GENETICS OF COMMITMENT TO CELL DIVISION IN S. CEREVISIAE

by

JIAN ZHANG, B.S.

A DISSERTATION

IN

CELL & MOLECULAR BIOLOGY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Advisory Committee

Brandt Schneider (Chairperson)
Elmus Beale
Martine Coue
Jim Hutson
Simon Williams

Accepted

Dean of the Graduate School of Biomedical Sciences
Texas Tech University Health Sciences Center

December, 2004

ACKNOWLEDGMENTS

This major accomplishment in my life was made possible by many people who helped me in many different ways. First of all, I am most grateful to my mentor Dr. Brandt Schneider, who taught me not only academic knowledge and techniques in my research field, but also the scientific way of thinking. Furthermore, it was his encouragement and scholarly advice that were so helpful when I was so frustrated by failures in my experiments.

My work was also greatly facilitated with kindly help and cooperation from my present and former coworkers. Ms. Jody Markwardt helped me with lots of cell sizing experiments. Ms. Uma Thippeswamy and Ms. Kimberly Martin provided great assistance on yeast transformations and northern blots. Ms. Colette Schneider always kept the lab in good order and spent much time on correcting my dissertation. I also thank Dr. Audra Day and Mr. Rolando del Aguila for their constructive discussion and advice on my research project and student life.

My thanks are also due to all other members in my committee, Dr. Elmus Beale, Dr. Martine Coue, Dr. Jim Hutson and Dr. Simon Williams. Their academic advice kept me on track of my study and research plan. Moreover, their pertinent critiques on my presentations and exams helped me a lot in both scientific thinking and expressing myself in a better way.

Furthermore, I would like to thank all other faculty members and staff in our department, who were always there to help without any hesitation.

I am grateful to Dr. Chong Xu, who greatly aroused my interests in scientific research. Finally, I owe my gratitude to my family and friends. Their long time support and understanding, especially from my beloved wife Ying Yao, have always been and will be a strong encouragement in my entire life.

TABLE OF CONTENTS

ABSTRACT

In all eukaryotes, proliferation is regulated by cell cycle controls. Elucidating the intrinsic mechanism whereby these control mechanisms modulate proliferation is essential for understanding the role of the cell cycle in development, aging and cancer. Our laboratory uses budding yeast *S. cerevisiae* as a model system. In budding yeast, proliferation is dependent upon cell growth, cell size, and the expression of G1 phase cyclins (Clns). However, the relationship among these requirements is poorly understood.

In this study, the relationship between cell growth, cell size, Cln expression, and proliferation was analyzed. It is found that rapidly growing cells express, and require, more Cln protein to divide than do slowly growing cells. To clarify the role of cell size, defined amounts of *CLN* mRNA were expressed in cells of different sizes. It is found that a critical threshold of Cln protein was required for proliferation, and that Cln1 protein expression was strongly modulated by cell size. In addition, expression of high levels of *CLNs* promoted proliferation in a size-independent manner suggesting that Clns are rate-limiting. To examine the relationship between cell growth and the ability of cells to proliferate, a systematic genome-wide genetic screen was conducted to identify mutants that dramatically altered the proliferative capacity of cells. In so doing, 49 gene deletions that dramatically changed cell size were identified. Twenty of these made cells abnormally small (*whi* mutants), and 29 made cells abnormally large (*uge* mutants). Nearly all of these genes have putative human homologues. Interestingly, five *uge* gene products are components of Ccr4-Not transcriptional complexes. Furthermore, it is found that *CCR4* positively regulates *CLN1* mRNA expression. In *ccr4*Δ strains, *CLN1* mRNA expression was decreased in asynchronous cultures and delayed in synchronized cultures, but restoration of *CCR4* expression induces *CLN1* mRNA expression and rescues the size phenotype of *ccr4*Δ. My results suggest that *CCR4* modulates the ability of the Bck2 protein to induce *CLN1* and *CLN2* transcription. In summary, my research has identified new gene products involved in cell cycle control and has helped elucidate the mechanism whereby cells coordinate cell growth with proliferation.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C – Celcius degree

µCi – microcurie

µg – microgram

µM – micromolar

4E-BP – eIF4E binding protein

5'-TOP –5' - terminal oligopyrimidine tract

Ace2 – activation of *CUP1* expression

Ada1 – adaptor

ATP – adenosine triphosphate

Azf1 – asparagine-rich zinc-finger protein

Bck2 – bypass of C-kinase

Bcy1 – bypass of cyclic AMP requirement

Caf1 – Ccr4 associating factor

Cak1 –Cdk activating kinase

cAMP – adenosine- 3', 5'- cyclic monophosphate

CCR4 – carbon catabolite repression

CDC – cell division cycle

CDK – cyclin dependent kinase

CKI – Cdk inhibitor

CLN1 – G1 phase cyclin 1

CLN2 – G1 phase cyclin 2

CLN3 – G1 phase cyclin 3

CTP – cytidine triphosphate

Ctr9 – Cln Tree Requirement

Cyr1 – cyclic AMP requirement

Dbf – dumbbell former

DEPC – diethylpyrocarbonate

DNA – deoxyribonucleic acid

DTT – dithiothreitol

E2F – adenovirus E2 promoter binding factor

ECB – early cell cycle box

EDTA – deoxyribonucleic acid

eIF4E – eukaryotic translation initiation factor 4E

ERK – extracellular signal-regulated kinase

fL – femtoliter

g – gram

G1 phase – gap 1 phase

G2 phase – gap 2 phase

G418 – geneticin

GDP – guanosine diphosphate

GSK – glycogen synthase kinase

GTP – guanosine triphosphate

GYPD –geneticin (G418) yeast extract peptone dextrose

HA – influenza hemagglutin

HO gene – homothallic gene

hr – hour

IGF – insulin-like growth factor

INK4 – inhibitors of Cdk4

IRS – insulin receptor substrate

kb – kilobase

kDa – kilodalton

kg – kilogram

LB – Luria-Bertani Broth

M phase – mitosis phase

MAP kinase – Mitogen-activated protein

MAT – mating type

MBF –MCB binding factor

MCB – MluI cell cycle box

mg – miligram

min – minutes

ml – microliter

mM – milimolar

MOPS – morpholinepropanesulfonic acid

mRNA – messenger ribonucleic acid

Msn1 – multicopy suppressor of *snf*

NaCl – Sodium Chloride

NaF – Sodium Fluoride

NaOAc – Sodium Acetate

ng –nanogram

NLS – nuclear localization signal

NOT – non-canonic TATA

OD – optical density

ORF – open reading frame

PC3 – pheocromocytoma cell-3

PCR – polymerase chain reaction

PDK1 – phosphoinositide-dependent kinase-1

PEG – polyethylene glycol

PEST – proline-aspartic acid-serine-threonine rich motif

PH domain – pleckstrin-homology domain

PI – propidium iodide

PI3K – phosphotidylinositol-3-OH kinase

PKA – protein kinase A

PKB – protein kinase B

PKC – Protein kinase C

pM – picomolar

Pph2 – protein phosphatase

pRB – retinoblastoma tumor suppressor

PTEN – phosphatase and tensin homolog

RNA – ribonucleic acid

RNAi – RNA interference

RRE – Rme1 response elements

RRM – RNA-recognition motif

Rtg1 – *CIT2* retrograde regulation

S phase – synthetic phase

S6K – ribosomal protein S6 kinase

SBF – SCB binding factor

SCB – Swi4 cell cycle box

SDS – sodium dodecylsulfate

SDS-PAGE – SDS-polyacrilamide gel eletrophoresis

SGD – *Saccharomyces* genome database

Sic1 – substrate/subunit/inhibitor of Cdk

siRNA – small interference RNA

Sit4 – suppressor of initiation of transcription

Slt2 – suppressor of the lytic phenotype

STRE – stress responsive elements

Swi4/6 – mating type switching

Taf1 – TBP associating factor

TBP – TATA-box binding protein

Tip1 – temperature shock-inducible protein

TOR – target of rapamycin

TSC1/2 – tuberous sclerosis complex

UAS – upstream activating sequence

uORF – upstream ORF

UTR – 5'-untranslated region

UV – ultraviolet

YEP – yeast extract peptone

YPD – yeast extract peptone dextrose

CHAPTER I
BACKGROUND AND INTRODUCTION

"The most obvious differences between different animals are differences in size, but for some reason the zoologists have paid singularly little attention to them."

J. B. S. Haldane, On Being the Right Size, 1927

1.1 Summary

All cellular organisms propagate by means of division. Following fertilization, metazoan cells proliferate by a process known as mitosis to ultimately form a mature organism. Similarly, in unicellular organisms, unabated proliferation can result in vast populations of cells. However, research indicates that mitotic proliferation is tightly modulated by cell cycle controls (Vermeulen, Van Bockstaele et al. 2003). In order to proliferate, a cell must double its mass, faithfully replicate its chromosomes, and accurately segregate half of each into two cells. Moreover, within a given cell type, cells are remarkably similar in size. This suggests that cells coordinate growth with proliferation (Neufeld, de la Cruz et al. 1998; Stocker and Hafen 2000; Potter and Xu 2001). While it is known that cell growth and proliferation are strongly regulated by both intrinsic and extrinsic factors, many of the molecular and genetic mechanisms involved in coordinating cell growth with proliferation are not known.

Several decades ago, Hartwell and his coworkers achieved the first insight into understanding the mechanisms that coordinate cell growth with proliferation (Hartwell, Culotti et al. 1970; Hartwell, Culotti et al. 1974). By analyzing temperature-sensitive mutants in the yeast, *S. cerevisiae*, they found that inactivation of some genes essential for proliferation resulted in cells that arrested in specific phases of the cell cycle. Because these mutants blocked progression through the cell division cycle (*cdc* mutants), the genes encoding these mutants were called *CDC* genes (Figure 1.3). Analysis of the function of *CDC* genes has greatly elucidated the genetic and biochemical pathways that control cell cycle progression (Hartwell 1974; 1978).

Today, more than 50 *CDC* genes have been identified. Most of these have been cloned and nearly all have human homologues (Murray 1993). While all *cdc* mutants result in specific cell cycle arrests, the largest group consisting of 22 *cdc* mutants, arrest in G1-phase (Murray 1993) (Table 1.1). Careful analysis of these mutants revealed three fundamental details of the basic architecture of the cell cycle.

First, it was discovered that the cell cycle is composed of a series of interdependent steps that are initiated at the transition point between G1- and S-phase. Because of the relationship between this transition and cell cycle progression, this point was named Start in yeast (Figure 1.1) (Hartwell 1974; Hartwell, Culotti et al. 1974). Subsequently, it was shown that Start is analogous to the "restriction point" in mammalian cells (Pardee 1974) (Figure 1.2).

Second, it was found that progression past Start is dependent upon cell growth and the attainment of a minimum cell size (Johnston, Pringle et al. 1977) (Figure 1.2). A subset of G1-phase *cdc* mutants was shown to block cell growth. In this manner, cells smaller than the required minimum cell size arrested before Start (Johnston, Pringle et al. 1977). Little is known about the biochemical mechanisms responsible for linking cell growth and cell size to proliferation.

Third, while it was shown that proliferation is dependent upon cell growth, it was found that the converse is not true. Most *cdc* mutants that arrested in G1-phase fail to proliferate, but cell growth continues at the normal rate (Hartwell 1974; Johnston, Pringle et al. 1977). The manner in which these *cdc* mutants prevent proliferation despite normal cell growth is not well understood.

1.2 Research Questions

The long-term goal of my research is to investigate the intrinsic genetic and biochemical pathways involved in promoting proliferation and to examine the interaction of these pathways with extrinsic signals and stimuli. Thus, in the simplest sense, I am interested in how cell division is regulated. More specifically, my thesis research addressed the question "How are cell growth and proliferation coordinated?"

1.3 Thesis Outline

In this chapter, I will introduce what is known about the molecular, genetic, and biochemical mechanisms that cells use to coordinate cell growth with proliferation. To illustrate this coordination, I will start with the cell division machinery and then discuss how cell growth is coupled with cell division. This chapter culminates in highlighting the key questions in this field. In the second chapter, I detail the techniques and protocols used during my thesis research. Subsequently, in chapters 3-5, I detail the progress that I have made towards addressing these questions. In the final chapter, I discuss the significance and biological relevance of my research and point out what I feel are the major challenges for this field in the near future.

1.4 Historical Perspectives: Mitosis and the Cell Cycle

The first major milestone in the study of cell division came in 1838 when Schleiden and Schwann proposed the cell theory (Reviewed in Sharp 1926). This theory maintained that organisms are composed of individual proliferating units which they called cells. In 1869, Frederick Miescher discovered nucleic acids (Reviewed in Sharp 1926). Using aniline dyes, Walther Flemming in 1882 discoverd "threadlike structures" in the cell nucleus, which Heinrich Waldeyer later called chromosomes meaning colored bodies (Reviewed in Sharp 1926). These staining techniques allowed Flemming to visualize movements of chromosomes during cell division, which he named 'mitosis' from the Greek for thread. From 1887 to 1890, Theodor Boveri studied the actions of chromosomes during mitosis (Balmain 2001). He found that chromosomes remained organized as individual structures during mitosis. He coined the terms 'centrosome', 'centriole' and 'interphase', and showed that during interphase, chromosomes were not visible. Boveri concluded that each cell needed a full set of chromosomes for normal development (Balmain 2001). Most importantly, he suggested that chromosomes were involved in heredity. In the early 1950s, the idea that chromosomes are made of DNA had been generally accepted, but the number of molecules per chromosome and the nature of their arrangement remained a mystery. Thus, Hewson Swift set out to address

this question in 1950. He asked whether or not plants also cells contained the same amount of DNA, as in animal tissues. Swift showed that "DNA occurs in well-marked units characteristic of the strain or species" by photometric measurements on individual Feulgen-stained corn and *Tradescantia fluminensis* (a ground-cover plant) nuclei (Swift 1950). Years later, the duplication of DNA was studied in more detail by Alma Howard and Stephen Pelc in 1953 (Howard 1953). Their autoradiography showed that DNA synthesis occurs only within a certain limited period in the middle of interphase. This discovery ultimately led to the division of the eukaryotic cell cycle into the S, G1, M and G2 phases.

The mechanism behind DNA synthesis was unraveled five years later. Matthew Meselson and Franklin Stahl used density-gradient sedimentation and heavy nitrogen isotope ^{15}N-labeling of DNA molecules in *Escherichi coli*. They found that DNA replication is semi-conservative (Meselson and Stahl 1958).

The prosperous era of cell-cycle studies started with genetics in budding yeast *Saccharomyces cerevisiae* pioneered by Hartwell and colleagues in the 1970s and with biochemistry with Xenopus eggs by Hunt in 1980s (Hartwell, Culotti et al. 1970; Hartwell, Culotti et al. 1974; Hartwell 1978; Evans, Rosenthal et al. 1983; Murray 1993). In the following sections, I will discuss how their findings and other important work led to our current understanding of cell cycle regulation.

1.4.1 Yeast as a Model System for Cell Cycle Research

The budding yeast *Saccharomyces cerevisiae* has been widely used as a genetic model system since Hartwell's pioneering work because some of its properties make yeast particularly suitable for biological studies. For example, yeast is nonpathogenic and proliferates rapidly (90 min doubling time in rich medium). As a well-defined genetic system, mutants can be easily isolated and characterized. Genetic analysis in yeast is remarkably powerful in part because yeast can be propagated as both haploids and diploids. In addition, yeast can be straightforwardly transformed with exogenous DNA to create transgenic mutant or knock-out strains in a matter of weeks as compared

to months for higher eukaryotes. Importantly, *S. cerevisiae* has numerous genetic and phenotypic markers which greatly aid genetic approaches such complementation, meiotic analysis and molecular cloning.

Most significantly, yeast was the first eukaryotic organism to have a completely sequenced genome (Dujon 1996; Goffeau, Barrell et al. 1996). *S. cerevisiae* contains a haploid set of 16 well-characterized chromosomes, ranging in size from 200 to 2,200 kb. There are 6,183 open reading frames (ORF) of over 100 amino acids long and approximately 5,800 of them were predicated to correspond to actual protein-coding genes (Dujon 1996; Goffeau, Barrell et al. 1996). Additionally, the yeast genome is highly compact, with ORFs representing 72% of the total sequence (http://dbb.urmc.rochester.edu/labs/sherman_f/yeast/5.html). Even more importantly, yeast is the first organism in which nearly every ORF was systematically deleted (Winzeler, Shoemaker et al. 1999). Thus, unlike any other organism nearly every individual ORF knock-out is readily available. This has made *S. cerevisiae* one of the most convenient and powerful genetic model systems.

1.4.2 Yeast Nomenclature

The gene names used throughout the dissertation can be found in either the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces) or Proteome (www.proteome.com). The standard *S. cerevisiae* genetic conventions state that dominant or wild-type genes and their mRNAs are three letter capitalized and italicized acronyms (e. g. *CLN3* indicates the wild type gene or its mRNA). Recessive mutants are in lowercase italics (e.g. *cln3Δ* denotes a complete deletion, $cdc28^{ts}$ denotes temperature sensitive mutation, and *ura3-11* denotes a recessive loss of function mutation). Finally, when referring to yeast proteins the first letter of the acronym is capitalized as in Cln3 or Cln3p. Unfortunately, these nomenclature conventions are not shared by all model organisms.

1.5 How Is Cell Division Regulated in Yeast: Lessons Learned From the *CDC* Genes

Research indicates that nearly 1,100 of the ~6,300 genes in yeast are essential (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). This means that loss of function of these genes results in inviability. Because of this, it is not possible to make or study complete knock-outs (i.e. deletions) of these genes. Rather, conditional mutants (i.e. temperature sensitive alleles) must be made to examine the function of essential genes. The vast majority (>96%) of conditional alleles of essential genes do not have a specific cell cycle phenotype. These mutants, unlike *cdc* mutants, which cause a homogenous arrest at specific phases of the cell cycle, result in a random and heterogeneous cell cycle phenotypes (Figure 1.1).

Detailed analysis of *cdc* mutants has enabled the construction of a mechanistic wiring map of the cell cycle. From this map, two fundamental characteristics of cell cycle regulation were deduced. First it was found that *cdc* mutants could be broadly grouped into two categories: *CDC* genes that are integral parts of the cell cycle engine and *CDC* genes that are involved in signal transduction cascades involved in monitoring cell cycle progression (Hartwell 1974; Hartwell, Culotti et al. 1974). Mutants in the first category arrest the cell cycle because they directly disrupt the cell cycle engine. In contrast, mutants in the second category are now called "checkpoints" Checkpoints involve feedback loops that monitor the integrity and completion of downstream events. Thus, cell cycle is arrested upon disruption of the feedback loops, i.e. decreases in the integrity of some structures (e.g. DNA damage) or failure to complete downstream events (e.g. DNA replication) (Murray 1993).

At the G1/S-phase transition, yeast initiates three independent cell cycle events: DNA replication, budding, and spindle-pole body duplication (analogous to centrosome replication in higher eukaryotes) (Murray 1993). A number of *cdc* mutants were found to specifically disrupt each of these processes. For example *cdc7*, *cdc24*, and *cdc31* mutants prevent the initiation of DNA replication, budding, and spindle-pole body duplication, respectively (Murray 1993). In addition, a small number of *cdc* mutants were found to block all three of these events simultaneously. Of these, the most intensely studied has

been the *CDC28* gene. Of all the *cdc* mutants identified, *cdc28* mutants arrested cells earliest in the cell cycle at a point just prior to the G1/S-phase transition. For this reason, yeast geneticists named this point Start (Hartwell, Culotti et al. 1974) (Figures 1.2).

1.6 Cell Size and Cdks: Diverging Observations But Converging Mechanisms

Early on, cell cycle research was dominated by three organisms, two yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the frog *Xenopus laevis*. Yeast were popular because of they were extremely versatile genetic systems. Likewise, frog eggs were a biochemical dynamo (de Robertis, Gurdon et al. 1977; Dawid and Sargent 1988; Charbonneau and Grandin 1989). Like *S. cerevisiae* (the budding bakers yeast), *S. pombe* (the fission yeast) derives its name from the African beer *pombe* as it has been widely used for brewing beer and fermenting grapes into wine. Because of the tremendous success the *CDC* genes garnered for the budding yeast, fission yeast researchers began conducting identical studies (Bartlett and Nurse 1990). Unfortunately, researchers used the similar genetic nomenclature (i.e. *S. pombe* genes are named with lower case italicized three letter acronyms), but they did not attempt to use same numbers. Thus, to date there are 28 fission yeast *cdc* genes (i.e. *cdc1-cdc28*) (Nurse and Thuriaux 1980). However, this lack of foresight has generated a good deal of unnecessary confusion. For example, the fission yeast *cdc28* gene is in no way related to budding yeast *CDC28* gene.

In the course of studying cell cycle genetics in fission yeast, researchers identified three key regulators of mitosis: *cdc2*, *cdc25*, and *wee1* (Nurse, Thuriaux et al. 1976). Subsequent studies suggested that *cdc2* was a key regulator of cell cycle progression (Simanis and Nurse 1986; Langan, Gautier et al. 1989; Norbury and Nurse 1989). For example, a dominant gain of function mutation in *cdc2* (*cdc2-3w*) advanced entry into mitosis and made cells smaller than normal, and loss of function mutations in *wee1* had the same phenotype (Sveiczer, Novak et al. 1996). In contrast, loss of function mutations in both *cdc25* and *cdc2* resulted in a cell cycle arrest (MacNeill, Warbrick et al. 1991; Sveiczer, Novak et al. 1996). Moreover, creation of double mutants proved to be

informative. While the *cdc2 cdc25* and *cdc2 wee1* double mutants still resulted in a cell cycle arrest, *cdc25 wee1* had a remarkably normal cell cycle phenotype. This suggested that *cdc25* and *wee1* mutations were self-canceling. Finally, it was shown that the *wee1* mutation was synergistic with *cdc2-3w* mutations (MacNeill, Warbrick et al. 1991; Sveiczer, Novak et al. 1996). From these genetic interactions, it was proposed that Cdc2 promoted cell cycle progression in a manner that was stimulated by Cdc25 and inhibited by Wee1 (Fleig and Gould 1991; Berry and Gould 1996; Kellogg 2003).

During this same time period, researchers using biochemical experiments in frog eggs were beginning to identify and purify factors involved in promoting cell cycle progression. Treatment of isolated frog eggs with progesterone stimulates meiotic cell cycle progression. Using this system, Masui and colleagues conducted a number of elegant experiments. Briefly, Masui et al. showed that the factors activated by progesterone could be transmitted from one cell to another via cytoplasmic transfer experiments (Masui and Markert 1971). In these, experiments ~5% of the cytoplasm from a progesterone treated cells was injected into a naïve cell. This induced cell cycle progression. Moreover, if the injected eggs were subsequently used as donors, 5% of this cytoplasm was sufficient to induce cell cycle progression in another naïve cell (Masui and Markert 1971). They named this transferable factor(s) maturation promoting factor (MPF) (Masui and Markert 1971). Because the reaction was auto-catalytic, they proposed that MPF activated itself by turning pools of pre-MPF in naïve cells into active MPF (Masui and Markert 1971).

Subsequently, a number of investigators showed that MPF activity rises and falls during each cell cycle (Evans, Rosenthal et al. 1983; Gerhart, Wu et al. 1984). Moreover, this activity was dependent upon fresh protein synthesis in each new cycle (Evans, Rosenthal et al. 1983; Gerhart, Wu et al. 1984). Biochemical studies in sea urchin eggs identified a protein whose levels oscillated in parallel with MPF activity. Hunt et al. named this protein cyclin in reference to its periodic appearance and disappearance. Based upon this discovery, Hunt et al. proposed that the function of cyclins was to activate MPF complexes (Evans, Rosenthal et al. 1983).

As discussed above, *S. pombe* researchers identified two mutations, *cdc2-3w* and *wee1*, that resulted in abnormally small cells (MacNeill, Warbrick et al. 1991). In fact, *wee1* was so named in reference to the small cell size of these mutants. More importantly, experiments showed that in normal cells commitment to proliferation was dependent upon the achievement of a minimum cell size. This cell size threshold is now known as the critical cell size (Figure 1.2). The *cdc2-3w* and *wee1* mutations resulted in abnormally small cells because they reduced critical cell size. Genetic screens for small cell mutants in *S. cerevisiae* initially identified only a single mutant, *whi1-1* (Sudbery, Goodey et al. 1980).

Using genetic complementation experiments, Nurse et al. demonstrated that *S. pombe* cdc2 was a kinase that was highly conserved in all eukaryotes (Simanis and Nurse 1986). In fact, the *S. cerevisiae* homologue of cdc2 was found to be Cdc28, the protein required for progression past Start (Hartwell, Culotti et al. 1974). Importantly, all of these independent discoveries converged after several remarkable discoveries in 1988. First, Futcher, and colleagues cloned the *whi1-1* mutant and demonstrated that it encoded a cyclin (Cross 1988; Nash, Tokiwa et al. 1988; Cross 1989). The *whi1-1* mutant had an in frame stop mutation that removed part of the C-terminus of the protein (Nash, Tokiwa et al. 1988). This region contained a sequence rich in prolines, aspartic acids, serines, and threonines that was responsible for greatly destabilizing the protein (Salama, Hendricks et al. 1994). These motifs are now known as PEST motifs and are widely recognized as being involved in protein stability (Rogers, Wells et al. 1986). In addition, biochemical purification of MPF revealed that it consisted of a cyclin bound to Cdc2 (Dunphy, Brizuela et al. 1988; Gautier, Norbury et al. 1988; Labbe, Lee et al. 1988). Finally, Reed and colleagues showed using a genetic suppressor screen that over-expression of two cyclin genes rescued the temperature sensitive phenotype of *cdc28* mutations. These genes were named *CLN1* and *CLN2*, for cyclin 1 and 2 (Reed, Hadwiger et al. 1989), and the *WHI1* gene was renamed *CLN3* (Richardson, Wittenberg et al. 1989). Thus, in a very short period of time it became clear that cyclins were

positive regulators of Cdks, and that this complex was essential for cell cycle progression (Cross 1990).

1.7 Role of Cdks in the Commitment oo Cell Division

The function of Cdks in commitment to cell division turned out to be highly conserved among all eukaryotes from yeast to man (Figure 1.3) (Hartwell and Kastan 1994; Murray 1994; Nurse 1997; Murray 2004) (Lee and Nurse 1987; Murray 1994; Woollard and Nurse 1995; Blagosklonny and Pardee 2002). Cdks are proline-directed kinases that phosphorylate serine or threonines residues in S/T-P motifs (Langan, Gautier et al. 1989). Cdks are so highly conserved that mammalian Cdks can complement their yeast counterparts (Lee and Nurse 1987). A cross-species alignment of Cdks is shown in Figure 1.4. Cdks are bi-lobed proteins with a β-sheet at the N- terminus and an α-helix at the C-terminus (De Bondt, Rosenblatt et al. 1993). In between the two lobes, there is an ATP binding domain (De Bondt, Rosenblatt et al. 1993). Whereas mammalian cells have at least seven Cdks involved in cell cycle regulation, yeast probably only have one, Cdc28 in *S. cerevisiae* and Cdc2 in *S. pombe* (Murray 1994; Miller and Cross 2001).

In yeast, progression past Start requires Cdc28 binding to Cln3, a G1-phase cyclin, and phosphorylating a cell cycle inhibitor Whi5 (Figure 1.5) (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004; Schaefer and Breeden 2004). This phosphorylation relieves its association and thus repression of two transcription factors, SBF and MBF (Breeden and Nasmyth 1987; Nasmyth and Dirick 1991; Moll, Dirick et al. 1992; Koch, Moll et al. 1993; Sidorova and Breeden 1993; Baetz and Andrews 1999). These two transcription factors in turn, up-regulate expression of other genes including the two other G1 phase cyclins, *CLN1* and *CLN2* (Figure 1.5) (Koch, Moll et al. 1993; Sidorova and Breeden 1993; Koch, Schleiffer et al. 1996; Sidorova and Breeden 1997). Subsequently, Cdc28-Cln1/2 complexes phosphorylate another cell cycle inhibitor, Sic1 (Schneider, Yang et al. 1996; Tyers 1996; Verma, Annan et al. 1997). Phosphorylation of Sic1 targets it for ubiquitination and degradation (Schneider, Yang et al. 1996; Verma, Annan et al. 1997). The degradation of Sic1 activates S-phase cyclin

Cdk complexes (e.g. Clb5-Cdc28 and Clb6-Cdc28) that initiate S-phase entry (Schneider, Yang et al. 1996; Tyers 1996; Verma, Annan et al. 1997).

Cell cycle progression is remarkably similar in mammalian cells (Figures 1.3 and 1.5). Cyclin D, the mammalian orthologue of Cln3, forms a complex with Cdk4 and activates E2F and related transcription factors by phosphorylating pRB and relieving its inhibitory effect on a transcription factor E2F, which induces genes for S phase entry, including the cyclin E (Mittnacht 1998; Stevens and La Thangue 2003; Schaefer and Breeden 2004) (Figure 1.5). Subsequently, Cyclin E-Cdk complexes phosphorylate p27 an inhibitor of S-phase Cdks. Because the basic cell cycle machinery is highly conserved between yeast and mammalian cells, for the remainder of this chapter, I will focus on important details of cell cycle regulation in yeast.

1.8 Regulation of Cdc28 at Start

The protein and mRNA levels of Cdc28 remain constant throughout the cell cycle (Mendenhall, Jones et al. 1987). The Cdc28 protein alone has little or no kinase activity, because its substrate binding site is blocked by a T-loop (Mendenhall and Hodge 1998). Therefore, Cdc28 activity is modulated at different cell cycle positions primarily by posttranslational mechanisms. These include activating events such as stimulatory phosphorylation by Cak1 (cyclin activating kinase) at threonine 169 and binding of cyclins or repressing events such as inhibitory phosphorylation at tyrosine 19 (Wu, Yee et al. 1994; Levine, Tinkelenberg et al. 1995; Kaiser, Sia et al. 1998; Mendenhall and Hodge 1998).

1.8.1 Activation of Cdc28 by Cak1

In *S. cerevisiae*, Cdc28 is activated by phosphorylation of threonine 169 by monomeric Cak1 (Cyclin-dependent kinase-activating kinase) (Cismowski, Laff et al. 1995; Kaldis, Sutton et al. 1996). Because *CAK1* is an essential gene (Cross and Levine 1998), deletion of *cak1* is lethal to cells. However, Cak1 was dispensable in a *cdc28*

mutant, in which T169 was mutated to glutamic acid, which mimics constitutive phosphorylation status (Cross and Levine 1998).

1.8.2 Inhibition of Cdc28 Activity

In fission yeast, the activity of Cdc2, the Cdc28homolog, can be inhibited by Wee1 kinase (Russell and Nurse 1987). Wee1 inhibits Cdc2 by phosphorylating Y15 at the N-terminus. This phosphorylation is antagonized by a phosphatase Cdc25 (Gautier, Solomon et al. 1991). This mechanism is well conserved in all eukaryotes (Kellogg 2003). In budding yeast, Cdc28 can be repressed by Swe1 kinase, the homolog of Wee1 which phosphorylates Cdc28 on Y19 (Booher, Deshaies et al. 1993). This phosphorylation can be reversed by a phosphatase Mih, the homolog of Cdc25 (Russell and Nurse 1986).

1.8.3 Activation of Cdc28 by G1-Phase Cyclins

There are two groups of cyclins in *S. cerevisiae* that bind to and activate Cdc28: G1-phase cyclins and B-type cyclins (Mendenhall and Hodge 1998). G1-phase cyclins include Cln1-3 (Clns) that have redundant but crucial functions (Hadwiger, Wittenberg et al. 1989; Richardson, Wittenberg et al. 1989). Loss of any two G1-phase cyclins is not lethal. However, loss of all three Clns results in inviable cells. In addition, there are six B-type cyclins, Clb1-6, which govern the remaining portion of the cell cycle (Mendenhall and Hodge 1998). None of the B-type cyclins are required for progression past Start.

Research indicates that one of the major limiting factors for the activation of Cdc28 prior to Start is the abundance and availability of G1-phase cyclins (Cross 1990). Studies have shown that the timing of Start is tightly modulated by G1-phase cyclin levels (Stuart and Wittenberg 1995). Increased or premature expression of G1-phase cyclins advances Start while decreased or delayed expression postpones Start (Cross 1990). Importantly, Cln expression is tightly controlled both transcriptionally and post-transcriptionally in a manner that is very sensitive to environmental stimuli (Yaglom,

Linskens et al. 1995; Gallego, Gari et al. 1997; Radcliffe, Trevethick et al. 1997; Hall, Markwardt et al. 1998). Moreover, Cln1 and Cln2 abundance peaks at Start (Figure 1.6).

1.9 Transcriptional Regulation of *CLN* mRNA

1.9.1 Regulation of *CLN3* mRNA Transcription

The regulation of transcription is the one of the very first steps of gene expression. *CLN3* mRNA expression is relatively constitutive and low during the cell cycle (Cross and Blake 1993; Tyers, Tokiwa et al. 1993). However, a modest peak of *CLN3* mRNA levels occurs in late M phase or early G1 phase (Dirick and Nasmyth 1991; Cross and Blake 1993; Tyers, Tokiwa et al. 1993). This peak of expression is dependent upon two ECB (Early Cell Cycle Box) sites between -968 and –916 relative to the transcription initiation site in the *CLN3* promoter. The Mcm1 transcription factor promotes *CLN3* transcription by binding to these two sites (McInerny, Partridge et al. 1997; Simon, Barnett et al. 2001; Mai, Miles et al. 2002). In addition, the Ace2 and Swi5 transcription factors bind to several consensus sites in the *CLN3* promoter that is located at -1185 to -1016 upstream of the transcription initiation site. Binding to these consensus sites occurs in late M phase (Simon, Barnett et al. 2001). Unlike Mcm1, Ace2 represses *CLN3* transcription as confirmed by the observations that expression of Ace2 delays Start by inhibiting *CLN3* mRNA expression (Laabs, Markwardt et al. 2003). Interestingly, this occurs only in daughter cells (Laabs, Markwardt et al. 2003). It is postulated that Ace2 may repress a *CLN3* activator, through a motif CCATTGCATTTC called DDE (daughter delay element) (Laabs, Markwardt et al. 2003).

In addition to its cell cycle regulated transcription, *CLN3* mRNA abundance is regulated by glucose. *CLN3* mRNA abundance is high in glucose-grown log-phase cells and lower in post-diauxic cells (cells that have depleted glucose from the medium and are growing on ethanol) (Parviz, Hall et al. 1998; Parviz and Heideman 1998; Newcomb, Diderich et al. 2003). However, *CLN3* mRNA can also be induced in post-log cells when fresh glucose medium is added (Parviz, Hall et al. 1998; Parviz and Heideman 1998; Newcomb, Diderich et al. 2003). This induction is specific to glucose metabolites,

because non-metabolized glucose analogs (2-deoxyglucose, L-glucose and 6-deoxyglucose) failed to induce *CLN3* mRNA transcription (Parviz, Hall et al. 1998; Parviz and Heideman 1998). This glucose response is dependent upon Azf1, a zinc finger transcription factor that recognizes two AAGAAAA motifs between -626 and -570 of the *CLN3* promoter. Azf1 specifically mediates the glucose-dependent transcription of *CLN3* because deletion of *AZF1* has no effect on *CLN3* mRNA abundance in cells growing in ethanol (Parviz and Heideman 1998; Newcomb, Hall et al. 2002). Furthermore, this response is dependent upon Ada2 and Ada3, two transcriptional adaptors that are components of the SAGA (Spt/Ada/Gcn5/Acetylase) complex, because loss of these two genes abolishes the induction (Wu, Newcomb et al. 1999).

1.9.2 Regulation of *CLN1* and *CLN2*

1.9.2.1 Regulation by SBF and MBF

When yeast cells reach critical cell size (Figures 1.2 and 1.5), Cln3-Cdc28 complexes activate two transcription factors SBF and MBF (Koch, Schleiffer et al. 1996), which in turn, promote expression of *CLN1* and *CLN2* mRNA (Figure 1.6). SBF is a heterodimer of Swi6 and Swi4 while MBF is a heterodimer of Swi6 and Mbp1 (Andrews and Herskowitz 1989; Koch, Moll et al. 1993). The DNA binding subunit of SBF is Swi4, and Mbp1 in MBF. SBF recognizes CACGAAAA (SCB sites) (Figure 1.5). Interestingly, this site is remarkably similar to the mammalian E2F site (GCGCAAA) (Weinmann, Yan et al. 2002). MBF binds to ACGCGTNA (MCB sites) (Dirick, Moll et al. 1992; Lowndes, Johnson et al. 1992; Iyer, Horak et al. 2001). Koch et al. showed that SBF binds to SCB elements in the *CLN2* promoter in a cell cycle regulated manner. Moreover, this binding occurs prior to Start (Koch, Schleiffer et al. 1996). Cln3 function is largely dependent on Swi6 (Wijnen, Landman et al. 2002), a common subunit for two transcription factors SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (MluI binding factor) (Andrews and Moore 1992; Dirick, Moll et al. 1992; Koch, Moll et al. 1993; Baetz and Andrews 1999). Microarray analysis revealed that 76 genes are induced by Cln3 at mid-G1 phase (Spellman, Sherlock et al. 1998). Of these, 58% have at least

one MCB site and 52% of them have at least one SCB site (Spellman, Sherlock et al. 1998). Moreover, genomic-wide chromatin immunoprecipitation analysis of SBF and MBF indicate that these transcription factors bind the promoters of 235 genes (Iyer, Horak et al. 2001). Because of this, it is not surprising that *swi4Δ swi6Δ* (SBF null mutants) and *swi4Δ mbp1Δ* double mutants are inviable (Nasmyth and Dirick 1991). However, it is intriguing that constitutive expression of *CLN2* under a spADH promoter rescues these phenotypes (Nasmyth and Dirick 1991), suggesting that *CLN2* expression is entirely dependent upon SBF and MBF.

1.9.2.2 Regulation of *CLN1* and *CLN2* by Bck2

CLN3 is not the only gene that induces *CLN1* and *CLN2* transcription. Genetic studies have indicated that the Bck2 (Bypass of C Kinase) protein also activates *CLN1* and *CLN2* transcription in an SBF and MBF dependent manner (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). Like *cln3*Δ mutants, *bck2*Δ mutants are larger than the wild type (Wijnen and Futcher 1999). While deletion of either *bck2*Δ or *cln3*Δ results in viable cells, the *bck2*Δ *cln3*Δ double mutant is inviable and arrests in G1-phase (Wijnen and Futcher 1999). This suggests that Bck2 and Cln3 function in independent parallel pathways (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). The lethality of *bck2*Δ *cln3*Δ double mutant can be rescued by constitutive expression of *CLN2* (Wijnen and Futcher 1999), suggesting that inducing the transcription of *CLN2* (and *CLN1*) is the essential role of these pathways. Interestingly, loss of the Start inhibitor, Whi5, rescues the phenotype of *bck2*Δ *cln3*Δ double mutants (Costanzo, Nishikawa et al. 2004). This suggests that Bck2 and Cln3 induce *CLN1* and *CLN2* transcription by repressing Whi5 function. It is known that Cln3-Cdc28 complexes can phosphorylate Whi5 (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). However, the biochemical nature and function of Bck2 remain unknown. Moreover, the lack of an obvious mammalian Bck2 homologue or orthologue complicates the elucidation the function of Bck2.

1.9.2.3 Other Transcription Factors Regulating *CLN1* and *CLN2* Transcription

Additional transcription factors affect *CLN1* and *CLN2* transcription. For example, a major upstream activating sequence (UAS1) in the *CLN2* promoter contains two Rme1 Response Elements (RRE) (-561 to -551 and -672 to -662) (Frenz, Johnson et al. 2001). Rme1 is a zinc-finger transcription factor that stimulates *CLN2* transcription. Also, there are factors that negatively regulate *CLN1* and *CLN2* transcription. Upon nutrient exhaustion, *CLN1* and *CLN2* mRNA expression is repressed by Whi2 (Saul and Sudbery 1985; Radcliffe, Trevethick et al. 1997). Moreover, *whi2*Δ mutants have higher levels of *CLN1* and *CLN2* mRNA than the wild type at stationary phase (Saul and Sudbery 1985; Radcliffe, Trevethick et al. 1997). In addition, over-expression of *CLN1* mimics the *whi2*Δ phenotype (Radcliffe, Trevethick et al. 1997). In contrast, *CLN3* mRNA expression is not affected by Whi2 (Radcliffe, Trevethick et al. 1997). However, the mechanism whereby Whi2 represses *CLN1* and *CLN2* expression is not clear. Finally, as previously mentioned, Whi5 is also a negative regulator of *CLN1* and *CLN2* expression (Figure 1.5) (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004).

1.9.2.4 General Transcriptional Machinery

General transcription factors also play important roles in *CLN1* and *CLN2* transcription. For example, Taf1 (yTAF$_{II}$145) is a component of the TFIID general transcription factor (Mal, Masutomi et al. 2004). Inactivation of Taf1 in temperature sensitive mutants leads to G1-phase arrest, resulting from decreased *CLN1* and *CLN2* transcription (Danaie, Altmann et al. 1999). The mechanism for this observation is not known, but one possibility is that inactivation of Taf1 dramatically decreases expression of Ctr9 (Koch, Wollmann et al. 1999). Ctr9 is a component of the RNA polymerase II holoenzyme with other proteins such as Cdc73, Hpr1, Ccr4, Rtf1, Paf1 and Leo1 (Koch, Wollmann et al. 1999; Mueller and Jaehning 2002). Inactivation of Ctr9 or Paf1 or Cdc73 abolishes or delays *CLN2* expression (Koch, Wollmann et al. 1999). Many genes

whose transcription is modulated by Paf1 are induced at Start in an SBF and MBF-mediated manner (Chang, French-Cornay et al. 1999; Koch, Wollmann et al. 1999). However, a biochemical interaction between SBF/MBF and the RNA polymerase II holoenzyme remains to be established.

1.10 Posttranscriptional Regulation of *CLN* mRNA and Cln Proteins

Post-transcriptional mechanisms also have critical roles in regulating Cln expression. These mechanisms include mRNA and protein localization, protein stability and translation efficiency of Cln proteins.

1.10.1 Localization of *CLN3* mRNA by Whi3

In order to be efficiently translated, *CLN3* mRNA must be recruited to polysomes. However, Whi3 binds *CLN3* mRNA specifically via a RNA-recognition motif (RRM) at its C-terminus, and confines a subset of *CLN3* mRNAs to distinct cytoplasmic foci (Gari, Volpe et al. 2001). This binding may block *CLN3* mRNA accessibility to the translational machinery, and therefore negatively modulates Cln3 protein expression (Gari, Volpe et al. 2001).

1.10.2 Localization of Cln2 and Cln3 Protein

Cln protein localizations are also critical to their functions. For example, nuclear localization of Cln3 is required for its ability to activate the SBF and MBF transcriptions factors (Miller and Cross 2000; Edgington and Futcher 2001). Research has demonstrated that Cln3 localization is directed by a bipartite NLS at the C-terminus (Edgington and Futcher 2001; Miller and Cross 2001). In early G1-phase, Whi3 retains the Cln3 protein in the cytoplasm (Wang, Gari et al. 2004). In contrast, throughout the cell cycle, Cln2 is localized in both cytoplasmic and nuclear compartments (Miller and Cross 2000; Edgington and Futcher 2001). Cytoplasmic Cln2 is associated with sites of budding, consistent with Cln2 functions required for budding (Miller and Cross 2000; Edgington and Futcher 2001). Additionally, Cln2 protein shuttles between the

cytoplasmic and nuclear compartments, implying that Cln2 nuclear localization is also required for its normal functions (Edgington and Futcher 2001; Miller and Cross 2001). However, a nuclear function for Cln2 remains to be established.

1.10.3 Cln3 Translation

Cln3 translation is very sensitive to global translation rate (Hall, Markwardt et al. 1998; Parviz, Hall et al. 1998). For example, moderate inhibition of protein synthesis with 35uM cycloheximide decreases protein synthesis by 50% and causes cell division arrest, due to a dramatic fall in Cln3 protein levels (Hall, Markwardt et al. 1998). Mutations in *CDC33*, *CDC63*, or in TOR genes block cell growth (Hanic-Joyce, Johnston et al. 1987; Barbet, Schneider et al. 1996; Danaie, Altmann et al. 1999; Newcomb, Diderich et al. 2003). In each case, cells arrest as non-growing and non-proliferating G1-phase cells. Each of these genes is involved in ribosome biogenesis and function. Interestingly, constitutive expression of *CLN3* rescues this phenotype (Polymenis and Schmidt 1997). These data suggest that That is, when growth is high at the optimal condition, Cln3 protein translation increases, leading to fast division.

In the 5' leader of the *CLN3* mRNA, there is a short upstream open reading frame, which represses effective translation of Cln3 protein when protein synthesis and cell growth rate are low (Polymenis and Schmidt 1997). When growth conditions are favorable, Cln3 protein is efficiently translated by a leaky scanning mechanism that increases Cln3 protein abundance (Polymenis and Schmidt 1997). When the ATG was mutated to TTG to eliminate the uORF, the translational efficiency was increased, with decreased critical cell size and increased resistant to α factor similar to that in the dominant *whi1-1* mutants (Cln3-1 mutants) (Polymenis and Schmidt 1997). Taken together, these data are consistent to the above conclusion that cells use Cln3 protein levels to coordinate cell growth and cell division.

1.10.4 Stability of Cln Proteins

1.10.4.1 Cln3 Stability

Cln3 protein abundance is constitutively during the cell cycle, similar to its mRNA(Tyers, Tokiwa et al. 1993). The protein abundance is determined not only by its production, but also by its turnover. Cln3 is very unstable with a half life of several minutes (Tyers, Tokiwa et al. 1992; Schneider, Patton et al. 1998). Therefore, its abundance is extremely sensitive to its turnover. This instability is conferred by the PEST-rich domains in its C-terminus that is found in all three yeast G1-phase cyclins (Salama, Hendricks et al. 1994; Rechsteiner and Rogers 1996). Loss of these sequences leads to stabilization and accumulation of Cln3 proteins. For example, in *CLN3-1* mutants, Cln3-1 proteins are stabilized and hyperactive due to a truncation at the C-terminus resulting from a nonsense mutation (Nash, Tokiwa et al. 1988).

1.10.4.2 Cln1 and Cln2 Protein Stability

The cyclic accumulation of Cln1 and Cln2 proteins in late G1-phase (Figure 1.6) is controlled by both periodic transcription of *CLN1* and *CLN2* and rapid degradation of Cln1 and Cln2 proteins. Like Cln3, the Cln1 and Cln2 degradation is also determined by the PEST sequences in the C-terminus since truncation of this domain causes stabilization of Cln2 (Rowley, Singer et al. 1991). Under physiological conditions, the half-lives of Cln proteins are less than 5 minutes (Tyers, Tokiwa et al. 1992; Schneider, Patton et al. 1998).

The C terminus of Cln1 and Cln2 contains seven consensus Cdc28 phosphorylation sites. Mutation of all these sites stabilizes Cln proteins, suggesting that Cdc28-dependent auto-phosphorylation is responsible for their instability (Lanker, Valdivieso et al. 1996). Subsequently, the phosphorylated Cln proteins are targeted for ubiquitination by the SCF^{Grr1} and degradation by 26S proteosome (Li and Johnston 1997; Kishi and Yamao 1998; Berset, Griac et al. 2002). Interestingly, a similar model has been proposed for mammalian G1-phase cyclins D1 and E, suggesting that the precise expression and destruction of G1 cyclins are crucial for proper regulation of the cell cycle

and that this mechanism is highly conserved from yeast to humans (Diehl, Zindy et al. 1997).

1.11 How Are Cell Growth and Cell Division Coordinated?

Under optimal conditions, external stimuli, such as nutrients and growth factors, promote cell growth and division. In this case, the term cell growth is not synonymous with the term proliferation but instead refers primarily to the collection of processes responsible for the addition of cell mass. Genetic studies have revealed that several conserved pathways are implicated in integrating external signals into cell growth and division machinery and coordinating these processes appropriately.

