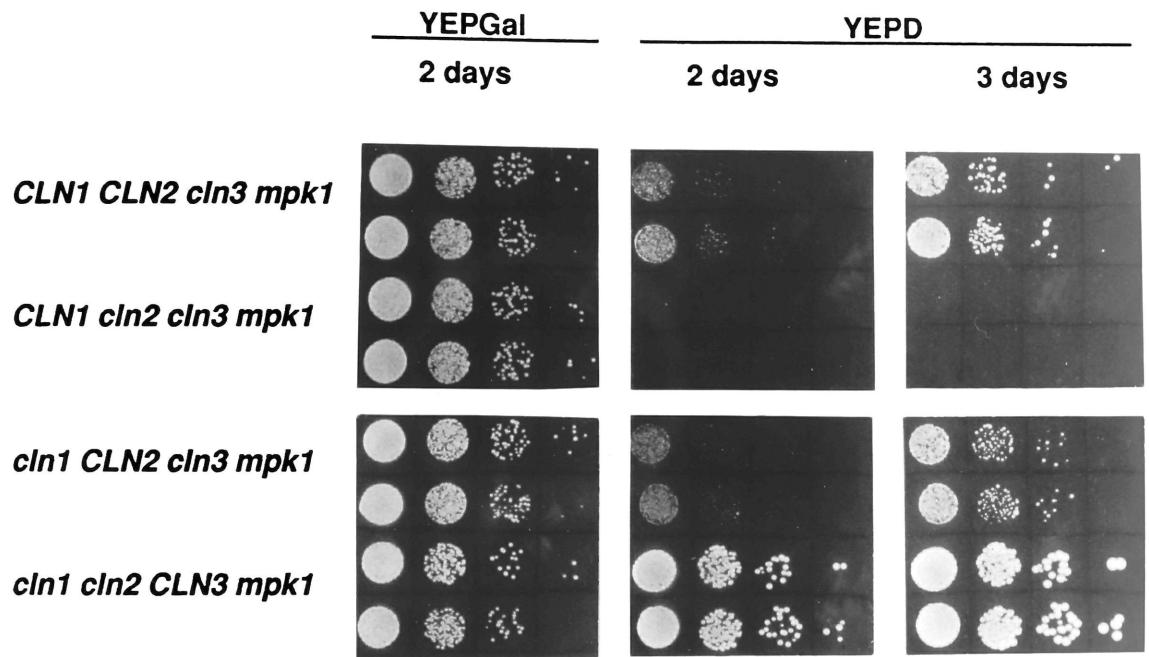
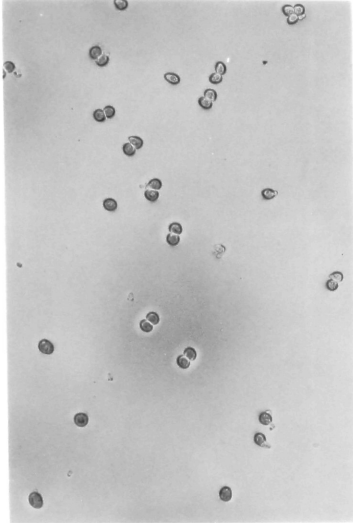


Figure 30. *CLN1 cln2 cln3 mpk1::ARG4* W16 strains arrest without budding in the first cycle after plating on YEPD.

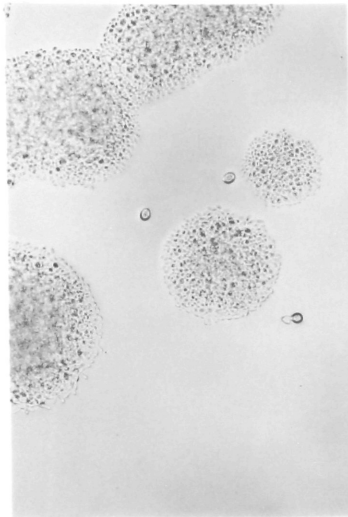
Genotype	Strain
<i>CLN1 cln2 cln3 mpk1</i> W16	1052-2D
<i>cln1 CLN2 cln3 mpk1</i> W16	1052-3D

The scoring of genotypes at *CLN3* in cross 1052 progeny is described in Materials and methods. The 1052-2D culture consisted of 38% budded cells when plated, hence the failure to detect groups of more than two cell bodies, and the finding of many single cell bodies, are both evidence for first cycle arrest.

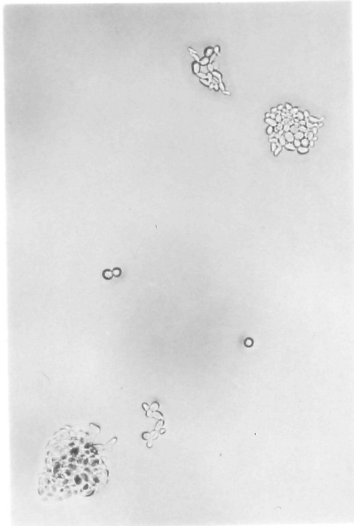
YEPD



YEPGal



CLN1 cln2 cln3 mpk1
GAL1::CLN3



cln1 CLN2 cln3 mpk1
GAL1::CLN3

End