1.11.1 The TOR Pathway Coordinates Cell Growth and Division

1.11.1.1 Summary of the TOR Pathways

The anti-fungal agent, rapamycin, prevents cell growth and proliferation in *S. cerevisiae* and results in a G1-phase arrest. A genetic screen for mutants that conferred resistance to rapamycin identified two genes *TOR1* and *TOR2* (Target of Rapamycin) (Heitman, Movva et al. 1991). Cloning of these genes revealed that TOR proteins are highly homologous to the phosphatidylinositol kinases PI-3K and PI-4K. It is now recognized that TOR proteins belong to a family of phosphatidylinositol kinase-related kinases (PIKKs) (Brunn, Hudson et al. 1997). Despite their homology to lipid kinases TOR proteins are Serine/Threonine kinases (Brown, Beal et al. 1995). TOR proteins are well conserved in eukaryotic cells, including yeast, worms, flies, plants and mammals (Shamji, Nghiem et al. 2003).

In yeast, the TOR signaling pathway stimulates cell growth and represses autophagy (Schmidt, Kunz et al. 1996; Schmelzle and Hall 2000). Autophagy is a process whereby cells recycle unneeded cytoplasmic mass by degrading cellular components into their basic constituents. Genetic analyses of the TOR function in yeast have identified at least five independent signaling pathways (Figure 1.7A). These pathways include mechanisms that promote ribosome biogenesis and stimulate

translation initiation, promote mRNA stability, modulate transcription, modulate phosphatase activity, and repress signaling through the cell integrity pathway. Each of these pathways is discussed briefly below.

1.11.1.2 TOR Pathways Stimulate Growth via Ribosomal Protein S6 Kinase

The quality and availability of nutrients modulates cell growth. Under optimal conditions and in the presence of rich nutrients, cell growth is rapid. Conversely, under starvation conditions, cell growth is minimal. Evidence suggests that the TOR signaling pathways respond to both the abundance and quality of extra-cellular nutrients. In addition, inactivation of these pathways (e.g. with rapamycin or in *tor1 tor2* double mutants) mimics nutrient starvation (Loewith, Jacinto et al. 2002).

One manner in which TOR stimulates cell growth is by promoting ribosome biosynthesis and increasing protein translation rates (Figure 1.7B). This is accomplished by increasing the transcription of ribosomal protein genes (Schmelzle and Hall 2000; Fingar and Blenis 2004; Fingar, Richardson et al. 2004). Interestingly, the RSC chromatin-remodeling complex is recruited to ribosomal protein gene promoter in a Tor-dependent manner (Ng, Robert et al. 2002). However, it is not yet known if this complex is a direct target of TOR.

TOR signaling increases protein translation by at least two independent pathways. One of these pathways involves the ribosomal protein S6 kinase (S6K). *In vitro*, TOR phosphorylates threonine 389 of S6K (Kim and Chen 2000). However, whether S6K is phosphorylated directly by TOR *in vivo* is controversial. The phosphorylation of S6K increases the translational efficiency of a class of mRNA transcripts that contain a terminal oligopyrimidine tract at their 5'-end (5'-TOP) (Jefferies, Fumagalli et al. 1997; Dennis, Fumagalli et al. 1999). Many of these 5'-TOP mRNAs encode components of the translation machinery, such as ribosomal proteins and elongation factors (Sonenberg and Gingras 1998). Therefore, S6K up-regulates the general translational capacity of the cell by enhancing the translation of components required for protein synthesis.

1.11.1.3 TOR Pathways Stimulate Growth via 4E-BP

A second pathway whereby TOR increases protein translation rates involves interaction with translation initiation factors (Figure 1.7B). 4E-BP1 (eIF4E Binding Protein) is a repressor of the translation initiation factor eIF4E. Observations have demonstrated that eIF4E is the rate-limiting translation initiation factor (Gingras, Raught et al. 2001). In yeast, eIF4E is encoded by the *CDC33* gene, and *cdc33* mutants arrest at Start (Danaie, Altmann et al. 1999). eIF4E binds to the Cap structure (m7GpppN) at the 5'-end of mRNA transcripts to initiate cap-dependent translation (Gingras, Raught et al. 2001).

In the absence of nutrients or growth factors, eIF4E function is repressed by the binding of hypo-phosphorylated 4E-BP1 (Lawrence and Abraham 1997). In response to rich nutrients, TOR phosphorylates N-terminal residues of 4E-BP1 at threonine 37 and threonine 46, and possibly at threonine 70 and serine 65 (Brunn, Hudson et al. 1997; Gingras, Raught et al. 2001). Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E (Sonenberg and Gingras 1998). Dissociation of this complex facilitates formation of the eIF4F complex (Sonenberg and Gingras 1998). The eIF4F complex is thought to initiate translation of mRNAs with extensive secondary structure in the 5'-untranslated region (UTR). As a result, ectopic expression of eIF-4E in mammalian cells results in the enhanced translation of poorly translated mRNAs that usually encode growth related genes (Lawrence and Abraham 1997; Sonenberg and Gingras 1998).

1.11.1.4 TOR Pathways Promote mRNA Stability

Cell growth and protein synthesis rates can also be modulated by the rate of mRNA turnover (Figure 1.7A). Genes with long mRNA half-lives will generate more protein per message than genes with short mRNA half-lives. In rapidly growing yeast, a deadenylation-dependent pathway is the major mechanism whereby mRNA decay rates are controlled (Jacobson 2004). In this case treatment with rapamycin or inactivation of TOR shortens the half-life of specific mRNAs (Albig and Decker 2001). While the mechanisms responsible for these observations are not known, it is interesting to note that

the Ccr4 protein, a component of the RNA PolII complexe, is also a major deadenylase in yeast (Chen, Chiang et al. 2002; Viswanathan, Chen et al. 2003).

1.11.1.5 TOR Pathways Modulate Transcription

As discussed above, TOR stimulates the transcription of RNA PolI-dependent rRNA genes and RNA PolIII-dependent tRNA genes. In addition, TOR inhibits at least four transcription factors: Rtg1, Rtg3, Msn2 and Msn4 (Figure 1.7A). Rtg1 and Rtg3 are involved in retrograde interorganelle communication between the mitochondria, peroxisomes, and the nucleus (Sekito, Thornton et al. 2000). This pathway has been associated with the control of apoptosis and aging (Jiang, Jaruga et al. 2000). Msn2 and Msn4 are transcription factors involved in a stress response signal transduction pathway, but like the Rtg1 and Rtg3 transcription factors, these factors are also involved in the control of aging (Jiang, Jaruga et al. 2000). It is not known if there are mechanisms that link cell growth to aging, but this possibility is discussed later in this chapter.

1.11.1.6 TOR Pathways Regulate Growth via Regulation of Phosphatases

Like kinases, phosphatases play important roles in cell growth and division, and some are regulated by TOR (Figure 1.7A and B). Under poor nutrient condition, TOR phosphorylates Tip41 to enhance its binding to Tap42, which represses two phosphatases Sit4 (PP6-like) and Pph21/22 (PP2A-like) (Jiang and Broach 1999). Moreover, treatment of cells with calyculin A, an inhibitor of type 1 and 2 protein phosphatases, prevents rapamycin-induced dephosphorylation of 4E-BP1, strongly suggesting that the phosphorylation status of serine 65 and threonine 70 of S6K are dependent upon phosphatases that are inhibited by TOR (Peterson, Desai et al. 1999). Similarly, in mammalian cells, phosphatases PP2A, PP4 and PP6 have also been identified as targets of the TOR signaling pathway in mammalian cells (Nanahoshi, Nishiuma et al. 1998; Peterson, Desai et al. 1999). Evidence showed that the binding of Tap42 homolog α4 with PP2A, PP4, and PP6 has been linked to rapamycin sensitivity (Nanahoshi, Nishiuma et al. 1998).

1.11.1.7 TOR Pathways Regulate Cln Expression

In *S. cerevisiae,* TOR activity is sensitive to the quality and abundance of nutrients (Fingar and Blenis 2004). Inhibition of TOR by rapamycin or by TOR deletions triggers a G1-phase arrest similar to nutrient starvation (Kunz, Henriquez et al. 1993; Helliwell, Wagner et al. 1994). Cln3 protein synthesis is also sensitive to both nutrients and rapamycin, suggesting that its translation can also be controlled by the TOR pathway (Barbet, Schneider et al. 1996). Genetic evidence has shown that eIF4F, a downstream target of the TOR pathway is involved in Cln3 translation (Barbet, Schneider et al. 1996; Polymenis and Schmidt 1997). As discussed above, eIF4E was identified as *CDC33*, a gene essential for progression past Start (Table 1). Interestingly, expression of stabilized Cln3 (Cln3-1) rescues this phenotype (Danaie, Altmann et al. 1999). In addition, expression of a *CLN3* ORF construct fused to the 5'UTR of *UBI4* gene allows the *CLN3* mRNA to be translated in a Cdc33-independent manner (Danaie, Altmann et al. 1999). Importantly, this construct rescues the G1-phase arrest of *cdc33* mutants (Danaie, Altmann et al. 1999).

TOR regulation of G1-phase cyclins is not restricted to yeast. In flies, cyclin E protein abundance is also low in TOR null mutant tissue, and ectopic expression of TOR rescues this phenotype (Zhang, Stallock et al. 2000). In mammalian cells, association of cyclin D1 mRNA with polysomes and consequently their translational efficiency is decreased by rapamycin (Gera, Mellinghoff et al. 2004). In addition to its function in translation initiation, it was found that when eIF4E is over-expressed in NIH3T3 cells, the majority of cyclin D1 mRNA is localized in the cytoplasm. However, in untransformed cells, the majority of cyclin D1 mRNA was confined to the nucleus (Topisirovic, Culjkovic et al. 2003). This result suggests that eIF-4E can somehow affect nucleo-cytoplasmic transportation of cyclin D1 mRNA, in addition to its role in translation initiation.

1.11.2 Coordination of Growth and Division by Ras/ PKA Pathway

In eukaryotes, Ras is a conserved small GTPase protein that transmits regulatory signals by converting GTP to GDP (Broach and Deschenes 1990; Coleman and Marshall 2001; Coleman, Marshall et al. 2004). Moreover, Ras is essential for proliferation. Microinjection of Ras proteins into mammalian G_0 cells induces S phase entry while microinjection of anti-Ras antibodies arrests cell proliferation (Dobrowolski, Harter et al. 1994). Ras is also strongly oncogenic (Coleman and Marshall 2001; Halfar, Rommel et al. 2001; Coleman, Marshall et al. 2004). Unlike mammalian cells, in yeast, there are two redundant genes, *RAS1* and *RAS2* (Breviario, Hinnebusch et al. 1986). Downstream of Ras, there are several interacting pathways involved in coordinating cell growth with proliferation, such as protein kinase A (PKA), PI3K, Raf and Myc pathways (Figure 1.8).

1.11.2.1 Summary of the Ras/PKA Pathway

In the budding yeast, glucose stimulates Ras (Breviario, Hinnebusch et al. 1986). As in other organisms, yeast Ras proteins transmit regulatory signals by shuttling between an inactive GDP-bound form and an active GTP-bound form (Weeks and Spiegelman 2003). The GTP-bound Ras proteins stimulate Cyr1 (a.k.a. *CDC35*), the adenylyl cyclase that contains a conserved Ras-binding site (Ras-associating domain). Activation of Cyr1 increases the intracellular cAMP level. In turn, cAMP activates PKA by liberating the yeast catalytic subunits, Tpk1, Tpk2 or Tpk3 from their inhibitory subunit Bcy1. Activated PKA can phosphorylate a number of proteins involved in transcription, energy metabolism and cell cycle progression (Thevelein and de Winde 1999; Weeks and Spiegelman 2003).

1.11.2.2 Ras/PKA Pathway Regulates Growth

Inactivation of adenylyl cyclase Cyr1 (Cdc35) leads to a decrease in cAMP and concomitantly a decrease in protein synthesis (Casperson, Walker et al. 1985). In contrast, increases in cAMP stimulate protein synthesis (Hall, Markwardt et al. 1998). For example, incubating cells in the presence of exogenous cAMP increased protein

synthesis rates nearly three fold (Hall, Markwardt et al. 1998). By comparing gene expression patterns in the presence and absence of cAMP in yeast, Neuman et al. showed that many genes for ribosomal synthesis such as PPL16A, RP28-1, were activated by cAMP (Neuman-Silberberg, Bhattacharya et al. 1995). This activation was dependent upon Rap1 sites in the promoters of these ribosomal genes. Rap1 is a transcription factor that becomes unstable upon cAMP depletion (Neuman-Silberberg, Bhattacharya et al. 1995).

1.11.2.3 PKA Regulates Cln Expression

As discussed above, loss of function of Cyr1 (*CDC35*) or the GTP exchange factor for Ras (*CDC25*), results in a G1-phase arrest (Casperson, Walker et al. 1985). Interestingly, G1-phase cyclin protein levels are strongly influenced by cAMP and by PKA activity (Polymenis and Schmidt 1997; Thevelein and de Winde 1999). For example, in the presence of glucose, cAMP levels and PKA activity are high as are Cln protein levels (Polymenis and Schmidt 1997; Thevelein and de Winde 1999). In addition, decreasing cAMP levels or PKA activity dramatically reduces Cln protein levels (Polymenis and Schmidt 1997; Thevelein and de Winde 1999). In fact, over-expression of *CLN3* can rescue the G1-phase arrest of *cyr1* mutants (Hall, Markwardt et al. 1998).

The C-terminus of Cln3 contains three phosphorylation sites for cAMP-dependent kinase (Hall, Markwardt et al. 1998). However, mutations of these sites did not affect Cln3 protein abundance (Hall, Markwardt et al. 1998). Thus, it is unlikely that PKA increases Cln3 protein levels directly by phosphorylation. Rather, it seems that 5' and 3' untranslated regions of Cln3 are important for cAMP regulation of Cln3 translation. Removal of these regions abolished this regulation (Hall, Markwardt et al. 1998). As previously discussed, there is an upstream open reading frame (uORF) at –315 of Cln3 mRNA. The presence of this uORF reduces translational efficiency under sub-optimal conditions (Polymenis and Schmidt 1997). Cln3 translation is very sensitive to the global translation rate. Because cAMP concentration reflects a general metabolic level, the presence of the uORF ensures that Cln3 levels sufficient to pass Start will not accumulate

when overall growth conditions are not optimal (Polymenis and Schmidt 1997; Hall, Markwardt et al. 1998).

1.11.3 Ras/PI3K Pathway Coordinates Cell Growth and Division

Another downstream target of Ras is PI3 kinase (PI3K), a heterodimer composed of a p85 regulatory and a p110 catalytic subunit (Figure 1.8) (Foster, Traer et al. 2003). Ras can activate PI3K directly and recruit it to the cell membrane. Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits PKB/Akt (v-Akt is the PKB homolog in murine retrovirus AKT8) and phosphoinositide-dependent kinase-1 (PDK1), two serine/threonine kinases, by binding to their pleckstrin-homology (PH) domains. Akt in turn stimulates a wide range of target proteins that control growth, cell proliferation, survival, and other processes. Given its important role, the level of PIP3 must be strictly regulated. One such mechanism that modulates the levels of PIP3 involves the tumor suppressor, PTEN. PTEN is a phosphatase that converts PIP3 back to PIP2 and thus inactivates PI3K signaling (Zhang, Stallock et al. 2000; Foster, Traer et al. 2003; Fingar and Blenis 2004). This pathway is better understood in higher eukaryotes than in yeast. In metazoans, the PI3K pathway can also be activated by the binding of insulin or insulin-like peptides to the insulin receptor (Inr), which activates insulin receptor substrates 1–4 (IRS1–4) that promotes PI3K activity (Oldham, Stocker et al. 2002; Oldham and Hafen 2003).

1.11.3.1 PI3K Regulates Growth via TOR Pathway

One major target of the PI3K pathway is the TOR pathway (Figure 1.8). The TOR pathway is repressed by TSC1 and TSC2 (Tuberous Sclerosis Complex). TSC1 and TSC2 are tumor suppressor genes in mammalian cells involved in growth signaling (Potter, Huang et al. 2001; Gao, Zhang et al. 2002; Potter, Pedraza et al. 2003). In *Drosophila* and mammalian cells, over-expression of TSC1 or TSC2 inhibits the phosphorylation of S6K and 4E-BP1 (Marygold and Leevers 2002; Tee, Fingar et al. 2002). In contrast, depletion of TSC1 or TSC2 with siRNA, leads to increased

phosphorylation of S6K and 4E-BP1 (Marygold and Leevers 2002; Tee, Fingar et al. 2002). Moreover, this increased phosphorylation is rapamycin-sensitive indicating that it is Tor-dependent (Inoki, Li et al. 2002; Li, Corradetti et al. 2004). Over-expression of TSC1 or TSC2 blocks the ability of amino acids to activate S6K1 in nutrient-deprived cells (Tee, Fingar et al. 2002). Moreover, amino-acid deprivation fails to reduce the increased phosphorylation of S6K observed in TSC1 or TSC2 null mammalian cells, or in *Drosophila* cells where siRNA has been used to reduce TSC1 or TSC2 expression (Stocker, Radimerski et al. 2003). However, this repression can be relieved by the phosphorylation of Akt that is activated by PI3K. Thus, PI3K can promote translation by stimulating TOR (Marygold and Leevers 2002; Li, Corradetti et al. 2004).

1.11.3.2 PI3K Regulates Growth Independent of TOR

PI3K can also regulate cell growth by promoting protein synthesis in a TOR-independent pathway (Figure 1.8). One mechanism is to activate PDK1. In vitro, PDK1 directly phosphorylates threonine 252 in the activation loop of the S6K (Rintelen, Stocker et al. 2001; Lawlor, Mora et al. 2002). This phosphorylation is stimulated by PI3K *in vivo* and is indispensable for p70 activity (Rintelen, Stocker et al. 2001; Lawlor, Mora et al. 2002). Finally, in addition to controlling TOR and S6K, PI3K also regulates cell growth by controlling translation of 5' TOP mRNAs *via* a S6K-independent mechanism (Rintelen, Stocker et al. 2001).

1.11.3.3 PI3K Regulates Expression of G1 Cyclins

Studies in *Drosophila* and mammalian cells also revealed the important role of the Ras/PI3K pathway in the commitment to cell division (Figure 1.8). Thus, this pathway bifurcates into one controlling cell growth via TOR and another regulating cell cycle machinery. The Ras/PI3K pathway induces expression of cyclin D1 upon stimulation of growth factors and mitogens by various mechanisms (Figure 1.8). First, this pathway can increase transcription of cyclin D1. For example, the Rac protein, a member of the Ras superfamily, induces cyclin D1 mRNA expression (Joyce, Bouzahzah et al. 1999).

Second, Ras promotes translation of cyclin D1 by preferentially recruiting cyclin D1 and D2 mRNA onto polysomes (Rajasekhar, Viale et al. 2003). Blocking components of this pathway seems to have only moderate effects on global gene transcription, but dramatic effects on polysomal mRNAs (Rajasekhar, Viale et al. 2003). Third, Ras/PI3K can also increase the protein stability of cyclin D1. Phosphorylation of cyclin D1 by glycogen-synthase kinase-3 (GSK3) results in the ubiquitination and degradation of cyclin D1 (Diehl, Zindy et al. 1997; Diehl, Cheng et al. 1998). However, PI3K/Akt inhibits GSK3, thereby stabilizing cyclin D1 protein (Diehl, Cheng et al. 1998). Finally, Ras may also down-regulate the cell cycle inhibitor p27, because expression of dominant-negative Ras prevents this down-regulation (Takuwa and Takuwa 1997). Consistently, abnormally high activity of this pathway is found in many tumors.

1.11.4 Raf-MAP Kinase

In mammalian cells, activated Ras can bind to and stimulate Raf-MAP kinase pathway. Raf (MEKK) is a serine/threonine kinase that stimulates MAPK Kinase (MEK) and in turn MAP kinase (ERK) (Gustin, Albertyn et al. 1998; Crespo and Leon 2000). Activated MAP kinase migrates into the nucleus and phosphorylates its substrates (Crespo and Leon 2000).

1.11.4.1 MAP Kinase Pathway Regulates eIF4E

As mentioned earlier, a key factor for translation initiation is eIF4E. This 24kDa protein binds to the 5' m7G Cap to facilitate subsequent assembly of ribosomes (Sonenberg and Gingras 1998; Gingras, Raught et al. 2001). It is noted that eIF4E is phosphorylated at serine 209 by Mnk1/2 (MAP kinase signal-integrating kinase) (Pyronnet 2000; Ueda, Watanabe-Fukunaga et al. 2004). This phosphorylation may promote translation initiation, because it is correlated with external stimulations such as treatment with serum, growth factors and insulin (Pyronnet 2000; Ueda, Watanabe-Fukunaga et al. 2004).

1.11.4.2 MAP Kinase Pathway Modulates G1-Phase Cyclin Expression

Like the Ras/PI3 pathway, the Raf-MEK-ERK pathway stimulates the expression of cyclin D1 and p21 family members that facilitate assembly of cyclinD-CdK2/4 complexes (Figure 1.8) (Cheng, Sexl et al. 1998). In fact, expression of cyclin D1 and its assembly into a complex with CDK4 or CDK6 requires RAS activation of the RAF–MEK–ERK/MAPK pathway (Cheng, Sexl et al. 1998). Moreover, these pathways increase the abundance of transcription factors such as FRA1, FRA2, c-JUN and JUNB, which in turn, promote the transcription of cyclin D1 from the AP-1 site in the cyclin D1 promoter (Balmanno and Cook 1999).

In the budding yeast, a similar MAP kinase pathway (the cell integrity pathway) is also known to positively regulate *CLN1* and *CLN2* mRNA expression by modulating SBF activity (Madden, Sheu et al. 1997; Baetz, Moffat et al. 2001). This pathway consists of a MAPK kinase kinase, Bck1/Slk1, a pair of redundant MAPK kinases, Mkk1/2, and a MAPK, Slt2p/Mpk1 (Gustin, Albertyn et al. 1998). The activated Slt2p MAPK activates in turn downstream transcription factors and consequently promotes gene expression (Gustin, Albertyn et al. 1998). Among the well characterized Slt2p targets are SBF and Rlm1p transcription factors, which control the expression of G1/S-phase cell cycle-regulated genes (Madden, Sheu et al. 1997; Gustin, Albertyn et al. 1998). This illustrates that the maintenance of cell integrity is dependent on coordination between cell wall biogenesis and cell proliferation (Madden, Sheu et al. 1997; Baetz, Moffat et al. 2001). Whether and how Ras can specifically regulate this pathway is unknown.

1.11.5 Myc Pathway Coordinates Cell Growth and Division

Myc is another target of Ras in higher eukaryotic cells (Figure 1.8). Myc is a strong inducer of proliferation (Leone, DeGregori et al. 1997; Nasi, Ciarapica et al. 2001). In *Drosophila* and mice, over-expression of Myc increases growth rate by activating the expression of eIF4E, nucleolin (involved in ribosome synthesis) and CAD (pyrimidine synthesis), and other genes involved in protein synthesis (Nasi, Ciarapica et al. 2001). This increases cell size (Stocker and Hafen 2000; Potter and Xu 2001). In

addition, Myc reduces the length of G1-phase by increasing cyclinE-Cdk2 kinase activity, repressing Cdk inhibitors p21 and p27, up-regulating Cdc25a and possibly bypassing pRB (Neufeld and Edgar 1998; Nasi, Ciarapica et al. 2001). Ras can up-regulate c-Myc at a post-transcriptional level by activating S6K and Akt/PKB as well as MAP kinase (Sears, Nuckolls et al. 2000). S6K and Akt/PKB inhibit Myc (Cross, Alessi et al. 1995). In addition, activated MAPKK can stabilize Myc by phosphorylating serine 62. This is confirmed by the observation that cells treated with specific inhibitors of either ERK or PI3K impair c-Myc accumulation. Thus, several pathways influence Myc expression and abundance which in turn affects the coordination of cell growth with division.

1.11.6 Summary of The Mechanisms That Coordinate Cell Growth and Cell Division

Data presented in the above sections highlight the many biochemical pathways involved in coordinating cell growth with division. Interestingly, quite a lot of details are known regarding the pathways that influence proliferation. For the most part, these pathways affect G1-phase cyclin activity or abundance. In contrast, considerably less is known about the general control of cell growth. In nearly all cells, coordinating cell growth with division results in cell size homeostasis. As discussed above, cells in general are remarkably homogenous in size. While it is not known how this homeostasis is achieved or maintained, a number of intriguing genetic studies have demonstrated that unlinking proliferation from cell growth can have profound effects on cell size. The major observations regarding the control of cell size are summarized below.

"I did lots of experiments in those days, looking at cell size. But the world wasn't interested. Then when things went molecular, research on cell size and cell growth sort of stopped."

Paul Nurse, Nobel Prize recipient 2001

1.12 Insights into Cell Size: Size Control or Passive Regulation

Cell populations tend to maintain a constant and relatively homogenous cell size even across species. The somatic cells of tiny fruit flies are very similar in size to somatic cells from enormous grey whales. However, the means by which this is accomplished are not known. In fact, this topic has been a source of debate for more than a century. On the subject of cell size control, debaters tend to fall into two camps. In the first camp are those who hypothesize that a mechanism exists that actively and directly regulates the size of cells. This hypothesis is largely based on observations that cells can divide faster than their mass doubling times. It is this phenomenon that returns extremely large oocytes to the normal size of somatic cells. Based on this rationale, these theorists propose that cells must have a mechanism that prevents somatic cells from dividing faster than their mass doubling time as this would result in a mitotic catastrophe (Figure 1.9). To solve this problem, it is proposed that cells are unable to commit to cell division until a minimum cell size is attained (Figure 1.2). In the second camp are those who suggest that cell size is the unavoidable passive consequence of cell division. These theorists point out that cell size is remarkably homeostatic and that cells accomplish this through unknown biochemical mechanisms that ensure that cells divide at regular intervals (Figure 1.10). In so doing, it is proposed that cells adjust their mass doubling rates appropriately to avoid becoming too large or too small. The cases for each of these perspectives are detailed below.

This debate centers around three questions. First, does cell size affect the ability of cells to divide? Second, do cells divide at regular intervals? Finally, do cells grow (e.g. in size by mass doubling) in a linear or exponential manner?

Using amoebae, Hartmann and Prescott demonstrated that cytoplasmic amputation could prevent cell division (Fantes, Grant et al. 1975). By preventing cell growth through cytoplasmic reductions, these investigators prevented cell division. Remarkably, continual repetition of this process prevented a single amoeba from dividing for six months while the control cell divided 65 times (Fantes, Grant et al. 1975). From this experiment, it was concluded that amoebae were continually monitoring their cell

mass and not the amount that they had grown nor the time that had elapsed since their last division (Fantes, Grant et al. 1975). Subsequently, researchers using mouse cells showed that the probability that a given cell would divide was proportional to its cell size. These studies have been greatly expanded upon in both budding and fission yeast where numerous experiments have revealed that cell size correlates closely with the probability that a cell will divide (Fantes and Nurse 1977; Fantes and Nurse 1978; Tyson, Lord et al. 1979; Tyson and Diekmann 1986). Nonetheless, size is a rather amorphous characteristic and because of this, it has proved very difficult to extend these observations from a correlative to causative relationship. In general this timing mechanism is referred to as the "critical cell size" model (Figure 1.2). Today, a great deal of research is ongoing in an attempt to identify the biochemical mechanism or factors responsible for promoting cell division in a size-dependent manner.

While the evidence presented above illustrates cases where size correlates with proliferative capacity, an equally large body of evidence exists that suggests that cells divide at regular intervals. For example, most embryonic cell divisions occur so regularly that scientists can predict with uncanny accuracy the number of cells that will be present at any given time. Moreover, it is well established in virtually all cell culture systems under homeostatic conditions that mean cell division times become remarkably constant and predictable. Thus, if cells vary from the mean division time, there must be an equivalent number of rapidly and slowly dividing cells. These observations suggest that mechanisms dependent upon time regulate cell division. However, it should be noted that embryonic cells are larger than normal and require no cell growth in order to divide. Once these cells reach the size of normal somatic cells, proliferative potential becomes asynchronous and unpredictable. In addition, attempts to synchronize the proliferation of cells have revealed that despite the fact that mean division times are constant and predictable, individual cell division times are inconstant and quite variable. This is illustrated by the fact that experimentally synchronized cells lose this synchrony in less than two cell divisions. In general this timing mechanism is referred to as the "cell cycle clock" model. As discussed above, a great deal of research is also ongoing in an

attempt to identify biochemical mechanisms or factors responsible for promoting cell division in a time-dependent manner.

As is the case with many questions in biology, it frequently becomes necessary to use mathematical models to simulate and test biological theories. In this manner, these approaches have been applied to the above debate. While it is widely accepted that cells proliferate in an exponential manner, it is not clear if all or any cells grow exponentially. In thinking about this question, consider the following example: exponential cell proliferation means that in a given period of time, cell number doubles (e.g. 2, 4, 8, etc); linear proliferation means that in a given period of time, cell number increases at a constant rate (e.g. 2, 4, 6, etc). In practice in the laboratory, it is frequently difficult to differentiate linear from exponential rates. But closer examination reveals striking differences between each rate (Figure 1.11). If cells divide every hour, then after the second hour during exponential proliferation, 4 cells divide to yield 8 cells. In contrast, after the second hour during linear proliferation, 4 cells divide to yield 6 cells. This clearly is impossible and does not happen. In fact, it is widely accepted that cells could grow with either linear or exponential rates. Indeed, both types of cell growth have been reported (Nurse, Thuriaux et al. 1976; Conlon and Raff 2003). However, importantly, mathematical models have revealed that patterns of linear cell growth are compatible with the "cell cycle clock" model, but exponential cell growth is not.

The central issue that differentiates the "cell cycle clock" model from the "critical cell size" model involves how each model handles the few cells that are considerably smaller or large than the mean size of the population. Experimental observations illustrate that these cells exist but are a small fraction of the population (Amon, Tyers et al. 1993). In this regard, the "critical cell size" model proposes that the frequency of these size outliers are kept low because cells smaller than a minimum cell size must grow in order to divide. This brings these cells closer to the mean (Figure 1.12). For abnormally large cells, division in the absence of cell growth also brings the daughter cells closer to the mean. In this case, it does not matter if cell growth is linear or exponential. In contrast, the "cell cycle clock" model proposes that the frequency of size

outliers is kept low because cells divide at regular intervals. In this case, because both growth rate and division rate are constant (e.g. as shown in Figure 1.12, all cells grow 5.5 mass units per hour and divide once per hour). This mechanism maintains cell size homeostasis, but demands that cell growth be linear. If cell growth is exponential, then cells grow at a rate proportional to their size which results in large cells getting continually larger and small cells getting continually smaller (Figure 1.10). However, as with the proliferation case described above, linear growth is not intuitive. Linear growth means that cells grow at the same rate regardless of their size. Thus, in the example shown in Figure 1.12, the initial small cell is incredibly efficient as it grows 550% in one hour. In contrast, the large cell is remarkably inefficient as it grows only 55% in one hour. While possible, no metabolic or biochemical studies currently exist to support this prediction.

Despite a wealth of empirical observations, it is still unclear if either the "critical cell size" or "cell cycle clock" models are correct. Indeed, it is possible that under certain circumstances, either both or neither model is correct. However, one of the goals of my research has been to identify and characterize the genetic and biochemical pathways involved in promoting cell division and to examine my findings in the light of each of these models.

1.13 Genetic Mechanisms That Affect Cell Size

Despite the fact that it is currently not known how cell size homeostasis is maintained, an increasing number of genes and genetic pathways have been shown to affect cell size homeostasis. Not surprisingly, disruption of many of the pathways involved in the coordination of cell growth with proliferation results in cell size defects. Importantly, these pathways include a number of genes involved in development of cancer and aging.

1.13.1 Ras and Myc

Deletion of Ras and Myc in mammalian cells inhibits cell growth and makes cells smaller than normal. In contrast, over-expression of Ras and Myc promotes cell growth and increases cell size (Halfar, Rommel et al. 2001). As a result, Ras over-expression causes cardiac hypertrophy in mice (Montagne 2000; Potter and Xu 2001).

In the fission yeast *Schizosaccharomyces pombe,* the Ras-like gene *Rheb* (Ras homolog enriched in brain) is required for growth (Fingar, Richardson et al. 2004). Like Ras, the *rheb* mutants (*rhb1*) arrest as small, rounded cells, indicating a role for *Rheb* in cell cycle progression and cell growth. In *Drosophila*, over-expression of *dRheb* results in tissue overgrowth and increased cell size in the whole organism, and promotes cell growth and transition from G1-phase into S-phase in cultured cells. Conversely, reduction of *dRheb* activity results in reduced tissue growth and smaller cell size in the whole organism, as well as a G1-phase arrest and smaller cell size in culture (Fingar, Richardson et al. 2004). Both Ras and Myc stimulate G1-phase cyclin expression (Diehl, Zindy et al. 1997; Diehl, Cheng et al. 1998; Rajasekhar, Viale et al. 2003) (Neufeld and Edgar 1998; Nasi, Ciarapica et al. 2001). However, the mechanism whereby Ras and Myc stimulate cell growth is not known.

1.13.2 PI3K Pathway

Inactivation of positively acting molecules in this pathway decreases organ and cell size concomitant with a slowing down of the cell cycle, while over-expression of PI3K, Akt and IRS (Insulin receptor substrate) increases cell and organ sizes (Shioi, Kang et al. 2000; Alvarez, Garrido et al. 2003; Fingar and Blenis 2004). In *Drosophila*, heterozygotic *Inr* mutations (insulin-like receptor) lead to small flies with fewer and smaller cells (Bohni, Riesgo-Escovar et al. 1999; Stocker and Hafen 2000; Oldham, Stocker et al. 2002). Mutants in either insulin receptor or *chico* (insulin-like receptor substrate) are 50% smaller in cell and body size. Similarly, knockout mice lacking any one insulin-like growth factor (IGF-I, IGF-II) or Inr, IRS1 or IRS2 have a reduced body size. In addition, treatment of myotubes with IGF-I results in hypertrophy, and *igf-I*

knockout mice has revealed a reduced muscle fiber size in the diaphragm (Bohni, Riesgo-Escovar et al. 1999; Stocker and Hafen 2000; Oldham, Stocker et al. 2002).

Over-expression of PI3K or Akt increases cell size (Shioi, Kang et al. 2000; Alvarez, Garrido et al. 2003; Fingar and Blenis 2004). In addition, knockouts of Akt1 (PKB) or PDK1 in mice results in a small cell size and thus small animal phenotype (Verdu, Buratovich et al. 1999). Modulation of PI3K, or Akt expression in the heart affects heart size as a result of changes in cardiomyocyte size but not cell number (Shioi, Kang et al. 2000).

Furthermore, cells devoid of the lipid kinase PI3K show a pronounced impairment of growth and a reduction in size whereas over-expression of a wild-type or of an activated version of the catalytic subunit of PI3K stimulates cellular growth, resulting in bigger cells (Leevers, Weinkove et al. 1996). Loss of *Drosophila* PTEN, the PI3K antagonist, results in enhanced growth and larger cells. Conversely, overexpression of PTEN reduces cell number and cell size (Komarnitsky, Chiang et al. 1998; Oldham and Hafen 2003).

1.13.3 TOR Pathway

Treatment with rapamycin also reduces cell size, indicating that TOR is an important mediator of cell growth. In support of this, reduction of TOR expression with RNAi also reduces cell size (Zhang, Stallock et al. 2000; Oldham and Hafen 2003; Fingar and Blenis 2004). Moreover, over-expression of PI3K or Akt increases cell size in a rapamycin-sensitive manner, indicating that the ability of the PI3K/Akt pathway to drive cell growth is TOR dependent. Over-expression of S6K1 or eIF4E also increases cell size, while over-expression of a dominantly acting mutant of 4E-BP1 decreases cell size (Fingar, Salama et al. 2002). Moreover, over-expression of rapamycin-resistant mutants of S6K1 or over-expression of eIF4E partially rescues the small cell size phenotype induced by rapamycin, indicating S6K1 and eIF4E to be downstream mediators of mTOR-dependent cell growth (Oldham, Bohni et al. 2000; Zhang, Stallock et al. 2000; Fingar and Blenis 2004).

As the repressor of TOR pathway, the *Drosophila* gene *gigas* encodes a homolog of the human tumor suppressor gene *TSC2* (tuberous sclerosis complex gene 2). The *gigas* mutants are very large (Gao, Zhang et al. 2002; Potter, Pedraza et al. 2003). In addition, overexpression of a dominant mutant of 4E-BP1 with high affinity for eIF4E reduces cell and organ size (Schalm, Fingar et al. 2003).

1.13.4 MAP Kinase

The ERK-MAP kinase pathway plays an important role in myocyte hypertrophy, an adaptive response to a number of heart diseases including myocardial infarction. Myocytes transfected with constitutively activate c-Raf or MKK1 displayed an enlarged and expression genes with hypertrophic pattern (Clerk, Pham et al. 2001). Hyperactivation of p42/44 (ERK) leads to myocyte hypertrophy in mouse (Cohen, Park et al. 2003), possibly via AP-1 regulated gene expression (Santalucia, Christmann et al. 2003; Tu, Bahl et al. 2003).

1.13.5 Cell Size Is Proportional to DNA Ploidy

In spite of the ongoing debate over how cell size is modulated, it is widely appreciated that cell size is proportional to DNA content. Nearly a century ago, it was first suggested that the amount of nuclear chromatin (DNA) per cell determined its size. In support of this, it is known that in nearly all cells there is a direct proportionality between cell volume and DNA content (Figure 1.13). Moreover, cell size is also directly proportional to ploidy (Melaragno, Mehrotra et al. 1993; Galitski, Saldanha et al. 1999). Diploid cells are twice the size of haploid cells, and tetraploid cells are four times as large and so on (Melaragno, Mehrotra et al. 1993; Galitski, Saldanha et al. 1999). Interestingly, transplantation studies have revealed that size is intrinsically programmed (Conlon and Raff 1999). Importantly, genetic studies in yeast have implicated G1-phase cyclins as biochemical molecules intimately involved in the control of cell size (Cross 1988; Nash, Tokiwa et al. 1988; Flick, Chapman-Shimshoni et al. 1998; Rupes 2002). Increased abundance of G1-phase cyclins decreases cell size while decreased abundance

increases cell size (Cross 1988; Nash, Tokiwa et al. 1988; Flick, Chapman-Shimshoni et al. 1998; Rupes 2002). Interestingly, ploidy-dependent repression of G1-phase cyclin expression may explain the increased size of cells with a higher ploidy (Galitski, Saldanha et al. 1999).

1.14 Does Cell Size Correlate with Commitment to Proliferation in Mammalian Cells

In some mammalian cells, for example, fibroblasts, the answer is yes (Zetterberg and Killander 1965; Yen, Fried et al. 1975; Dolznig, Grebien et al. 2004). However, a recent study by Conlon and Raff challenged this idea (Conlon, Dunn et al. 2001; Conlon and Raff 2003). They showed that in Schwann cells, cell growth was linear and was not the limiting factor for cell division. From this they inferred that the "critical cell size model" does not operate in Schwann cells (Conlon, Dunn et al. 2001; Conlon and Raff 2003). However, this view has been recently challenged. Two groups using glial cells or ethyrocytes claim that the "critical cell size model" does operate in mammalian cells (Conlon and Raff 1999; Dolznig, Grebien et al. 2004). Therefore, this is still a controversial issue requiring further investigation.

1.15 The Big Picture: Biological Significance and Relevance

The appropriate coordination of cell growth with proliferation is essential to development and differentiation. This coordination is strongly implicated in normal physiological responses like immune responses, tissue regeneration and wound healing. In addition disruption of this coordination can result in pathologies like cancer, heart disease, immune deficiencies and premature aging.

Examination of the retinoblastoma tumor suppressor pathway (Figure 1.5) highlights the significance of coordinating cell growth with proliferation. In mammalian cells, this pathway is essential for this process. Importantly, it is disrupted in nearly every type of cancer, and the disruption of this pathway causes dramatic effects on cell cycle proliferation and cell size. For example, mutation of upstream inputs into this pathway (e.g Ras, Myc, PI3K, TOR, etc discussed above) stimulates G1-phase cyclin

expression (Mitchison, Novak et al. 1997; Montagne 2000; Stocker and Hafen 2000; Potter and Xu 2001). Thus, G1-phase cyclins are frequently over-expressed in cancer resulting in smaller than normal cells (Hartwell and Kastan 1994; Sherr 1996; McDonald and El-Deiry 2000; Vermeulen, Van Bockstaele et al. 2003). Similarly, loss of the tumor suppressor genes pRb, PTEN, NF-1, TSC1, and TSC2 all affect cell size. These results suggest that disruption of the coordination of cell growth with proliferation is an integral part of the initiation of carcinogenesis. As discussed throughout this chapter, research has also indicated that the proper coordination is also essential in preventing premature aging, heart disease, and tissue regeneration pathologies (Sherr 1996; Garrett and Fattaey 1999; Cohen, Park et al. 2003). Thus, a thorough understanding how cell growth and proliferation are coordinated is integral to expanding our understanding of normal and pathological cell development. Importantly, the strong conservation of the pathways involved in these processes in the budding yeast have allowed the research progress made in this model organism to be directly translated and related to mammalian cells.

1.16 Research Questions and Goals

Elegant studies have shown clearly that cell growth (protein synthesis) and cell division are coordinated. Under optimal conditions, external signals such as growth factors and nutrients can simultaneously activate several interacting pathways that not only increase protein synthesis capacity, but also directly or preferentially up-regulate G1 phase cyclins. Though early studies have shown that cell division is dependent upon growth, the mechanisms responsible for this dependence are not completely understood. The long-term goal of my research is to investigate the intrinsic genetic and biochemical pathways involved in promoting proliferation and to examine the interaction of these pathways with extrinsic signals and stimuli. Thus, in the simplest sense, I am interested in how cell division is regulated. More specifically, my thesis research addresses the question "How are cell growth and proliferation coordinated?"

1.17 Thesis Summary

This chapter has served to introduce my thesis research questions and goals. In chapter 2, I describe the methods and materials used in my project. In my third chapter, I address the biochemical mechanisms and factors involved in coordinating cell growth with proliferation. In particular, I focus on the role of G1-phase cyclins in this process. Results presented in this chapter were recently accepted for publication in *Molecular and Cellular Biology*. In my fourth chapter, I detail a systematic genome-wide genetic screen I conducted to identify genes involved in coordinating cell growth with proliferation. Results presented in this chapter were recently accepted for publication in *Current Biology*. In my fifth chapter, I examine the function of a subset of genes identified in Chapter 4. This subset of genes codes for proteins that physically interact in the Ccr4-Not complex that is strongly conserved throughout evolution. In this chapter, I detail the interactions of this complex with G1-phase cyclins. Finally, in chapter 6, I will discuss the significance and biological relevance of my results in the context of their implications on this field.

Cdc Genes	Biological or molecular functions	Human Homologs
Cdc4	Ubiquitination	F-box Cdc4
Cdc7	Initiation of DNA replication	Cdc7Hs
Cdc19	Glycolysis	pyruvate kinase
Cdc24	Guanine nucleotide exchange factor	DBL family protein
Cdc25	Ras-signaling	son-of-sevenless (SOS)-1
Cdc28	Cyclin dependent kinase	Cdc2
Cdc31	Microtubule nucleation	Calmodulin 2
Cdc32	Not clone, function unknown	Not Cloned
Cdc33	Translation initiation	eIF4E
Cdc35	Adenylate cyclase	KIAA0606
Cdc36	Transcription; mRNA poly(A) deadenylation	hNOT2
Cdc37	Chaperone for protein folding	Cdc37
Cdc39	Transcription; mRNA poly(A) deadenylation	hNOT1
Cdc42	Signal transduction	Cdc42
Cdc45	Initiation of DNA replication	Cdc45L
Cdc46	Initiation of DNA replication	huMCM5
Cdc47	Initiation of DNA replication	p85Mcm
Cdc54	Initiation of DNA replication	Cdc21
Cdc60	Translation elongation	leucyl-tRNA synthetase
Cdc63	Translation elongation	Prt1
Cdc65	Translation elongation	Not cloned
Cdc68	Transcription elongation	SPT16/CDC68

Table 1.1. The list of the cdc mutants that arrest in G1 phase at the restrictive temperature. Except for Cdc32 and Cdc65 that haven't been cloned, all others are conserved and have human counterparts, indicating conserved mechanisms in cell cycle regulation.

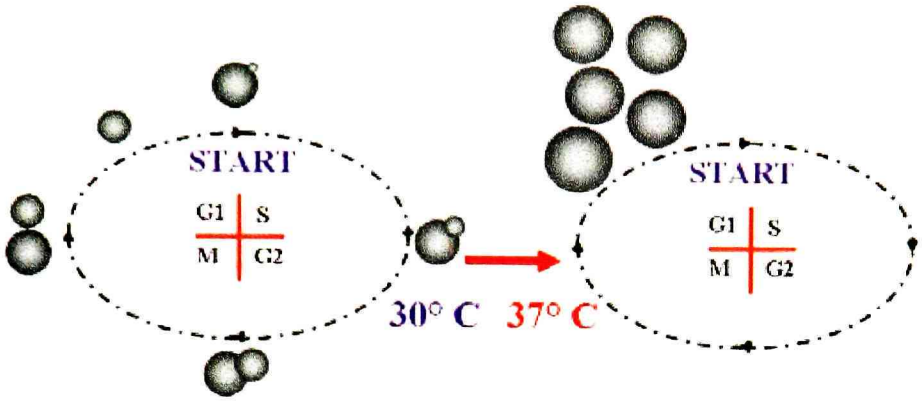


Figure 1.1. Cell cycle morphology in yeast and the characterization of *cdc* mutants. Unlike any other organism, cell cycle phases in yeast can be determined morphologically as indicated in the center of the oval. The left panel of this figure illustrates how an asynchronous culture grown at 30°C would appear under the microscope. G1-phase cells are small and unbudded. At the G1/S-phase boundary (Start), cells bud. As cells progress through the cell cycle, the bud preferentially grows larger. Thus, by cytokinesis when the bud/daughter cell separates from the mother cell, it is more than half the size of the mother. By mutagenizing cultures and identifying mutants that were unable to proliferate at high temperatures (e.g. 37 C), Hartwell and colleagues generated a panel of conditional mutants. By analyzing the cell cycle morphologies of individual mutants, they found that most (>96%) essential genes in yeast are not specifically involved in cell cycle regulation. That is upon shifting to the restrictive temperature of 37 C, these mutants arrested randomly throughout the cell cycle and appeared identical to 1N asynchronous culture (left panel above). In contrast, a small subset of mutants gave a uniform terminal arrest morphology (e.g. large unbudded cells as seen in the right panel above). Hartwell and coworkers called these mutants cell division cycle (*cdc*) mutants. Thus, the wild type genes became known as *CDC* genes.

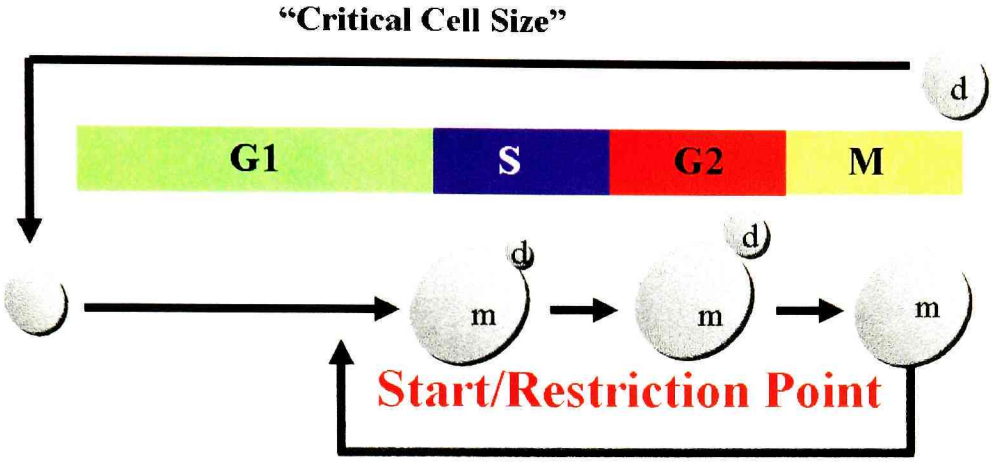


Figure 1.2. Start and critical cell size. A mother cell (m) gives rise to a daughter cell (d). Because the division of budding yeast is asymmetric, the daughter cell is typically smaller than the mother cell. In the next round of cell division, the daughter cell must grow to reach a certain size (the critical cell size) before it can progress past Start. Meanwhile, the mother cell can progress past Start almost instantly because it has already reached the critical cell size in the last round. Thus, the daughter cell has a longer G1 phase than its mother.

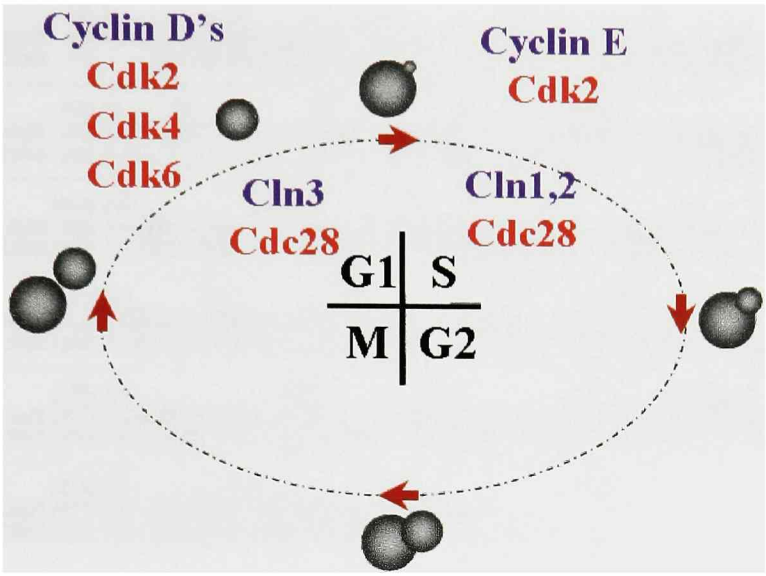


Figure 1.3. Cell cycle machinery is well conserved. Both budding yeast and mammalian cells commit to cell division at G/S boundary. It has been established that the key molecules of this commitment are G1-phase cyclins (blue), which bind to and activate cyclin-dependent kinases (Cdk, red) to activate downstream cascades. In the mammalian cells, Cyclin D binds to Cdk2, 4 and 6 to activate expression of Cyclin E that in turn activates Cdk2. Similarly, the yeast homolog of Cyclin D, Cln3 binds to Cdc28 to activate expression of Cln1, 2 as the key step for Start.

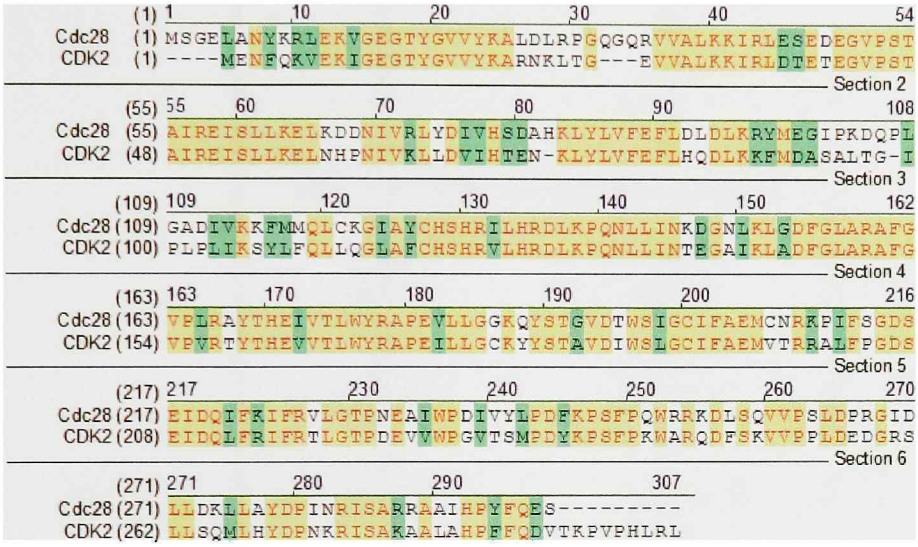


Figure 1.4. Conservation of cyclin dependent kinases. The amino acid sequences of yeast Cdc28 and human Cdk2 are aligned with VectorNTi. Their similarity is ~60%, indicating their homology.

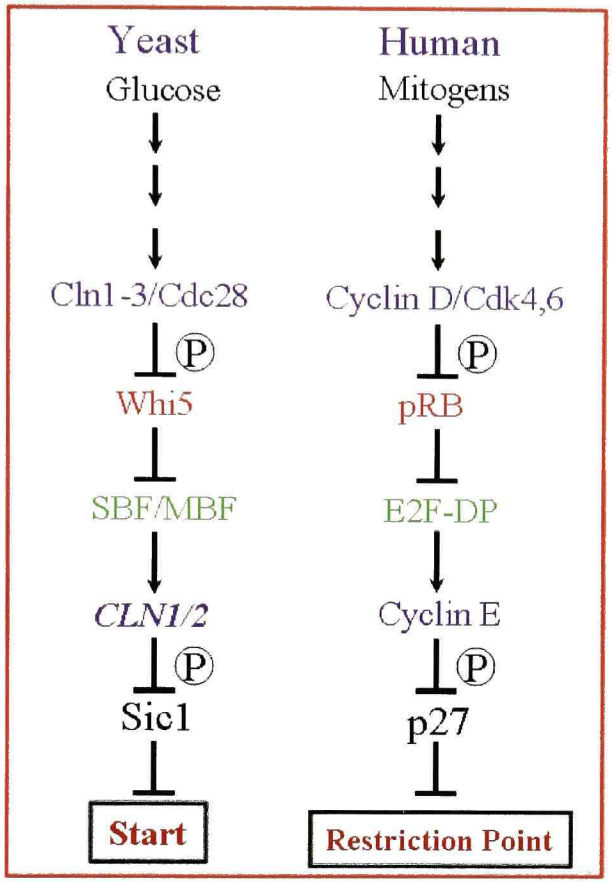


Figure 1.5. Comparison of yeast and mammalian cell cycle machinery. In budding yeast, nutrients are the primary mitogenic signals to stimulate expression of Cln3 that activates Cdc28 in order to phosphorylate Whi5, an ortholog of pRb. Like pRb, Whi5 inhibits two transcription factors, SBF and MBF. Phophorylation of Whi5 dissociates it from SBF and MBF, thus relieving its inhibitory effect. Like, E2F, SBF and MBF are responsible for the expression of the G1-phase cyclins Cln1 and Cln2, orthologues of Cyclin E. Accumulated G1-phase cyclin/Cdk complexes phosphorylate Sic1, and orthologue of p27, and promote progression past Start. Mammalian cells adopt a very similar strategy. Mitogens (i.e. growth factors) stimulate the expression of Cyclin D that binds to Cdk4 to phosphorylate pRB, an E2F inhibitor. Hyperphosphoryalted pRB dissociates from E2F and can no longer repress E2F transcriptional activity. The E2F targets include Cyclin E which phosphorylates p27 to promote progression past the restriction point. Importantly, research has shown that the mammalian pathway is mutated or disrupted in nearly every known cancer. 'P' denotes directphosphorylation.

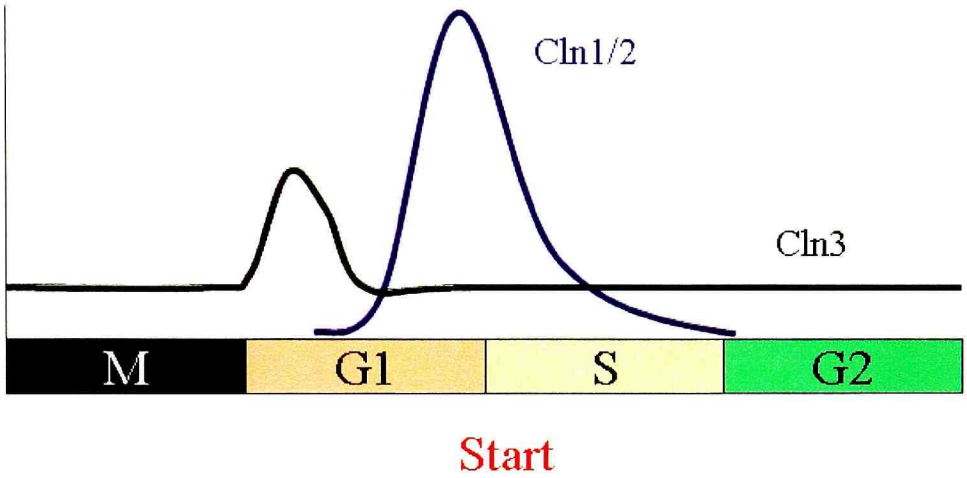


Figure 1.6. Cell cycle regulated expression of Cln1-3. *CLN3* mRNA and protein are expressed constitutively throughout the cell cycle with moderate peak in early G1-phase. In contrast, *CLN1* and *CLN2* mRNA and proteins are induced at Start by Cln3-Cdc28 and other factors. This leads to an increase in their abundance which reaches a maximal level in late G1-phase.

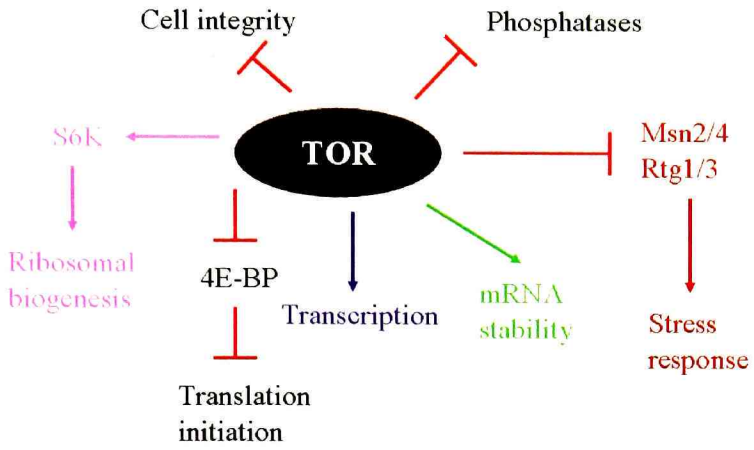


Figure 1.7A. Physiological roles of TOR. TOR coordinates multiple physiological functions in cells, such as promoting ribosome biogenesis, increasing translation initiation, stabilizing mRNA, modulating transcription, inhibiting phosphatase activity, repressing the cell integrity pathway and stress responses.

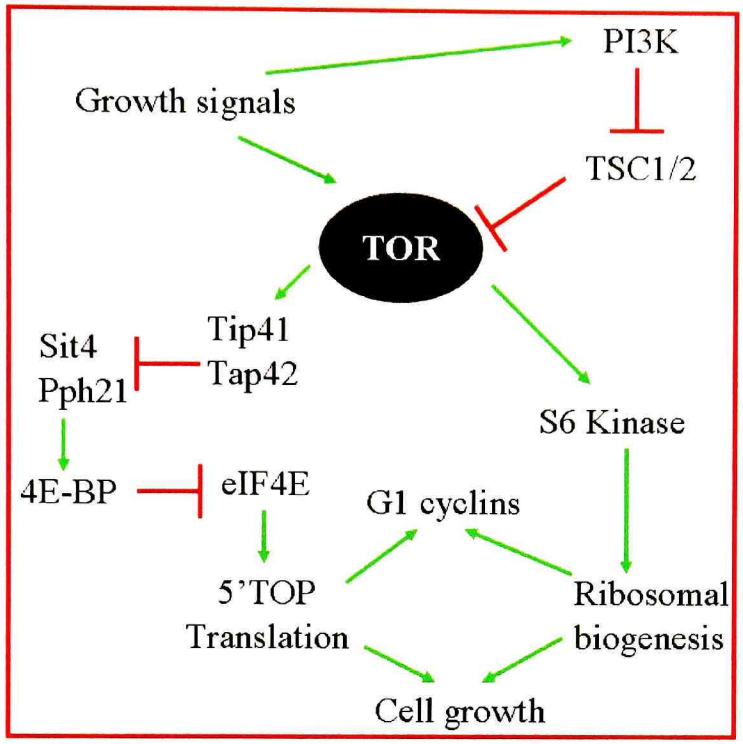


Figure 1.7B. TOR pathway plays an important role coordinating cell growth and cell division. TOR can be activated by nutrients dependent and independent of PI3K. Upon activation, the TOR signal bifurcates into promoting translation and ribosomal biogenesis. TOR promotes Tip41 and Tap42 binding that represses Sit4 and Pph21 phosphatases activity which promote the inhibition of eIF-4E by 4E-BP. Thus, TOR promotes preferential translation of genes with 5'TOP including G1 phase cyclins by relieving 4E-BP inhibition of eIF-4E function. Meanwhile, TOR can increase ribosomal biogenesis by stimulating ribosomal S6 kinase. As a result, the TOR signal coordinates cell growth (protein synthesis) and cell division.

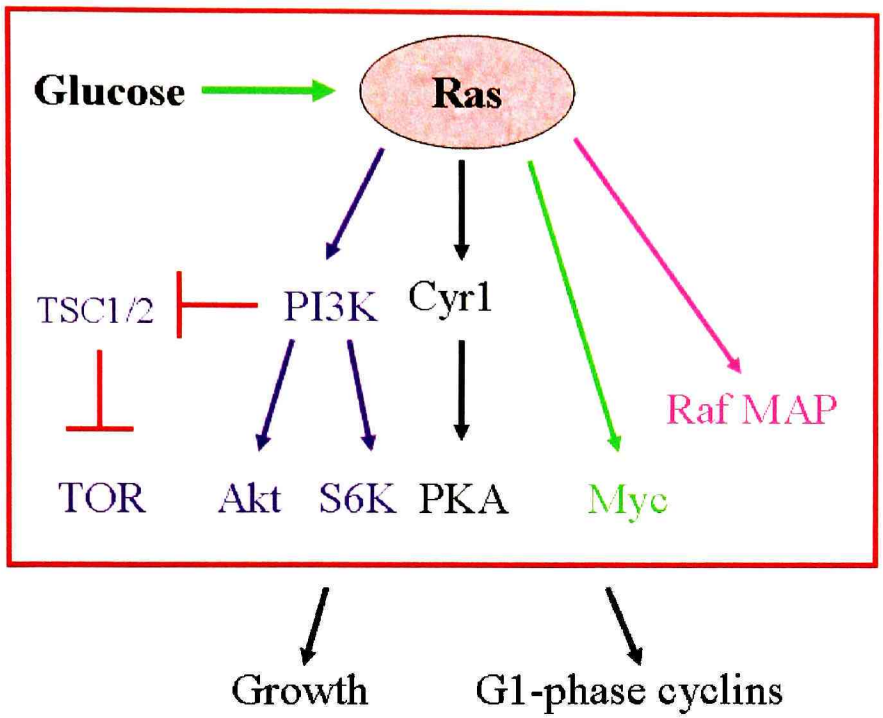


Figure 1.8. Ras plays a central role coordinating cell growth and division. Downstream of Ras, there are TOR, PI3K, PKA, Myc and Raf MAP kinase pathways promoting growth (protein synthesis and metabolism) and expression of G1 phase cyclins. These pathways also exhibit extensive cross-talk.

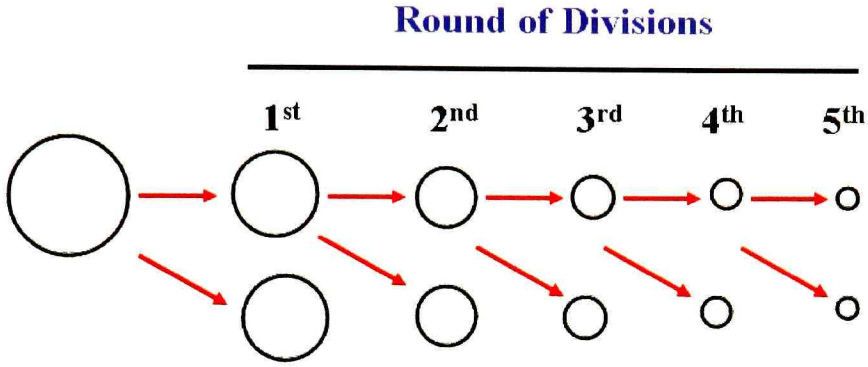


Figure 1.9. Cell growth and cell division must be coordinated. If cells complete the cell cycle in a shorter time than they double their mass, the subsequent daughter cells will become progressively smaller, resulting in catastrophe (Adapted from Introduction of Cell cycle by Murray and Hunt).

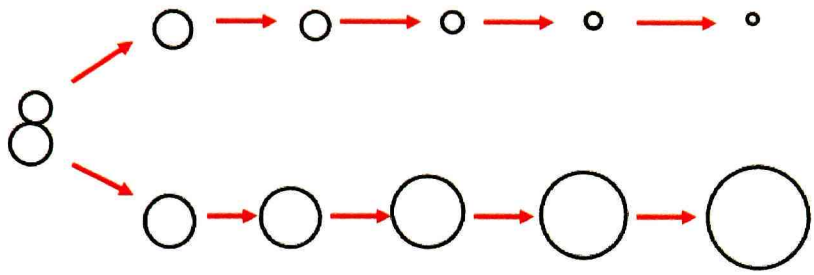


Figure 1.10. The problem of unequal cell division. The budding yeast divides unequally, producing one large mother cell and a small daughter cell. Obviously, if cells simply double their birth mass before dividing, the mother cell and the daughter cell will eventually producing progressively larger or smaller cells, ending up with catastrophe. In this case, a critical cell size requirement can prevent the crisis (Adapted from Introduction of Cell cycle by Murray and Hunt).

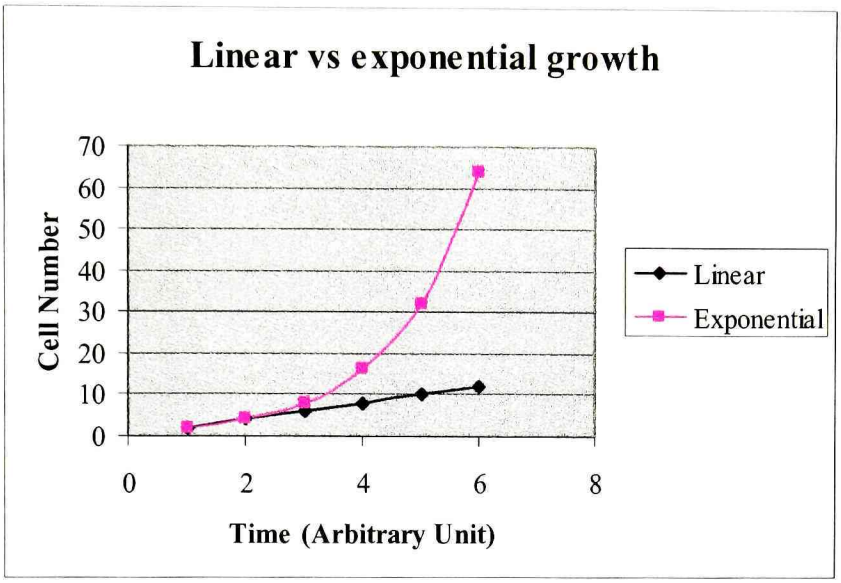


Figure 1.11. Exponential vs linear cell proliferation. For example, if cells divide every hour, then after the second hour during exponential proliferation, 4 cells divide to yield 8 cells. On the other hand, after the second hour during linear proliferation, 4 cells divide to yield 6 cells. However, in the laboratory, it is difficult to distinguish linear from exponential rates. It is more likely that cells grow with both linear and exponential rates. Actually, both the growth types have been observed.

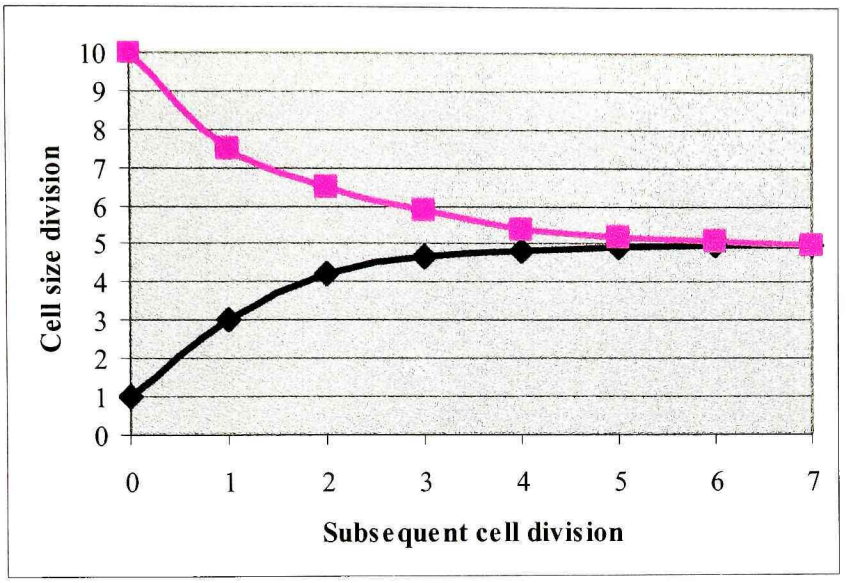


Figure 1.12. A hypothetic model showing how small and large daughter cells eventually return to the mean population size after several doublings if small and large cells grow and divide at the same rates. The initial division is unequal and produces one cell of 10 units of mass and one of 1 unit of mass. The subsequent 7 divisions of progeny cells are equal. Following the first division, each cell grows 5.5 units of mass in each division. Thus, the initial small daughter cell grow to 6.5 units before giving rise to two daughters of about 3.2 units each, while the initial large daughter cell grows to 15.5 units before producing two cells of 7.8 units (Adapted from Conlon and Raff, 2003. J Biol 2(1):7)

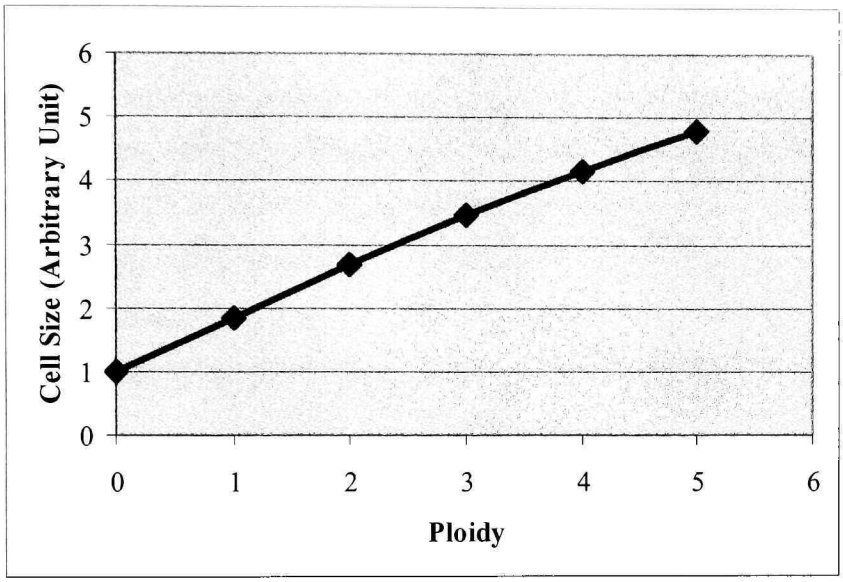


Figure 1.13. Cell size positively correlates with ploidy. For example, haploid yeast cells are about half the size of diploids. Moreover, this correlation has been observed in different organisms. Similarly, cell size has the same relationship with DNA content. Moreover, the wet weight of cells also correlates with DNA per cell (Adpated from Ycas et al. 1965, 9:444).

CHAPTER II
MATERIAL AND METHODS

2.1 Media and Strains

2.1.1 Media for Bacteria

Bacterial cultures were grown in in Luria-Bertani Broth (LB). It was made with 5g of yeast extract (Difco-Bacto), 10g of tryptone (Difco-Bacto) and 10g of NaCl dissolved in 1 liter of H_2O. The medium was autoclaved under high temperature (120°C) and pressure (1.41×10^4 kg/m^2) for at least 20 min. Heat labile materials were filter-sterilized through 0.2μm filter apparatus (Millipore). Antibiotics were added to the autoclaved medium after it was cooled down to 50°C. For solid media, 20g of agar (Difco-Bacto) was added prior to autoclaving.

2.1.2 Media for Yeast

Yeast cultures were grown in YEP-based media (20g Difco Bacto peptone and 10g Difco Bacto yeast extract were dissolved in 900ml of water and autoclaved) or YNB-based media (6.7g Difco-Bacto yeast nitrogen base lacking amino acids and ammonium sulfate were added to 900ml of water and autoclaved). Required amino acids were supplemented to 50mg/L except for L-tryptophan (80mg/L), adenine sulfate (32mg/L) and p-aminobenzoic acid (5mg/L)). After autoclaving, sterile filter carbon sources were added to a final concentration of 2% (glucose, sucrose, or raffinose) or 3% (ethanol and glycerol).

2.1.3 Bacteria Strain

TOP10 F' traD36 lacIΔ (lacZ) M15 proA+B+/e14-(McrA-)Δ(lac-proAB) thi gyrA96 (Nalr) endA1 hsdR17 (rK-mK+) relA1 supE44 recA1

2.1.4 Yeast Strains and Plasmids

Please refer to the Table 2.1 and Table 2.2.

2.1.5 Determination of Measurement of Cell Density

The density of bacteria was determined by measuring OD units at 600nm with a BioRad Smart Spec 3000. One unit of OD_{600} corresponded to 5 X 10^8 cells/ml. The density of yeast was measured with a Beckman Coulter Counter that measures cell density and size simultaneously.

2.2 Centrifugal Elutriation

A two-liter culture was grown to log phase at 30°C. The cells were concentrated to 100ml by centrifugation. Cells were sonicated 10 seconds for three times on ice with intervals of 30 seconds, and then loaded onto JE5.0 rotor (Beckman). The cells were eluted at 2600 rpm and with an increasing pump speed of 1ml/min at 30°C. Elutriated fractions were monitored on microscopy. Small unbudded cells were collected and sized with a Z2 Coulter Counter Channelyzer (Beckman-Coulter). The cells were pelleted and re-suspended with fresh medium for further analysis.

2.3 Cell Size Analysis

Cell cycle synchronizations were performed using centrifugal elutriation as described above. Analysis of the cell size distribution of yeast strains was done using cultures in mid-log phase. Samples of the cultures were re-suspended in 10ml of Isoton buffer, briefly sonicated, and immediately analyzed using a Coulter Counter Channelyzer Model ZM or Model Z2.

2.4 Light Microscopy

For analysis of budding indexes and sporulation rates, a Ziess Axial Lab light microscope was applied. Normally, a minimum of 200 cells were counted to ensure randomness. The percent of budded cells were verified in at least two independent experiments.

2.5 Cell Cycle Analysis

For flow cytometry, yeast cells were harvested, washed, sonicated, and fixed overnight in 70% ethanol at 4°C. Cells were re-suspended and washed with 50mM sodium citrate, sonicated, treated with RNAse A (final concentration 0.25 mg/ml) for one hour at 50°C, and treated with Proteinase K (final concentration 1 mg/ml) for an additional hour at 50°C. Before analysis the yeast cells were stained with propidium iodide at a final concentration of 16mg/ml. Flow cytometry were performed on yeast cells stained with propidium iodide with Epics XL (Beckman-Coulter) flow cytometer as described. The samples were analyzed with EPICS XL ADC Analysis software version 5.1.

2.6 Sporulation and Tetrad Dissecting

To make double mutants, a haploid single deletion strain i.e. *ccr4Δ MATa* was mated with another haploid single deletion strain *e.g. bck2Δ MATalpha*, to generate heterozygous diploids. The heterozygous cells were then sporulated in 3ml of Sporulation Medium (1% KOAc, 0.005% ZnOAc) for 3~7 days at room temperature. The tetrads were dissected with a Singer MSM Series 300 micromanipulator. The spores were plated onto YPD for further selection of double deletions.

2.7 Molecular Cloning

2.7.1 Extraction of Plasmid DNA

Small scale plasmids from 2ml of bacteria were prepared with BioRad Mini-prep kits according to the manufacturer's instruction.

2.7.2 Restriction Digestion

All restrictive endonucleases and necessary reagents were purchased from NEB. Restriction digestions were done following the manufacturer's instruction. Typically, 1μg of DNA was incubated with 10 units of enzyme, 1X digestion buffer and 1X BSA at 37°C for 1hr.

2.7.3 Purification of DNA Fragments from Agarose Gels

DNA fragments of endonuclease digestion were separated with 1% agarose gels with 0.5μg/ml ethidium bromide. Gels were run in Tris-acetate EDTA (TAE) or Tris-borate EDTA (TBE) buffer. A 1-kilobase ladder (NEB) served as a size standard. DNA fragments of expected sizes were sliced out of the gel and extracted with QIAEX kits from Qiagen following the manufacturer's instructions.

2.7.4 Ligation

DNA ligations were done using T4 DNA ligase (NEB) according to the manufacture's specifications. A typically 5:1 molar ratio between DNA fragments for insertion and precut vectors was adjusted to achieve optimal ligation efficiency.

2.7.5 Polymerase Chain Reastions

Oligonucleotides for primers were synthesized by Sigma-Genosys and reconstituted in distilled water to a final concentration of 100μM. All PCR reagents were purchased from NEB. A typical 100μl PCR reaction contained 10ng DNA template, 1μl of Taq polymerase, 100 picomoles of each primer, 1μl of 25mM dNTPs and 10μl 10X Taq buffer. A reaction normally had 30 cycles consisting of denaturation for 1min at 95°C, annealing at 50C~58°C for 1 min, and 1~3 min extension at 72°C (1 min for 1kb fragment). The reactions were performed with Eppendorf Mastercycler.

2.7.6 Preparation of Yeast Genomic DNA

Yeast genomic DNA was obtained from 10ml overnight culture. Cells were harvested by centrifugation and washed with 500μl of sterile water. The cells were pelleted by centrifugation and resuspended in a lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl, pH 8.0, and 1mM EDTA). 200μl of glass beads (0.5mm) and 200μl of 1:1:1 phenol:chloroform:isoamylalcohol were added and vortexed vigorously. After centrifugation, the aqueous phase (upper) was transferred to a 1.8ml

Eppendorf tube. The nucleic acids were precipitated with 1ml of ice-cold ethanol and centrifuged. The pellet was resuspended in 400μl of water and treated with DNase-free RNase for 5 min at 37°C. The DNA was then precipitated with 100mM ammonium acetate and 100% ethanol. The pellet was air-dried and resuspended in 100μl of water.

2.7.7 Transformation of Bacteria

Bacteria were transformed by electroporation. 40μl of the electro-competent cells (TOP10) were incubated with 10ng of plasmids on ice for 10min, before being transferred to a chilled 0.2mm electroporation cuvette (BioRad) and pulsed at 1.8kV with a BioRad GenePulser. The cells were immediately washed out with 1ml of SOC and shaken at 37°C for at least one hour. Typically, 2 ~ 20μl of cells were spread onto appropriate selection plates and incubated overnight at 37°C. Several colonies were then selected for further analysis.

2.7.8 Transformation of Yeast

Fifty ml of yeast cells were grown to log phase (OD_{600} ~1.0) and washed once with sterile water. The pellet was resuspended with 1ml 0.1M lithium acetate (LiAc). An aliquot of 300μl of cells were transferred to a 1.5ml Eppendorf tube. The cells were pelleted and the supernatants were removed. The cells were resuspended with the transformation mixture (240μl of 50% PEG (MW 3350), 36μl of 1M LiAc, 50ul of 10mg/ml boiled salmon sperm DNA, up to 10μg of DNA for transformation, and water to final volume to 360μl). The cells were incubated at 30°C for 30 min and 42°C for 30 min. The cells were pelleted and resuspended in 1ml of sterile H_2O. 200μl of the cells were spread onto appropriate selective plates and incubated at 30°C for 2 to 4 days until colonies formed.

2.7.9 DNA Sequencing

All the DNA sequencing was performed by the Core Facility of Genomics at Texas Tech University.

2.8 Analysis of RNA

2.8.1 Preparation of Yeast RNA

Yeast cells were quickly chilled on ice and washed once with ice cold water before being frozen at -80°C for at least one hour. The pellets were resuspended in 600μl of TES buffer (100mM Tris, 10mM EDTA, 0.2% SDS, pH7.5) and 400μl of water-equilibrated phenol were added. The mixture was heated to 65°C and vortexed vigorously with for 10 seconds. The mixture was then incubated at 65°C for 45 min with occasional inversion. The mixture was then chilled on ice for 1 min and centrifuged at 4°C with maximum speed for 5 min. The aqueous phase was transferred to clean 1.5ml Eppendorf tubes and vortexed with another 400μl water-equilibrated phenol for 10 seconds. The mixtures were chilled on ice for 1 min and centrifuged at 4°C for 5 min. The aqueous phase was transferred to a clean 1.5ml tube and vortexed again with 400μl chloroform. Again, the mixtures were chilled and centrifuged at 4C. The aqueous phase was incubated with 2 volumes of ethanol and 0.1 volume NaOAc, and incubated at – 20°C for at least 30 min, before being pelleted at maximum speed at 4°C for 10 min. The pellet was dissolved in 50μl DEPC H_2O, after being washed once with 70% ethanol and air-dried on ice.

2.8.2 Quantitation of RNA

The RNA samples were diluted 100 ~ 500 fold and quantitated by UV absorbance at 260nm with BioRad SmartSpec 3000. OD 1.0 at 260nm was converted to 40ug/ml RNA.

2.8.3 Northern Blot

10μg of RNA were added to 17.5μl of RNA loading buffer (12.5 mM MOPS, pH 7.1, 2.5mM NaOAC, 0.25mM EDTA, 3.1% formaldehyde, 25% formamide, 2% glycerol dye, 4mg/ml bromphenol blue, 4mg/ml xylene blue and 50 μg/ml ethidium bromide) and heated to 55°C for 15 min. The samples were loaded onto 1% agarose gel containing 6.7% formaldehyde, and run at 100 volts for 1hr at 4°C. The gel was then soaked in

distilled H_2O for 30 min to remove formaldehyde, before it was transferred onto a non-charged nylon membrane (Hybond N+, Amersham) with VacuGene XL vacuum blotting system (Pharmacia) according to the manufacturer's instruction. The wet membrane was then UV-crosslinked with 150mJoule UV exposure in GS Gene Linker UV Chamber (BioRad), before a pre-hybridization with 15 ml of Church buffer (0.3M Na_2HPO_4, pH 7.2 and 6.25% SDS) at 65°C for at least one hour in Hybridization Oven (Amersham Pharmacia).

2.8.4 Radioactive Labeling of Probes

The probes were synthesized by PCR, and purified with spin columns (BioRad). 100ng of the probe were boiled and labeled with α-32P dCTP (Deoxycytosine 5′-[α-^{32}P] triphosphate 3000 Ci/mmol, MP Biomedicals), following the protocol of Promega Prime-A-Gene kit.

2.8.5 Hybridization

The radio-labeled probe was boiled for 5 min to denature the DNA and chilled on ice immediately before being added to 15ml prewarmed Church buffer at 65°C. The probe was incubated with the membrane overnight at 65°C. The membrane was washed twice with 1X SSC plus 0.1% SDS before being exposed to a blank phosphoimager screen (Molecular Probes) that was scanned with the Typhoon Scanner (Typhoon 9410, Amersham Phamacia). The image was digitally stored and quantitated with the FluorChem 2.0 spot densitometry analysis program (Alpha Innotech).

2.9 Analysis of Protein

2.9.1 Preparation of Cell Lysates

The cell cultures were quickly chilled and pelleted at 4°C. The cells were then washed with 1ml of ice cold water and frozen at -80°C for at least 1hr before being lysed. One volume of the NEB Buffer 3 (0.1% NP40, 250mM NaCl, 50mM NaF, 5mM EDTA and 50mM Tris-HCl, pH7.5) plus protease inhibitors (Roche) and of 0.5mm glass bead

were added to the pellet and shaken vigorously with a bead beater (Biospecs) for 1min at 4°C. The lysates were cleared by centrifugation at max speed for 15 min at 4°C, and transferred to clean tubes.

2.9.2 Quantitation of Proteins

The lysates were quantitated by Bradford assay. 1µl of the lysates was mixed with 1.8ml of H_2O, and 200µl of Bradford Reagent (BioRad). The mixture was incubated for 5 min at room temperature. The OD was measured at 595nm and converted to a concentration based on the standard curve created each time.

2.9.3 Western Analysis

For immunoblot (western) analysis, 50 µg of protein lysates were mixed with an equal volume of 2x protein sample and samples were boiled for two minutes. Samples were loaded onto small 10% SDS-PAGE gels and were run at 75-100 volts. Protein gels were transferred to nitrocellulose using a semi-dry transfer apparatus (Millipore) and probed with appropriately diluted primary and secondary antibodies. The antibody binding were visualized using the Pierce Supersignal system according to the manufacturer's specifications.

Quantification of western data was conducted using the FluorChem 2.0 spot densitometry analysis program (Alpha Innotech). Images captured on film were digitized and analyzed. To ensure linearity of the signal, three to seven exposures were analyzed in each case, but avoiding saturated exposures. Because of the wide dynamic range in some experiments, the film exposures used for quantitation of some lanes do not necessarily include the exposure shown in the Figure. To control for loading, Cln protein signals were normalized to β-tubulin.

2.10 Expression of a Controlled Amount of *CLN1* mRNA from the *GAL* Promoter

To create a strain where the *GAL* promoter could be induced by very low concentrations of galactose, I deleted the promoters of the divergently-transcribed *GAL1*

and *GAL10* genes in a conditional *CLN* strain (*cln1 cln2 cln3 GAL-CLN1*), yielding *gal1 gal10* mutants (BS111). To express a controlled amount of *CLN1* mRNA, strain BS111 were grown in YEP + 1% raffinose + 1% galactose to mid-log phase, washed thoroughly with YEP + 1% raffinose, re-suspended in YEP + 1% raffinose for several hours to ensure that the *GAL-CLN1* were shut off. For northern analysis, cultures were grown to 1-3 x 10^7 cells per ml. To confirm that cell concentration did not had an effect on the ability of galactose to induce the *GAL-CLN1* gene at low doses of galactose, experiments were repeated at cell concentrations of ~1 x 10^5 cells per ml. To express defined amounts of *CLN1* in different sized cell populations, centrifugal elutriation were used to obtain small, unbudded cells. These small unbudded cells (~10-15 fL) were re-suspended in fresh YEP + 1% raffinose and split into eight fractions at a concentration of 5-10 x 10^6 cells per ml. In each fraction different amounts of galactose were added (final percentage: 0, 0.001%, 0.003%, 0.01%, 0.03%, 0.1%, 0.3% and 1%) and cells were incubated at 30°C. Samples were taken at regular intervals for cell size and budding. To produce larger unbudded G1-phase cells, initial elutriated fractions were re-suspended in fresh YEP + 1% raffinose and incubated at 30°C until the desired size were achieved.

Table 2.1 Yeast Strains used in the study

Strain	Genotype
W303	*MATa ade2Δ can1Δ his3Δ leu2Δ trp1Δ ura3Δ*
GT104	*MATa ade2Δ can1Δ his3Δ leu2Δ trp1Δ ura3Δ CLN1-HA3*
GT108	*MATa ade2Δ can1Δ his3Δ leu2Δ trp1Δ ura3Δ CLN3-HA3*
BS111	*MATa ade2Δ can1Δ ura3Δ cln1::GALCLN1-HA3 cln2::TRP1 cln3::HIS3 gal1,gal10::hisg*
MT263	*MATa ade2Δ can1Δ his3Δ leu2Δ trp1Δ ura3Δ CLN2-HA3*
BY4741	*MATa his3Δ leu2Δ met15Δ ura3Δ*
BY4742	*MATα his3Δ leu2Δ lys2Δ ura3Δ*
BY4743	MAT a/α 4741/4742
ccr4Δ	*isogenic to BY4743, except for ccr4::KanMX*

Table 2.2 Plasmids used in the study

Plamid	**Source**
GAL-CLN1	This study
GAL-CLN2	This study
GAL-CLN3	This study
GAL-SWI4	This study
GAL-SWI6	This study
GAL-BCK2	This study
GAL-CCR4HA	Komarnitsky SL et al, 1998, Mol Cell Biol, 18:2100-2107.
CCR4-FLAG	Chen J et al, 2002. EMBO J, 21:1414-1426.

CHAPTER III
START AND *CLN* EXPRESSION

3.1 Summary

In *S. cerevisiae*, commitment to cell cycle progression occurs at Start. Progression past Start requires cell growth and protein synthesis, a minimum cell size, and G1-phase cyclins. The relationships between these factors were examined. Rapidly growing cells expressed, and required, dramatically more Cln protein than did slowly growing cells. To clarify the role of cell size, defined amounts of *CLN* mRNA were expressed in cells of different sizes. When Cln was expressed at near-physiological levels, a critical threshold of Cln expression was required for cell cycle progression, and this critical threshold varied with both cell size and growth rate: as cells grew larger, they needed less *CLN* mRNA, but as cells grew faster, they needed more Cln protein. At least in part, large cells had a reduced requirement for *CLN* mRNA because large cells generated more Cln protein per unit mRNA than small cells. When Cln was over-expressed, it was capable of promoting Start rapidly, regardless of cell size or growth rate. In summary, the amount of Cln required for Start depends dramatically on both cell size and cell growth rate. Large cells generate more Cln1 or Cln2 protein for given amount of *CLN* mRNA, suggesting the existence of a novel, post-transcriptional size-control mechanism.

3.1.1 Research Objective

The budding yeast coordinates cell growth and division by reaching a threshold size before commitment to cell division (Johnston, Pringle et al. 1977). This coordination establishes cell size homeostasis by preventing yeast cells from becoming either progressively smaller or larger. However, the molecular mechanism underlying a critical cell size requirement is not clear.

As discussed in Chapter 1, the timely expression of G1-phase cyclins is required for the commitment to cell division in eukaryotes. fIn budding yeast, right before Start, Cln3-Cdc28 phosphorylates Whi5 and hence activates two transcription factors SBF and

MBF that in turn lead to transcription of many genes including two other G1-phase cyclins *CLN1* and *CLN2* (Figure 1.5) (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). One crucial function of Cln1 and Cln2 is to inactivate Sic1 by Cdc28 phosphorylation and subsequent ubiquitination (Schneider, Yang et al. 1996; Tyers 1996). Sic1 is a cell cycle inhibitor that represses functions of the S-phase cyclins Clb5 and Clb6 (Schwob, Bohm et al. 1994). Phosphorylated Sic1 is then specifically recognized by the F-box protein Cdc4 that targets Sic1 for ubiquitination by the Cdc34–SCF complex (Feldman, Correll et al. 1997). There are nine Cdc28 consensus sites on Sic1 (Nash, Tang et al. 2001). However, phosphorylation of any one of the nine sites on Sic1 did not lead to Cdc4 binding (Nash, Tang et al. 2001). Rather, efficient Cdc4 recognition requires phosphorylation of at least six sites (Nash, Tang et al. 2001). This experiment and other elegant studies suggested that Cln1 and Cln2 must reach a critical level so that it can completely eliminate Sic1 for S phase entry (Schwob, Bohm et al. 1994; Schneider, Yang et al. 1996; Verma, Annan et al. 1997), but this idea has not been quantitatively tested. Thus, the objectives for this research were to address two questions: Is there a Cln threshold for Start? Why is a critical cell size required for Start?

3.1.2 Hypothesis

I hypothesized that there was a Cln threshold required for Start. I further hypothesized that cell size modulated Cln expression to ensure that at critical cell size cells produce enough Cln to exceed the Cln threshold.

3.1.3 Rationale and Expected Results

The idea of a *CLN* threshold was simple: at or above a certain *CLN* level, all cells past Start could be microscopically monitored. Below that *CLN* level, no cell executed Start. Therefore, two conditions must be satisfied. First, it was important to construct a strain in which *CLN* expression can be finely tuned from an inducible promoter. Second, the endogenous *CLN*s must be completely removed to avoid their interference with the result.

To test whether or not *CLN* expression (both mRNA and protein) was modulated by cell size, *CLN* would be induced in cells of different sizes. If the hypothesis was right, a size-dependent expression of *CLN* mRNA and/or protein would be observed.

3.2 There Is a Cln Threshold

3.2.1 A System for Controlled Cln Expression

To quantitatively evaluate whether a threshold amount of Cln was needed for Start, a system to express controllable amounts of *CLN* mRNA was developed. Strains containing integrated *GAL-CLN* constructs give high, non-physiological levels of *CLN* expression when incubated with 1% galactose. On the other hand, very low concentrations of galactose could not be used, because the cells metabolize galactose, leading to its rapid depletion. To create a strain where the *GAL* promoter could be induced by very low concentrations of galactose without such depletion, the promoters of the divergently-transcribed *GAL1* and *GAL10* genes were deleted in a conditional *CLN* strain (*cln1Δ cln2Δ cln3Δ GAL-CLN1*), yielding *gal1Δ gal10Δ* strains. *GAL1* encodes galactokinase, which is required for the first step in galactose metabolism, so this strain had little or no ability to metabolize galactose (Hovland, Flick et al. 1989).

Initially I needed to determine if the *cln1Δ cln2Δ cln3Δ GAL-CLN1 gal1Δ gal10Δ* strain (BS111) could be used to express controlled levels of *CLN1*. To accomplish this, the BS111 strain was grown in YEP + 1% raffinose + 1% galactose to mid-log phase, washed thoroughly with YEP + 1% raffinose, re-suspended in YEP + 1% raffinose, and then the culture was split into seven aliquots. Different amounts of galactose (final percentage: 0, 0.001%, 0.002%, 0.003%, 0.01%, 0.03%, and 0.1%) were added, and after 90 min, cells were harvested and the level of *CLN1* mRNA was assayed by northern analysis. As shown in Figure 3.1A and 3.1B, there was a fairly direct relationship between the amount of galactose added and the level of expression of *GAL-CLN1*. In fact, between 0.001% and 0.03% galactose, the relationship was close to linear. At concentrations greater than 0.03% galactose, the response was partially saturated (Figure 3.1B).

A graded response like the one shown in Figure 3.1 could be explained if each cell in the population had a graded response to increasing galactose concentrations, or if any one cell had an all-or-none response, but the proportion of responding cells increased with galactose concentration. However, the second explanation was not correct, as in this strain, viability depended on expression of *GAL-CLN1*. At very low concentrations of galactose, cells failed to bud and proliferate, and at slightly higher concentrations, nearly all of the cells budded and divided (Figure 3.2). Thus, I concluded that the *GAL* promoter had a graded response to galactose concentration. A graded response for the *GAL* promoter had also been seen by Biggar and Crabtree (Biggar and Crabtree 2001).

The response to low concentrations of galactose as a function of time was also measured. After 6 hours of induction, the amount of *CLN1* mRNA had decreased to about half the level of the 90 min. measurement (data not shown). There were many potential reasons for this loss of response with time; for instance, galactokinase may be expressed at low levels despite loss of its promoter. Alternatively, since measurements were being made per unit of total cellular RNA, this "decrease" in *CLN1* mRNA could simply reflect the fact that the cells continue to grow in size, so the amount of normalizing, total RNA increases. In any case, because the loss of response was the same for 0.01% and 0.003% galactose over a six hour period, it was reasonable to compare cellular responses to these levels of galactose over this time period.

3.2.2 A Cln1 Threshold Was Required for Start

To determine if cells required a discrete level of *CLN* expression for Start, the strain BS111 (*cln1Δ cln2Δ cln3Δ GAL-CLN1 gal1Δ gal10Δ*) was used. The strain BS111 was grown in YEP + 1% raffinose + 1% galactose to mid-log phase, washed thoroughly with YEP + 1% raffinose, and re-suspended in YEP + 1% raffinose to shut off *GAL-CLN1*. Subsequently, centrifugal elutriation was used to obtain small, unbudded cells. These small unbudded cells (10~15 fL) were re-suspended in fresh YEP + 1% raffinose and split into eight fractions. In each fraction, a different amount of galactose was added (final percentage: 0, 0.001%, 0.003%, 0.01%, 0.03%, 0.1%, 0.3% and 1%) and cells

were incubated at 30°C. Samples were taken at regular intervals, and cell size and percentage budding were measured. Results were shown in Figure 3.2. In Figure 3.2A, the budding data were plotted as a function of time; in Figure 3.2B, the same budding data were plotted as a function of cell size.

There were three especially noteworthy points. First, it appeared that there was a threshold requirement for *CLN1*, which was achieved at a galactose concentration of 0.01%. A three-fold lower level of galactose (0.003%) gave lower but detectable levels of *CLN1* expression, and yet cells failed to progress past Start. The cells in 0.003% and 0.001% galactose were also examined after 9 and 24 hours, and there was still no budding (however it should be noted that the amount of *CLN1* mRNA in asynchronous BS111 cultures decreased two-fold from 1.5 to 6 hours and even more after 9 and 24 hours, after normalization to total RNA). On the other hand, three-fold higher levels of galactose (0.03%) rapidly promoted progression past Start.

Second, when *GAL-CLN1* expression was relatively high (0.03% galactose or more), budding occurs very rapidly, and occurs at very small cell sizes (less than 20 fL). Under these conditions, a wild-type cell would not bud until a size of about 27 fL. Budding at very small cell sizes suggests that Cln was either the major or perhaps the only, limiting factor for Start.

Third, at the threshold level (0.01% galactose), the budding profiles versus size were remarkably similar to budding profiles in wild-type cells, even though the normal wave of SBF and MBF-dependent transcription (which depends on Cln3) was presumably largely absent, and even though the transcriptional regulation of *CLN1* had an entirely different basis. Perhaps most strikingly, the cells grown in 0.01% galactose did not bud during the first two hours (even though this was the period of maximal *GAL-CLN1* expression), but then began to bud in the third hour (when *GAL-CLN1* expression was beginning to wane in asynchronous BS111 cultures!), a time when they achieved a size of about 25 fL, which was similar to the critical size for wild-type cells under these growth conditions. In other words, some mechanism was implementing relatively normal

size control, despite the absence of *CLN2* and *CLN3* and the constitutive expression of *CLN1*.

3.3 The Cln Threshold Was Modulated by Cell Growth Rates

Yeast cells live in a variety of environments. When the environment was optimal, cells grow and proliferate rapidly. Under hostile conditions, the cells grow very slowly. Thus, their division time may vary greatly from 90 minutes to 600 minutes, and the slowly growing cells had a longer G1 phase than fast growing ones. Considering a Cln threshold is required for Start, I asked if cells with different growth rates had to achieve the same Cln threshold or different thresholds. Both *CLN* mRNA and protein are very unstable, and their synthesis is extremely sensitive to external environmental changes, such as heat shock and nutrients (Rowley, Johnston et al. 1993; Hall, Markwardt et al. 1998; Newcomb, Diderich et al. 2003). Moreover, the instability of *CLN* mRNAs and proteins is unaffected by growth rate (Figure 3.3A). Therefore, I hypothesized that there were different Cln thresholds for cells with different growth rates.

3.3.1 Abundance of Cln Protein Was Correlated With Growth Rate

To understand how cell growth may affect the expression of *CLN*s, *CLN1* and *CLN2* mRNA and protein levels were determined in asynchronous cultures growing at different rates. In budding yeast, the growth rates can be modulated by growing cells in different carbon sources. For example, cells growing in 2% glucose, 2% raffinose, or 3% ethanol at 30°C had mass doubling times of about 90 min, 130 min, or 220 min respectively. Wild type cells were harvested at log phase to extract total RNA and proteins for Northern or Western blot. As shown in Figure 3.3A and C, the abundance of *CLN1*, *CLN2* and *CLN3* mRNAs were surprisingly similar in all three growth conditions. However, Cln1 and Cln2 protein levels varied greatly (Figure 3.3B and D). The cells in glucose expressed five to seven times more Cln1-2 than those in ethanol, whereas the cells growing in raffinose had an intermediate abundance of Cln1-2 (Figures 3.3B and 3.3D). To determine if this posttranscriptional effect was specific to carbon sources, cell

growth was also modulated by using different nitrogen sources in synthetic medium. Cells in YNB-proline, YNB-arginine or YNB-ammonium sulfate had fast, moderate or slow growth respectively. As expected, Cln2 protein levels were high when growth was rapidly, and low when growth was slow. Thus, the modulation of Cln expression by cell growth was not limited to carbon sources (Figure 3.3E, bottom panel).

3.3.2 The Requirement for Cln Protein at Start Was Correlated With Growth Rate

The experiments discussed above were conducted on asynchronous cells, but the critical issue was the amount of Cln expressed at Start. Thus, it was possible that the small amounts of Cln in slowly-growing cells reflect a long period in early G1-phase where there was no Cln, followed by a short period in late G1-phase where Cln levels might be comparable to Cln levels in rapidly proliferating cells. Indeed, Silje et al. had shown in very slowly proliferating cells with a G1-phase of 580 min that *CLN1* and *CLN2* mRNAs were not significantly expressed until slightly more than half of G1-phase had passed (this was not true for *CLN3* mRNA, however, which was expressed at relatively constant levels throughout G1-phase) (Tyers, Tokiwa et al. 1993; Stuart and Wittenberg 1995; Sillje, ter Schure et al. 1997). To address this issue, centrifugal elutriation was used to fractionate cultures according to cell size and cell cycle position, and then Cln levels were measured just before Start, at Start, and just after Start.

The experiments were then performed with cells growing in YEP plus 2% sucrose, or 2% raffinose, or 3% ethanol, with the doubling times of about 100 min, 130 min, or 220 min, respectively. In these cases, selected fractions around Start were chosen for analysis by western. Synchrony and cell cycle position were determined by the percent of budded cells, cytometry, and alpha factor resistance assays. As shown in Figure 3.4, the pre-Start cells growing in sucrose (lanes 7-9) had more Cln2 than cells growing in ethanol (lane 3) or raffinose (lanes 5 and 6) at Start (Figure 3.4A and B). This showed that the rapidly-growing cells required more Cln for Start than the slowly-growing cells. Thus, the amount of Cln required for Start varied dramatically according to growth conditions (see Discussion).

3.3.3 A Model How This Threshold Works

In summary, the above results clearly showed that the fast growing cells had or require more Cln protein for Start. Hence rapidly-growing cells must have a higher Cln threshold than slowly growing cells do. Based on this conclusion, a variable threshold model was proposed here: in slowly growing cells, the abundance of Cln protein was low, but the threshold level required for progression past Start was also low. Right before Start, Cln proteins accumulated to a level higher than the threshold level and lead to execution of Start. In contrast, in fast growing cells, the Cln threshold for Start was higher. This required production of more Cln proteins for the execution of Start (Figure 3.5).

3.4 Cln Expression Is Modulated by Cell Size

In the previous experiments, the cells with 0.01% galactose did not progress past Start until they achieved a size of about 27fL (Figure 3.2B), similar to the critical size for wild-type cells under the same growth condition. It was well known that in budding yeast, there was a size requirement for Start. That is, a minimal size must be reached before cells can execute Start. Coincidentally, *CLN1* and *CLN2* mRNA and protein abundance peaks right before Start. So the next issue I addressed was the relationship between cell size and Cln expression before Start. I hypothesized that cell size can modulate Cln protein expression.

3.4.1 Cln Expression Was Size Dependent

Why did the cells grown in 0.01% galactose delay budding for two hours, until they achieved a size of 27fL? One possibility was that Cln1 works over time, and that the cells required exposure to Cln1 for a substantial period of time before its work was done; i.e., the activity of Cln was integrated over time. A second possibility was that as cells became larger, they became more sensitive to *CLN1* mRNA (e.g., by synthesizing more protein, or, alternatively, by loss of an inhibitor of Cln function). To distinguish these

possibilities, an experiment was designed to obtain unbudded, Cln-less G1-phase cells of different sizes, and then inducing *GAL-CLN1* in these cells. To do this, strain BS111 was grown to mid-log phase in YEP + 1% galactose and 1% raffinose, washed with YEP + 1% raffinose, and re-suspended in YEP + 1% raffinose to shut off *GAL-CLN1*. Subsequently, centrifugal elutriation was used to obtain small, unbudded cells, exactly as for Figure 3.6. These G1-phase cells were re-suspended in YEP + 1% raffinose and split into three fractions, labeled "Small", "Medium", and "Large." The "Small" cells (20fL) were further split into three aliquots, and galactose was immediately added to a final concentration of 0%, 0.003%, or 0.01%. The "Medium" cells were incubated in YEP + 1% raffinose at 30°C until they grew to 30fL, and then galactose was added to a final concentration of 0%, 0.003%, or 0.01%. The "Large" cells were incubated in YEP + 1% raffinose at 30°C until they grew to 40fL, and then they were treated as above. Thus, *GAL-CLN1* was turned on in the "Small", "Medium", and "Large" cells at sizes of 20, 30, and 40fL, respectively.

None of the cells budded at any time in 0% or 0.003% galactose (data not shown). However, cells exposed to 0.01% galactose budded after a time that depended on their initial size (Figure 3.6). The "Large" was 50% budded after 45 min; the "Medium" cells was 50% budded after 75 min; and the "Small" was 50% budded after 150 min, by which time they had achieved a size of 31fL. These results suggest that cell size, rather than the length of exposure to Cln1, was important.

It was possible that cell size affected the amount of *CLN1* mRNA made in response to a given dose of galactose, or, alternatively, that cell size affected the amount of Cln1 protein made in response to a given dose of mRNA. To investigate this, the level of *CLN1* mRNA and Cln1 protein in the smallest fraction of cells was examined as a function of time. During the time course (0-180 min), these cells (Figure 3.6 closed diamond) grew to 38 fL. By measuring cell size at each time point, it was found that the ratio of *CLN1*/*ACT1* mRNAs was unaffected by cell size (Figure 3.7). In contrast, the level of Cln1 protein varied considerably with cell size (Figure 3.7). The amount of Cln1 protein expressed per unit *CLN1* mRNA increased dramatically as cells got larger (Figure

3.7). A 50% increase in cell size resulted in a four-fold increase in the amount of Cln1 protein expressed per unit *CLN1* mRNA; similarly, a 100% increase in cell size resulted in an eight-fold increase in the amount of Cln1 protein expressed per unit *CLN1* mRNA (Figure 3.7B).

To investigate this observation further, I transformed strain BS111 with a plasmid containing an HA epitope tagged *CLN2* gene under the control of *S. pombe ADH* promoter and largely lacking the natural 5' UTR of *CLN2*. The *S. pombe ADH* promoter was constitutively expressed in *S. cerevisiae* and was not regulated by cell size or cell cycle position. Asynchronous cultures of BS111 transformed with this plasmid were grown to mid-log phase in YEP + 2% glucose. Centrifugal elutriation was used to fractionate cells by size. Western analysis showed that larger cells contained significantly more Cln2 protein than small cells (Data not shown). Thus, the greater sensitivity of larger cells to *CLN* mRNA was because these larger cells maintain more Cln protein per unit mRNA.

3.4.2 The Stability of Cln Protein Was Not Affected by Cell Size

What might be the mechanism by which larger cells had more Cln protein? One possibility was that in larger cells the Cln proteins were more stable than they were in smaller cells so that more Cln1 protein was able to accumulate rapidly as the cells got larger.

In order to examine this possibility, the unbudded cells of BS111 were again collected as previously. The cells were grown in YEP plus 1% raffinose until they reached 30fL or 40fL. Galactose was added to a final concentration of 0.01%, and the cells were incubated for 30 minutes. The gal-promoter was shut off by adding glucose to the cultures to a final concentration of 1%. The samples were collected at 0, 5, 10, 15, 30 and 60 minutes after glucose addition. The total RNA and proteins were prepared from each time point for detecting *CLN1* mRNA and protein.

As shown in Figure 3.8, *CLN1* mRNA was very unstable. The *CLN1* mRNA disappeared within 5 minutes in the cells of both sizes. Its half-life was too short to

measure. This was consistent with the previous result that the steady level of *CLN1* mRNA did not vary with cell size (Figure 3.8). It seems that Cln1 protein had a longer half-life than its mRNA. Thus, I was able to measure the half-lives in cells of both sizes. However, in both cases, the half-life was about 5 minutes (Figure 3.8).

The results clearly showed that the stability of both *CLN1* mRNA and protein did not vary much in cells with different sizes (Figure 3.8A and B). Thus, the *CLN1* mRNA and protein stability did not account for the accumulation of Cln1 protein. Rather, the result suggests that some other posttranscriptional mechanisms were implicated in the modulation of Cln protein expression of cell size.

3.5 Discussion

3.5.1 Models and Mechanisms Linking Cln Thresholds to Start

There have been two simple models of how Cln activity might be related to Start. First was the "Critical Threshold" model, which asserts that Start occurs when the amount of Cln rises to some critical threshold. However, because of the instability of Cln mRNA and protein, it was not clear how slowly-growing cells could ever attain the same levels of Cln as rapidly growing cells, and indeed, I showed that they did not (Figure 3.3 and 3.4). Rather, the low level of Cln in slowly-growing cells was the inevitable consequence of both an unstable protein and a low rate of protein synthesis. Nevertheless, the cells could still execute Start in a Cln-dependent manner, indicating a compensatory mechanism. Thus, the "Critical Threshold" model in its simplest form was incorrect, since slowly-growing and rapidly growing cells go through Start with very different amounts of Cln. Related results had previously been obtained by Heideman and co-workers (Hall, Markwardt et al. 1998; Parviz, Hall et al. 1998; Parviz and Heideman 1998; Newcomb, Diderich et al. 2003).

A second potential model was the "Integrated Activity" model, which asserts that Clns act over time during G1-phase, and their cumulative effect, integrated over time, must reach some minimum to induce Start. In other words, a large amount of Cln could induce Start after a short G1-phase, or a small amount of Cln could induce Start after a

longer G1-phase. A similar idea had been proposed by Heideman and co-workers (Hall, Markwardt et al. 1998; Parviz, Hall et al. 1998; Parviz and Heideman 1998; Newcomb, Diderich et al. 2003). This model was also wrong in its simplest form; Figure 3.2 showed that when Cln1 expression was reduced to one-third of its original level, Start never occurred, even after very long incubations. If the "Integrated Activity" model was true, this reduction in Cln1 expression would simply expand G1-phase by three-fold. In addition, our results suggested that absolute cell size was important, and not just the time spent expressing Cln.

Both models can be modified to fit the facts. With respect to the "Critical Threshold" model, one could imagine that the Cln-Cdc28 complexes were opposed by some phosphatase or other activity, and that the level of the phosphatase activity constitutes the threshold. If this phosphatase, like Cln1 and Cln2, had a half-life of about 10 min., then the level of the threshold would naturally go up and down with growth rates, more-or-less paralleling the changes in Cln activity. This would be a "Variable Threshold" model. With respect to the "Integrated Activity" model, one could imagine that an inhibitor of Start (e.g., Sic1, or the hypothetical phosphatase) was expressed in a peak in late M phase, but also expressed at a lower level throughout the cell cycle. The inhibitor made in M phase could be phosphorylated (and then degraded?) by a large amount of Cln over a short time, or by a small amount of Cln over a long time, but in either case, a critical minimum level of Cln would be needed to deal with the ongoing synthesis of the inhibitor.

3.5.2 The Effect of Size

When *GAL-CLN1* cells of different sizes were induced with galactose, they made the same amount of *CLN1* mRNA ("Same amount" here means after normalization to *ACT1* mRNA) (Figure 3.7A and B). In absolute terms, the large cells had more *ACT1* and *CLN1* mRNA than small cells). However, the larger cells went through Start much earlier than the smaller cells, showing, in some sense, a heightened sensitivity to *CLN* (Figure 3.6). Larger cells also contained a larger amount of Cln protein than the smaller

cells (Figure 3.7) (again, after normalization to a control protein). Similar results were obtained with *CLN2* expressed from the *S.p.ADH* promoter. Thus, larger cell size increased the relative amount of Cln protein by some post-transcriptional mechanism, and this might be essential for the accumulation of a critical amount of Cln.

In a wild-type cell, the ability of *CLN3* to activate SBF and MBF transcription depends on cell size, even when the amount of *CLN3* transcript remains relatively constant (Wittenberg, Sugimoto et al. 1990; Tyers, Tokiwa et al. 1993), and it had been suggested that Cln3 protein expression was also modulated by cell size (Dirick, Bohm et al. 1995). Thus, all three Cln proteins may increase in abundance with increased cell size, perhaps by a common post-transcriptional mechanism. The nature of this mechanism was unclear, but it could be increased translation. However, if increased translation was responsible, the effect did not seem to depend on the natural 5'mRNA leaders of *CLN1* and *CLN2*, which were absent in these experiments. Alternatively, it was possible that the Cln proteins were somehow selectively stabilized in larger G1-phase cells.

3.5.3 Redundant Cell Size Controllers

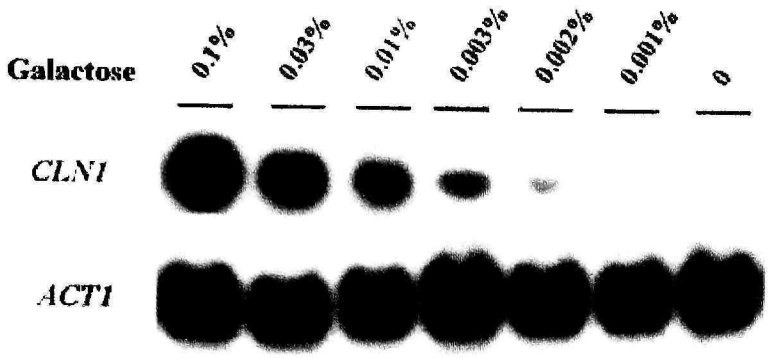
Previous work clearly showed that *CLN1-3* were important for cell size control (Nash, Tokiwa et al. 1988; Cross 1989; Mountain and Sudbery 1990). Yet, in the experiments shown in Figure 3.2 and Figure 3.6, cell size control was relatively normal, despite the fact that *CLN3* and *CLN2* had been deleted, and *CLN1* was being expressed at a constant level from the *GAL* promoter. Bck2 was partially redundant with Cln3 in providing size control, but it was also thought to work at least in part through the transcription of *CLN1* and *CLN2* (Epstein and Cross 1994; Di Como, Chang et al. 1995; Wijnen and Futcher 1999), and it was not clear how Bck2 could play a role.

Figure 3.2 and 3.6 showed cell size control at Start that was certainly independent of *CLN3*, and of the *CLN1* and *CLN2* promoters, and probably independent of *BCK2*. The result in Figure 3.7 showed that some of this size control came from the ability of large cells to generate relatively large amounts of protein from a given amount of *CLN* mRNA.

This protein synthesis based size control would be independent to the known pathways for *CLN* transcription. Recent genome-wide screens had found many new genes that help control cell size, and many of these appear to work independently of *CLN3* (Jorgensen, Nishikawa et al. 2002). Strikingly, many of these size control genes were affecting ribosome biogenesis, and therefore protein synthesis (e.g., *SFP1*, *SCH9*) (Jorgensen, Nishikawa et al. 2002). One or more of these new size control/protein synthesis genes might be responsible for this redundant control mechanism.

As a wild-type cell grows through G1-phase, it transcribes increasingly larger amounts of *CLN1*, *CLN2*, and other SBF- and MBF-dependent genes (Dirick, Bohm et al. 1995). This increase in transcription with cell size is largely dependent on Cln3 and helps a cell pass through Start (Dirick, Bohm et al. 1995; Stuart and Wittenberg 1995; Hall, Markwardt et al. 1998). At the same time, there could be a novel Cln3-independent mechanism rendering Start more sensitive to a given amount of *CLN1* or *CLN2* transcript. That is, as cells grow, they not only make more *CLN1* and *CLN2* transcripts, but also a larger amount of Cln1 and Cln2 protein per transcript. These two effects may convert moderate, gradual changes in size into a sharp, switch-like change in cell fate.

A.



B.

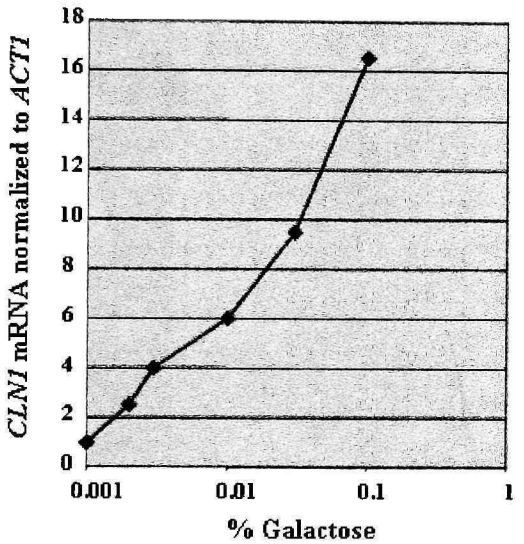
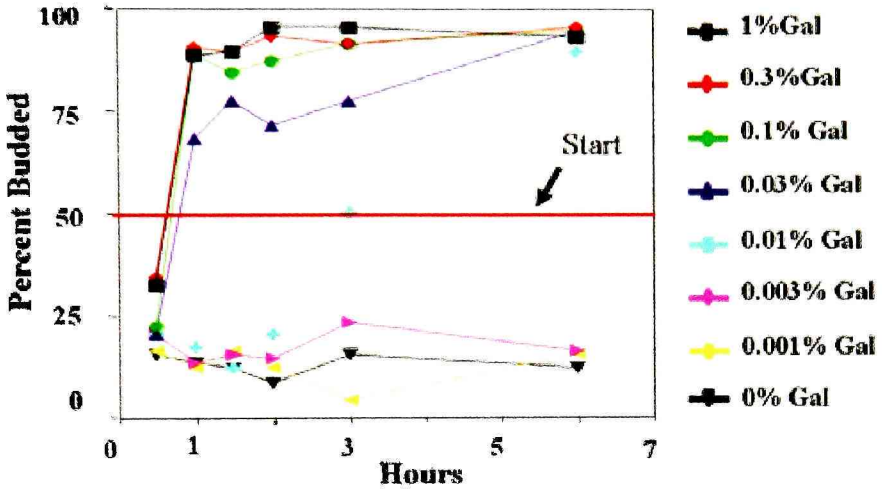


Figure 3.1. An experimental system for expressing controlled amounts of *CLN1* mRNA. BS111 (*gal1Δ gal10Δ cln1Δ cln2Δ cln3Δ GAL-CLN1*) was used to express controlled levels of *CLN1*. The cells were grown in YEP plus 1% raffinose and 1% galactose to mid-log phase, then washed thoroughly with YEP plus 1% raffinose three times, and resuspended in YEP plus 1% raffinose. The cells were split into seven aliquots and different amounts of galactose were added to the final concentration of 0, 0.001%, 0.002%, 0.003%, 0.01%, 0.03%, and 0.1%. The cells were incubated at 30°C for 90 min and harvested for *CLN1* mRNA expression by northern analysis. As shown in A, there was a near dose-dependent relationship between the amount of galactose and the abundance of *CLN1* mRNA. At galactose concentrations higher than 0.1%, the response is partially saturated. B. *CLN1* abundance was quantitated and normalized to *ACT1*, the loading control. The values were plotted as a function of galactose concentration.

A.


100
75
50
25
0
Percent Budded
0
1
3
5
7
Hours
Start
1%Gal
0.3%Gal
0.1% Gal
0.03% Gal
0.01% Gal
0.003% Gal
0.001% Gal
0% Gal

B.

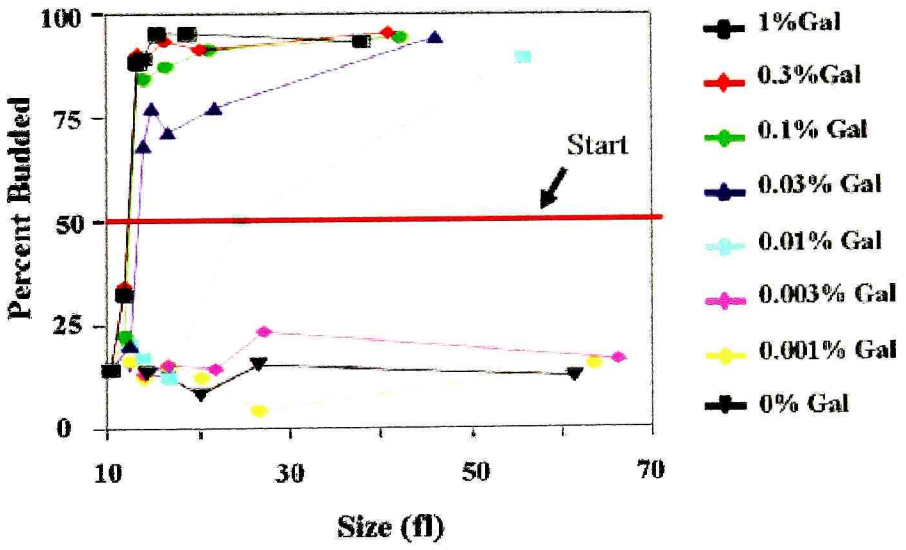

100
75
50
25
0
Percent Budded
10
30
50
70
Size (fl)
Start
1%Gal
0.3%Gal
0.1% Gal
0.03% Gal
0.01% Gal
0.003% Gal
0.001% Gal
0% Gal

Figure 3.2. A critical Cln threshold for Start. The cells were grown in YEP plus 1% raffinose and 1% galactose to mid-log phase. Small, unbudded G1 cells were obtained by centrifugal elutriation and divided into eight aliquots. Different concentrations of galactose were added to each aliquot as indicated. The cell size and budding indexes, the indicator for Start, were examined at regular time intervals. Start was defined as the point where 50% of the cells have budded (red line). The percentage of budded cells was plotted as a function of time (A) or cell size (B). The cells incubated with 0.01% galactose budded at 3 hours (A) or at 27fL (B), similar to the wild type (data not shown). A three fold decrease (0.003%) of galactose failed to induce budding even after 6 hours (A) or larger than 60fL (B). In contrast, a three fold higher (0.03%) galactose concentration induced progression past Start in less than one hour (A) or at 15fL, much earlier or at much smaller cell sizes than the wild type cells.

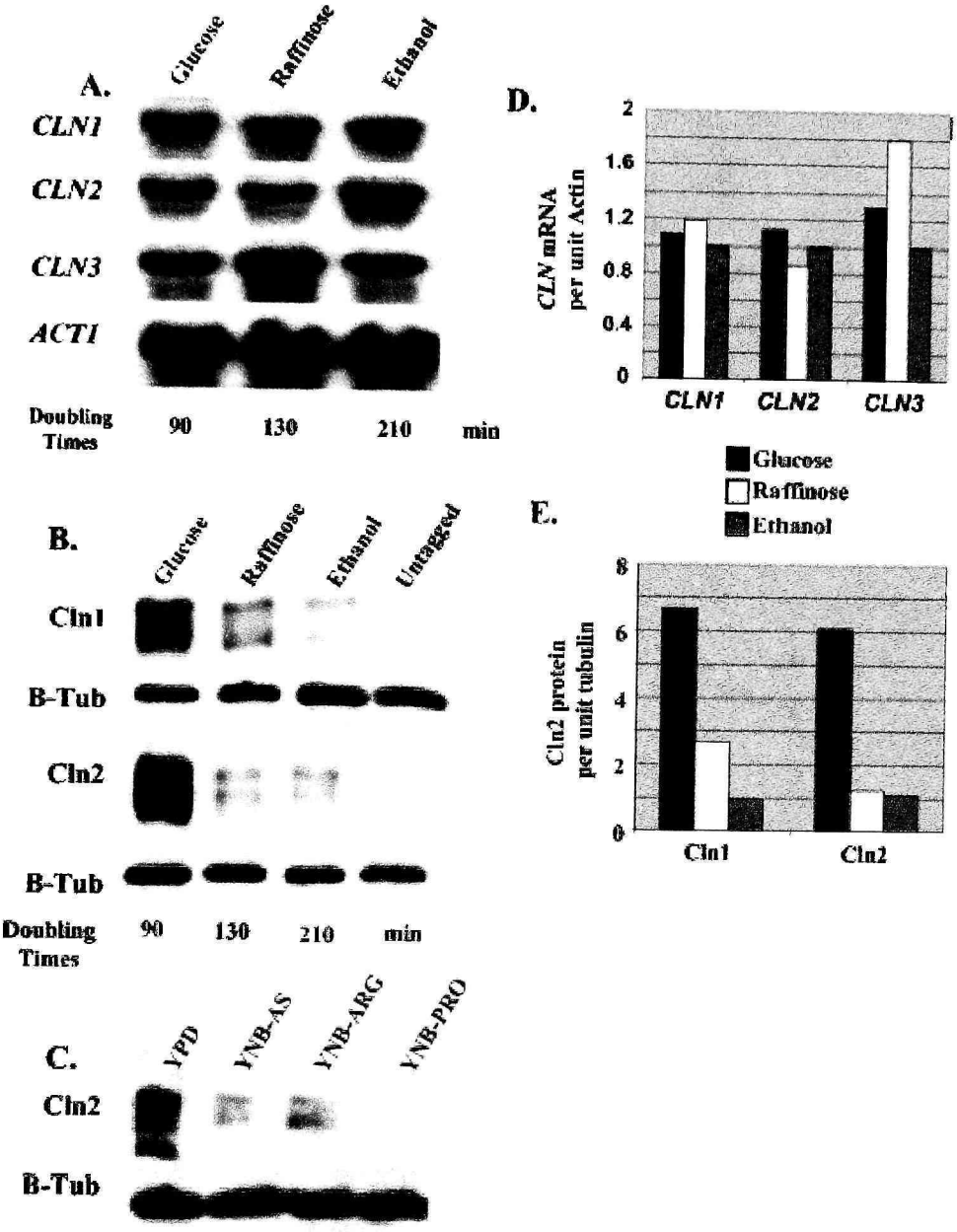

A.
Glucose
Raffinose
Ethanol
CLN1
CLN2
CLN3
ACT1
Doubling Times
90
130
210
min
B.
Glucose
Raffinose
Ethanol
Untagged
Cln1
B-Tub
Cln2
B-Tub
Doubling Times
90
130
210
min
C.
YPD
YNB-AS
YNB-ARG
YNB-PRO
Cln2
B-Tub
D.
CLN mRNA per unit Actin
2
1.6
1.2
0.8
0.4
0
CLN1
CLN2
CLN3
Glucose
Raffinose
Ethanol
E.
Cln2 protein per unit tubulin
8
6
4
2
0
Cln1
Cln2

Figure 3.3. Cln protein levels are modulated by growth rate. Asynchronous cultures of HA-epitope tagged Cln1 (GT104) or Cln2 (MT263) were grown in YEP plus 2% glucose (fast), 2% raffinose (moderate) or 3% ethanol (slow) respectively. The cells were harvested in mid-log phase and total RNA and proteins were extracted. **A.** *CLN1-3* mRNA did not vary in cells with different growth rates. The *ACT1* mRNA was used as a loading control. **B.** Cln1 and Cln2 proteins revealed that glucose grown cultures expressed more Cln protein than cultures grown in raffinose, or ethanol. Lysates from an untagged strain were used as a negative control. β-tubulin served as a loading control. **C.** growth rates were modulated by different nitrogen sources. Cells were grown in synthetic YNB medium with glucose. Nitrogen sources were ammonium sulfate (YNB-AS), arginine (YNB-ARG), or proline (YNB-PRO). **D.** Quantitation of *CLN* mRNAs of Figure A. *CLN* mRNAs were quantified normalized to *ACT1* mRNA. Subsequently, these values were normalized to the signal in the ethanol cultures, which was set equal to 1, and plotted. **E.** Quantitation of Cln proteins of Figure B. Cln proteins were quantitated and normalized to β - tubulin. Subsequently, these values were normalized to the signals in the ethanol cultures, which were set equal to 1, and plotted. Black bar, fast growing cells; White bar, moderately growing cells; and grey bar, slowly growing cells.

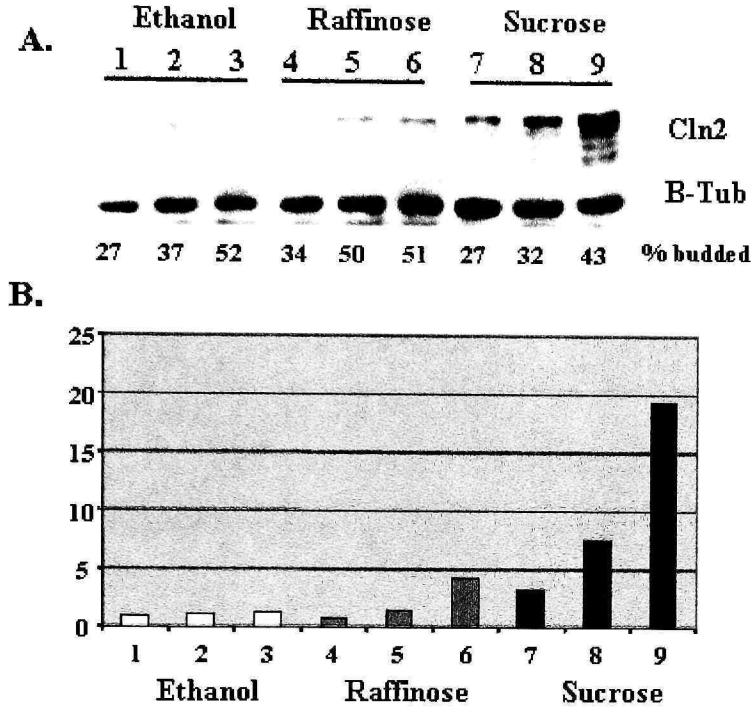


Figure 3.4. Slowly growing cells require less Cln2 to progress past Start. Asynchronous cultures of HA-epitope tagged Cln2 (MT263) were grown in YEP plus 3% ethanol (lanes 1-3), 2% raffinose (lanes 4-6), or 2% sucrose (lanes 7-9). Cultures were grown to mid-log phase, harvested by centrifugation at 0°C and elutriated. Fractions were collected on ice. The percentage of budded cells in each fraction was counted. A. Proteins from fractions before Start and right at Start were extracted and Cln2 was detected. Lane assignment: lanes 1-3, 3% ethanol; 4-6, 2% raffinose; 7-9, 2% sucrose. B. Quantitation of Cln2 protein. Cln2 protein was quantitated and normalized to β-tubulin. Subsequently, these values were normalized to the signal in the first ethanol fraction (lane 1), which was set equal to 1, and plotted. These data revealed that rapidly growing cells that have not yet progressed past Start (e.g. sucrose fractions 7 and 8) express considerably more (2-16 fold) Cln2 protein than slowly growing cells at Start (e.g. ethanol fraction 3 and raffinose fractions 5 and 6).

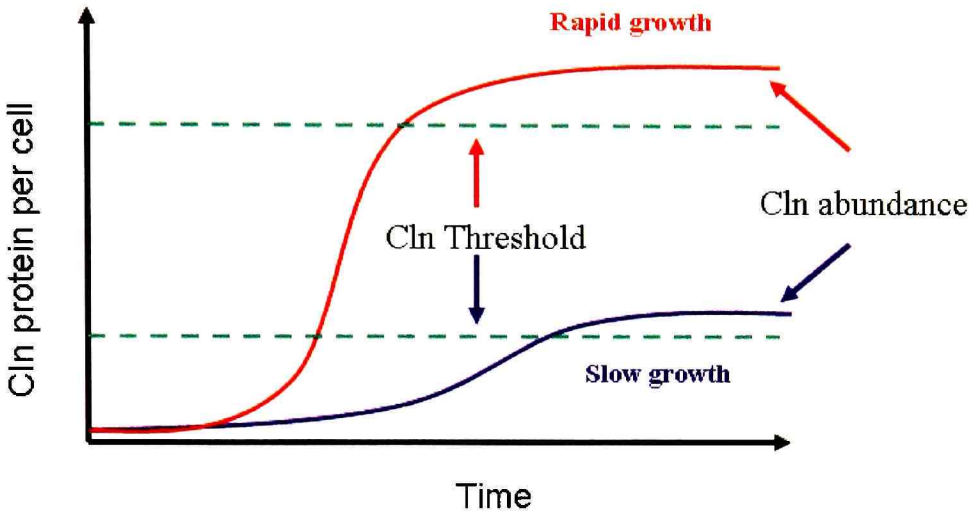


Figure 3.5. A variable threshold model was proposed here: in slowly growing cells, the abundance of Cln protein is low, but the threshold level required for progression past Start is also low. Meanwhile, expression of Cln protein in fast growing cells is high, but the threshold for Start execution is also high. Therefore, slowly growing cells require less Cln to execute Start than fast growing cells (Figure 8). Solid lines represented Cln protein abundance. Dashed lines represented Cln thresholds.

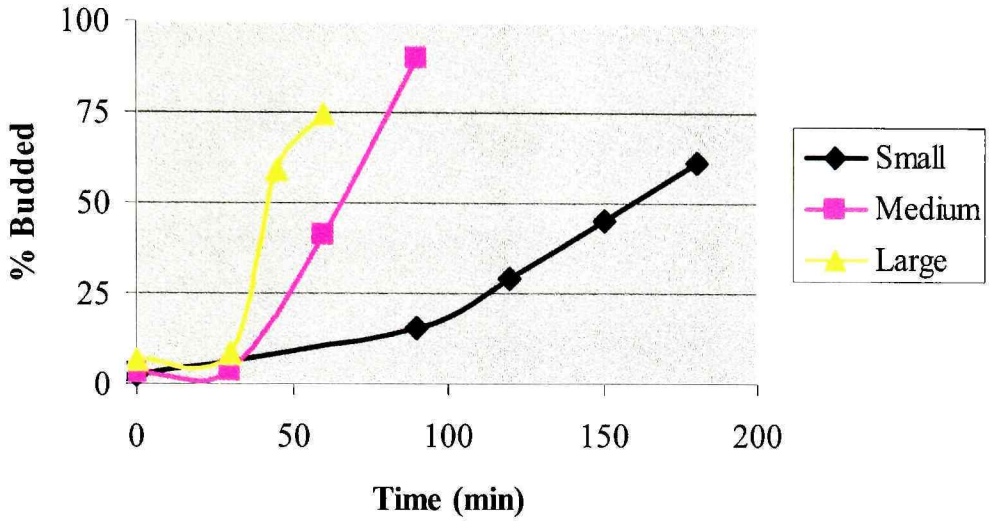
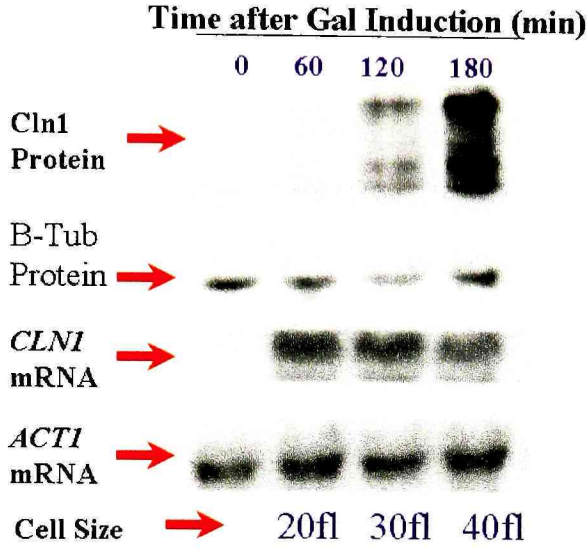


Figure 3.6. Cells with different sizes had different sensitivity to Cln expression. BS111 cells were grown to mid-log phase in YEP plus 1% galactose and 1% raffinose, washed with YEP plus 1% raffinose, and resuspended in YEP plus 1% raffinose to shut off *GAL-CLN1*. Next, homogeneously sized unbudded cells were collected by centrifugal elutriation, as for Figure 4. These G1-phase cells were split into three fractions and incubated in YEP plus 1% raffinose at 30°C. Because there is no galactose to induce *CLN1*, the cells would grow in size without division. When the cells reached 20fL, 30fL or 40fL, they were labeled as "Small" (20fL), "Medium" (30fL) and "Large" (40fL) respectively. Next, 0.01% of galactose was added to each fraction. While the cells continued to be incubated, samples were taken for cell size and budding indexes.

A.



B.

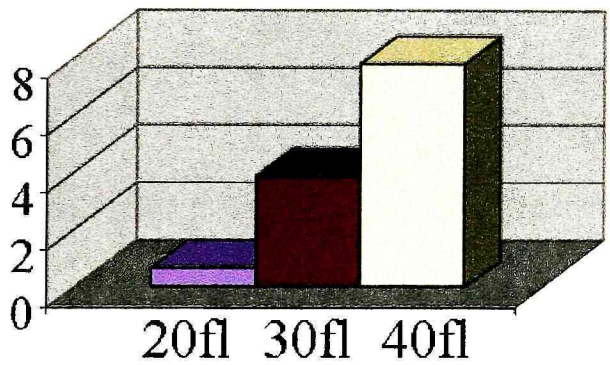
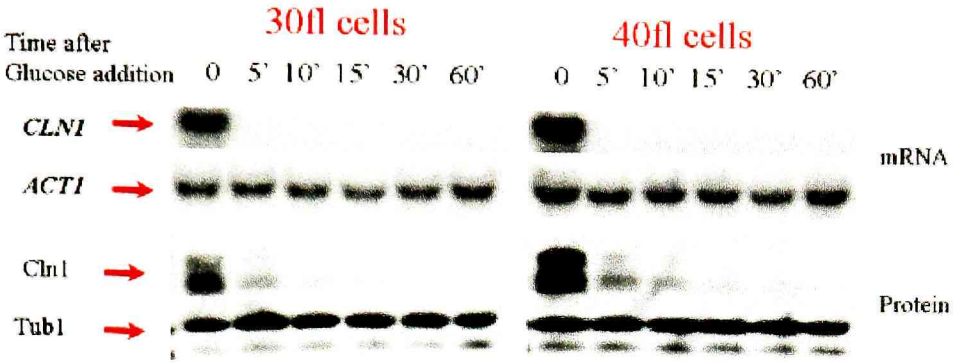


Figure 3.7. The BS111 samples were taken from the "Small" fraction of Figure 3.6 at 0 min, 60 min, 120 min and 180 min after the galactose addition. Both RNA and protein were extracted from these samples for Cln expression. A. Northern blot showed that the abundance of *CLN1* mRNA did not vary as a function of cell size. In contrast, the Cln1 protein level varied dramatically with cell size. B. The amount of Cln1 protein expressed per unit *CLN1* mRNA increased exponentially as cell size increased. A 50% increase in cell size resulted in a four-fold increase in the amount Cln1 protein expressed per unit *CLN1* mRNA. Similarly, a 100% increase in cell size resulted in an eight-fold increase in the amount Cln1 protein expressed per unit *CLN1* mRNA.

A.



B.

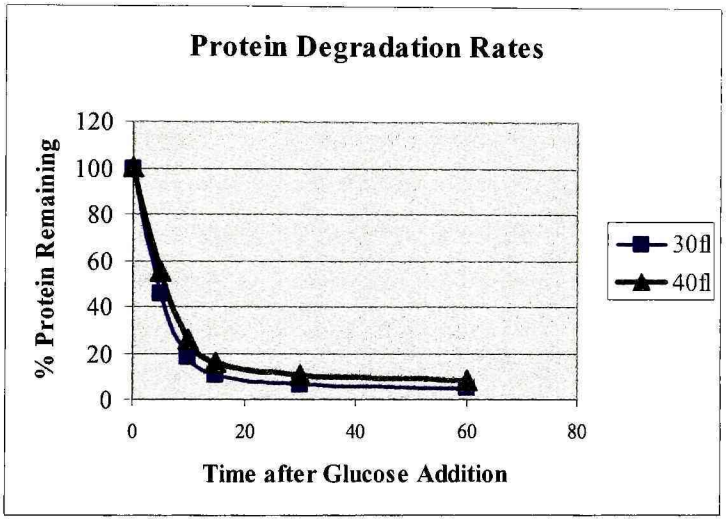


Figure 3.8. *CLN1* mRNA and protein stability was not affected by cell size. The unbudded cells of BS111 were again collected as Figure 3.6. The cells of 20fL or 30fL were incubated with 0.01% of galactose for 30 minutes. The galactose induction was stopped by adding glucose to 1% into the culture. The samples were collected at 0, 5, 10, 15, 30 and 60 minutes after glucose addition. The total RNA and proteins were prepared from each time point for detecting *CLN1* mRNA and protein. A. No significant differences between the stability of *CLN1* mRNA and protein in the cells of 30fL or 40fL. In both cases, the half life of mRNA and protein is very short. B. Graph of the half life of the Cln1 protein. The Cln1 protein was quantitated and normalized to β-tubulin. The values were plotted as a function of time.

CHAPTER IV
GENOME-WIDE SCREENING FOR CELL SIZE MUTANTS

4.1 Summary

In most eukaryotic cells, there is a relationship between cell size and proliferative capacity. For example, in order to commit to cell division, the yeast *Saccharomyces cerevisiae* must attain a "critical cell size." This mechanism coordinates growth with cell division to maintain cell size homeostasis. Because very few cell size control genes are known, the genetic pathways responsible for cell size homeostasis remain obscure. Furthermore, elucidation of the mechanism of cell size homeostasis has been recalcitrant to genetic analysis primarily due to the difficulty in cloning cell size control genes. To identify new size control genes, the effect of 5,958 single gene deletions (4,792 homozygous and 1,166 heterozygous gene deletions) on cell size in yeast grown to saturation was systematically determined. From these data, 49 genes were identified that dramatically altered cell size. Thirty-five of these are involved in transcription, signal transduction, or cell cycle control; 89% of these genes have putative human homologues. Sixteen genes regulate cell size in a dosage dependent manner, and the majority of mutants identified fail to correctly exit the cell cycle. Many of these genes are components of Ccr4-Not transcriptional complexes or function in the PKC-Map kinase pathway. These genes may modulate cell size by altering the expression or activity of G1-phase cyclins. These results illustrate how systematic genetic screens can be used to dissect intricate biological processes that are refractory to classic genetic approaches. Further, these results confirm the conservation and complexity of cell size control mechanisms.

4.1.1 Objective

In wild type yeast, it is known that the ability of Cln3 to induce *CLN1* and *CLN2* transcription is modulated by cell size (Tyers, Tokiwa et al. 1993; Stuart and Wittenberg 1995). In small cells, Cln3 at normal levels is unable to induce this transcription but

gains this ability as cells attain a minimum cell size (Tyers, Tokiwa et al. 1993; Stuart and Wittenberg 1995). The molecular details of this mechanism are not known. Moreover, in the previous chapter, I demonstrated that cells required a threshold level of Cln activity to progress past Start (Figure 3.2). However, the substrates of Cln3-Cdc28 involved in the activation of *CLN1* and *CLN2* transcription are not known. Furthermore, the mechanism whereby Bck2 induces the activation of *CLN1* and *CLN2* transcription is also not known (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). In addition, I have demonstrated that Cln protein expression is also modulated by cell size (Figure 3.7). However, these results have created a paradox. Clns alter cell size and cell size alters Cln expression. To examine this problem in more detail and with the hopes of learning more about the mechanism responsible for these observations, I conducted a genome-wide genetic screen to identify cell size mutants in yeast. The goals of this screen were to find genes from those mutants that dramatically altered cell size. Thus, my research objective was to further understand the mechanisms whereby cell growth and division is coordinated.

4.1.2 Hypothesis

Because of the relationship between cell size and Cln expression, I hypothesized that some or all of these mutants might affect Cln expression.

4.1.3 Rationale and Expected Results

As discussed in Chapter 1, the coordination of cell growth and cell division is essential for cell size homeostasis. Therefore, loss of this coordination would lead to changes in cell size. These types of changes can be easily visualized under the microscope and quantitated with a Coulter Counter (Figure 4.1). For example, loss-of-function of positive cell cycle regulators delays cell cycle progression, leading to large cells. Conversely, deletion of cell cycle inhibitors accelerates cell cycle and thus results in smaller cells (Figure 4.2).

There are about 6,200 ORFs (open reading frame) in *S. cerevisiae*. One great advantage in screening budding yeast was that nearly every single ORF had been systematically deleted, and the whole set of single deletion strains was available commercially making it possible to conduct systematic genome-wide genetic screens (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). It was expected that this screen would identify many new cell size mutants that help elucidate the mechanism whereby cells coordinate cell growth with proliferation.

4.2 Genomic-Wide Genetic Cell Size Screen

The complete set of homozygous and heterozygous *S. cerevisiae* deletions strains was obtained from Research Genetics (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). In each of the strains, the ORF of a known gene or a putative gene was replaced by KanMX module that confers resistance to geneticin (G418). To screen for mutants with cell size phenotypes, 5,958 single deletion strains (~96% of the genome) were patched onto GYPD plates (YPD plus G418) and incubated at 30°C for 3 days to reach the stationary phase before sizing with a coulter counter (Figure 4.1B). The majority of known cell size control genes have size phenotypes in logarithmic and saturated cultures, but a few have phenotypes only in saturated cultures (Radcliffe, Trevethick et al. 1997). Thus, it was reasoned that by initially sizing saturated cultures, both types of mutants would be detected. In fact, 14% (7/50) of the mutants identified demonstrated phenotypes only in saturated cultures.

The cell size of each mutant strain was measured individually, and the cell size distribution was plotted as a histogram. The distribution of cell size in wild type yeast is rarely a normal curve, but rather a right-skewed curve (Figure 4.3B). In normal curves, mean (average size), median (size at which half of the distribution is smaller and half is larger), and mode (most frequently occurring size) cell sizes are identical. However, in right-skewed curves mode and median values can be smaller than mean values. To correct for these differences, mean, median, and mode cell sizes were obtained for all

mutants. From these data, the average mean, median, and mode sizes were calculated (Figure 4.3A). Wild type cells grown to saturation had similar cell sizes (Figure 4.3A). The mean, median, and mode cell sizes of the entire mutant population were plotted (Data not shown). From these data, the smallest 2.5% and largest 2.5% of the 5,958 deletion mutants was identified.

The average mean, median, and mode sizes of all the mutants (5,958) were calculated as 50.8fL, 52.3fL and 46.9fL, with the standard deviation (SD) of 6.7fL, 5.8fL and 8.9fL respectively (Figure 4.3A). These parameters were similar to those of the wild type cells under the same condition (Figure 4.3A), indicating most of the mutants didn't have a size phenotype. These values therefore served as the bases for statistics. The smallest 2.5% and largest 2.5% of the 5,958 strains was identified by ± 2 SD by either mean or median or modal size. Only those cells which appeared in at least two groups (either mean or median or mode) were considered as size mutants.

Altering the rate of cell division can have dramatic effects on cell size (Potter and Xu 2001; Rupes 2002). For example, delaying cell division indirectly makes cells abnormally large. These types of mutants would be less informative. To eliminate mutants that act indirectly, the doubling times of all potential cell size mutants were measured and mutants with doubling times greater than 125 minutes were excluded from this initial screen but are discussed later. In this manner, 49 cell size mutants were identified, and an example of the cell size plot of a *whi* mutant, *ada1*Δ, and an *uge* mutant, *spt10*Δ is shown (Figure 4.3B). Of these, 21 mutants were dramatically smaller (*whi*) and 29 mutants were dramatically larger (*uge*) than wild type cells (Table 4.1 and 4.2). Thus, less than one percent (50/5958 = 0.8%) of genes in the yeast genome severely affected cell size. Microscopic analysis of mutants confirmed the cell size defects (Figure 4.4).

4.3 General Description of the Identified Mutants

All three of the known cell size genes, *BCK2*, *CLN3,* and *WHI2* were identified in our screen as expected. Their identification validated the efficacy of this screening.

CLN1 and *CLN2* were not included, because their redundant functions led to less severe size phenotype than *CLN3* deletion, so tthey were excluded by our statistical criteria.

I searched the database (http://www.yeastgenome.org/) to exam how the genes identified may function in yeast. Of the 49 genes identified, five genes or putative ORFs *YJR054W, YBR134W, YDR526C, YGL047W* and *YKL037W* had no known functions. Of note, *YJR054W* is very closely related (2.3 E^{-67}) to the yeast *PRM6* gene that is involved in a pheromone-dependent MAPK signal transduction pathway. In addition, *YGL047W* has homology to glycosyltransferases, but its biological role remains unknown (http://www.yeastrc.org/cgi-bin/unknown_orfs/listGO.cgi).

Of the remaining 44 mutants, 86% (38/44) could be functionally grouped as involved in: transcription, signal transduction, and cell cycle (Table 4.1 and 4.2). BLAST searches were performed in order to determine if these cell size regulators were conserved in mammalian cells (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=euk). All eight genes in the signal transduction group had putative human homologues (Table 4.1 and 4.2), and 90% (21/23) and 87% (7/8) of the mutants in the transcription and cell cycle regulation groups had putative human counterparts too (Table 4.1 and 4.2). Notably, many genes identified function in clusters or complexes as discussed later.

4.3.1 HO Gene Cluster

The *HO* gene encodes an endonuclease that creates a double-stranded break at the *MAT* locus for mating-type switching in *S. cerevisiae* (Breeden and Nasmyth 1985; Breeden and Nasmyth 1987). The transcription of the *HO* gene is asymmetric, as it is only transcribed in mother cells right before budding and DNA replication (Breeden and Nasmyth 1985; Breeden and Nasmyth 1987). Activation of the *HO* transcription depends on at least 10 genes, named *SWI1* through *SWI10.* Amonng them, *SWI4* and *SWI6* encode subunits of a cell cycle-regulated transcription factor SBF (SCB-binding factor) that activates a number of genes at the G1/S-phase boundary (Nasmyth 1993). Nine mutants identified in our genetic screen, *ada1Δ, ace2Δ, anc1Δ, ccr4Δ, hpr1Δ, pho2Δ, she4Δ,*

*spt3*Δ, and *srb8*Δ, affect the expression of the *HO* (Dohrmann, Voth et al. 1996; Porter, Washburn et al. 2002). The mechanism whereby these gene products modulate the *HO* transcription is not known. However, the fact that these genes encode proteins for RNA polII holoenzyme or transcription regulators implied that they might directly control transcription of the *HO* gene. Additionally, several other mutants identified in this screen, *bck2*Δ, *cln3*Δ, *paf*Δ*1*, *whi2*Δ and *whi5*Δ affected the transcription of the G1-phase cyclins *CLN1* and *CLN2* that were co-regulated with the *HO* gene in yeast (Di Como, Chang et al. 1995; Koch, Wollmann et al. 1999).

4.3.2 Cell Integrity Pathway

The cell integrity pathway (Figure 4.5) mediates cell cycle-regulated cell wall synthesis and responds to different signals such as temperature and osmolarity (Madden, Sheu et al. 1997; Baetz, Moffat et al. 2001). These signals activate Slg1 (Wsc1), Wsc2 and Wsc3 on the cell wall. These two sensors relay the signals to a GTP binding protein Rho1, which in turn activates Pkc1, the protein kinase C (Heinisch, Lorberg et al. 1999). Downstream of Pkc1, there is a well conserved module of MAP kinase pathway. This module is composed of Bck1 (MAP kinase kinase kinase), Mkk1 and Mkk2 (redundant MAP kinase kinase), and Slt2 (MAP kinase) (Heinisch, Lorberg et al. 1999). Upon activation, MAP kinase Slt2 migrates into nuclei to phosphorylate its substrates, among which there are two transcription factors Rlm1p and SBF for the cell wall synthesis (Madden, Sheu et al. 1997; Gustin, Albertyn et al. 1998).

In this screening, I found that deletion of *slg1, bck1* and *slt2* made cells smaller. This was unexpected because a previous study showed that Slt2 phosphorylation activated SBF and promoted *CLN1* and *CLN2* expression (Madden, Sheu et al. 1997). From their results, it would be expected that loss of function of this pathway should reduce *CLN1* and *CLN2* expression and subsequently lead to enlarged cells. However, our data did not support this conclusion. Rather, they implied that Slt2 might repress SBF activity and *CLN1* and *CLN2* expression, because *slt2*Δ phenotype was similar to those mutants that have elevated Cln expression, such as in *whi2*Δ mutant. Further

studies will be required to assess the relationship between *slg1, bck1* and *slt2* mutants and *CLN* mRNA expression.

Additionally, the cell integrity pathway has genetic interactions with other cell cycle regulators, strongly suggesting their involvement in the cell cycle regulation. For example, *BCK1* and *SLT2* interact with three other mutants identified: *bck2Δ*, *cln3Δ*, and *whi2Δ* (Lee, Hines et al. 1993). Interestingly, the unknown gene *YKL037W* also interacts genetically with this pathway. Double mutants of *ykl037wΔ* and *slt2Δ*, or *ykl037wΔ* and *bck1Δ* were synthetically lethal, suggesting the gene product encoded by *YKL037W* is required in *slt2Δ*, and *bck1Δ* strains (http://db.yeastgenome.org/cgi-bin/locus.pl?locus=ykl037w).

In mammalian cells, protein kinase C plays very important roles in growth control, differentiation, tumorigenesis and apoptosis (Dempsey, Newton et al. 2000). For example, several members of the protein kinase C (PKC) family serve as substrates for caspases and PKC-∂ isozyme has been intimately associated with DNA damage-induced apoptosis (Basu 2003). PKC-α overexpression is also implicated in tumorigeneis of pancreatic, gastric and colorectal cancer, as well as hepatocellular carcinoma (Lahn, Sundell et al. 2003). Thus, the above findings were consistent with the conserved roles of PKC that links various external signals to the cell cycle machinery in both yeast and mammals.

4.3.3 Ccr4-Not Complexes, Signal Transduction Pathways and Cell Size Homeostasis

Surprisingly, the gene products of five *uge* mutants, *ccr4Δ, hpr1Δ, paf1Δ, pop2Δ*, and *rlr1Δ* are components of Ccr4-Not complexes (Figure 4.6). Ccr4-Not complexes have at least two *in vivo* forms of 1.9 MDa and 1.0 MDa respectively (Denis and Chen 2003). Ccr4-Not complexes have multiple functions in mRNA metabolism. For example, Ccr4-Not complexes are involved in transcription initiation, elongation and mRNA stability (Collart 2003; Denis and Chen 2003). In addition, Ccr4-Not complexes are also involved in protein ubiquitination and chromatin remodeling (Albert, Hanzawa et al. 2002; Krogan, Dover et al. 2003; Ng, Dole et al. 2003). The physiological roles of

Ccr4-Not complexes in yeast include the transcription of genes for nonfermentative growth and cell cycle regulation, as well as DNA damage checkpoints (Collart 2003; Denis and Chen 2003). In addition to the cell size genes I identified, four essential gene products are also components of Ccr4-Not complex (Figure 4.6). They are Not1 (Cdc39), Not2 (Cdc39), Cdc68 and Cdc73 that are required for G1- to S-phase cell cycle progression (Reed 1980). In Chapter 5, I would focus on the role of Ccr4-Not complexes in cell cycle progression and modulating cell size.

4.3.4 Chromatin Remodeling Clusters

Our genetic cell size screen identified seven gene products involved in chromatin and chromatin remodeling, Ada1, Asf1, Htb1, Rsc1, Spt3, Spt10 and Ycr020w-b. *HTB1* is an essential gene that encodes histone H1. Asf1, Spt10 and Rsc1 are responsible for the repression of *HTB1* transcription (Dollard, Ricupero-Hovasse et al. 1994; Sutton, Bucaria et al. 2001; Ng, Robert et al. 2002). Interestingly, *htb1*Δ strain was a *whi* mutant, while *asf1*Δ, *spt10*Δ and *rsc1*Δ strains were *uge*. Ycr020w-b was recently identified as Htl1 that physically interacts with RSC nucleosome remodeling complex (Lu, Lin et al. 2003). Additionally, deletion of *ada1* made cells smaller (Table 4.1A and B). Ada1 is part of the ADA/GCN5 complex, involved in general transcription. One target of Ada1 is *HAP2*, because in an *ada1*Δ strain, *HAP2* expression was reduced by nine fold (Horiuchi, Silverman et al. 1997). This agreed with the fact that the *hap2*Δ strain was also a *whi* mutant (Table 4.2 A and B). Moreover, Ada2 and Ada2 are also involved in the rapid induction of *CLN3* mRNA expression in glucose stimulation, as deletion of either *ADA2* or *ADA3* inhibited expression of *CLN3* transcription when fresh glucose medium was added to post-log cells (Wu, Newcomb et al. 1999).

4.3.5 Miscellaneous Genes

Deletion of a number of genes involved in transcriptional regulation also changed cell size. For example, nine transcription factors, Ace2, Gal80, Hap2, Hap4, Met32, Pho2, Srb8, Taf14 and Yap3 were in this group (Tables 4.1 and 4.2). Ace2 was shown to

be a daughter cell-specific inhibitor of *CLN3* expression (Laabs, Markwardt et al. 2003). Deletion of *ace2* enabled daughter cells to bypass the size requirement for Start (Laabs, Markwardt et al. 2003). However, *ace2*Δ was larger than the wild type in our screen. The reason for such a discrepancy is not clear at this time. However, it is possible that Ace2 transcription factor may also have a mother-specific function, and loss of this function can increase cell size.

Four genes, *MCK1, RPL34B,* and *UBP15, YML014W* are involved in protein synthesis or abundance. For example, *YML014W* was recently found to encode a methyltransferase Trm9, modifying tRNA (Kalhor and Clarke 2003). Ubp15 is involved in deubiquitination and therefore stability of proteins (Hochstrasser 1996). Rpl34B encodes ribosomal subunit responsible for translation directly (http://db.yeastgenome.org/cgi-bin/locus.pl?locus=YIL052C). Mck1 is the yeast homolog of GSK-3 of mammalian cells, involved in the PKA signaling pathway and therefore cell growth (Rayner, Gray et al. 2002).

In this screening, however, only one phosphatase was identified as a *whi* mutant. *SAC1,* an inositol-phosphatidylinositol phosphatase, is implicated in the PI3K (phosphatidylinositol-3-OH kinase) signaling pathway. Finally, *YAL056W* was found recently to encode Gpb2, functioning in G protein signaling, involved in glucose sensing and filamentous growth (Harashima and Heitman 2002). Its deletion led to the *whi* phenotype. This might be caused by redundant expression of the phosphatases.

4.3.6 Dosage Dependent Regulation of Cell Size

The most physiologically relevant cell size control genes are likely to be those that regulate cell size in a dosage dependent manner (Jorgensen, Nishikawa et al. 2002). Several previously isolated cell size control genes have this phenotype (Nash, Tokiwa et al. 1988; Cross 1989). Only five of 1166 essential genes exhibited dramatic cell size phenotypes as heterozygotes in saturated cultures. Of these genes, *HTB1*encodes histone H1; *SRM1* one is a GTP exchange factor,. Another is a component of the 19S regulatory particle of the proteasome, *RPT2,* and two are genes of unknown function (Tables 4.1 and

4.2). In addition, the size of heterozygotes for remaining mutants was measured to determine how many affected cell size in a dosage dependent manner (Tables 4.1 and 4.2). It was found that 33% (7/21) of the *whi* mutants and 31% (9/29) of the *uge* mutants displayed a dosage dependent effect (Tables 4.1 and 4.2).

4.4 Cell Cycle Effects of *whi* and *uge* Mutants

All previously known cell size genes affect cell size, cell cycle distributions, and the percent of budded cells in culture (Di Como, Chang et al. 1995; Stuart and Wittenberg 1995; Danaie, Altmann et al. 1999; Gari, Volpe et al. 2001). To establish the effect of *whi* and *uge* mutants on cell cycle distributions, the percentages of budded and G1-phase cells in logarithmically growing and saturated cultures were determined (Tables 4.1A and 4.2A). Four *whi* mutants, *sac1*, *she4*, *spt3*, and *whi2*, had considerably fewer 2N cells than wild type cells (Tables 4.1B). In addition, a number of *whi* mutants also had fewer unbudded cells as compared to wild type cells (Tables 4.1B). This suggests that in these mutants, cells might progress past Start at a smaller than normal cell size. Thus, the genes for these mutants might encode protein products that normally inhibit progression past Start. However, not all *whi* mutants displayed these characteristics. A number of *whi* mutants had increased numbers of unbudded cells despite having numbers of 2N cells comparable to wild type cells (Tables 4.1B). In wild type cells, the initiation of budding and DNA synthesis are coupled at Start. These results suggest that these *whi* mutants might uncouple the initiation of budding from DNA synthesis.

More than one-third of the *uge* mutants had increased numbers of 2N cells or unbudded cells as compared to wild type cells (Tables 4.2B). These genes may be necessary to promote progression past Start. However, as was the case with *whi* mutants, a number of *uge* mutants displayed characteristics suggestive of uncoupling of the initiation of budding from the initiation of DNA replication. In this case, nearly half of the *uge* mutants had elevated numbers of budded cells as compared to wild type cells

(Tables 4.2B). These results confirm that the majority of these mutants disrupt normal cell cycle progression.

In summary, some of the *whi* genes may encode cell cycle inhibitors, because loss-of-function of these genes caused cells to enter the cell cycle prematurely. On the other hand, some of the *uge* genes may play a role as positive cell cycle regulators. In addition, some of these genes in both groups seem to coordinate initiation of budding and DNA synthesis. These results indicated that the majority of these mutants altered normal cell cycle progression.

4.5 Mutants That Affect Cell Growth

Wild type cells modulate their size according to growth conditions. Slowly growing cells tend to be smaller than rapidly growing cells (Johnston, Ehrhardt et al. 1979). In fission yeast, *wee1* mutants are unable to modulate their size in response to a change in environmental conditions suggesting that *wee1* mutants disrupt cells nutrient sensing pathways (Russell and Nurse 1987; Kellogg 2003). Because the mechanism that links nutrient-sensing pathways to cell size homeostasis is not well understood, I attempted to determine if any mutants identified here might function in these pathways. Thus, the ability of these mutants to modulate their size in response to nutrient quality was investigated. Growth on glycerol decreases the growth rate and the size of wild type cells. Nearly all of the glycerol grown *uge* mutants were larger than glycerol grown wild type cells (Table 2). Moreover, the majority of *uge* mutants logarithmically growing in glycerol were smaller than the same mutants grown in glucose. This suggests that these mutants can still modulate their size in response to changes in nutrient quality. In contrast, the response of *whi* mutants to growth on glycerol was more complex. Approximately one-third of the glycerol grown *whi* mutants were smaller, one-third were equally sized, and one-third were larger than glycerol grown wild type cells. In addition, a number of *whi* mutants logarithmically growing in glycerol were larger than comparably growing wild type cells. This suggests that these mutants are defective in their ability to modulate their size in response to changes in nutrient quality. Finally, it

was found that the majority of both *whi* and *uge* mutants were unable to exit the cell cycle appropriately. When grown to saturation, these mutants arrested with dramatically higher numbers of budded and 4N cells as compared to wild type cells. These results indicate that the majority of these mutants disrupt the normal coordination between cell growth and division and frequently disrupt the ability of cells to appropriately modulate their size in response to changing environmental nutrient conditions.

4.6 Cell Size Mutants Modulate "Critical Cell Size"

Commitment to cell division is dependent upon the attainment of a "critical cell size" which is an indicator of Cln activation. Normally, a large "critical size" usually means that Cln activation is delayed or decreased. In contrast, a small "critical size" correlates to precocious Cln activation. To test if the critical size of some mutants was changed, I selected three *whi* mutants and three *uge* mutants. These strains were first grown in YPD to mid-log phase. Then, small unbudded cells were collected by centrifugal elutriation. These cells were released into fresh YPD and their progression past Start was monitored at regular intervals as described previously. Critical cell size was defined operationally as the point at which 50% of the cells in the culture budded. As shown in Figure 4.7A and B, the critical cell sizes of six mutants were altered. In the three *whi* mutants analyzed, *mck1*Δ, *rip1*Δ, and *sac1*Δ, the critical cell size was lowered by 10fL (Figure 4.7A). Conversely, the critical cell size increased dramatically (20-60fL) in three *uge* mutants, *cln3*Δ, *met32*Δ, and *rpt2*Δ (Figure 4.7B). Thus, the cell sizes in these 6 mutants are modulated by altering critical cell size.

4.7 Complementary Data

In addition to have identified 49 genes described in Table 4.1 and 4.2, I also found 181 mutants that dramatically changed cells size (See Appendix I). These were called petite mutants as they had defects in mitochondria and therefore energy production. As the energy production has broad effect on many cellular processes, these mutants grew very slowly with doubling time longer than 120 minutes in log phase. Therefore, they

were not included in the list, because their size phenotype might be caused by indirect effect on cell growth, rather than on the coordination of cell growth and cell division. Furthermore, a list of mutants with growth defect (i.e. groing very slowly) was included in Appendix II. These mutants may have affected cell size indirectly.

4.8 Discussion

Elegant work from Killander, Zetterberg, Hartwell, Mitchison, Sudbery, Fantes, Nurse, and colleagues laid the foundation for the theory that proliferation in most eukaryotic cells is dependent upon cell growth (Zetterberg and Killander 1965; Hartwell 1974; Fantes, Grant et al. 1975; Nurse, Thuriaux et al. 1976; Fantes and Nurse 1978; Nurse and Thuriaux 1980; Sudbery, Goodey et al. 1980; Mitchison, Novak et al. 1997). The tethering of proliferation to growth is achieved in yeast by preventing commitment to cell division until a "critical cell size" is attained (Hartwell and Unger 1977). Cells may use G1-phase cyclins to coordinate cell growth with proliferation (Gallego, Gari et al. 1997; Polymenis and Schmidt 1997; Hall, Markwardt et al. 1998; Danaie, Altmann et al. 1999). However, the molecular details of the mechanism of cell size homeostasis remain largely undiscovered. For example, it is not known how cell growth is regulated, how cell size is sensed, or how cells use G1-phase cyclins to coordinate cell growth with cell division. More specifically, the concept of "critical cell size" has only been operationally defined and needs to be mechanistically explained both molecularly and biochemically. The goal of these present studies was to use a systematic genomic-wide screen in yeast to identify the genetic tools necessary to begin answering these questions.

The molecular mechanism of cell size control has been a long-standing fundamental biological problem whose significance in normal development and in human disease is becoming increasingly apparent (Coelho and Leevers 2000; Conlon, Dunn et al. 2001; Potter, Huang et al. 2001; Wang, Tokarz et al. 2002). Dissection of this mechanism has historically been difficult to genetic analysis primarily due to the difficulty in cloning cell size mutants. This problem was circumvented by using a systematic genetic screen to identify 49 mutants that resulted in abnormally small (*whi*)

or abnormally large (*uge*) cells (Tables 4.1 and 4.2). Importantly, this set of mutants included three of four previously known cell size control genes. Thus, 46 new cell size mutants were identified. The gene products for five of these genes, Ccr4, Hpr1, Paf1, Pop2, and Rlr1, are components of Ccr4-Not complexes. Deletion of these genes resulted in abnormally large cells. Moreover, loss of function of three essential gene products associated with Ccr4-Not complexes, Cdc36, Cdc39, and Cdc68, resulted in a dramatic decrease in *CLN2* mRNA transcription. This suggested that loss of function of Ccr4-Not complex components might increase the size of cells by decreasing *CLN* expression. Previous studies have demonstrated that there are complex genetic interactions between Ccr4-Not complexes and the PKC-MAP pathway. It seems that the complexes may function in a linear genetic pathway with PKC, because *paf1Δ slt2Δ* and *paf1Δ pkc1Δ* double mutants did not have additive phenotype relative to the single mutants (Chang, French-Cornay et al. 1999). Loss of function of three genes, *bck1, slg1*, and *slg1* that function in the PKC-MAP kinase pathway resulted in abnormally small cells. The Slt2 MAP kinase phosphorylates the SBF transcription factor, which regulates *CLN1* and *CLN2* transcription (Nasmyth and Dirick 1991; Andrews and Moore 1992; Madden, Sheu et al. 1997). It has been proposed that the PKC-MAP kinase signal transduction pathway stimulates the transcription of some G1-phase genes (Madden, Sheu et al. 1997). However, the *whi* phenotype observed is more consistent with the hypothesis that Slt2 phosphorylation of SBF may repress *CLN1* and *CLN2* transcription. The latter hypothesis is supported by the recent observation that *slg1* mutants, an upstream effector in the Slt2 pathway, exhibit a phenotype consistent with Cln hyperactivation (Nash, Tokiwa et al. 1988; Mountain and Sudbery 1990; Radcliffe, Trevethick et al. 1997).

A number of lines of evidence suggested that Clns are integral to cell size homeostasis (Mitsuzawa 1994; Wijnen, Landman et al. 2002; Laabs, Markwardt et al. 2003). Many of the cell size mutants identified here interact genetically with Clns. For example, the gene products of two *uge* mutants, Cln3 and Bck2 altered cell size by regulating *CLN1* and *CLN2* transcription (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). Nuclear localization of Cln3 is essential for its function (Miller and Cross

2000; Miller and Cross 2001). In this light, it is interesting to note that deletion of *MSN5*, whose gene product functions in nuclear transport, results in abnormally large cells (Table 4.2) (Queralt and Igual 2003). Since Msn5 has a role in SBF regulated transcription and is synthetically lethal with the deletion of *cln1* and *cln2*, it is possible that Msn5 is involved in the nuclear localization of Cln3 (Queralt and Igual 2003). Alternatively, Msn5 may positively regulate *CLN* expression via PKA pathway. As discussed in Chapter 1, PKA pathway positively regulates *CLN* expression and represses Msn2/4 trans-activation activity. Msn5 is a nuclear transporter responsible for Msn2 nuclear export, as Msn2 accumulated abnormally in the nucleus in *msn5*Δ cells (Chi, Huddleston et al. 2001). Therefore, it is reasonable to postulate that Msn2/4 may directly or indirectly repress *CLN* expression in the nucleus, so high cAMP increases *CLN* expression by positively regulating Msn5 that exports Msn2/4 out to the cytoplasm.

Finally, it was found that loss of function of a glycogen synthase kinase (GSK) homologue, Mck1, resulted in small cells (Table 4.1). It has been shown that GSK regulates the protein stability of mammalian cyclin D, a Cln homolog (Diehl, Cheng et al. 1998). Phosphorylation of cyclin D by GSK greatly destabilized the protein (Diehl, Cheng et al. 1998). If Mck1 functioned similarly in yeast, then *mck1*Δ mutants might stabilize Cln proteins. This would predictably make cells small. A number of detailed mechanistic and genetic studies are needed to elucidate the functional significance of these observations.

Recently in a similar systematic cell size screen, Jorgensen et al. identified a number of novel genes involved in the coordination of cell growth with division in proliferating haploid cells (Jorgensen, Nishikawa et al. 2002). With elegant genetic experiments, they demonstrated that a number of their mutants interacted genetically with the transcription factors SBF and MBF, required for the transcription of *CLN1* and *CLN2* (Jorgensen, Nishikawa et al. 2002). Most importantly, they identified a transcription factor, Sfp1, which affects the expression of genes required for ribosome biogenesis (Fingerman, Nagaraj et al. 2003; Jorgensen, Rupes et al. 2004). Despite the elegance of this result, the mechanism whereby ribosome biogenesis affects cell size is still not clear.

In comparing the two systematic genetic screens, 42% (24/57 total; 18/29 *uge* mutants and 6/28 *whi* mutants) of the cell size mutants reported here were also identified by Jorgensen et al. (Jorgensen, Nishikawa et al. 2002) (Tables 4.1 and 4.2). The majority of the mutants in common between these two screens altered cell size in a dosage dependent manner (Tables 4.1 and 4.2). Several observations pointed to the complementary nature of these two screens. First, the majority of mutants reported here failed to exit the cell cycle properly and may have roles involved in nutrient-sensing (Tables 4.1A and 4.2A). Second, the stationary phase acting *WHI2* gene was identified in the current screen but not by Jorgensen et al. Similarly, by initially sizing only saturated cultures, it is likely that some genes that function predominately in logarithmically growing cultures may have been missed in this screen. Moreover, the criteria chosen for the definition of cell size mutants (+/- two standard deviations) was subjective and did not infer that mutants with slightly weaker cell size phenotypes are not physiologically significant in cell size homeostasis. In fact, it is likely that many mutants with slightly less dramatic cell size phenotypes will have physiologically significant roles in cell size homeostasis. However, the observation that many mutants identified here affect cell size more drastically than some of the previously known cell size mutants affirms the validity of this approach. Thus, the genes reported here may have a more predominant role in the regulation of cell cycle entry and exit rather than cell cycle progression or cell size homeostasis in logarithmically growing cells. These two systematic genome-wide genetic screens will provide the cell cycle field with a number of genetic tools necessary for addressing a number of unanswered mechanistic questions about cell size homeostasis. For example, it is not known how "critical cell size" is measured or how reaching this threshold promotes commitment to cell division. Cln3 and Bck2 promote cell cycle progression by inducing the transcription of G1-phase genes, including *CLN1* and *CLN2* (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). This function is essential for cell size control. However, the molecular nature of the transcriptional activation event is poorly understood. In this screen, 22 genes that function as transcriptional regulators were identified. Further analysis of the role of these genes in

cell size control may help elucidate the molecular details of "critical cell size" and the mechanism whereby G1-phase cyclins promote commitment to cell division.

In addition, the mechanism whereby nutrient-sensing signal transduction pathways, mitochondrial function, and ribosome biogenesis modulate cell size is not well understood. In particular, it is not known how these pathways affect the expression and the activity of G1-phase cyclins. Cells can rapidly adapt to changing environmental conditions and do so in part by modulating cell size and proliferation rates. While the relationship between cell size and proliferation rates is poorly understood, it is likely that each of the above stated pathways are involved in the mechanism that coordinates cell growth with division. However, the dissection of this mechanism awaits detailed genetic and biochemical analysis of the newly identified cell size mutants to reveal and elucidate the significant functional interactions and relationships.

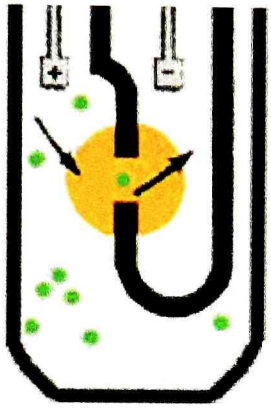
Finally, it is not known how the activity of G1-phase Cdk complexes is modulated or measured. A number of observations suggest that the activity of G1-phase Cdk complexes is integral to cell size homeostasis. To begin to address this question, two things need to be accomplished. First, it will be necessary to identify more G1-phase Cdk substrates. Second, proteins that modulate G1-phase Cdk activity or respond to this activity need to be identified. In this light, it is interesting to note that neither of the systematic cell size screens identified protein phosphatases that strongly modulate cell size (Jorgensen, Nishikawa et al. 2002). This may be due to functional redundancy of some phosphatases. The identification of these phosphatases and key substrates will be a major step forward in understanding the relationship between nutrient sensing, cell growth, and commitment to cell division.

4.9 Conclusions

The regulation of cell division is a fundamental biological function and the appropriate coordination of cell growth with cell division is a prerequisite for normal development and differentiation. Observations suggest that the coordination of cell growth with cell division is essential for cell size homeostasis. Because the relationship

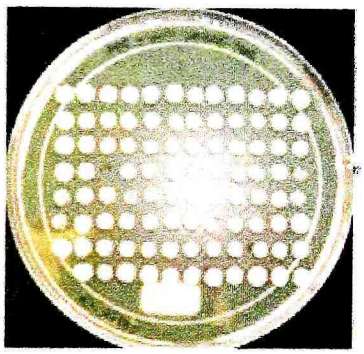
between cell size and proliferation is not mechanistically understood, a systematic genome-wide genetic screen in yeast was conducted to identify mutants that disrupt the coordination of cell growth with division. Here, the effect of 5,958 single gene deletions on cell size in saturated cultures is reported. This study identified 46 new genes that function to coordinate cell growth with division. Thirty-five of these are involved in transcription, signal transduction, or cell cycle control, 89% of which have putative human homologues. These results illustrate how systematic genetic screens can be used to dissect intricate biological processes that are refractory to classic genetic approaches.

A.



Coulter Counter

B.



A plate of deletion mutants

Figure 4.1. A Coulter counter was used to measure both cell size and number simultaneously. The single deletion strains were patched onto GYPD plates and incubated at 30°C for three days. The cells were sized with the Coulter counter as described in Chapter 2.

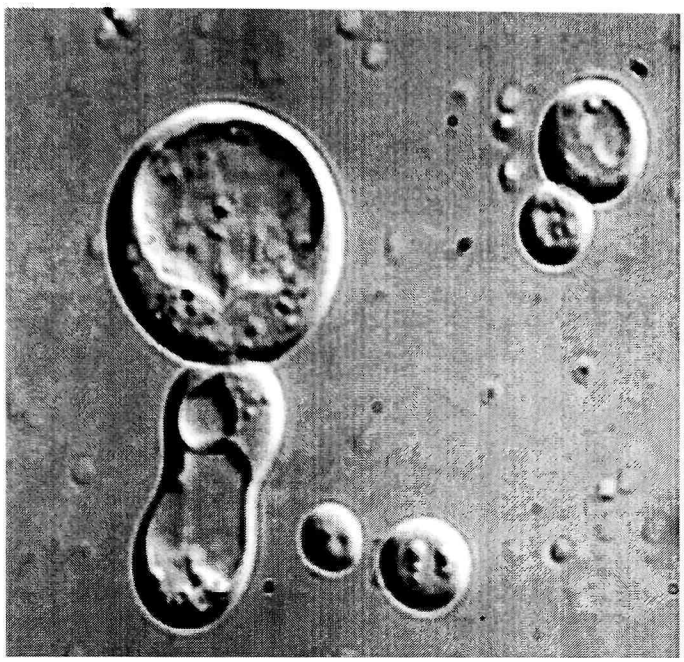


Figure 4.2. Yeast cell size mutants. Wild type haploid yeast are remarkably homogenous in cell size with average size of ~ 40fL (see upper right) and rarely vary more than two-fold. I have identified cell size control mutants that can dramatically affect cell size. These mutants can make cells abnormally small (see bottom middle) or abnormally large (see upper left). The diameter of the largest cell is nearly 4 times greater and its volume is 32 times larger than the smallest cell.

A.

Mutants	Mean (fL)	Median (fL)	Mode (fL)
Avg.	50.8	52.3	46.9
SD	6.7	5.8	8.9
Wild Type			
Avg.	58.2	52.3	47.1
SD	4.9	4.1	7.6

B.

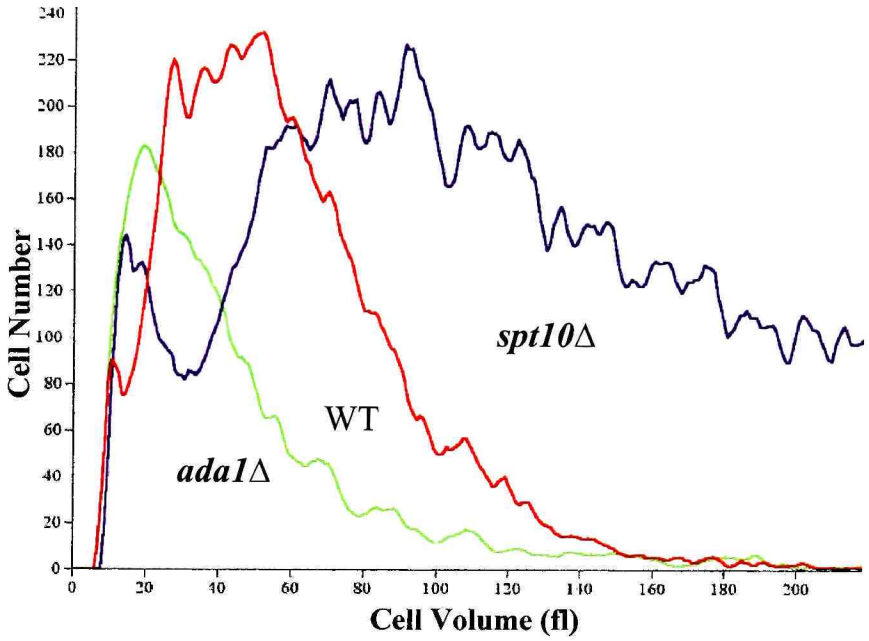


Figure 4.3. Statistical determination of *whi* and *uge* mutants. A. The cell size data from 5,958 individual strains was used to calculate the average mean, median, and mode cell sizes. Standard deviations were also shown. For reference, the average mean, median, and mode cell sizes with their standard deviations of 40 independent wild type colonies were shown. B. A representative graph of size mutants. Red line was the wild type. Blue line representsed a *uge* mutant *spt10*Δ, and green line a *whi* mutant, *ada1*Δ.

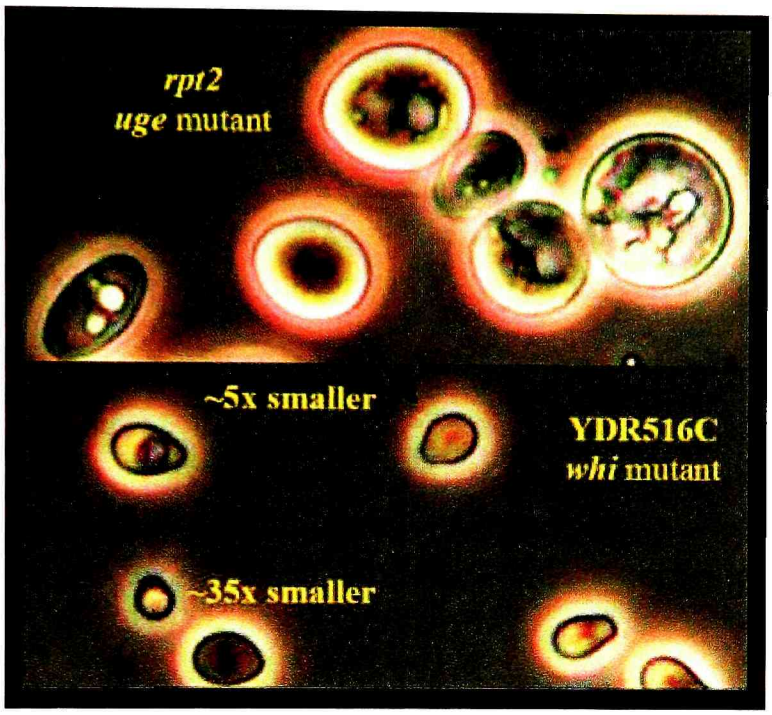


Figure 4.4. Microscopic comparison of a representative *uge* and *whi* mutant. All cell size mutants were confirmed with microscopic examination. Shown were the micrographs of two mutants for comparison. Micrographs were taken at a 100x with a Kodak DC290 camera on a Zeiss Axiolab phase contrast microscope. Measurements indicated that the labeled cells were either approximately 5x smaller or 35x smaller by volume (i.e. comparison between smallest cell and largest cell).

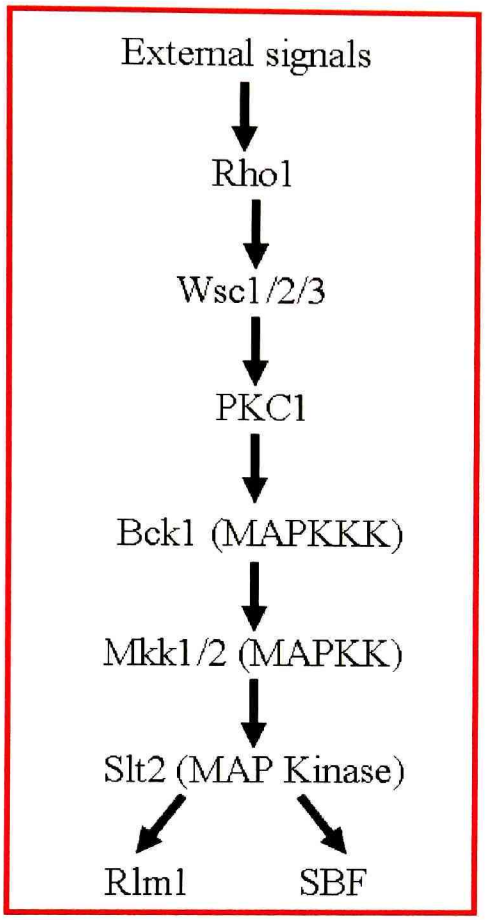


Figure 4.5. Current scenario of the cell integrity pathway in budding yeast. This pathway is activated by external signals such as nutrients, osmolarity and temperature. These signals activate Pkc1 (protein kinase C), which in turn, activate the conserved MAP kinase module. Pkc1 activates Bck1, a MAP kinase kinase kinase. Bck1 phosphorylates and actives Mkk1/2, a MAP kinase kinase. Mkk1/2, upon activation, phosphorylates and activates a MAP kinase Slt2, which migrates into the nucleus to activate two transcription factors Rlm1 and SBF. Rlm1 is responsible for the transcription of genes responsible for cell wall synthesis. SBF is a positive regulator of cell cycle progression.

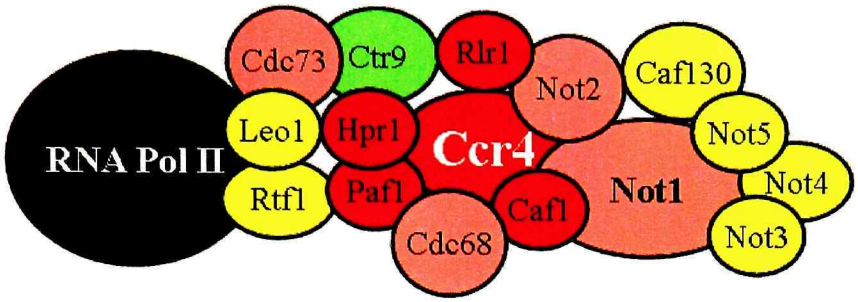


Figure 4.6. Ccr4 is the core component of huge complexes (Ccr4-Not and PAF). The components in red have been identified as the *uge* mutants as described in Chapter 4. Those in pink were initially identified as the essential genes in budding yeast. Not1 is also called Cdc39 and Not2, Cdc36. The organization of the components shown is not necessarily reflecting the real interactions.

A. B.

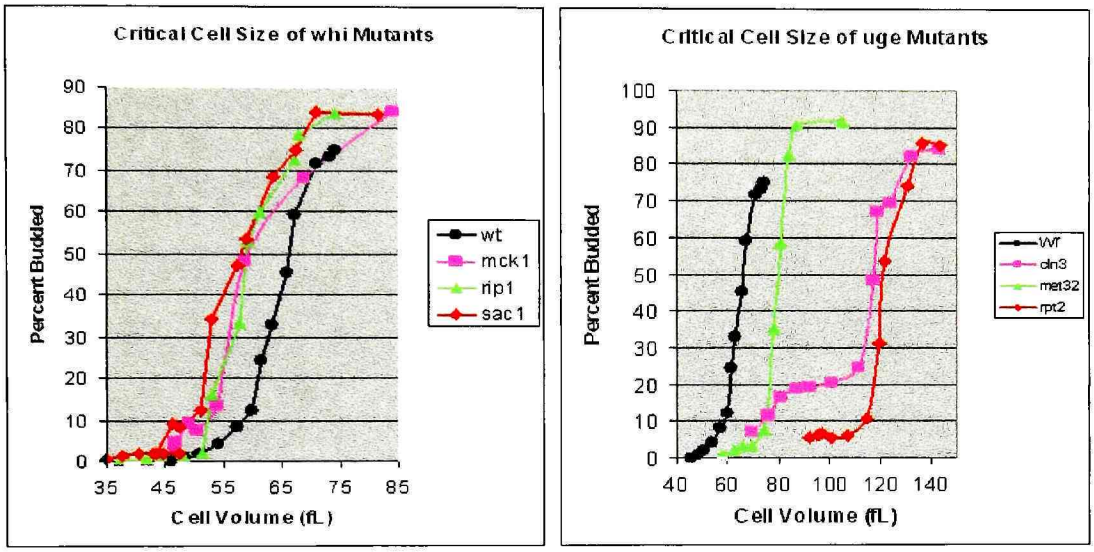


Figure 4.7. Cell size was regulated by changes in "critical cell size". A. Critical sizes of three *whi* mutants. The percentage of budded cells was plotted as a function of cell size. The result showed that each of these mutants executed Start (50% budded) at ~55fL as compared to ~65fL for the wild type cells. This reduction in "critical cell size" was consistent with that observed in other previously identified *whi* mutants or through the over-expression of *CLN* genes. B. Critical sizes of three *uge* mutants. The percentage of budded cells was plotted as a function of cell size. These data showed that each of these mutants executed Start at a much larger size that the wild type: *met32*Δ at ~ 80fL, *cln3*Δ at ~115fL, and *rpt2*Δ at ~120fL. These data suggested that cell size might be regulated primarily through changes in "critical cell size."

ORF	gene	Glucose		Glycerol	
		log size	sat size	log size	sat size
	WT	96 ± 3	52 ± 4	60	39
YOR083W	**WHI5**	91 ± 5	34 ± 3	50	34
YNL307C	MCK1	83 ± 7	34 ± 4	57	35
YPL254W	ADA1	65 ± 6	34 ± 4	43	33
YGL237C	HAP2	82 ± 3	35 ± 2	68	39
YHR030C	SLT2	97 ± 9	36 ± 5	34	52
YLL016W	SDC25	86 ± 9	37 ± 5	76	44
YML051W	GAL80	88 ± 14	37 ± 5	90	32
YKL109W*	**HAP4**	88 ± 5	37 ± 5	67	41
YKL212W	SAC1	80 ± 2	38 ± 11	68	47
YKL037W		77 ± 4	38 ± 4	62	41
YIL052C	RPL34B	79 ± 3	38 ± 4	42	34
YDR392W	SPT3	81 ± 8	38 ± 5	61	38
YMR304W	UBP15	76 ± 8	39 ± 3	71	42
YIL098C	FMC1	81 ± 7	39 ± 5	76	32
YOR043W	**WHI2**	99 ± 5	40 ± 3	74	30
YJL095W	BCK1	84 ± 5	40 ± 4	37	37
YAL056W	**KRH1**	97± 8	40 ± 7	61	42
YGL097W*	**SRM1**	64 ± 2	40 ± 7	53	NA
YOR008c	SLG1	78 ± 7	40 ± 9	58	57
YDR224C	**HTB1**	81 ± 3	44 ± 5	77	50

Table 4.1A. Cellular functions of the *whi* mutants. Deletion of 20 yeast genes made cells abnormally small (*whi*). Mutants in this table were grouped by average cell size in saturated cultures. Shown here were the median cell size (fL) in saturated glucose cultures, the gene, the systematic name, the median cell size (fL) in logarithmically growing glucose cultures, the median cell size (fL) in logarithmically growing glycerol cultures, the median cell size (fL) in saturated glycerol cultures, the percent of cells with 2N DNA content in logarithmically growing glucose cultures, the percent of unbudded cells in logarithmically growing glucose cultures, the percent of cells with 2N DNA content in saturated glucose cultures, the percent of unbudded cells in saturated glucose cultures, the best putative human homology, the doubling time of glucose grown cultures, and the assigned molecular function of the gene product. Haploinsufficient mutants are indicated in bold. *Cell size mutants identified in Jorgenson et al's sytematic genetic screen

gene	ORF	%2N log	unbudded log	%2N sat	unbudded sat	human homologue	doubling time	function
WT		31 ± 0	29	96 ± 3	99		90	
WHI5	**YOR083W**	28 ± 2	43	85 ± 9	57		77	transcription
MCK1	YNL307C	30 ± 1	52	93 ± 2	48	GSK 3	86	signal transduction
ADA1	YPL254W	36 ± 4	12	65 ± 18	88		83	transcription
HAP2	YGL237C	35 ± 2	51	95 ± 3	49	AAA35950	81	transcription
SLT2	YHR030C	32 ± 3	44	96 ± 2	56	ERK5	79	signal transduction
SDC25	YLL016W	30 ± 1	34	92 ± 1	66	H-GRF55	82	signal transduction
GAL80	YML051W	37 ± 5	74	87 ± 9	26		79	transcription
HAP4	**YKL109W***	34 ± 1	62	92 ± 1	38	DSP	81	transcription
SAC1	YKL212W	23 ± 3	24	90 ± 6	76	SYNJ1	125	signal transduction
	YKL037W	27 ± 4	48	92 ± 6	52		74	unknown
RPL34B	YIL052C	22 ± 1	48	88 ± 4	52	RPL34	82	protein synthesis
SPT3	YDR392W	15 ± 2	31	95 ± 2	69	TFIID	118	transcription
UBP15	YMR304W	30 ± 1	52	87 ± 7	48	CAA96580	90	Ubiquitination
FMC1	YIL098C	30 ± 2	21	86 ± 1	79		84	energy generation
WHI2	**YOR043W**	30 ± 4	4	78 ± 1	96		92	cell cycle
BCK1	YJL095W	27 ± 2	39	96 ± 1	61	MEKK3	90	signal transduction
KRH1	**YAL056W**	32 ± 3	47	92 ± 4	53		82	signal transduction
SRM1	**YGL097W***	53 ± 1	59	89 ± 7	41	RCC1	123	protein transport
SLG1	YOR008c	31 ± 4	46	89 ± 12	54	KIAA0523	113	signal transduction
HTB1	**YDR224C**	29 ± 2	14	95 ± 0	86	H2B	84	transcription

Table 4.1B. Cellular functions of the *whi* mutants. Deletion of 20 yeast genes made cells abnormally small (*whi*). Mutants in this table were grouped by average cell size in saturated cultures. Shown here were the median cell size (fL) in saturated glucose cultures, the gene, the systematic name, the median cell size (fL) in logarithmically growing glucose cultures, the median cell size (fL) in logarithmically growing glycerol cultures, the median cell size (fL) in saturated glycerol cultures, the percent of cells with 2N DNA content in logarithmically growing glucose cultures, the percent of unbudded cells in logarithmically growing glucose cultures, the percent of cells with 2N DNA content in saturated glucose cultures, the percent of unbudded cells in saturated glucose cultures, the best putative human homology, the doubling time of glucose grown cultures, and the assigned molecular function of the gene product. Haploinsufficient mutants are indicated in bold. *Cell size mutants identified in Jorgenson et al's sytematic genetic screen

ORF	gene	Glucose		Glycerol	
		log size	sat size	log size	sat size
	WT	96 ± 3	52 ± 4	60	39
YLR131C*	ACE2	68 ± 37	100 ± 15	NA	100
YJL127C*	**SPT10**	137 ± 6	97± 6	131	93
YAL040C*	CLN3	118 ± 4	88 ± 5	91	91
YDL007W*	**RPT2**	137 ± 6	87± 8	57	90
YGR056W*	RSC1	140 ± 5	87 ± 9	71	96
YDR264C*	AKR1	124 ± 9	87 ± 8	NA	93
YER167W*	BCK2	102 ± 3	87 ± 2	93	95
YDL066W	IDP1	118 ± 12	86 ± 7	69	74
YCR020W-B*	**HTL1**	133 ± 0	85 ± 6	66	89
YDR138W*	HPR1	145 ± 3	83 ± 9	62	93
YDR335W	**MSN5**	105 ± 10	82 ± 5	66	79
YAL021C	CCR4	116 ± 14	79 ± 7	65	90
YKL114C	**APN1**	142 ± 12	79 ± 7	85	105
YNL139C*	RLR1	128 ± 4	78 ± 3	68	96
YJL080C*	**SCP160**	129 ± 2	77± 5	73	74
YJR054W*		113 ± 4	77 ± 8	77	66
YBR279W*	PAF1	120 ± 4	76± 7	57	84
YBR134W		99 ± 4	76 ± 8	106	76
TCR077C	PAT1	112 ± 7	76 ± 4	NA	79
YNR052C*	POP2	98 ± 2	76 ± 2	83	82
YPL129W*	**ANC1**	113 ± 5	76 ± 10	70	62
YJL115W	ASF1	117 ± 3	75 ± 5	75	65
YDR253C	MET32	115 ± 4	73 ± 8	60	65
YDL106C	BAS2	104 ± 3	71± 4	73	69
YCR081W	SRB8	103 ± 2	70 ± 9	107	76
YHL009C	YAP3	110 ± 3	70 ± 4	67	60
YML014W		114 ± 11	69 ± 3	94	57
YDR526C*		100 ± 1	66 ± 1	83	69
YGL047W*		78 ± 3	60 ± 4	69	37

Table 4.2A. Cellular functions of the *uge* mutants. Deletion of 29 yeast genes made cells abnormally large (*uge*). Please refer to the legend of Table 4.1A for details.

gene	ORF	%2N log	unbudded log	%2N sat	unbudded sat	human homologue	doubling time	General function
WT		31 ± 0	29	96 ± 3	99		90	
ACE2	YLR131C*	18 ± 16	5	21 ± 3	95	EGR alpha	161	transcription
SPT10	**YJL127C***	21 ± 5	8	95 ± 2	92	XM_003947	101	transcription
CLN3	YAL040C*	50 ± 5	46	93 ± 4	54	cyclin B3	72	cell cycle
RPT2	**YDL007W***	40 ± 4	28	97 ± 6	72	S4		cell cycle
RSC1	YGR056W*	33 ± 7	52	92 ± 2	48	SNF2	94	transcription
AKR1	YDR264C*	19 ± 1	14	88 ± 4	86	KIAA0379	114	signal transduction
BCK2	YER167W*	42 ± 7	50	91 ± 4	50	DSP	82	cell cycle
IDP1	YDL066W	35 ± 2	36	88 ± 2	64	CAA49208	81	energy generation
HTL1	**YCR020W-B***	38 ± 2	27	83 ± 4	73		90	transcription
HPR1	YDR138W*	28 ± 4	13	95 ± 6	87	AK092672	122	transcription
MSN5	**YDR335W**	35 ± 3	46	99 ± 0	54	RANBP21	84	cell cycle
CCR4	YAL021C	36 ± 1	17	88 ± 5	83	RSU-1	101	transcription
APN1	**YKL114C**	35 ± 7	85	91 ± 2	15		92	DNA repair
RLR1	YNL139C*	34 ± 3	11	53 ± 5	89		81	transcription
SCP160	**YJL080C***	28 ± 1	25	94 ± 1	75	HDLBP	95	cell cycle
	YJR054W*	40 ± 2	11	88 ± 1	89		87	unknown
PAF1	YBR279W*	33 ± 1	38	96 ± 1	62	PD2	103	transcription
	YBR134W	23 ± 3	20	96 ± 2	80		81	unknown
PAT1	TCR077C	42 ± 1	30	88 ± 1	70	AK094193	114	cell cycle
POP2	YNR052C*	31 ± 6	17	97 ± 1	83	CALIFp	117	transcription
ANC1	**YPL129W***	36 ± 5	25	90 ± 1	75	AF-9	127	transcription
ASF1	YJL115W	23 ± 1	18	94 ± 2	82	HSPC146	112	cell cycle
MET32	YDR253C	36 ± 5	33	92 ± 0	67	PMLZ-4	91	transcription
BAS2	YDL106C	34 ± 2	35	93 ± 2	65	HOX-C8	95	transcription
SRB8	YCR081W	43 ± 5	23	86 ± 7	77	TRAP230	112	transcription
YAP3	YHL009C	44 ± 4	47	82 ± 9	53	TREB	81	transcription
	YML014W	42 ± 9	49	89 ± 3	51	KIAA1456	102	translation
	YDR526C*	42 ± 8	10	97 ± 1	90		87	unknown
	YGL047W*	29 ± 4	54	94 ± 2	46		78	unknown

Table 4.2B. Cellular functions of the *uge* mutants. Deletion of 29 yeast genes maded cells abnormally large (*uge*). Please also refer to the legend for Table 4.1B for details.

CHAPTER V
REGULATION OF *CLN1* and *CLN2* EXPRESSION BY CCR4

5.1 Summary

In Chapter 4, I described a genome-wide genetic screen and the identification of 49 genes responsible for maintaining cell size homeostasis. Among them, I found that 5 deletion strains, *ccr4*Δ, *hpr1*Δ, *paf1*Δ, *pop2*Δ, and *rlr1*Δ, were significantly larger than the wild type cells. These genes are well conserved from yeast to human, and their gene products physical interact to form the Ccr4-Not complexes (Albert, Lemaire et al. 2000; Collart 2003).

This chapter summarized the experiments I conducted to elucidate the role of Ccr4 in the coordination of cell growth with proliferation. The large cell size phenotype of *ccr4*Δ suggested that they might commit to proliferation at a larger cell size than wild type cells (Figure 5.1A). I confirmed that this was indeed the case. Moreover, I showed that in *ccr4*Δ cells, *CLN1* and *CLN2* transcription was delayed. Genetic analyses revealed that Ccr4 functions downstream of Bck2 and upstream of Swi4, Cln1, and Cln2. Thus, I have elucidated some of the mechanisms whereby cells induce the transcription of *CLN1* and *CLN2* in a size dependent manner. These data expanded our understanding of how cell growth is coordinated with proliferation.

5.1.1 Research Objective

The large cell size phenotype of the *ccr4*Δ strain was similar to that of *cln3*Δ or *bck2*Δ strains (Figure 5.1B). In strains lacking either Cln3 or Bck2, the expression of *CLN1* and *CLN2* was mildly reduced but greatly delayed. This was because the transcription of *CLN1* and *CLN2* was induced by Cln3 and Bck2 in a manner dependent upon the two transcription factors SBF and MBF (Figure 5.2). While the molecular details involved in these steps are still not completely understood, examination of one of the mutants I identified, Whi5, indicated that it functioned as an inhibitor of *CLN1* and *CLN2* transcription (Figure 5.2). Based on these data, I proposed that Ccr4 was an activator of *CLN1* and *CLN2* transcription.

5.1.2 Hypothesis

Based on the similarity of the large cell size phenotypes of the *ccr4*Δ, *cln3*Δ and *bck2*Δ strains, I hypothesized that Ccr4, like Cln3 and Bck2, was a positive regulator of *CLN1* and *CLN2* transcription.

5.1.3 Rationale and Expected Results

The rationale for the studies conducted here was shown in (Figure 5.3). Specifically, I would examine the genetic interactions between *CCR4* and known cell cycle regulators (e.g. Cln3, Bck2, Swi4, Swi6, Mbp1, Cln1 and Cln2) (Figure 5.2). Genetic epistatic assays would be conducted to clarify the relationship between *CCR4* and each of these other genes. Genetically, *CCR4* might have three possible relationships with each of the genes (e.g. referred to as gene X for simplicity) shown in the pathway (Figure 5.3): parallel (1), upstream (2) or downstream (3) (Figure 5.3). To test if *CCR4* and gene X were functional in parallel, both *CCR4* and gene X would be deleted simultaneously to make a double mutant, the cell size of which would be compared with the single deletion strains of either the *ccr4*Δ or the *genex*Δ. If the double mutants were larger or inviable, this would reveal an additive effect. This would suggest that *CCR4* and gene X function in independent pathways. Conversely, if there was no additive effect, this would suggest that they were functioning in the same genetic pathway. In this case, the next step was to determine the genetic hierarchy between the two genes. To test this, ectopic expression of gene X in the *ccr4*Δ deletion or ectopic expression of *CCR4* in gene X deletion strains helped establish the genetic hierarchy. If gene X was downstream of *CCR4*, ectopic expression of gene X in the *ccr4*Δ strain would be able to complement loss of *CCR4* function, and thus would rescue the phenotype of the *ccr4*Δ strains. If gene X was upstream of *CCR4*, ectopic expression of gene X in the *ccr4*Δ strain would be unable to complement loss of *CCR4* function, and thus would not rescue the phenotype of the *ccr4*Δ strains. Using these types of analyses, I have placed Ccr4 within the known cell cycle regulatory pathways.

5.1.4 Introduction to the Function and Role of Ccr4-Not Complexes

The yeast *CCR4* (Carbon Catabolite Repression) gene product was first identified as a general transcription factor with both positive and negative effects on gene transcription (Denis 1984; Denis and Malvar 1990). The yeast *CCR4* gene encodes a 2.8 kb mRNA and a 95 KDa protein (Draper, Liu et al. 1994). Ccr4 protein itself doesn't have a DNA binding domain, but does have a trans-activation domain at its N-terminus (Figure 5.4A). Thus, when fused to a LexA DNA binding domain, Ccr4 enhanced the expression of a reporter gene (Draper, Liu et al. 1994). The protein possesses five tandem leucine-rich repeats between residue 350 and 473, suggesting interactions with other proteins (Figure 5.4A) (Malvar, Biron et al. 1992; Draper, Liu et al. 1994). Indeed, it was the core protein associated with functionally related PAF RNA PolII holoenzyme and Not poly(A) deadenylase complexes (Figure 5.4B) (Bai, Salvadore et al. 1999; Chang, French-Cornay et al. 1999; Chen, Rappsilber et al. 2001). These complexes were involved in several aspects of mRNA metabolism, including transcription initiation and elongation or deadenylation and recently, ubiquitination (Collart 2003; Denis and Chen 2003).

In vivo, Ccr4 and other components form huge complexes with a molecular weight up to 1.9 MDa (Chen, Rappsilber et al. 2001). Understanding the physiological roles of this complex has been greatly handicapped because of its biochemical complexity and the complicated genetic interactions among the components. However, mounting evidence has implicated Ccr4 complexes in cell cycle regulation. For example, several components were initially identified as Cdc genes in budding yeast, such as Cdc39 (Not1), Cdc36 (Not2), Cdc68 and Cdc73 (Figure 5.4B) (Reed 1980). Dbf2, a kinase responsible for cytokinesis was also a component of Ccr4-Not complex (Liu, Toyn et al. 1997). Some components of the Ccr4 complexes played key roles in a DNA damage checkpoint (Westmoreland, Olson et al. 2003; Westmoreland, Marks et al. 2004). Furthermore, it has been shown that Paf1 and Ctr9, two proteins associated with Ccr4, were implicated in *CLN1* and *CLN2* expression, as in the temperature sensitive strain of

paf1^{ts} *or ctr9*^{ts}, expression of these two genes was largely abolished at the restrictive temperature (Koch, Wollmann et al. 1999).

5.2 Ccr4 Positively Regulates *CLN1* and *CLN2* mRNA Expression

5.2.1 Ccr4 Affects *CLN1* and *CLN2* mRNA Abundance

Like deletion of *CLN3* and *BCK2* (Figure 5.1B), deletion of *CCR4* made cells larger than the wild type (Figure 5.1A). To address my hypothesis, that Ccr4 was an activator of *CLN1* and *CLN2* transcription, I first compared the abundance of *CLN1* and *CLN2* mRNA during mid-log phase in the *ccr4*Δ and wild type strain (Figure 5.5A). Quantitation revealed a modest ~20% decrease in *CLN1* and *CLN2* mRNA expression in asynchronous *ccr4*Δ cells in log phase as compared to wild type cells. In addition, ectopic expression of *CCR4* in the *ccr4*Δ cells elevated *CLN1* and *CLN2* mRNA expression more than 1.5 fold (Figure 5.5B). These data supported the idea that Ccr4 positively regulated *CLN1* and *CLN2* mRNA expression.

5.2.2 *CLN1* and *CLN2* Are Epistatic to *CCR4*

If the larger cell size phenotype of *ccr4*Δ was due to reduced *CLN1* and *CLN2* mRNA expression, then ectopic expression of *CLN1* or *CLN2* should be able to rescue this phenotype. To test this idea, *ccr4*Δ cells were transformed with *GAL-CLN1* and *GAL-CLN2*. Transformed cells were then grown in presence of galactose to induce the *GAL-CLN* gene. Log phase cells were sized and compared with control cells transformed with the empty vector alone. As expected, the cells with *GAL-CLN1* and *GAL-CLN2* were smaller than control (empty vector) (Figure 5.5C). This indicated that *CLN1* and *CLN2* were epistatic to *CCR4* (Figure 5.5C). The result strongly supported the positive role of Ccr4 in *CLN1* and *CLN2* mRNA expression.

5.2.3 Ccr4 Affects Cell Cycle Regulation of *CLN1/2*

As shown in Chapter 4, three *uge* mutants (larger than the wild type) had a larger "critical cell size: operationally defined as the size at which 50% of the cells were

budded" than the wild type (Figure 4.5B). To establish if the *ccr4*Δ strains have the same phenotype, the critical cell size of *ccr4*Δ was determined and compared to wild type cells as previously described. Briefly, small unbudded G1-phase cells were collected by centrifugal elutriation before they were released into fresh YPD. Cells were incubated at room temperature and time points were collected for cell size and budding indexes. The budding indexes were plotted as a function of time or cell size. As shown in (Figure 4.6A and B), the critical cell size for the wild type was ~65fL as previously reported (Figure 4.6). The *ccr4*Δ cells executed Start at ~75fL (Figure 5.6A). Meanwhile, Start was also delayed in the *ccr4*Δ strains. Wild type cells executed Start at ~90 min after being released into the fresh medium whereas, in the *ccr4*Δ cells Start was delayed to 130 min (Figure 5.6B).

Critical cell size and the timing of Start execution were dependent upon the timely expression of Cln1 and Cln2 expression (Dirick, Bohm et al. 1995). The large critical cell size and the delayed Start of *ccr4*Δ cells strongly suggested that *CLN* expression might also be delayed. To examine this possibility, RNA was isolated from the same time points from for budding and cell size. The expression of *CLN1* and *CLN2* mRNA were then measured by northern blot (Figure 5.7). Consistent with the timing of Start, in the wild type cells, the expression of *CLN1* and *CLN2* mRNA was undetectable until the 60 min time point and peaked between 75-90 min (Figure 5.7A). In contrast, in the *ccr4*Δ cells *CLN1* and *CLN2* mRNA was undetectable until the 90 min time point and peaked between 110~125min (Figure 5.7B). From the above data, I concluded that Ccr4 positively regulated *CLN1* and *CLN2* mRNA expression and determines the timing of their expression.

5.3 *CCR4* Is Upstream of *SWI4*

Next, I addressed the mechanism whereby Ccr4 positively regulates *CLN1* and *CLN2* mRNA expression. The timely expression and the abundance of *CLN1* and *CLN2* mRNA expression were dependent upon two transcript factors, SBF and MBF (Figure 5.2). As previously mentioned, both SBF and MBF were heterodimers. SBF was

composed of Swi4 and Swi6 while MBF contains Mbp1 and Swi6. To understand the mechanism whereby Ccr4 positively regulated *CLN1* and *CLN2* mRNA expression, I examined the genetic interactions between *CCR4* and *SWI4*, *SWI6* and *MBP1*.

5.3.1 *CCR4* Functions in a Linear Pathway with *SWI4*

First, I attempted to determine if *CCR4* and *SWI4* function in the same or different pathway. To do this, I constructed a *ccr4*Δ *swi4*Δ double mutant to assess if the addition of a *swi4*Δ deletion would make *ccr4*Δ cells larger. As discussed in Figure 5.3, the additive size effect or synthetic lethality of double mutants would indicate that the two genes function in independent pathways. I found that the *ccr4*Δ *swi4*Δ double mutant was viable and was not larger than *ccr4*Δ alone (Figure 5.8A). Moreover, by comparing the cell size of the *ccr4*Δ *swi4*Δ double mutant with another haploid mutant *rox2*Δ, a gene involved in RNA PolII, I demonstrated that this result was not because the *ccr4*Δ *swi4*Δ mutants had reached maximal size of a haploid cell (Figure 5.8B). Thus, these results suggested that Ccr4 and Swi4 functioned in the same genetic pathway.

5.3.2 *SWI4* Is Epistatic to *CCR4*

Next, I addressed the genetic hierarchy between *CCR4* and *SWI4*. If *SWI4* functioned downstream of *CCR4*, ectopic expression of *SWI4* would made *ccr4*Δ cells smaller (Figure 5.3, possibility 2). Conversely, if *SWI4* functioned upstream of *CCR4*, ectopic expression of *SWI4* would not reduce the size of *ccr4*Δ cells (Figure 5.3, possibility 3). To address this question, the *ccr4*Δ deletion strain was transformed with *GAL1-SWI4* or an empty vector as a control. The log phase sizes of the transformed cells were compared. As shown in Figure 5.8C, the cell size of *GAL1-SWI4* transformed cells was dramatically smaller those transformed with the empty vector. This result suggested that *SWI4* functions downstream of *CCR4* and was thus epistatic to *CCR4*.

5.4 *CCR4* and *SWI6* Are Synthetically Lethal

To determine if *CCR4* and *SWI6* function in the same or different pathway, I attempted to construct *ccr4*Δ *swi6*Δ double mutants. However, I was unable to recover any viable *ccr4*Δ *swi6*Δ double mutants. After sporulation, most of tetrads (>90%) contained only 3 or fewer spores. These results suggested that the *ccr4*Δ *swi6*Δ double mutants were inviable, and these two genes functioned in the complementary pathways. This type of relationship was referred to as synthetic lethality because strains were only dead when two genes having redundant functions were lost.

5.5 *CCR4* and *BCK2* May Function in a Linear Pathway

BCK2 and *CLN3* function independently to activate *CLN1* and *CLN2* mRNA expression (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). Like the *ccr4*Δ *swi6*Δ double mutants, the *cln3*Δ *bck2*Δ double mutants were synthetically lethal (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). In addition, in the *cln3*Δ *bck2*Δ double mutants *CLN1* and *CLN2* mRNA were not expressed (Di Como, Chang et al. 1995; Wijnen and Futcher 1999). Thus, if *CCR4* functions independently of *CLN3*, it should fall into the *BCK2* pathway. To test this idea, the double mutant *ccr4*Δ *bck2*Δ was constructed. The double mutant *ccr4*Δ *bck2*Δ was viable, and its size was not larger than the parental strains *bck2*Δ or *ccr4*Δ (Figure 5.9A). This confirmed that *BCK2* and *CCR4* function in the same genetic pathway.

Next, I addressed the genetic hierarchy between *CCR4* and *BCK2*. If *BCK2* functions downstream of *CCR4*, ectopic expression of *BCK2* would make *ccr4*Δ cells smaller (Figure 5.3, possibility 2). Conversely, if *BCK2* functions upstream of *CCR4*, ectopic expression of *BCK2* would not reduce the size of *ccr4*Δ cells (Figure 5.3, possibility 3). To address this question, the *ccr4*Δ deletion strain was transformed with *GAL1-BCK2* or an empty vector as a control. The log phase sizes of the transformed cells were compared. As shown in Figure 5.9B, the cell size of *GAL1-BCK2* transformed cells was the same as those transformed with the empty vector. This result suggested that *CCR4* functioned downstream of *BCK2*. To exclude the possibility that the *GAL-BCK2*

plasmid was defective, the wild type cells were transformed with this plasmid. As shown in Figure 5.9C, the wild type cells become smaller. Thus, the *GAL-BCK2* plasmid was functional. To confirm that *CCR4* was downstream of *BCK2*, I transformed *bck2*Δ strains with *GALl-CCR4*. The *bck2*Δ cells become smaller (Data not shown). This corroborated the above result. In summary, several lines of evidence demonstrated that *CCR4* functions downstream of *BCK2*. First, the double mutant *ccr4*Δ *bck2*Δ did not have additive cell size phenotype. Second, over-expression of *BCK2* in the *ccr4*Δ mutants failed to complement its cell size phenotype. Third, ectopic expression of *CCR4* rescued the size phenotype of the *bck2*Δ cells.

5.6 *CCR4* and *CLN3* May Function Independently

The function of *CLN3* was dependent upon *SWI6* (Wijnen, Landman et al. 2002). As *CCR4* and *SWI6* were synthetically lethal, I expected that *CCR4* would also be synthetically lethal with *CLN3*. Indeed, I was unable to recover any viable *ccr4*Δ *cln3*Δ double mutants when trying to construct the double deletion strains. After sporulation, most of tetrads (>90%) contained only 3 or fewer spores, and no viable spores were double mutants. These results suggested that CCR4 and CLN3 are functionally complementary as the *ccr4*Δ *cln3*Δ double mutants were inviable.

To corroborate this result, I transformed *ccr4*Δ strains with a *GAL-CLN3* construct. If *CCR4* and *CLN3* functioned in independent pathways, then I would expect that over-expression of *CLN3* should reduce the size of *ccr4*Δ cells. As expected, the cell size of the *GAL-CLN3 ccr4*Δ transformed cells was smaller than that of the control (empty vector) (Figure 5.10).

5.7 Cdc39 (Not1) and Cdc36 (Not2) Also Regulate *CLN* Expression

Not1 and Not2 were integral components of Ccr4-Not complexes (Figure 5.4B). These two genes were first identified as *CDC39* and *CDC36* respectively, because the temperature sensitive mutants of $cdc39^{ts}$ and $cdc36^{ts}$ arrested at G1 phase as large unbudded cells at the restrictive temperature (Figure 5.11A). Because of their physical

association with Ccr4, and of their G1-phase arrest phenotype, I hypothesized that they might also be involved in *CLN1* and *CLN2* mRNA expression. To test this hypothesis, *cdc39*^{ts} and *cdc36*^{ts} strains were grown to log phase at room temperature and shifted to 37°C for six hours. Time points were taken every 2 hours and RNA was isolated from WT (negative control), *cdc28*^{ts} (positive control), *cdc36*^{ts}, and *cdc39*^{ts} cultures. Northern analysis revealed that *CLN1* mRNA expression was dependent upon Cdc36 and Cdc39 (Figure 5.11B). These data further implicate Ccr4-Not complexes in the activation of *CLN1* expression.

5.8 Discussion

5.8.1 Working Model for Ccr4 Function

CLN1 and *CLN2* mRNA expression can be activated by both Cln3 and Bck2 (Epstein and Cross 1994; Di Como, Chang et al. 1995; Wijnen and Futcher 1999). This activation is largely dependent upon two transcription factors SBF and MBF. *BCK2* is a gene without a known human homolog in the database, and the mechanism whereby it activates *CLN1* and *CLN2* expression is not known.

In this chapter, I provided evidence that *CCR4* was involved in regulating *CLN1* and *CLN2* mRNA expression, because in the *ccr4*Δ cells, *CLN1* and *CLN2* mRNA expression was delayed. *CCR4* was functional independently of *CLN3*, *SWI6* and *MBP1*, because the double mutants of *ccr4*Δ *cln3*Δ, *ccr4*Δ *swi6*Δ and *ccr4*Δ *mbp1*Δ were inviable. Rather, it worked upstream of *SWI4* and downstream of *BCK2*.

Thus, I proposed the following genetic model to explain how Ccr4 functioned in budding yeast. The external signals somehow activate Bck2, which in turn, activates Ccr4 (expression and/or activity). Ccr4 then positively regulates Swi4 that is responsible for *CLN1* and *CLN2* mRNA expression (Figure 5.12).

Several lines of evidence supported this model: 1. the *bck2*Δ *ccr4*Δ double mutants were the same size as the *ccr4*Δ single mutant; 2. ectopic expression of *CCR4* rescued the size phenotype of *bck2*Δ; 3.the *ccr4*Δ *swi4*Δ double mutants were the same size as the *ccr4*Δ single mutant; 4. ectopic expression of *SWI4* rescued the size phenotype of *ccr4*Δ; 5.

SWI6 was epistatic to *CLN3* (Wijnen, Landman et al. 2002). In addition, I showed that the *ccr4*Δ *cln3*Δ double mutant was inviable, indicating that Ccr4 functioned independently of *CLN3*. This synthetic lethality was also similar to that of *cln3*Δ *bck2*Δ or *bck2*Δ *swi6*Δ mutant (Di Como, Chang et al. 1995); 6. TAF1 ($yTAF_{II}145$), a yeast TATA-box binding protein (TBP) associated factor, was also under regulation of growth state. For example, the level of TAF1 decreased dramatically when cells entered stationary phase (Walker, Shen et al. 1997). This protein was responsible for transcription of G1-cyclins *CLN1* and *CLN2*, but not *CLN3*. TAF1 bound to Ccr4 complex both *in vitro* and *in vivo* (Walker, Shen et al. 1997), indicating that this complex may function downstream or in parallel of *CLN3*. 7. Ctr9 was a component of Ccr4 complex. In the $ctr9^{ts}$ mutants, *CLN1* and *CLN2* expression was abolished (Koch, Wollmann et al. 1999). Interesting, *CLN3* and *CTR9* were synthetic lethal (Wijnen, Landman et al. 2002), strongly suggesting that the Ccr4 complexes were functionally independent of *CLN3*. Finally, two other components of the complexes, Not1 (Cdc39) and Not2 (Cdc36) were also involved in the regulation of *CLN1* and *CLN2* (Figure 5.11), consistent to the fact that the temperature sensitive mutants of *CDC36* and *CDC39* arrested in G1-phase at the restrictive temperature (Reed 1980). These results strongly supported a role of Bck2 in *CLN1* and *CLN2* expression via Ccr4 complexes.

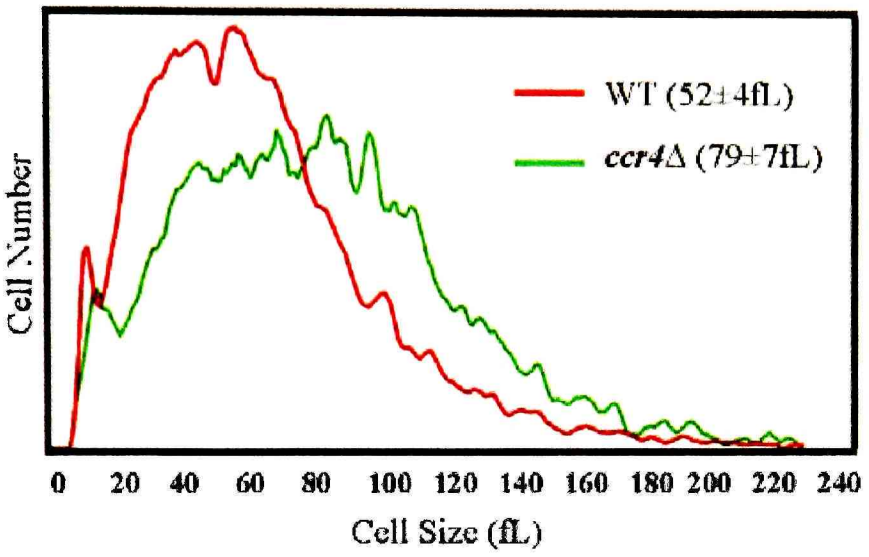
5.8.2 Biological Significance

The components of the Ccr4 complexes were well conserved in eukaryotic cells from yeast to human (Albert, Lemaire et al. 2000; Tirone 2001; Denis and Chen 2003), suggesting important roles of the Ccr4 complexes in the cell. Indeed, the current evidence has shown that the complexes are implicated in tumor suppression and DNA damage check point. However, their physiological roles are still unclear.

In this chapter, I have shown that Ccr4 positively regulated *CLN1* and *CLN2* mRNA expression in budding yeast. Because the cell cycle machinery is well conserved, Ccr4 may also positively regulate G1 phase cyclin expression in mammalian cells. Expression of G1 phase cyclins, as discussed in Chapter 1, has been implicated in the

important processes such as development, aging and tumorigenesis. Therefore, my data showed a novel way to regulate expression of G1 phase cyclins.

A



B

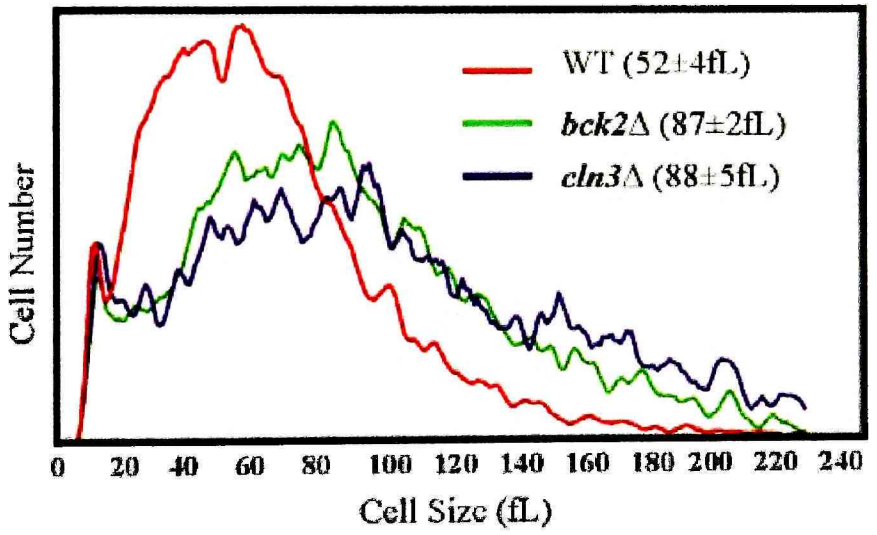


Figure 5.1. The cell size of *ccr4*Δ strain was larger than the wild type, similar to that of *bck2*Δ or *cln3*Δ strains. A. As described in Chapter 4, the *ccr4*Δ and the isogenic wild type cells (BY4743) were growing to the stationary phase on YPD plates and sized with the Coulter counter. B. *bck2*Δ and *cln3*Δ strains were grown to the stationary phase on YPD plates and their sizes are compared with that of the wild type (BY4743). The average cell sizes were calculated from at least five independent experiments.

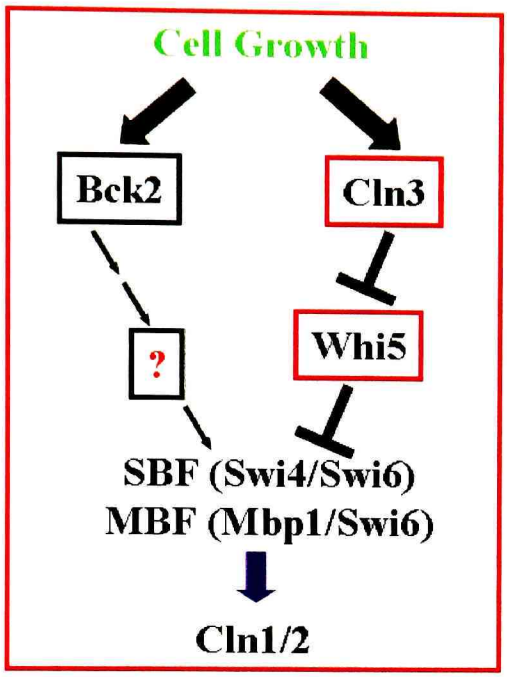


Figure 5.2. Current scenario of *CLN1* and *CLN2* expression. External cell growth signals stimulate two independent genetic pathways: *BCK2* and *CLN3*. It is known that Cln3 phosphorylates Whi5, which inhibits two heterodimeric transcription factors SBF (Swi4 and Swi6) and MBF (Mbp1 and Swi6). The phosphorylation causes dissociation of Whi5 and thus relieves its inhibition on SBF and MBF. These two transcription factors directly activate *CLN1* and *CLN2* mRNA expression. Bck2 pathway can also activate *CLN1* and *CLN2* mRNA expression dependent upon SBF and MBF. However, the biochemical mechanism is poorly understood.

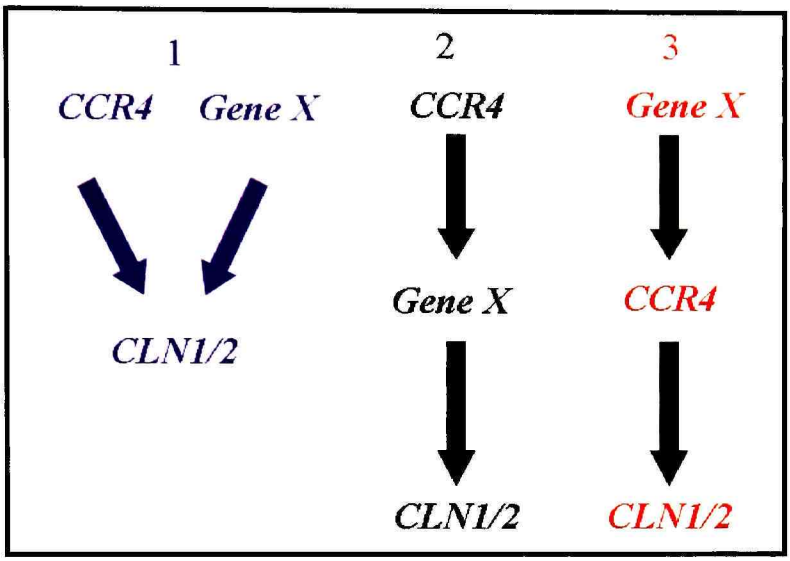


Figure 5.3. Rationale of analysis of genetic interactions between *CCR4* and gene X in the *BCK2 or CLN3* pathway. In general, there are three possible relationships between *CCR4* and another gene: parallel (1), upstream (2) and downstream (3). The strategy to distinguish these possibilities relationship is as follows. A. To examine if these two genes function in two independent pathways (1). If they do, inactivation of both genes simultaneously in the cells will have more severe phenotype i.e. cell size than either of the single mutant (additive effect). Conversely, if they function in the same pathway, there will be no additive phenotype i.e. cell size. If this is the case, the genetic hierarchy will be distinguished. To do this, gene X will be ectopically expressed in the *ccr4*Δ cells. If *CCR4* size phenotype is rescued, then gene X must be downstream of or epistatic to *CCR4* (2). If the *ccr4*Δ size phenotype persists, *CCR4* is downstream of gene X (3). To confirm the result, ectopic expression *CCR4* in a gene X deletion strain will be used.

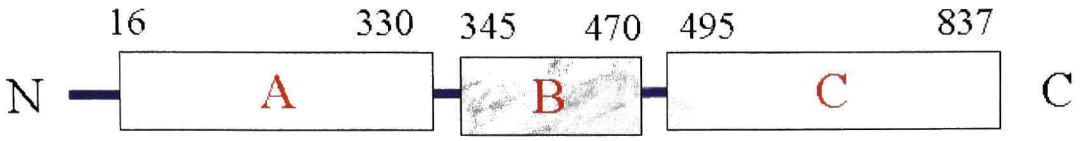
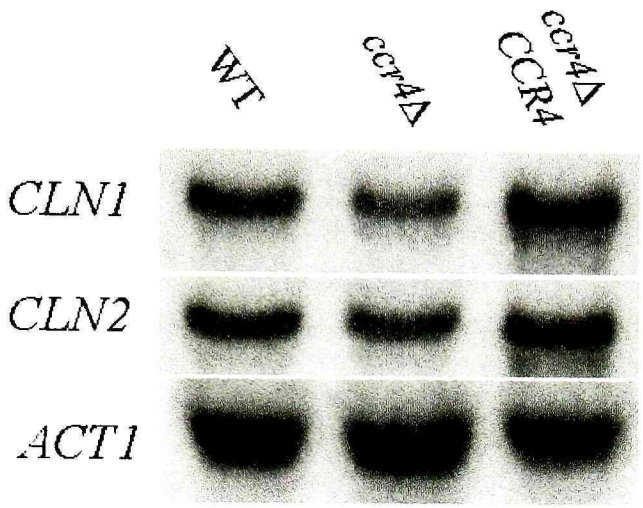
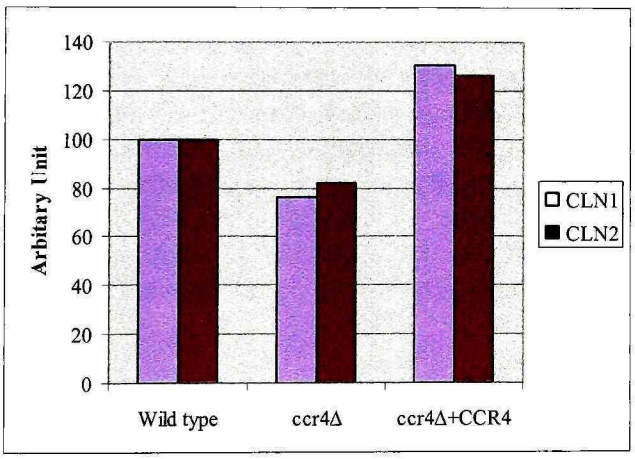


Figure 5.4. Illustration of Ccr4 domains. The yeast Ccr4 protein is 95 KDa, consisting of three functional domains: (A) trans-activation domain; (B) Leucine-rich domain and (C) mRNA poly(A) deadenylase domain.

A.


WT
ccr4Δ
ccr4Δ CCR4
CLN1
CLN2
ACT1

B.


Arbitary Unit
140
120
100
80
60
40
20
0
Wild type
ccr4Δ
ccr4Δ+CCR4
CLN1
CLN2

C.

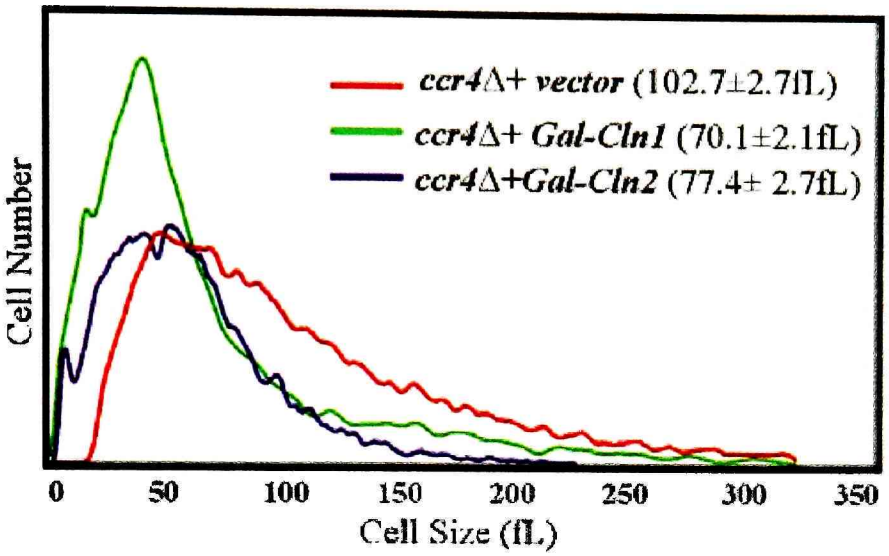
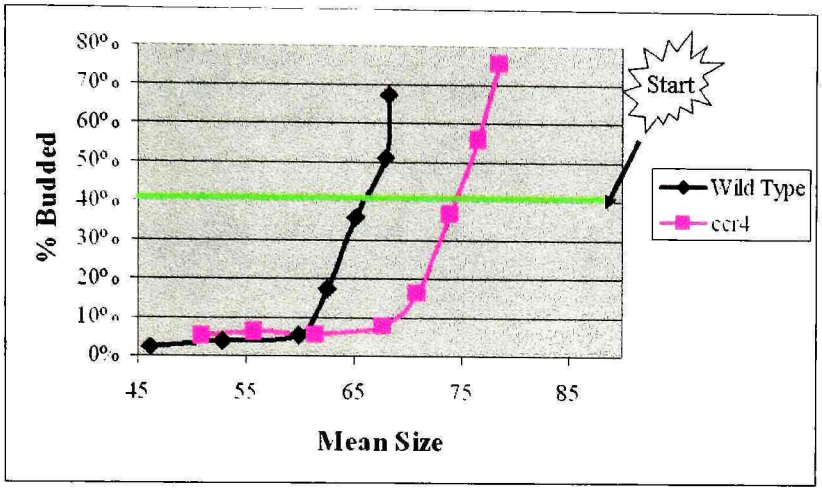


Figure 5.5. Ccr4 positively regulated *CLN1* mRNA expression. A. *CLN1* mRNA expression decreased in *ccr4*Δ cells, and ectopic expression of *CCR4* restored *CLN1* expression. The wild type (BY4743), the *ccr4*Δ cells (isogenic to BY4743) and the *ccr4*Δ transformed with *CCR4*-FLAG (gift from Dr. Denis) were grown in YPD at 30°C to log phase. RNA was extracted for *CLN1* expression by northern blot. B. Quantitation of *CLN1* expression above as described. C. *CLN1* and *CLN2* were epistatic to *CCR4*. The *ccr4*Δ cells were transformed with *GAL-CLN1* or *GAL-CLN2*. The cell size of the transformed cells was compared with the control (empty vector).

A.



B.

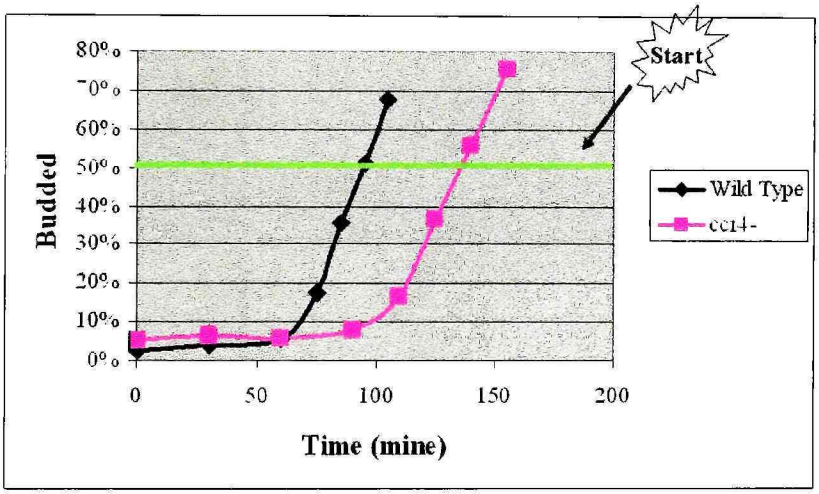
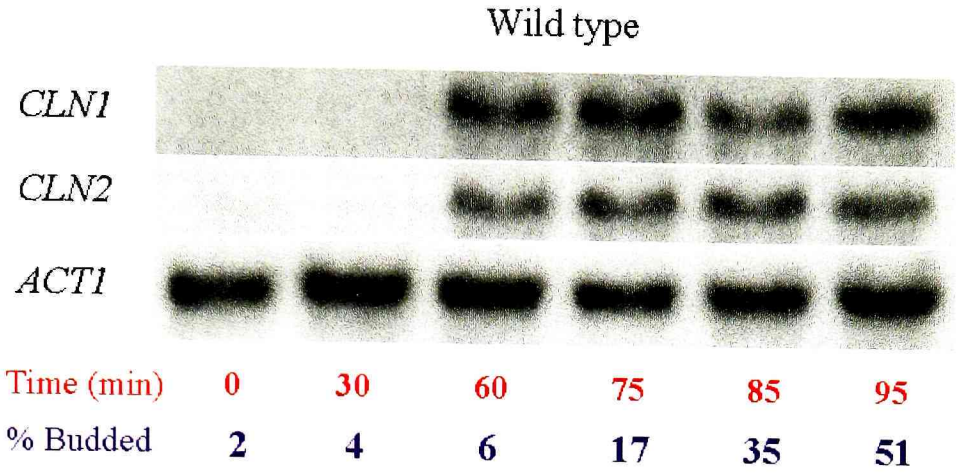


Figure 5.6. The Ccr4 regulated cell cycle progression. Small unbudded cells of the wild type (BY4743) and the *ccr4*Δ strain (isogenic to BY4743) were collected by centrifugal elutriation. The cells were then released into fresh YPD medium and incubated at room temperature. The samples were taken at regular intervals for budding and cell size. The budding indexes wer plotted as a function of cell size or time. A. The *ccr4*Δ cells had larger critical cell size. B. The execution of Start was delayed in the *ccr4*Δ cells. Start is technically defined as the point at which 50% of the cells have budded.

A.



B.

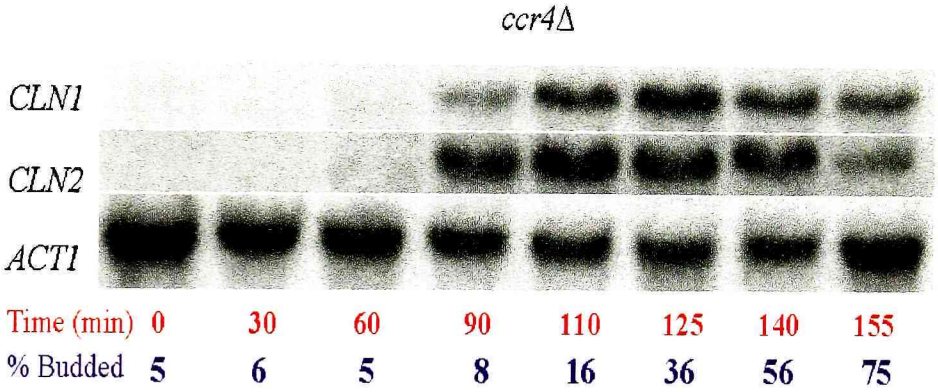
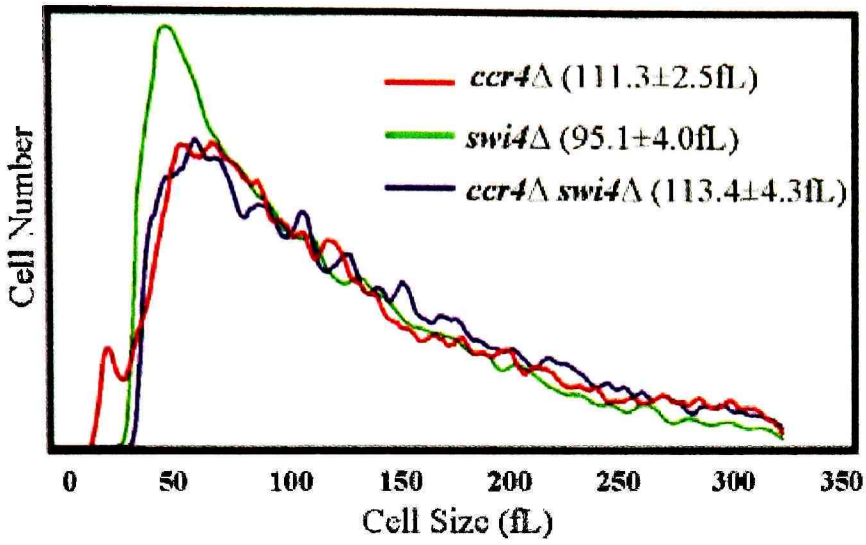
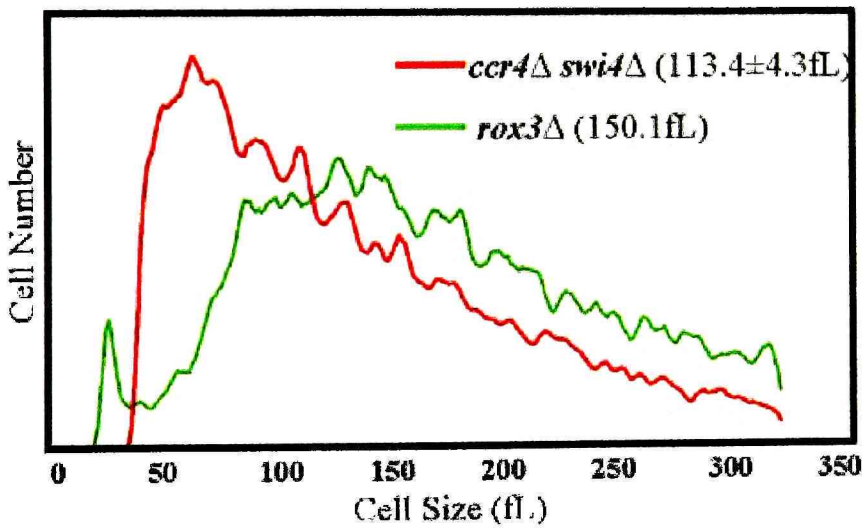


Figure 5.7. *CLN1* and *CLN2* mRNA expression was delayed in the *ccr4*Δ cells. The samples were the same as those for budding and sizing in Figure 5.6. The RNA was extracted and *CLN1* and *CLN2* mRNA expression was examined by northern blot. A. Timely expression of *CLN1* and *CLN2* in the wild type (BY4743) and B. in the *ccr4*Δ cells (isogenic to BY4743).

A.


ccr4Δ (111.3±2.5fL)
swi4Δ (95.1±4.0fL)
ccr4Δ swi4Δ (113.4±4.3fL)
Cell Number
0
50
100
150
200
250
300
350
Cell Size (fL)

B.


ccr4Δ swi4Δ (113.4±4.3fL)
rox3Δ (150.1fL)
Cell Number
0
50
100
150
200
250
300
350
Cell Size (fL)

C.

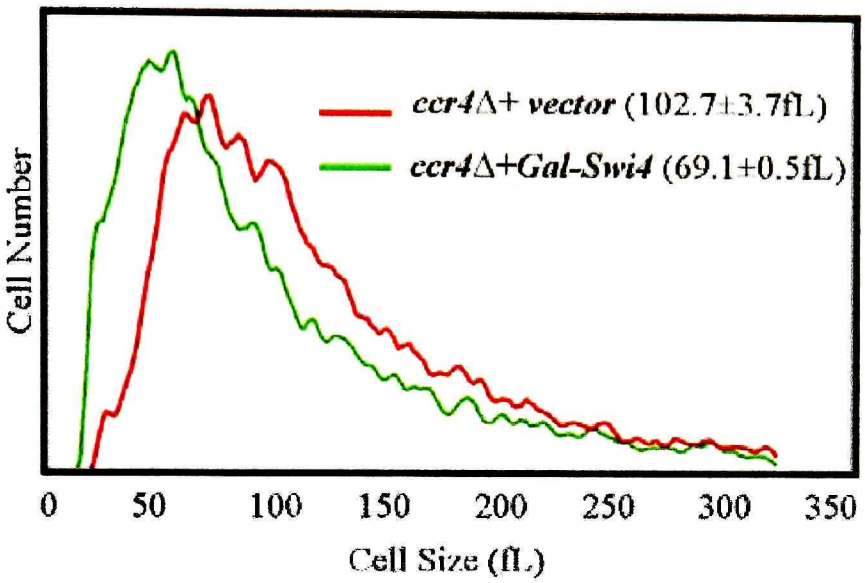
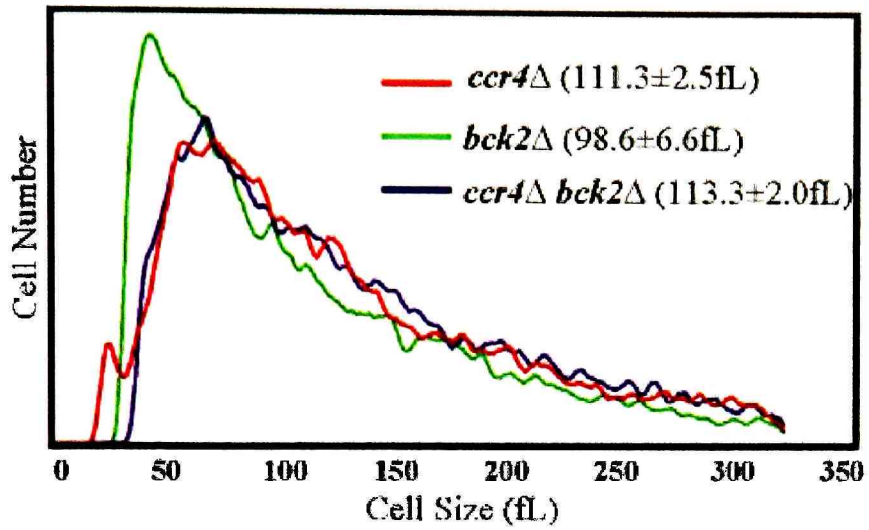
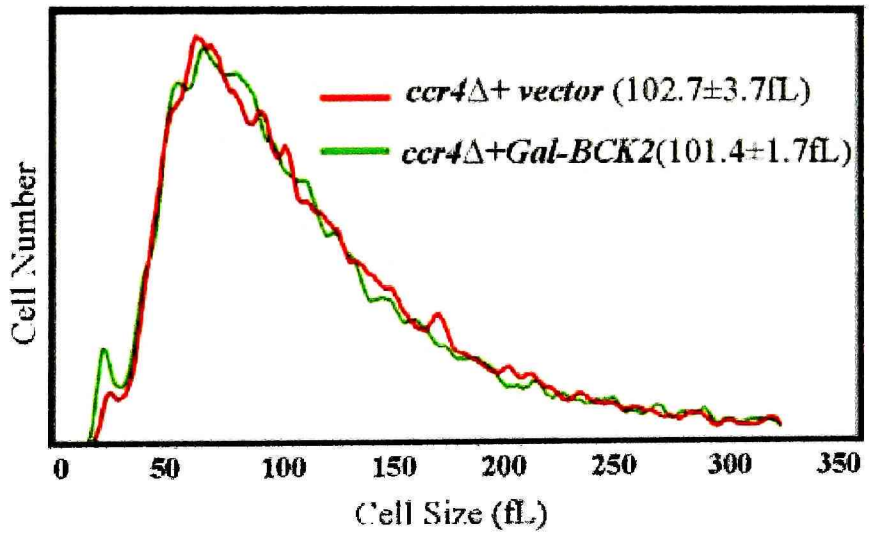


Figure 5.8. *CCR4* was functionally upstream of *SWI4*. A. No additive cell size phenotype in the *ccr4Δ swi4Δ* double mutant. The cell sizes of, *swi4Δ* and *ccr4Δ swi4Δ* double mutant were compared. The double mutant was not larger than the *ccr4Δ* cells. B. The cell size of the *ccr4Δ swi4Δ* double mutant did not reached the maximal size of a haploid. A haploid strain *rox2Δ* was compared with the *ccr4Δ swi4Δ* double mutant. C. Ectopic expression of *SWI4* rescued *ccr4Δ* phenotype, indicating *SWI4* was downstream of *CCR4*.

A.


ccr4Δ (111.3±2.5fL)
bck2Δ (98.6±6.6fL)
ccr4Δ bck2Δ (113.3±2.0fL)
Cell Number
0
50
100
150
200
250
300
350
Cell Size (fL)

B.


ccr4Δ+ vector (102.7±3.7fL)
ccr4Δ+Gal-BCK2(101.4±1.7fL)
Cell Number
0
50
100
150
200
250
300
350
Cell Size (fL)

C.

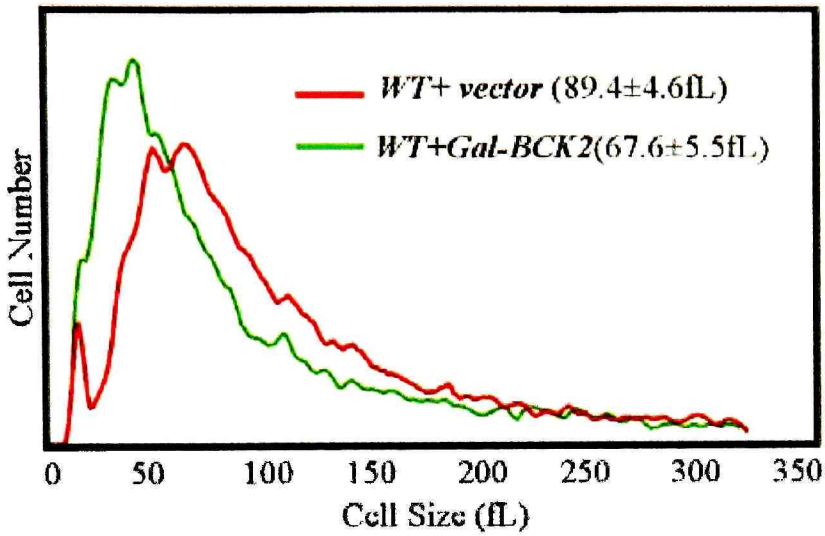


Figure 5.9. *CCR4* functioned downstream of *BCK2*. A. There was no additive cell size phenotype in the *ccr4*Δ *bck2*Δ double mutant. The cell sizes of *ccr4*Δ, *bck2*Δ and *ccr2*Δ *bck2*Δ double mutant were compared. The double mutant was not larger that *ccr4*Δ single deletion. Thus, *CCR4* and *BCK2* functioned in the same genetic pathway. B. Over-expression of *BCK2* did not rescue the cell size phenotype in the *ccr4*Δ cells. The *ccr4*Δ cells were transformed with *GAL-BCK2*. There was no difference between the cells transformed with Gal-*BCK2* and transformed with the empty vector. C. *GAL-BCK2* made the wild type cells smaller, indicating that the plasmid was functional.

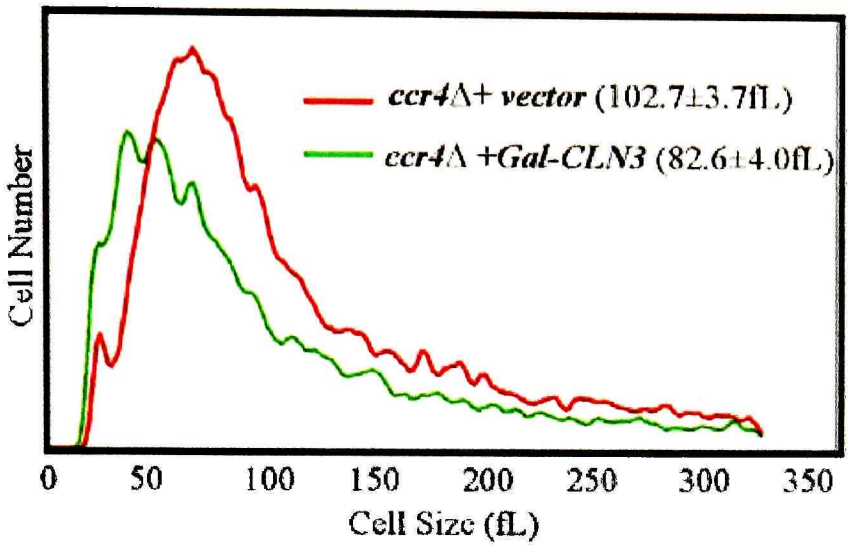
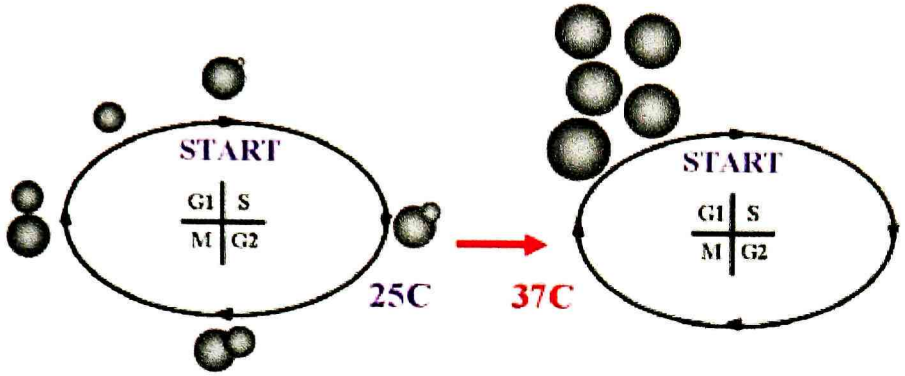


Figure 5.10. *CCR4* and *CLN3* function independently. A. the *ccr4*Δ *cln3*Δ double mutant was inviable. B. Overexpression of *CLN3* rescued*ccr4*Δ size phenotype. The *ccr4*Δ cells were transformed with Gal-*CLN3* or the empty vector (Control). The Gal-*CLN3* transformed cells were smaller than the control, supporting the conclusion that the two genes functioned in parallel.

A.



B.

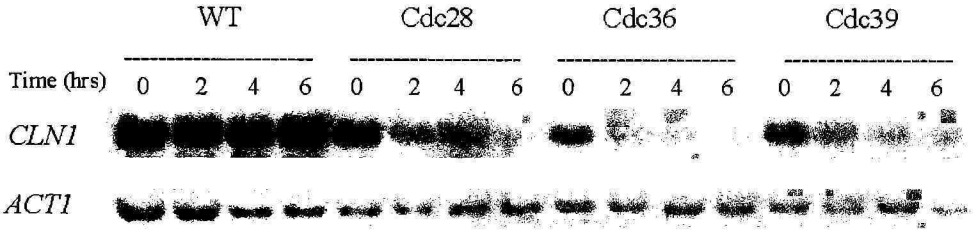


Figure 5.11. Cdc36 and Cdc39 also positively regulatd *CLN1* mRNA expression. The temperature sensitive mutants of Cdc28, Cdc36 and Cdc39 were grown to log phase, and shifted to 37°C (A). The cells eventually arrested G1 phase. The time points were taken as indicated after the temperature shift. The RNA was extracted for *CLN1* expression (B).

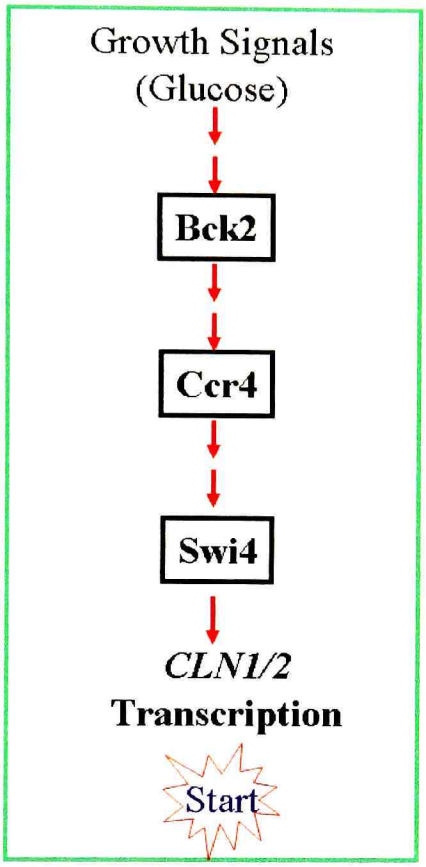


Figure 5.12. A model shows how *CCR4* functions to promote Start. The *CCR4* functions exclusively in *BCK2* pathway. *BCK2* somehow activates Ccr4, which in turn, activates Swi4. The Swi4 is a subunit of the heterodimeric transcription factor SBF that is responsible for *CLN1 and CLN2*transcription. When Cln1 and Cln2 reach a certain level, the cells commit to division at Start.

CHAPTER VI
DISCUSSION AND CONCLUSION

6.1 Summary

In my research I have analyzed the relationship between cell growth, cell size, Cln expression, and proliferation. I found that a critical threshold of Cln protein was required for proliferation. Moreover, I found that rapidly growing cells expressed, and required more Cln protein than did slowly growing cells. To clarify the role of cell size, I expressed defined amounts of *CLN* mRNA in cells of different sizes. In so doing, I found that Cln protein expression was strongly modulated by cell size. Finally, I showed that the expression of high levels of *CLNs* promoted proliferation in a size-independent manner suggesting that Clns were rate-limiting. The significance and implications of these findings were discussed.

To examine the relationship between cell growth and the ability of cells to proliferate, I conducted a systematic genome-wide genetic screen to identify mutants that dramatically altered the proliferative capacity of cells. In so doing, I identified 49 gene deletions that dramatically changed cell size. Twenty of these made cells abnormally small (*whi* mutants), and 29 made cells abnormally large (*uge* mutants). Nearly all of these genes have putative human homologues. Interestingly, five *uge* gene products were components of Ccr4-Not transcriptional complexes. The function and role of Ccr4-Not complexes were examined in detail. To this end, the significance and implications of these findings were also discussed.

6.2 What Is The Value of A Cln Threshold?

As discussed in the introduction, Start that was synonymous with the "restriction point," was so named because it appeared to be the beginning point of the cell cycle. It appears that perhaps the essential part of these two points was not that they signal the genesis of a new cycle, but rather that they present a barrier to proliferation. In Chapter 3, I showed that there was a Cln threshold required for proliferation that was associated with Start. Why do cells need a Cln threshold? Perhaps this threshold is part of the

mechanism that presents a barrier to proliferation. This barrier exists in G1-phase at a point before cells have begun any of the biochemical or metabolic steps (e.g. DNA replication) that commit cells to divide. Because of this, it may represent a "safety zone" or a position in the cell cycle where cells are not easily affected by harsh conditions or damaging agents. Moreover, the vast majority of cells under physiological conditions are not proliferating. Instead, nearly all cells spend the largest part of their existence just prior to Start. Because of this, it is perhaps more accurate to think of this point as Stop.

6.3 How Is the Minimum Cln Threshold Attained?

It was well established that in yeast, cell size correlates with the ability of cells to proliferate, but the mechanism for these observations have not been well explored. Yeast have three G1-phase cyclins (Clns). The transcription of two of these genes, *CLN1* and *CLN2*, was strongly modulated by cell size. As cells approach critical cell size, the expression of *CLN1* and *CLN2 was* rapidly induced. While this may be part of the mechanism that links cell size to proliferation, it cannot be the only one. Cells lacking *CLN1* and *CLN2* were not only viable, but they proliferate in a size-dependent manner despite the fact that *CLN3* transcription was constitutive and unaffected by cell size. To examine what other mechanisms might link cell size to proliferative capacity, a strain was constructed so that *CLN1* could be expressed from a promoter that was insensitive to cell size (Figure 3.1). Using this system, I showed that expression of Cln1 protein increased exponentially as the cells got larger (Figure 3.7), and this increase of Cln protein abundance was not due to changes in its half-life (Figure 3.8). Therefore, it was likely that *CLN* mRNAs were preferentially translated in a size-dependent manner. Thus, both *CLN* mRNA and Cln protein expression were size-dependent.

6.4 The Case for Size Control

As I discussed in my introduction, despite a number of observations that correlate cell size with the probability that cells divide, there was not a clear mechanism that links cell size to proliferative capacity. Both Cln3 and Bck2 induce G1/S-phase specific gene

transcription in a size-dependent manner. Interestingly, this is a co-dependent effect. That is, in diploid cells Cln3 normally induces the transcription of a set of nearly 100 genes when cells reach a size of ~65fL (Spellman, Sherlock et al. 1998). Until recently, little was known about this mechanism. While Cln3 was dependent upon Swi6 to activate the transcription of these genes, the details of the mechanism remained obscure (Wijnen, Landman et al. 2002). Swi6 is a phospho-protein that can be phosphorylated by Cln3-Cdc28 (Sidorova, Mikesell et al. 1995; Wijnen, Landman et al. 2002). However, mutagenesis of all consensus Cdc28 phosphorylation sites in Swi6 had no effect on the timing of Start (Sidorova, Mikesell et al. 1995). Interestingly, one of the mutants isolated in my systematic genome-wide genetic screen, *whi5*, had been shown to be integral to this process (Table 4.1). Deletion mutants of *WHI5* were abnormally small due to the premature expression of *CLN1* and *CLN2* (Jorgensen, Nishikawa et al. 2002). Elegant studies have shown that Whi5 encoded an inhibitor of SBF and MBF that functions similarly to the pRB tumor suppressor gene (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004; Schaefer and Breeden 2004). Like pRB, Whi5 prevented the expression of G1-phase cyclin genes by binding to and sequestering the transcription factors required for their expression. In mammalian cells, these were E2F-DP1 transcription factors, and in yeast these were SBF and MBF (Cosma, Panizza et al. 2001; Iyer, Horak et al. 2001). As cells approach critical cell size, Cln3-Cdc28 complexes phosphorylated Whi5. The hyperphosphorylated Whi5 was released from SBF and MBF, resulting in the activation of these two transcription factors (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). Subsequently, transcription of genes including *CLN1* and *CLN2* rapidly increased. Despite the fact that the abundance of Cln3-Cdc28 complexes is relatively constant throughout the cell cycle, it appears that these complexes are only capable of phosphorylating Whi5 when cells approach critical cell size in G1-phase. The reason or mechanism responsible for this observation is not known.

Interestingly, the ability of Cln3 to activate *CLN1* and *CLN2* transcription at critical cell size is dependent upon Bck2 and vice versa. In cells lacking either Cln3 or Bck2, the induction of this transcriptional cascade was greatly delayed. Nonetheless, the

mechanism whereby Cln3 and Bck2 cooperate is not known. Indeed, nothing was known about the mechanism whereby Bck2 activates *CLN1* and *CLN2* transcription. My work on the function of Ccr4-Not complexes helped fill this gap. The implications and significance of this work is discussed below.

6.5 What Is the Advantage of Having the Cln Threshold Vary With Growth Rate?

In order to maintain cell size homeostasis, rapidly proliferating cells must also be growing at an equal pace. In yeast, rapidly growing cells are considerably larger than slowly growing cells (e.g. the average size of the most rapidly growing cells can be nearly twice the size of the slowest growing cells). There was no known mechanism responsible for this observation. As discussed above, I have shown that a minimum Cln threshold was required for cells to proliferate (Figure 3.2). Thus, it was possible that rapidly growing cells were large because Cln expression was low in rapidly growing cells. Interestingly, I found that just the opposite was true. Rapidly growing cells expressed six to ten fold more Cln protein than did slowly growing cells (Figure 3.3 and 3.4). However, this observation created somewhat of a paradox. Over-expression of Clns normally reduces cell size. So why were rapidly growing cells with large amounts of Cln protein larger than slower growing cells with less Cln? The answer to this question is not known, but the observation alone suggests several intriguing points about cell cycle control.

First, because the Cln threshold varies with growth rate, these data provided the first concrete evidence that cells possess a mechanism that actively inhibits progression past Start. It is not known what this mechanism is or how it works, but there are several possibilities. The simplest and most obvious explanation for these observations is that cells have phosphatases that counteract the activity of Cln-Cdc28 kinase. It is likely that these phosphatases are more active (or more abundant) in rapidly growing cells. Interestingly, no Cln-Cdc28 phosphatases are known. This is in part due to the small number of known Cln-Ccd28 substrates. Alternatively, the abundance or accessibility of a putative Cln substrate can be growth rate-dependent. Another possibility is that there

are more cell cycle inhibitors in fast growing cells than in slowly growing cells. In mammalian cells, there are two major types of Cdk inhibitors in G1-phase, INK4 (inhibitors of Cdk4) and Cip/Kip families. The INK4 family includes p15, p16, p18 and p19, specifically inhibiting Cdk4 and Cdk6. The Cip/Kip family had $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which have broader inhibitory effects on Cdks. $p27^{Kip1}$ for example, might be regulated by cell growth (Sherr and Roberts 1999), as there was a uORF of ~400nt in the 5' UTR of the $p27^{Kip1}$ transcript (Gopfert, Kullmann et al. 2003). As mentioned in Chapter 1, Cln3 translation was repressed by a uORF when the growth was low. When cell growth was high, a leaky scanning mechanism skipped this uORF and initiated translation of Cln3 (Polymenis and Schmidt 1997). Similarly, this uORF may exert negative effect on $p27^{Kip1}$ translation. Thus, expression of $p27^{Kip1}$ may be high when cell growth rate is high. This seems to be counter-intuitive. However, considering its additional role in facilitating G1-phase cyclin and Cdk binding (Sherr and Roberts 1999), one may postulate that in a certain period in G1-phase, $p27^{Kip1}$ and some other inhibitors may play a positive role enhancing Cdk activity. Surprisingly, no sequence homologues of any of these inhibitors have been identified in budding yeast. Are there any yeast orthologues for these molecules? Functional studies will be needed for this question.

Regardless of the mechanism that determines the Cln threshold, my research has clearly demonstrated that this threshold varies with growth rate, being considerably higher in rapidly growing cells. Since Cln abundance is also dramatically higher in rapidly growing cells, perhaps having a higher Cln threshold prevents cells from immediately committing to another round of cell division right after cytokinesis. Such a mechanism could protect cells from sudden environmental changes or give rapidly growing cells more developmental options (e.g. allowing cells to mate or enter meiosis, both of which can only be done in cells that have not progressed past Start).

6.6 Do Mammalian Cells Require A Cyclin Threshold?

There is no experimental evidence for answering this question. However, mammalian cells may adopt a similar strategy for S-phase entry. Similar to Sic1 and

Whi5, the pRB protein in mammalian cells had sixteen Cdk consensus sites (Harbour, Luo et al. 1999). Thus, it is very likely that pRB also needs a simultaneous phosphorylation of multiple sites which can be used to set a G1-phase cyclin threshold. Furthermore, a moderate inhibition of protein synthesis by cycloheximide or the removal of serum arrested cells before the "restriction point" (Pardee 1974; Medrano and Pardee 1980). In this case, the capacity of protein synthesis was compromised and the production of G1-phase cyclins decreased accordingly. Given that the stability of G1-phase cyclins remains the same, their abundance can never reach the threshold in such a circumstance. Moreover, ectopic expression of G1-phase cyclins would promote premature entry into S-phase in some mammalian cells (Terada, Yamada et al. 1997). This reduces cell size (Terada, Yamada et al. 1997). In addition, loss of pRB reduces cell size (Fingar, Salama et al. 2002). These data suggested that like in yeast there might be a G1-phase threshold required for entry into S-phase in mammalian cells (Medrano and Pardee 1980; Resnitzky, Hengst et al. 1995; Cooper 1998).

6.7 What Are the Advantages of Linking Proliferative Capacity to Cell Size?

Definitely, this mechanism was beneficial for cells to maintain both cell size homeostasis and genomic stability. First, as discussed in Chapter 1, budding yeast divide asymmetrically, giving rise to smaller daughter cells. This mechanism prevents grand-daughter cells from becoming progressively smaller (Figure 1.10). Second, the cells duplicate DNA in S phase and thus are very sensitive to external changes. Therefore, the cells must monitor the environment to ensure the availability of enough nutrients and mitogenic signals in the environment in G1 phase. How do the cells monitor the environment? The answer may be simple: protein synthesis rate, which was mirrored by accumulation of cell mass, or cell size. Moreover, as I showed in Figure 3.7, the expression of Cln protein was also modulated by cell size. Thus, it was very likely that only when the cells reach a certain size, can they produce enough Cln proteins for S phase entry. In general, this cell size-dependent mechanism prevents the crisis in S phase in case that the external environment was not optimal.

6.8 Is Proliferative Capacity Linked to Cell Size in Higher Eukaryotes?

Currently, there is no direct experimental evidence to answer this question. However, several observations suggest that the same mechanism may exist in certain types of cells. First, there was evidence indicating that these cells required a minimum cell size for S phase entry (Zetterberg and Killander 1965; Cooper 2004; Dolznig, Grebien et al. 2004). Second, moderate inhibition of protein synthesis with cyclohexamide arrested cells at G1 phase, suggesting that a critical level of labile proteins (i.e. G1 phase cyclins) must be reached (Campisi, Medrano et al. 1982). Third, cyclin D and E contain PEST sequences like Cln1-3, indicating their instability (Diehl, Cheng et al. 1998). There might be exceptions. As argued by Raff and coworkers, there was no such critical size requirement and hence no size-dependent mechanisms for S phase entry in Schwann cells (Conlon, Dunn et al. 2001; Conlon and Raff 2003).

6.9 What May Be the Mechanism Whereby Ccr4 Regulates *CLN1* and *CLN2* mRNA Expression?

In my genetic screen reported in Chapter 4, I found that the gene products of several *uge* mutants physically associated with Ccr4-Not complexes. Ccr4 may function as a global regulator that had both positive and negative effects on gene transcription (Liu, Badarinarayana et al. 1998). Previous studies have shown that several components of this complex were implicated in cell cycle regulation. Consistent with these studies, I have shown that Ccr4 was a positive regulator of *CLN1* and *CLN2 mRNA* expression.

There are several possible mechanisms: 1. the Ccr4 complex binds to *CLN1* and *CLN2* promoters directly to initiate their transcription. Even though Ccr4 itself does not have a DNA binding domain, the complex may be recruited to the promoter via association with other transcription factors, i.e. Swi4. 2. Because Ccr4 has a transactivation domain in its N-terminus, it is possible that the Ccr4 complex activates SBF and thus indirectly promotes *CLN1* and *CLN2* transcription. 3. Ccr4 may indirectly activate *CLN* transcription by binding to cell cycle inhibitors and preventing them from inhibiting SBF transcriptional activity.

Studies in mammalian cells have unraveled some of the physiological roles of Ccr4 complexes. It has been found that Ccr4 complexes interacted with the PC3/BTG2 tumor suppressor gene product *in vivo* (Prevot, Morel et al. 2001). Ectopic expression of PC3/BTG2 led to G1-phase arrest due to accumulation of hypophosphorylated pRb (Tirone 2001). This resulted from PC3-mediated down-regulation of cyclin D1 transcription and the subsequent inhibition of Cdk2/4 activity (Guardavaccaro, Corrente et al. 2000; Tirone 2001). These interactions were dependent upon Caf1 whose localization was regulated in a cell cycle regulated manner (Ikematsu, Yoshida et al. 1999). In both G0 and G1-phase cells, Caf1 was localized in the nucleus but it was exported to the cytoplasm during S phase (Ikematsu, Yoshida et al. 1999). Furthermore, Caf1 might be able to regulate the cell cycle by modulating Cdk activity through their physical interaction with Cdk4 and Cdc2 (Ikematsu, Yoshida et al. 1999).

6.10 Ccr4 May Coordinate Cell Growth and Cell Division

The mRNA expression of Ccr4 and other components in Ccr4-Not complexes was not cell cycle regulated (Denis and Chen 2003). So how do these complexes integrate into cell cycle regulation machinery? In budding yeast, glucose is a strong regulator of both cell growth and division. Glucose positively regulates the expression of G1-phase cyclins *CLN1-3* and also regulates *BCK2* (Newcomb, Diderich et al. 2003). If Ccr4 complexes positively regulate *CLN* mRNA expression, they may also be subject to glucose regulation. Indeed, this is the case. For example, Draper et al. showed that when fused to a LexA DNA binding domain, Ccr4 activated transcription in a glucose repressible manner (Draper, Liu et al. 1994). There is evidence that these proteins might also be subject to post-translational modifications in a nutrient dependent manner. First, the binding partners of Ccr4 for cells growing in glucose were different than for cells growing in ethanol (Draper, Liu et al. 1994). Second, upon glucose depletion, Not3 and Not5 became phosphorylated and degraded (Albert, Hanzawa et al. 2002; Collart 2003). Third, Caf1 (Pop2) could be phosphorylated at threonine 97 by Yak1 kinase upon glucose deprivation (Moriya, Shimizu-Yoshida et al. 2001). Mutation of the Yak1

phosphorylation site allowed cells to continue dividing after glucose deprivation (Moriya, Shimizu-Yoshida et al. 2001). This phenotype was similar to *whi2Δ* cells. Because Caf1 was a Ccr4 binding partner, this phosphorylation may somehow generate a negative signal on Ccr4 regulation of *CLN1* and *CLN2* mRNA (Figure 6.1).

Yak1 expression is under the control of the PKA pathway, which is activated in the presence of glucose. PKA kinase repressed Yak1 expression by phosphorylating and thus inhibiting the two transcription factors Msn2 and Msn4 (Figure 6.1) (Smith, Ward et al. 1998; Moriya, Shimizu-Yoshida et al. 2001). Upon glucose depletion or other stress conditions, these two transcription factors bound to stress responsive elements (STRE) in target gene promoters and induced their transcription (Thevelein and de Winde 1999). Deletion of *YAK1* suppressed the growth arrest due to PKA inactivation (Smith, Ward et al. 1998).

Not surprisingly, *CLN1-3* expression was also under the control of the PKA pathway. Deletion of genes encoding PKA caused a G1-phase cell cycle arrest (Thevelein and de Winde 1999). This arrest was consistent with a decreased Cln3 protein expression and thus decreased *CLN1* and *CLN2* expression (Hall, Markwardt et al. 1998). Ectopic expression of *CLN1-3* rescued this arrest phenotype (Hall, Markwardt et al. 1998). Upon depletion of glucose and other stress conditions, lowered levels of cAMP down-regulated *CLN* expression and activated the genes for stress responses by inducing the phosphorylation of the Msn2/4 transcription factors (Thevelein and de Winde 1999; Lenssen, Oberholzer et al. 2002).

Genetic studies have shown that Ccr4-Not complexes were also involved in repressing stress induced genes (Lenssen, Oberholzer et al. 2002). In the *ccr4Δ* and *not5Δ* strains, two genes, *CTT1* and *SSA3,* were expressed at much higher levels than the wild type upon glucose depletion (Lenssen, Oberholzer et al. 2002). This was caused by hypo-phosphorylation of Msn2, the transcription factor responsible for stress inducible transcription (Smith, Ward et al. 1998). As mentioned above, Msn2-dependent gene transcription was negatively regulated by the PKA pathway (Smith, Ward et al. 1998).

Therefore, I postulate that Ccr4-associated complexes promote cell cycle progression by both activating *CLN* expression and repressing stress response genes.

6.11 Involvement of Ccr4 Complexes in Cell Cycle Regulation

Recent data have revealed important roles for Ccr4-Not complexes in cell cycle regulation. It has been implicated in the expression of G1 phase cyclins, chromatin remodeling, and binding to tumor repressors.

6.11.1 Ccr4 Complex and *CLN* Expression

Ccr4 can also form complexes containing the RNA PolII holoenzyme. These complexes contain Paf1, Ctr9, Leo1, Rtf1, Cdc73 and other proteins, and are responsible for the expression of a subset of genes. Ctr9 and Paf1 were positive regulators of *CLN1* and *CLN2* mRNA expression (Koch, Wollmann et al. 1999). *CTR9* (cln three requiring) was identified as a gene required for survival in the absence of *CLN3* (Di Como, Chang et al. 1995). In addition, *CTR9* was identified as *CDP1* (cbf1-dependent), which was involved in chromosome segregation (Foreman and Davis 1996), and later as *RST1* (regulation of start) in different genetic screenings (Koch, Wollmann et al. 1999). *CTR9* encoded a nuclear 125 KDa protein whose nuclear localization was not cell cycle regulated (Mueller, Porter et al. 2004). Temperature sensitive mutants of $ctr9^{ts}$ accumulated as large unbudded cells at the restrictive temperature (Koch, Wollmann et al. 1999). In these mutants, *CLN2* mRNA expression was abolished, indicating that Ctr9 positively regulates *CLN2* expression.

Ctr9 physically interacts with Paf1. Similarly, temperature sensitive mutants of *PAF1* did not express *CLN2* at the restrictive temperature. This result was consistent with our finding that inactivation of *PAF1* resulted in large cells. Additionally, other cell cycle regulated genes such as *RNR1, FKS1* and *CLN1* transcription were also reduced in *paf1*Δ mutants.

So what might be the mechanisms for the regulation of *CLN1* and *CLN2* by the Ccr4 complexes? One possibility is that these complexes are responsible for the basal

transcription of a subset of genes including *CLN1* and *CLN2*. This regulation may be transcriptional, as the Ccr4 complexes contained the RNA polymerase holoenzyme *in vivo* (Figure 6.2). Furthermore, Paf1 had a positive role in transcription elongation (Shi, Finkelstein et al. 1996; Squazzo, Costa et al. 2002; Ng, Dole et al. 2003). In addition, the Ccr4 complexes were involved in the formation or stability of full-length poly(A) tails (Figure 6.2) (Krogan, Kim et al. 2002). Loss of Paf1, Ctr9, Cdc73, or Rtf1 results in a dramatic decrease in poly(A) tail length (Krogan, Kim et al. 2002). It has been suggested that these complexes may affect the phosphorylation of the carboxyl terminus (CTD) of PolII. Decreased phosphorylation the CTD was observed in *paf1*Δ cells (Palancade and Bensaude 2003). CTD phosphorylation was required for the recruitment of poly-adenylation factors such as Pcf1 to RNA PolII, so this recruitment was blocked in *paf1*Δ cells (Palancade and Bensaude 2003). Loss of poly(A) tails was the first step in mRNA degradation (Dehlin, Wormington et al. 2000). *CLN* mRNAs were very unstable, with a half life of several minutes (Tyers, Tokiwa et al. 1992; Schneider, Patton et al. 1998). Hence, this complex may also regulate *CLN1* and *CLN2* expression by affecting their mRNA stability (Figure 6.2).

6.11.2 Ccr4-Paf1 and Chromatin Remodeling

Another possible explanation for the *CLN* regulation is that the Ccr4 complexes are implicated in transcriptional activation by remodeling chromatin (Figure 6.3) (Ng, Dole et al. 2003; Ng, Robert et al. 2003; Simic, Lindstrom et al. 2003). In budding yeast, methylation of lysine 4 (K4) in histone H3 correlated with gene activation, and Set1 was the primary methyltransferase responsible for this modification (Krogan, Dover et al. 2002). Loss of the components in the Ccr4 complexes decreases histone H3 methylation (Krogan, Dover et al. 2002). Moreover, the recruitment of Set1 to actively transcribed chromatin was dependent on Paf1 (Ng, Dole et al. 2003; Ng, Robert et al. 2003). In addition, ubiquitination of the histone H2B C-terminal tail was the prerequisite for methylation of H3 (Figure 6.3) (Ng, Robert et al. 2003). Deletion of *RAD6,* an E2 ubiquitin ligase, or mutation of a H2B ubiquitination site prevented methylation of K4 in

H3 (Ng, Robert et al. 2003). Rtf1 was required for ubiquitination of H2B, strongly suggesting that Rtf1 affects the methylation of K4 and K79 in H3 through its effect on H2B ubiquitination.

Even though the significance of this methylation on cell cycle regulation is still vague, research in mammalian cells indicates that methylation of histone is important for G1-phase regulation. For example, histone methylation of lysine 9 in the DHFR (dihydrofolate reductase) promoter was high in serum-starved 3T3 cells but low in cells re-entering the cell cycle (Nicolas, Roumillac et al. 2003). Interestingly, adenovirus promoted S-phase entry by repressing the methylation of lysine 9 in H3 in the promoters of E2F target genes (Narita, Nunez et al. 2003).

Ogawa et al. showed that a novel histone methyltransferase for histone lysine 9 in H3 methylation bound to E2F6 in HeLa cells (Ogawa, Ishiguro et al. 2002). E2F6 was a potent transcription repressor involved in neoplasia and in regulating chromatin, because over-expressed E2F6 could inhibit S-phase entry and delay the exit from S-phase (Ogawa, Ishiguro et al. 2002). One E2F6 target gene was BRCA1, involved in multiple mechanisms of DNA recombination, repair and checkpoint function (Oberley, Inman et al. 2003). Although there was no yeast homolog of BRCA1, Westermoreland et al. found that when over-expressed in budding yeast, BRCA1 caused G1-phase arrest of the cell cycle. This cell cycle arrest could be rescued by deletion of *dhh1*, which also physically and genetically interacted with Ccr4 (Westmoreland, Olson et al. 2003). Dhh1 was a cytoplasmic protein involved in mRNA turnover by increasing the efficiency of 5' decapping enzyme Dcp1 (Hata, Mitsui et al. 1998; Maillet and Collart 2002). The *dhh1*Δ deletion strain failed to arrest at G1-phase upon nitrogen starvation (Maillet and Collart 2002; Westmoreland, Olson et al. 2003). *dhh1*Δ deletion strains have a smaller cell size and decreased viability in comparison to the wild type (Maillet and Collart 2002; Westmoreland, Olson et al. 2003). This phenotype was very similar to *WHI1-1* and *whi2*Δ, in which *CLN* expression was elevated (Nash, Tokiwa et al. 1988; Radcliffe, Trevethick et al. 1997).

In addition to G1/S-phase control, the Ccr4 complexes may also be involved in other cell cycle stages, such as S/G2-phase DNA damage checkpoints, cytokinesis in M phase, and cell cycle regulated cell wall synthesis (Westmoreland, Olson et al. 2003; Westmoreland, Marks et al. 2004). With further investigations, I would better understand how Ccr4 associated complexes integrate external signals and perform diversified functions in the cell.

6.12 A Working Model on How G1-phase Cyclins Are Regulated in Budding Yeast

Based on my data and current knowledge, I hereby propose a genetic model summarizing how G1-phase cyclins are regulated by external signals, i.e. glucose, in budding yeast. In this model, glucose signal is relayed into the cell via several parallel but interacting pathways (Figure 6.4). First, glucose can activate TOR pathway directly with an unknown mechanism, and indirectly via PI3K pathway. Secondly, glucose activates Ras signal pathway, branching into PI3K, PKA and PKC pathways. Therefore, expression of G1 phase cylins increases, probably independent of SBF and MBF. Third, PKC pathway can activate SBF transcription factor to promote expression of *CLN1* and *CLN2*. Likewise, glucose can somehow activate Bck2 pathway to increase *CLN1* and *CLN2* expression via Ccr4 and SBF. Consequently, Cln1 and Cln2 expression exceeds the threshold requirement and the cells are then able to progress past Start (Figure 6.4).

6.13 Biological and Implications Significance

Cell division must be coordinated with cell growth in most cases. Several known signaling pathways have been implicated in this coordination. Deregulation of these pathways is responsible for both cell size homeostasis and diseases such as cardiac hypertrophy, aging and tumorigenesis (Hannan and Rothblum 1995; Sherr 1996; Clerk, Pham et al. 2001; Nasi, Ciarapica et al. 2001; Cohen, Park et al. 2003; Bjornsti and Houghton 2004). For example, hyperactivation of PI3K pathway (by over-expression of Akt kinase or loss of PTEN) could not only lead to larger cell size, but also cause cancer (Huang, Potter et al. 1999; Potter, Pedraza et al. 2003). Moreover, this pathway was

implicated in aging in *Drosophila* and *C. elegans* (Lee, Kennedy et al. 2003). Thus, studies investigating the mechanisms of this coordination may lead to significant insights into understanding and fighting these diseases.

In my research, I showed that a Cln threshold was required for proliferation. This observation may also be true in mammalian cells. Cancer is essentially a disease of uncontrolled division. In many cancer cells, protein synthesis rates were very high (Parsons 2004). Thus, high protein synthesis rates may lead to an excess of G1-phase cyclins and other necessary molecules for the commitment to cell division. Indeed, G1-phase cyclin levels were elevated in many tumors (Malumbres, Hunt et al. 2003). Additionally, others and I have shown that *CLN1* and *CLN2* mRNA can also be positively regulated by Ccr4 complexes. Currently, whether Ccr4 complexes have similar function in mammalian cells is not known. However, their binding to the tumor suppressor PC3, which represses expression of cyclin D, suggests their regulation of G1 phase cyclins may still be conserved. As discussed above, their natural involvement in the cell division and other essential processes in mammalian cells imply that the Ccr4 complexes may be involved in tumorigenesis. Further exploration of the Ccr4 complexes is necessary to confirm this postulation.

6.14 Unanswered Questions and Future Directions

I have established the genetic hierarchy as Bck2-Ccr4-Swi4. However, a number of unanswered questions remain. How does Ccr4 regulate *CLN1* and *CLN2* expression? How does Bck2 interact with Ccr4? Do Ccr4 and Swi4 physically interact? What are the genetic interactions between *CCR4* and *SWI6* or *MBP1*? How does Ccr4 regulate *CLN1* and *CLN2* mRNA expression, given that its mRNA itself is not cell cycle regulated? These questions cannot be answered without further study. Finally, the role of cell size in modulating proliferation needs to be examined more thoroughly. My research provides a good foundation for examining these questions mechanistically and for translating this research into mammalian cells.

6.15 Conclusions

I have analyzed the relationship between cell growth, cell size, Cln expression, and proliferation. I found that rapidly growing cells expressed, and required more Cln protein than do slowly growing cells. I found that a critical threshold of Cln protein was required for proliferation, and that Cln1 protein expression was strongly modulated by cell size. In addition, expression of high levels of *CLNs* promoted proliferation in a size-independent manner, suggesting that Clns were rate-limiting for Start. Furthermore, I conducted a systematic genome-wide genetic screen to identify mutants that dramatically altered the proliferative capacity of cells. In so doing, I identified 49 gene deletions that dramatically changed cell size. To elucidate one of the mechanisms that yeast cells use to coordinate cell growth with division, I investigated the function of the Ccr4 complexes. I found that *CCR4* positively regulated *CLN1* mRNA expression. In the *ccr4*Δ strains, *CLN1* mRNA expression was decreased in asynchronous cultures and delayed in synchronized cultures, but restoration of *CCR4* expression induced *CLN1* mRNA expression and rescued the size phenotype of the *ccr4*Δ. My results suggested that *CCR4* modulated the ability of the Bck2 protein to induce *CLN1* and *CLN2* transcription. In summary, my research has identified new gene products involved in cell cycle control and has helped elucidate the mechanism whereby cells coordinate cell growth with proliferation.

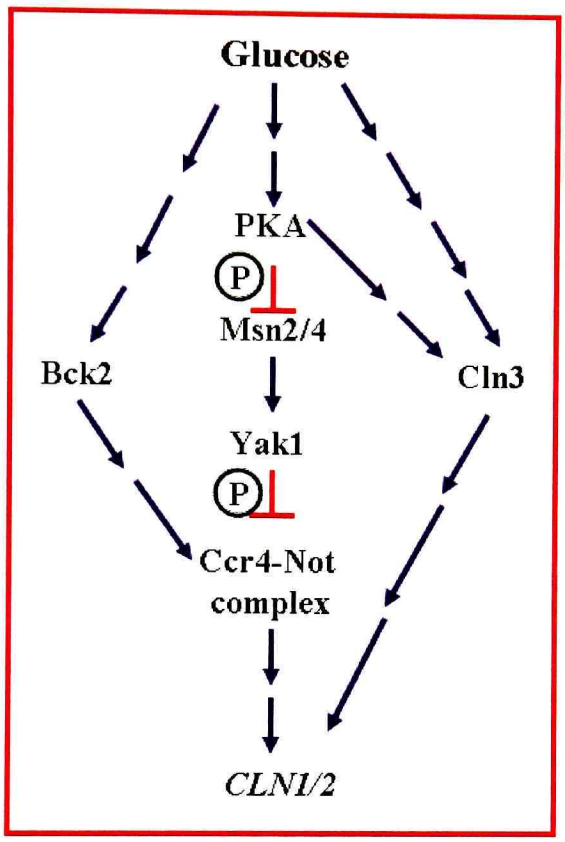


Figure 6.1. Genetic interactions between the PKA pathway and the Ccr4 complexes. The Ccr4 complexes are the integral part in regulating *CLN1* and *CLN2* mRNA expression via the PKA pathway. Except for the Bck2 and Cln3 pathways that positively regulate *CLN1* and *CLN2* mRNA expression, the Ccr4-Not complex may relay the signal from PKA. It is known that glucose stimulates PKA activity, which may repress two transcription factors Msn2/4 by inhibitory phosphorylation. It has been found that Msn2/4 is responsible for Yak1 activation. Yak1 can directly phosphorylate Caf1, a component of this complex, upon nutrient deprivation. Deletion of YAK1 causes a phenotype similar to overexpression of *CLN1* and *CLN2*. Therefore, Yak1 phosphorylation on Caf1 may repress *CLN1* and *CLN2* mRNA expression. Ⓟ denotes phosphorylation.

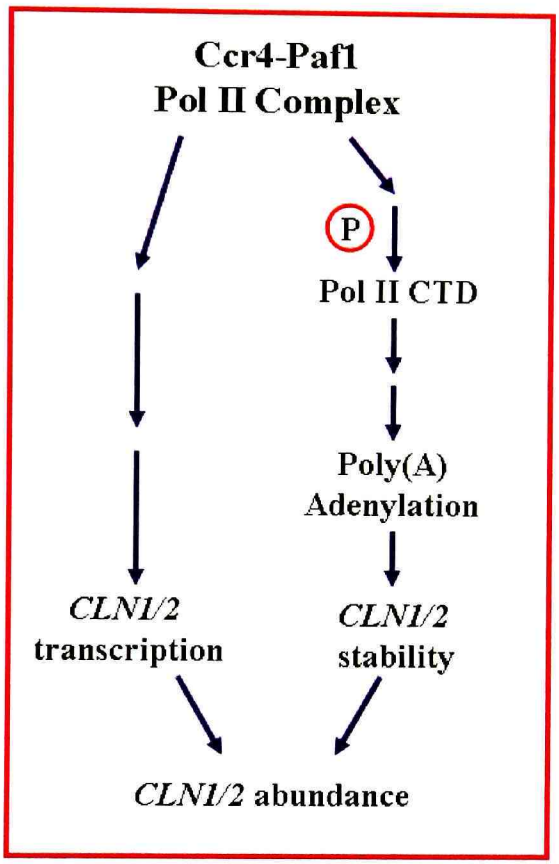


Figure 6.2. The Ccr4-Paf1 complex increases *CLN1* and *CLN2* mRNA abundance. This complex has two roles in increasing *CLN1* and *CLN2* mRNA abundance. First, this complex is a RNA PolII complex, responsible for transcription of *CLN1* and *CLN2* directly. Meanwhile, this complex may increase PolII CTD phosphorylation, which leads to poly(A) adenylation and therefore *CLN1* and *CLN2* mRNA stability. Ⓟ denotes phosphorylation.

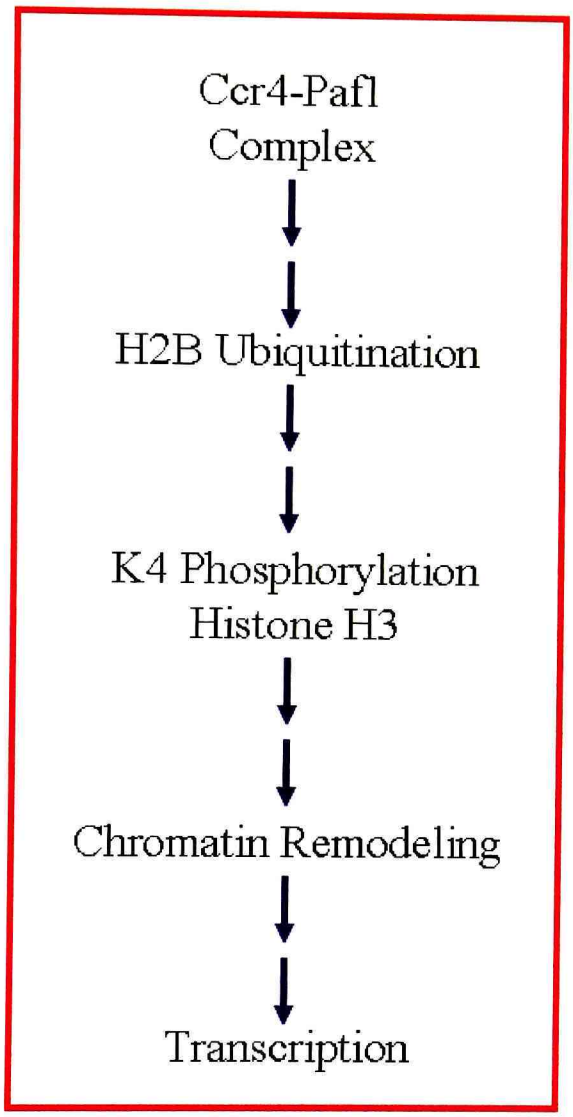


Figure 6.3. The Ccr4-Paf1 complex regulates chromatin remodeling. It is found that this complex is involved in histone H2B ubiquitination, which is required for the lysine 4 (K4) phosphorylation of the histone H3. This K4 phosphorylation is responsible for chromatin remodeling and subsequent transcription.

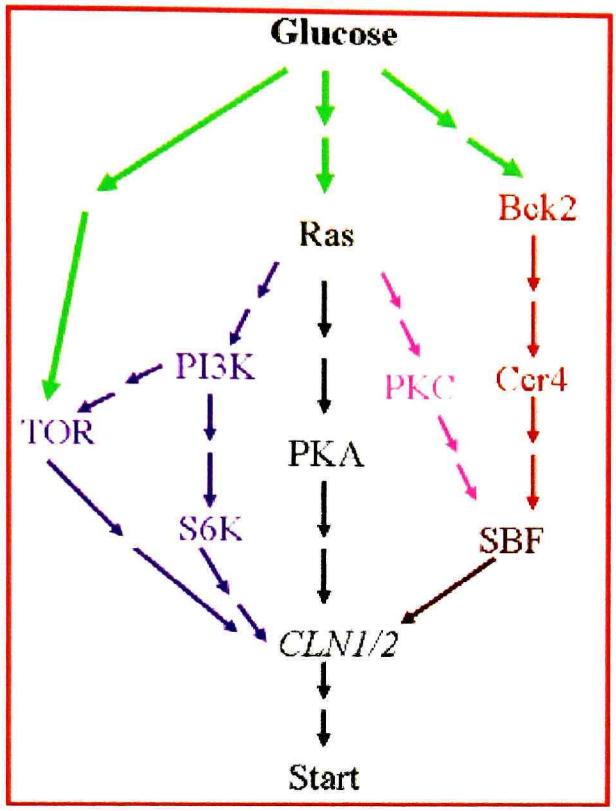


Figure 6.4. A Working model integrating glucose signal into regulation of *CLN1* and *CLN2* expression in budding yeast. Glucose stimulates activation of several independent, but interacting pathways: TOR, Ras and Bck2. The Ras signaling further splits into at least three pathways, PI3K, PKA and PKC. In general, the activation of these pathways leads to increase of Cln1-3. Once a threshold level has been reached, the cells are able to execute Start.

Reference:

Albert, T. K., H. Hanzawa, et al. (2002). "Identification of a ubiquitin-protein ligase subunit within the CCR4-NOT transcription repressor complex." Embo J **21**(3): 355-64.

Albert, T. K., M. Lemaire, et al. (2000). "Isolation and characterization of human orthologs of yeast CCR4-NOT complex subunits." Nucleic Acids Res **28**(3): 809-17.

Albig, A. R. and C. J. Decker (2001). "The target of rapamycin signaling pathway regulates mRNA turnover in the yeast Saccharomyces cerevisiae." Mol Biol Cell **12**(11): 3428-38.

Alvarez, B., E. Garrido, et al. (2003). "Phosphoinositide 3-kinase activation regulates cell division time by coordinated control of cell mass and cell cycle progression rate." J Biol Chem **278**(29): 26466-73.

Amon, A., M. Tyers, et al. (1993). "Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins." Cell **74**(6): 993-1007.

Andrews, B. J. and I. Herskowitz (1989). "The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription." Nature **342**(6251): 830-3.

Andrews, B. J. and L. A. Moore (1992). "Interaction of the yeast Swi4 and Swi6 cell cycle regulatory proteins in vitro." Proc Natl Acad Sci U S A **89**(24): 11852-6.

Baetz, K. and B. Andrews (1999). "Regulation of cell cycle transcription factor Swi4 through auto- inhibition of DNA binding." Mol Cell Biol **19**(10): 6729-41.

Baetz, K., J. Moffat, et al. (2001). "Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4." Mol Cell Biol **21**(19): 6515-28.

Bai, Y., C. Salvadore, et al. (1999). "The CCR4 and CAF1 proteins of the CCR4-NOT complex are physically and functionally separated from NOT2, NOT4, and NOT5." Mol Cell Biol **19**(10): 6642-51.

Balmain, A. (2001). "Cancer genetics: from Boveri and Mendel to microarrays." Nat Rev Cancer **1**(1): 77-82.

Balmanno, K. and S. J. Cook (1999). "Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells." Oncogene **18**(20): 3085-97.

Barbet, N. C., U. Schneider, et al. (1996). "TOR controls translation initiation and early G1 progression in yeast." Mol Biol Cell **7**(1): 25-42.

Bartlett, R. and P. Nurse (1990). "Yeast as a model system for understanding the control of DNA replication in Eukaryotes." Bioessays **12**(10): 457-63.

Basu, A. (2003). "Involvement of protein kinase C-delta in DNA damage-induced apoptosis." J Cell Mol Med **7**(4): 341-50.

Berry, L. D. and K. L. Gould (1996). "Regulation of Cdc2 activity by phosphorylation at T14/Y15." Prog Cell Cycle Res **2**: 99-105.

Berset, C., P Griac, et al. (2002). "Transferable domain in the G(1) cyclin Cln2 sufficient to switch degradation of Sic1 from the E3 ubiquitin ligase SCF(Cdc4) to SCF(Grr1)." Mol Cell Biol **22**(13): 4463-76.

Biggar, S. R. and G. R. Crabtree (2001). "Cell signaling can direct either binary or graded transcriptional responses." Embo J **20**(12): 3167-76.

Bjornsti, M. A. and P. J. Houghton (2004). "The TOR pathway: a target for cancer therapy." Nat Rev Cancer **4**(5): 335-48.

Blagosklonny, M. V. and A. B. Pardee (2002). "The restriction point of the cell cycle." Cell Cycle **1**(2): 103-10.

Bohni, R., J. Riesgo-Escovar, et al. (1999). "Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4." Cell **97**(7): 865-75.

Booher, R. N., R. J. Deshaies, et al. (1993). "Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins." Embo J **12**(9): 3417-26.

Breeden, L. and K. Nasmyth (1985). "Regulation of the yeast HO gene." Cold Spring Harb Symp Quant Biol **50**: 643-50.

Breeden, L. and K. Nasmyth (1987). "Cell cycle control of the yeast HO gene: cis- and trans-acting regulators." Cell **48**(3): 389-97.

Breviario, D., A. Hinnebusch, et al. (1986). "Carbon source regulation of RAS1 expression in Saccharomyces cerevisiae and the phenotypes of ras2- cells." Proc Natl Acad Sci U S A **83**(12): 4152-6.

Broach, J. R. and R. J. Deschenes (1990). "The function of ras genes in Saccharomyces cerevisiae." Adv Cancer Res **54**: 79-139.

Brown, E. J., P. A. Beal, et al. (1995). "Control of p70 s6 kinase by kinase activity of FRAP in vivo." Nature **377**(6548): 441-6.

Brunn, G. J., C. C. Hudson, et al. (1997). "Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin." Science **277**(5322): 99-101.

Campisi, J., E. E. Medrano, et al. (1982). "Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells." Proc Natl Acad Sci U S A **79**(2): 436-40.

Casperson, G. F., N. Walker, et al. (1985). "Isolation of the gene encoding adenylate cyclase in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **82**(15): 5060-3.

Chang, M., D. French-Cornay, et al. (1999). "A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling." Mol Cell Biol **19**(2): 1056-67.

Charbonneau, M. and N. Grandin (1989). "The egg of Xenopus laevis: a model system for studying cell activation." Cell Differ Dev **28**(2): 71-93.

Chen, J., Y. C. Chiang, et al. (2002). "CCR4, a 3'-5' poly(A) RNA and ssDNA exonuclease, is the catalytic component of the cytoplasmic deadenylase." Embo J **21**(6): 1414-26.

Chen, J., J. Rappsilber, et al. (2001). "Purification and characterization of the 1.0 MDa CCR4-NOT complex identifies two novel components of the complex." J Mol Biol **314**(4): 683-94.

Cheng, M., V Sexl, et al. (1998). "Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1)." Proc Natl Acad Sci U S A **95**(3): 1091-6.

Chi, Y., M. J. Huddleston, et al. (2001). "Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase." Genes Dev **15**(9): 1078-92.

Cismowski, M. J., G. M. Laff, et al. (1995). "KIN28 encodes a C-terminal domain kinase that controls mRNA transcription in Saccharomyces cerevisiae but lacks cyclin-dependent kinase-activating kinase (CAK) activity." Mol Cell Biol **15**(6): 2983-92.

Clerk, A., F. H. Pham, et al. (2001). "Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1." Mol Cell Biol **21**(4): 1173-84.

Coelho, C. M. and S. J. Leevers (2000). "Do growth and cell division rates determine cell size in multicellular organisms?" J Cell Sci **113 (Pt 17)**: 2927-34.

Cohen, A. W., D. S. Park, et al. (2003). "Caveolin-1 null mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts." Am J Physiol Cell Physiol **284**(2): C457-74.

Coleman, M. L. and C. J. Marshall (2001). "A family outing: small GTPases cyclin' through G1." Nat Cell Biol **3**(11): E250-1.

Coleman, M. L., C. J. Marshall, et al. (2004). "RAS and RHO GTPases in G1-phase cell-cycle regulation." Nat Rev Mol Cell Biol **5**(5): 355-66.

Collart, M. A. (2003). "Global control of gene expression in yeast by the Ccr4-Not complex." Gene **313**: 1-16.

Conlon, I. and M. Raff (1999). "Size control in animal development." Cell **96**(2): 235-44.

Conlon, I. and M. Raff (2003). "Differences in the way a mammalian cell and yeast cells coordinate cell growth and cell-cycle progression." J Biol **2**(1): 7.

Conlon, I. J., G. A. Dunn, et al. (2001). "Extracellular control of cell size." Nat Cell Biol **3**(10): 918-21.

Cooper, S. (1998). "On the interpretation of the shortening of the G1-phase by overexpression of cyclins in mammalian cells." Exp Cell Res **238**(1): 110-5.

Cooper, S. (2004). "Control and maintenance of mammalian cell size." BMC Cell Biol **5**(1): 35.

Cosma, M. P., S. Panizza, et al. (2001). "Cdk1 triggers association of RNA polymerase to cell cycle promoters only after recruitment of the mediator by SBF." Mol Cell **7**(6): 1213-20.

Costanzo, M., J. L. Nishikawa, et al. (2004). "CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast." Cell **117**(7): 899-913.

Crespo, P. and J. Leon (2000). "Ras proteins in the control of the cell cycle and cell differentiation." Cell Mol Life Sci **57**(11): 1613-36.

Cross, D. A., D. R. Alessi, et al. (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." Nature **378**(6559): 785-9.

Cross, F. R. (1988). "DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of Saccharomyces cerevisiae." Mol Cell Biol **8**(11): 4675-84.

Cross, F. R. (1989). "Further characterization of a size control gene in Saccharomyces cerevisiae." J Cell Sci Suppl **12**: 117-27.

Cross, F. R. (1990). "Cell cycle arrest caused by CLN gene deficiency in Saccharomyces cerevisiae resembles START-I arrest and is independent of the mating-pheromone signalling pathway." Mol Cell Biol **10**(12): 6482-90.

Cross, F. R. and C. M. Blake (1993). "The yeast Cln3 protein is an unstable activator of Cdc28." Mol Cell Biol **13**(6): 3266-71.

Cross, F. R. and K. Levine (1998). "Molecular evolution allows bypass of the requirement for activation loop phosphorylation of the Cdc28 cyclin-dependent kinase." Mol Cell Biol **18**(5): 2923-31.

Danaie, P., M. Altmann, et al. (1999). "CLN3 expression is sufficient to restore G1-to-S-phase progression in Saccharomyces cerevisiae mutants defective in translation initiation factor eIF4E." Biochem J **340**(Pt 1): 135-41.

Dawid, I. B. and T. D. Sargent (1988). "Xenopus laevis in developmental and molecular biology." Science **240**(4858): 1443-8.

De Bondt, H. L., J. Rosenblatt, et al. (1993). "Crystal structure of cyclin-dependent kinase 2." Nature **363**(6430): 595-602.

de Bruin, R. A., W H. McDonald, et al. (2004). "Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5." Cell **117**(7): 887-98.

de Robertis, E. M., J. B. Gurdon, et al. (1977). "Injected amphibian oocytes: a living test tube for the study of eukaryotic gene transcription?" Biochem Soc Symp(42): 181-91.

Dehlin, E., M. Wormington, et al. (2000). "Cap-dependent deadenylation of mRNA." Embo J **19**(5): 1079-86.

Dempsey, E. C., A. C. Newton, et al. (2000). "Protein kinase C isozymes and the regulation of diverse cell responses." Am J Physiol Lung Cell Mol Physiol **279**(3): L429-38.

Denis, C. L. (1984). "Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II." Genetics **108**(4): 833-44.

Denis, C. L. and J. Chen (2003). "The CCR4-NOT complex plays diverse roles in mRNA metabolism." Prog Nucleic Acid Res Mol Biol **73**: 221-50.

Denis, C. L. and T. Malvar (1990). "The CCR4 gene from Saccharomyces cerevisiae is required for both nonfermentative and spt-mediated gene expression." Genetics **124**(2): 283-91.

Dennis, P. B., S. Fumagalli, et al. (1999). "Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation." Curr Opin Genet Dev **9**(1): 49-54.

Di Como, C. J., H. Chang, et al. (1995). "Activation of CLN1 and CLN2 G1 cyclin gene expression by BCK2." Mol Cell Biol **15**(4): 1835-46.

Diehl, J. A., M. Cheng, et al. (1998). "Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization." Genes Dev **12**(22): 3499-511.

Diehl, J. A., F. Zindy, et al. (1997). "Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway." Genes Dev **11**(8): 957-72.

Dirick, L., T. Bohm, et al. (1995). "Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae." Embo J **14**(19): 4803-13.

Dirick, L., T. Moll, et al. (1992). "A central role for SWI6 in modulating cell cycle Start-specific transcription in yeast." Nature **357**(6378): 508-13.

Dirick, L. and K. Nasmyth (1991). "Positive feedback in the activation of G1 cyclins in yeast." Nature **351**(6329): 754-7.

Dobrowolski, S., M. Harter, et al. (1994). "Cellular ras activity is required for passage through multiple points of the G0/G1 phase in BALB/c 3T3 cells." Mol Cell Biol **14**(8): 5441-9.

Dohrmann, P. R., W. P. Voth, et al. (1996). "Role of negative regulation in promoter specificity of the homologous transcriptional activators Ace2p and Swi5p." Mol Cell Biol **16**(4): 1746-58.

Dollard, C., S. L. Ricupero-Hovasse, et al. (1994). "SPT10 and SPT21 are required for transcription of particular histone genes in Saccharomyces cerevisiae." Mol Cell Biol **14**(8): 5223-8.

Dolznig, H., F. Grebien, et al. (2004). "Evidence for a size-sensing mechanism in animal cells." Nat Cell Biol **6**(9): 899-905.

Draper, M. P., H. Y. Liu, et al. (1994). "CCR4 is a glucose-regulated transcription factor whose leucine-rich repeat binds several proteins important for placing CCR4 in its proper promoter context." Mol Cell Biol **14**(7): 4522-31.

Dujon, B. (1996). "The yeast genome project: what did we learn?" Trends Genet **12**(7): 263-70.

Dunphy, W. G., L. Brizuela, et al. (1988). "The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis." Cell **54**(3): 423-31.

Edgington, N. P. and B. Futcher (2001). "Relationship between the function and the location of G1 cyclins in S. cerevisiae." J Cell Sci **114**(Pt 24): 4599-611.

Epstein, C. B. and F. R. Cross (1994). "Genes that can bypass the CLN requirement for Saccharomyces cerevisiae cell cycle START." Mol Cell Biol **14**(3): 2041-7.

Evans, T., E. T. Rosenthal, et al. (1983). "Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division." Cell **33**(2): 389-96.

Fantes, P. and P. Nurse (1977). "Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division." Exp Cell Res **107**(2): 377-86.

Fantes, P. A., W. D. Grant, et al. (1975). "The regulation of cell size and the control of mitosis." J Theor Biol **50**(1): 213-44.

Fantes, P. A., W. D. Grant, et al. (1975). "The regulation of cell size and the control of mitosis." J Theor Biol **50**(1): 213-44.

Fantes, P. A. and P. Nurse (1978). "Control of the timing of cell division in fission yeast. Cell size mutants reveal a second control pathway." Exp Cell Res **115**(2): 317-29.

Feldman, R. M., C. C. Correll, et al. (1997). "A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p." Cell **91**(2): 221-30.

Fingar, D. C. and J. Blenis (2004). "Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression." Oncogene **23**(18): 3151-71.

Fingar, D. C., C. J. Richardson, et al. (2004). "mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E." Mol Cell Biol **24**(1): 200-16.

Fingar, D. C., S. Salama, et al. (2002). "Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E." Genes Dev **16**(12): 1472-87.

Fingerman, I., V. Nagaraj, et al. (2003). "Sfp1 plays a key role in yeast ribosome biogenesis." Eukaryot Cell **2**(5): 1061-8.

Fleig, U. N. and K. L. Gould (1991). "Regulation of cdc2 activity in Schizosaccharomyces pombe: the role of phosphorylation." Semin Cell Biol **2**(4): 195-204.

Flick, K., D. Chapman-Shimshoni, et al. (1998). "Regulation of cell size by glucose is exerted via repression of the CLN1 promoter." Mol Cell Biol **18**(5): 2492-501.

Foreman, P K. and R. W. Davis (1996). "CDP1, a novel Saccharomyces cerevisiae gene required for proper nuclear division and chromosome segregation." Genetics **144**(4): 1387-97.

Foster, F M., C. J. Traer, et al. (2003). "The phosphoinositide (PI) 3-kinase family." J Cell Sci **116**(Pt 15): 3037-40.

Frenz, L. M., A. L. Johnson, et al. (2001). "Rme1, which controls CLN2 expression in Saccharomyces cerevisiae, is a nuclear protein that is cell cycle regulated." Mol Genet Genomics **266**(3): 374-84.

Galitski, T., A. J. Saldanha, et al. (1999). "Ploidy regulation of gene expression." Science **285**(5425): 251-4.

Gallego, C., E. Gari, et al. (1997). "The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast." Embo J **16**(23): 7196-206.

Gao, X., Y. Zhang, et al. (2002). "Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling." Nat Cell Biol **4**(9): 699-704.

Gari, E., T. Volpe, et al. (2001). "Whi3 binds the mRNA of the G1 cyclin CLN3 to modulate cell fate in budding yeast." Genes Dev **15**(21): 2803-8.

Garrett, M. D. and A. Fattaey (1999). "CDK inhibition and cancer therapy." Curr Opin Genet Dev **9**(1): 104-11.

Gautier, J., C. Norbury, et al. (1988). "Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+." Cell **54**(3): 433-9.

Gautier, J., M. J. Solomon, et al. (1991). "cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2." Cell **67**(1): 197-211.

Gera, J. F., I. K. Mellinghoff, et al. (2004). "AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression." J Biol Chem **279**(4): 2737-46.

Gerhart, J., M. Wu, et al. (1984). "Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs." J Cell Biol **98**(4): 1247-55.

Gingras, A. C., B. Raught, et al. (2001). "Hierarchical phosphorylation of the translation inhibitor 4E-BP1." Genes Dev **15**(21): 2852-64.

Gingras, A. C., B. Raught, et al. (2001). "Regulation of translation initiation by FRAP/mTOR." Genes Dev **15**(7): 807-26.

Goffeau, A., B. G. Barrell, et al. (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-7.

Gopfert, U., M. Kullmann, et al. (2003). "Cell cycle-dependent translation of p27 involves a responsive element in its 5'-UTR that overlaps with a uORF." Hum Mol Genet **12**(14): 1767-79.

Guardavaccaro, D., G. Corrente, et al. (2000). "Arrest of G(1)-S progression by the p53-inducible gene PC3 is Rb dependent and relies on the inhibition of cyclin D1 transcription." Mol Cell Biol **20**(5): 1797-815.

Gustin, M. C., J. Albertyn, et al. (1998). "MAP kinase pathways in the yeast Saccharomyces cerevisiae." Microbiol Mol Biol Rev **62**(4): 1264-300.

Hadwiger, J. A., C. Wittenberg, et al. (1989). "A family of cyclin homologs that control the G1 phase in yeast." Proc Natl Acad Sci U S A **86**(16): 6255-9.

Halfar, K., C. Rommel, et al. (2001). "Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity." Development **128**(9): 1687-96.

Halfar, K., C. Rommel, et al. (2001). "Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity." Development **128**(9): 1687-96.

Hall, D. D., D. D. Markwardt, et al. (1998). "Regulation of the Cln3-Cdc28 kinase by cAMP in Saccharomyces cerevisiae." Embo J **17**(15): 4370-8.

Hanic-Joyce, P. J., G. C. Johnston, et al. (1987). "Regulated arrest of cell proliferation mediated by yeast prt1 mutations." Exp Cell Res **172**(1): 134-45.

Hannan, R. D. and L. I. Rothblum (1995). "Regulation of ribosomal DNA transcription during neonatal cardiomyocyte hypertrophy." Cardiovasc Res **30**(4): 501-10.

Harashima, T. and J. Heitman (2002). "The Galpha protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gbeta subunits." Mol Cell **10**(1): 163-73.

Harbour, J. W., R. X. Luo, et al. (1999). "Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1." Cell **98**(6): 859-69.

Hartwell, L. H. (1974). "Saccharomyces cerevisiae cell cycle." Bacteriol Rev **38**(2): 164-98.

Hartwell, L. H. (1978). "Cell division from a genetic perspective." J Cell Biol **77**(3): 627-37.

Hartwell, L. H., J. Culotti, et al. (1974). "Genetic control of the cell division cycle in yeast." Science **183**(120): 46-51.

Hartwell, L. H., J. Culotti, et al. (1970). "Genetic control of the cell-division cycle in yeast. I. Detection of mutants." Proc Natl Acad Sci U S A **66**(2): 352-9.

Hartwell, L. H. and M. B. Kastan (1994). "Cell cycle control and cancer." Science **266**(5192): 1821-8.

Hartwell, L. H. and M. W Unger (1977). "Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division." J Cell Biol **75**(2 Pt 1): 422-35.

Hata, H., H. Mitsui, et al. (1998). "Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from Saccharomyces cerevisiae." Genetics **148**(2): 571-9.

Heinisch, J. J., A. Lorberg, et al. (1999). "The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in Saccharomyces cerevisiae." Mol Microbiol **32**(4): 671-80.

Heitman, J., N. R. Movva, et al. (1991). "Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast." Science **253**(5022): 905-9.

Helliwell, S. B., P. Wagner, et al. (1994). "TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast." Mol Biol Cell **5**(1): 105-18.

Hochstrasser, M. (1996). "Ubiquitin-dependent protein degradation." Annu Rev Genet **30**: 405-39.

Horiuchi, J., N. Silverman, et al. (1997). "ADA1, a novel component of the ADA/GCN5 complex, has broader effects than GCN5, ADA2, or ADA3." Mol Cell Biol **17**(6): 3220-8.

Hovland, P., J. Flick, et al. (1989). "Galactose as a gratuitous inducer of GAL gene expression in yeasts growing on glucose." Gene **83**(1): 57-64.

Howard, A. a. P., S. (1953). "Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage." Heredity **6** (Suppl.): 261-273.

Huang, H., C. J. Potter, et al. (1999). "PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development." Development **126**(23): 5365-72.

Ikematsu, N., Y. Yoshida, et al. (1999). "Tob2, a novel anti-proliferative Tob/BTG1 family member, associates with a component of the CCR4 transcriptional regulatory complex capable of binding cyclin-dependent kinases." Oncogene **18**(52): 7432-41.

Inoki, K., Y. Li, et al. (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol **4**(9): 648-57.

Iyer, V. R., C. E. Horak, et al. (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF " Nature **409**(6819): 533-8.

Jacobson, A. (2004). "Regulation of mRNA decay: decapping goes solo." Mol Cell **15**(1): 1-2.

Jefferies, H. B., S. Fumagalli, et al. (1997). "Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k." Embo J **16**(12): 3693-704.

Jiang, J. C., E. Jaruga, et al. (2000). "An intervention resembling caloric restriction prolongs life span and retards aging in yeast." Faseb J **14**(14): 2135-7.

Jiang, Y. and J. R. Broach (1999). "Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast." Embo J **18**(10): 2782-92.

Johnston, G. C., C. W. Ehrhardt, et al. (1979). "Regulation of cell size in the yeast Saccharomyces cerevisiae." J Bacteriol **137**(1): 1-5.

Johnston, G. C., J. R. Pringle, et al. (1977). "Coordination of growth with cell division in the yeast Saccharomyces cerevisiae." Exp Cell Res **105**(1): 79-98.

Johnston, G. C., J. R. Pringle, et al. (1977). "Coordination of growth with cell division in the yeast Saccharomyces cerevisiae." Exp Cell Res **105**(1): 79-98.

Jorgensen, P., J. L. Nishikawa, et al. (2002). "Systematic identification of pathways that couple cell growth and division in yeast." Science **297**(5580): 395-400.

Jorgensen, P., I. Rupes, et al. (2004). "A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size." Genes Dev **18**(20): 2491-505.

Joyce, D., B. Bouzahzah, et al. (1999). "Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway." J Biol Chem **274**(36): 25245-9.

Kaiser, P., R. A. Sia, et al. (1998). "Cdc34 and the F-box protein Met30 are required for degradation of the Cdk-inhibitory kinase Swe1." Genes Dev **12**(16): 2587-97.

Kaldis, P., A. Sutton, et al. (1996). "The Cdk-activating kinase (CAK) from budding yeast." Cell **86**(4): 553-64.

Kalhor, H. R. and S. Clarke (2003). "Novel methyltransferase for modified uridine residues at the wobble position of tRNA." Mol Cell Biol **23**(24): 9283-92.

Kellogg, D. R. (2003). "Wee1-dependent mechanisms required for coordination of cell growth and cell division." J Cell Sci **116**(Pt 24): 4883-90.

Kim, J. E. and J. Chen (2000). "Cytoplasmic-nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation." Proc Natl Acad Sci U S A **97**(26): 14340-5.

Kishi, T. and F. Yamao (1998). "An essential function of Grr1 for the degradation of Cln2 is to act as a binding core that links Cln2 to Skp1." J Cell Sci **111 (Pt 24)**: 3655-61.

Koch, C., T. Moll, et al. (1993). "A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase." Science **261**(5128): 1551-7.

Koch, C., A. Schleiffer, et al. (1996). "Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Clb/Cdc28 kinases displace it from the promoter in G2." Genes Dev **10**(2): 129-41.

Koch, C., P. Wollmann, et al. (1999). "A role for Ctr9p and Paf1p in the regulation G1 cyclin expression in yeast." Nucleic Acids Res **27**(10): 2126-34.

Komarnitsky, S. I., Y. C. Chiang, et al. (1998). "DBF2 protein kinase binds to and acts through the cell cycle-regulated MOB1 protein." Mol Cell Biol **18**(4): 2100-7.

Krogan, N. J., J. Dover, et al. (2002). "COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression." J Biol Chem **277**(13): 10753-5.

Krogan, N. J., J. Dover, et al. (2003). "The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation." Mol Cell **11**(3): 721-9.

Krogan, N. J., M. Kim, et al. (2002). "RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach." Mol Cell Biol **22**(20): 6979-92.

Kunz, J., R. Henriquez, et al. (1993). "Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression." Cell **73**(3): 585-96.

Laabs, T. L., D. D. Markwardt, et al. (2003). "ACE2 is required for daughter cell-specific G1 delay in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **100**(18): 10275-80.

Labbe, J. C., M. G. Lee, et al. (1988). "Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2+." Nature **335**(6187): 251-4.

Lahn, M., K. Sundell, et al. (2003). "Targeting protein kinase C-alpha (PKC-alpha) in cancer with the phosphorothioate antisense oligonucleotide aprinocarsen." Ann N Y Acad Sci **1002**: 263-70.

Langan, T. A., J. Gautier, et al. (1989). "Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells." Mol Cell Biol **9**(9): 3860-8.

Lanker, S., M. H. Valdivieso, et al. (1996). "Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation." Science **271**(5255): 1597-601.

Lawlor, M. A., A. Mora, et al. (2002). "Essential role of PDK1 in regulating cell size and development in mice." Embo J **21**(14): 3728-38.

Lawrence, J. C., Jr. and R. T. Abraham (1997). "PHAS/4E-BPs as regulators of mRNA translation and cell proliferation." Trends Biochem Sci **22**(9): 345-9.

Lee, K. S., L. K. Hines, et al. (1993). "A pair of functionally redundant yeast genes (PPZ1 and PPZ2) encoding type 1-related protein phosphatases function within the PKC1-mediated pathway." Mol Cell Biol **13**(9): 5843-53.

Lee, M. G. and P. Nurse (1987). "Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2." Nature **327**(6117): 31-5.

Lee, S. S., S. Kennedy, et al. (2003). "DAF-16 target genes that control C. elegans life-span and metabolism." Science **300**(5619): 644-7.

Leevers, S. J., D. Weinkove, et al. (1996). "The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth." Embo J **15**(23): 6584-94.

Lenssen, E., U. Oberholzer, et al. (2002). "Saccharomyces cerevisiae Ccr4-not complex contributes to the control of Msn2p-dependent transcription by the Ras/cAMP pathway." Mol Microbiol **43**(4): 1023-37.

Leone, G., J. DeGregori, et al. (1997). "Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F " Nature **387**(6631): 422-6.

Levine, K., A. H. Tinkelenberg, et al. (1995). "The CLN gene family: central regulators of cell cycle Start in budding yeast." Prog Cell Cycle Res **1**: 101-14.

Li, F. N. and M. Johnston (1997). "Grr1 of Saccharomyces cerevisiae is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle." Embo J **16**(18): 5629-38.

Li, Y., M. N. Corradetti, et al. (2004). "TSC2: filling the GAP in the mTOR signaling pathway." Trends Biochem Sci **29**(1): 32-8.

Liu, H. Y., V. Badarinarayana, et al. (1998). "The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively." Embo J **17**(4): 1096-106.

Liu, H. Y., J. H. Toyn, et al. (1997). "DBF2, a cell cycle-regulated protein kinase, is physically and functionally associated with the CCR4 transcriptional regulatory complex." Embo J **16**(17): 5289-98.

Loewith, R., E. Jacinto, et al. (2002). "Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control." Mol Cell **10**(3): 457-68.

Lowndes, N. F., A. L. Johnson, et al. (1992). "SWI6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast." Nature **357**(6378): 505-8.

Lu, Y. M., Y. R. Lin, et al. (2003). "Dissecting the pet18 mutation in Saccharomyces cerevisiae: HTL1 encodes a 7-kDa polypeptide that interacts with components of the RSC complex." Mol Genet Genomics **269**(3): 321-30.

MacNeill, S. A., E. Warbrick, et al. (1991). "Controlling cell cycle progress in the fission yeast Schizosaccharomyces pombe." Curr Opin Genet Dev **1**(3): 307-12.

Madden, K., Y. J. Sheu, et al. (1997). "SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway." Science **275**(5307): 1781-4.

Mai, B., S. Miles, et al. (2002). "Characterization of the ECB binding complex responsible for the M/G(1)- specific transcription of CLN3 and SWI4." Mol Cell Biol **22**(2): 430-41.

Maillet, L. and M. A. Collart (2002). "Interaction between Not1p, a component of the Ccr4-not complex, a global regulator of transcription, and Dhh1p, a putative RNA helicase." J Biol Chem **277**(4): 2835-42.

Mal, T. K., Y. Masutomi, et al. (2004). "Structural and functional characterization on the interaction of yeast TFIID subunit TAF1 with TATA-binding protein." J Mol Biol **339**(4): 681-93.

Malumbres, M., S. L. Hunt, et al. (2003). "Driving the cell cycle to cancer." Adv Exp Med Biol **532**: 1-11.

Malvar, T., R. W. Biron, et al. (1992). "The CCR4 protein from Saccharomyces cerevisiae contains a leucine-rich repeat region which is required for its control of ADH2 gene expression." Genetics **132**(4): 951-62.

Marygold, S. J. and S. J. Leevers (2002). "Growth signaling: TSC takes its place." Curr Biol **12**(22): R785-7.

Masui, Y. and C. L. Markert (1971). "Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes." J Exp Zool **177**(2): 129-45.

McDonald, E. R., 3rd and W. S. El-Deiry (2000). "Cell cycle control as a basis for cancer drug development (Review)." Int J Oncol **16**(5): 871-86.

McInerny, C. J., J. F. Partridge, et al. (1997). "A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription." Genes Dev **11**(10): 1277-88.

Medrano, E. E. and A. B. Pardee (1980). "Prevalent deficiency in tumor cells of cycloheximide-induced cycle arrest." Proc Natl Acad Sci U S A **77**(7): 4123-6.

Melaragno, J. E., B. Mehrotra, et al. (1993). "Relationship between Endopolyploidy and Cell Size in Epidermal Tissue of Arabidopsis." Plant Cell **5**(11): 1661-1668.

Mendenhall, M. D. and A. E. Hodge (1998). "Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae." Microbiol Mol Biol Rev **62**(4): 1191-243.

Mendenhall, M. D., C. A. Jones, et al. (1987). "Dual regulation of the yeast CDC28-p40 protein kinase complex: cell cycle, pheromone, and nutrient limitation effects." Cell **50**(6): 927-35.

Meselson, M. and F. W Stahl (1958). "The replication of DNA." Cold Spring Harb Symp Quant Biol **23**: 9-12.

Miller, M. E. and F R. Cross (2000). "Distinct subcellular localization patterns contribute to functional specificity of the Cln2 and Cln3 cyclins of Saccharomyces cerevisiae." Mol Cell Biol **20**(2): 542-55.

Miller, M. E. and F. R. Cross (2001). "Cyclin specificity: how many wheels do you need on a unicycle?" J Cell Sci **114**(Pt 10): 1811-20.

Miller, M. E. and F. R. Cross (2001). "Mechanisms controlling subcellular localization of the G(1) cyclins Cln2p and Cln3p in budding yeast." Mol Cell Biol **21**(18): 6292-311.

Mitchison, J. M., B. Novak, et al. (1997). "Size control in the cell cycle." Cell Biol Int **21**(8): 461-3.

Mitsuzawa, H. (1994). "Increases in cell size at START caused by hyperactivation of the cAMP pathway in Saccharomyces cerevisiae." Mol Gen Genet **243**(2): 158-65.

Mittnacht, S. (1998). "Control of pRB phosphorylation." Curr Opin Genet Dev **8**(1): 21-7.

Moll, T., L. Dirick, et al. (1992). "SWI6 is a regulatory subunit of two different cell cycle START- dependent transcription factors in Saccharomyces cerevisiae." J Cell Sci Suppl **16**: 87-96.

Montagne, J. (2000). "Genetic and molecular mechanisms of cell size control." Mol Cell Biol Res Commun **4**(4): 195-202.

Moriya, H., Y. Shimizu-Yoshida, et al. (2001). "Yak1p, a DYRK family kinase, translocates to the nucleus and phosphorylates yeast Pop2p in response to a glucose signal." Genes Dev **15**(10): 1217-28.

Mountain, H. A. and P. E. Sudbery (1990). "Regulation of the Saccharomyces cerevisiae WHI2 gene." J Gen Microbiol **136 (Pt 4)**: 727-32.

Mountain, H. A. and P. E. Sudbery (1990). "The relationship of growth rate and catabolite repression with WHI2 expression and cell size in Saccharomyces cerevisiae." J Gen Microbiol **136 (Pt 4)**: 733-7.

Mueller, C. L. and J. A. Jaehning (2002). "Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex." Mol Cell Biol **22**(7): 1971-80.

Mueller, C. L., S. E. Porter, et al. (2004). "The Paf1 complex has functions independent of actively transcribing RNA polymerase II." Mol Cell **14**(4): 447-56.

Murray, A. W. (1994). "Cyclin-dependent kinases: regulators of the cell cycle and more." Chem Biol **1**(4): 191-5.

Murray, A. W. (2004). "Recycling the cell cycle: cyclins revisited." Cell **116**(2): 221-34.

Murray, A. W. a. H., T. (1993). The Cell Cycle: An Introduction, Oxford University Press.

Nanahoshi, M., T. Nishiuma, et al. (1998). "Regulation of protein phosphatase 2A catalytic activity by alpha4 protein and its yeast homolog Tap42." Biochem Biophys Res Commun **251**(2): 520-6.

Narita, M., S. Nunez, et al. (2003). "Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence." Cell **113**(6): 703-16.

Nash, P., X. Tang, et al. (2001). "Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication." Nature **414**(6863): 514-21.

Nash, R., G. Tokiwa, et al. (1988). "The WHI1+ gene of Saccharomyces cerevisiae tethers cell division to cell size and is a cyclin homolog." Embo J **7**(13): 4335-46.

Nasi, S., R. Ciarapica, et al. (2001). "Making decisions through Myc." FEBS Lett **490**(3): 153-62.

Nasmyth, K. (1993). "Regulating the HO endonuclease in yeast." Curr Opin Genet Dev **3**(2): 286-94.

Nasmyth, K. and L. Dirick (1991). "The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast." Cell **66**(5): 995-1013.

Neufeld, T. P., A. F. de la Cruz, et al. (1998). "Coordination of growth and cell division in the Drosophila wing." Cell **93**(7): 1183-93.

Neufeld, T. P. and B. A. Edgar (1998). "Connections between growth and the cell cycle." Curr Opin Cell Biol **10**(6): 784-90.

Neuman-Silberberg, F S., S. Bhattacharya, et al. (1995). "Nutrient availability and the RAS/cyclic AMP pathway both induce expression of ribosomal protein genes in Saccharomyces cerevisiae but by different mechanisms." Mol Cell Biol **15**(6): 3187-96.

Newcomb, L. L., J. A. Diderich, et al. (2003). "Glucose regulation of Saccharomyces cerevisiae cell cycle genes." Eukaryot Cell **2**(1): 143-9.

Newcomb, L. L., D. D. Hall, et al. (2002). "AZF1 is a glucose-dependent positive regulator of CLN3 transcription in Saccharomyces cerevisiae." Mol Cell Biol **22**(5): 1607-14.

Ng, H. H., S. Dole, et al. (2003). "The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B." J Biol Chem **278**(36): 33625-8.

Ng, H. H., F. Robert, et al. (2002). "Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex." Genes Dev **16**(7): 806-19.

Ng, H. H., F. Robert, et al. (2003). "Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity." Mol Cell **11**(3): 709-19.

Nicolas, E., C. Roumillac, et al. (2003). "Balance between acetylation and methylation of histone H3 lysine 9 on the E2F-responsive dihydrofolate reductase promoter." Mol Cell Biol **23**(5): 1614-22.

Norbury, C. J. and P. Nurse (1989). "Control of the higher eukaryote cell cycle by p34cdc2 homologues." Biochim Biophys Acta **989**(1): 85-95.

Nurse, P. (1997). "Regulation of the eukaryotic cell cycle." Eur J Cancer **33**(7): 1002-4.

Nurse, P. and P. Thuriaux (1980). "Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe." Genetics **96**(3): 627-37.

Nurse, P., P. Thuriaux, et al. (1976). "Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe." Mol Gen Genet **146**(2): 167-78.

Oberley, M. J., D. R. Inman, et al. (2003). "E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9." J Biol Chem **278**(43): 42466-76.

Ogawa, H., K. Ishiguro, et al. (2002). "A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells." Science **296**(5570): 1132-6.

Oldham, S., R. Bohni, et al. (2000). "Genetic control of size in Drosophila." Philos Trans R Soc Lond B Biol Sci **355**(1399): 945-52.

Oldham, S. and E. Hafen (2003). "Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control." Trends Cell Biol **13**(2): 79-85.

Oldham, S., H. Stocker, et al. (2002). "The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels." Development **129**(17): 4103-9.

Palancade, B. and O. Bensaude (2003). "Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation." Eur J Biochem **270**(19): 3859-70.

Pardee, A. B. (1974). "A restriction point for control of normal animal cell proliferation." Proc Natl Acad Sci U S A **71**(4): 1286-90.

Parsons, R. (2004). "Human cancer, PTEN and the PI-3 kinase pathway." Semin Cell Dev Biol **15**(2): 171-6.

Parviz, F., D. D. Hall, et al. (1998). "Transcriptional regulation of CLN3 expression by glucose in Saccharomyces cerevisiae." J Bacteriol **180**(17): 4508-15.

Parviz, F. and W. Heideman (1998). "Growth-independent regulation of CLN3 mRNA levels by nutrients in Saccharomyces cerevisiae." J Bacteriol **180**(2): 225-30.

Peterson, R. T., B. N. Desai, et al. (1999). "Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein." Proc Natl Acad Sci U S A **96**(8): 4438-42.

Polymenis, M. and E. V. Schmidt (1997). "Coupling of cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast." Genes Dev **11**(19): 2522-31.

Porter, S. E., T. M. Washburn, et al. (2002). "The yeast pafl-rNA polymerase II complex is required for full expression of a subset of cell cycle-regulated genes." Eukaryot Cell **1**(5): 830-42.

Potter, C. J., H. Huang, et al. (2001). "Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size." Cell **105**(3): 357-68.

Potter, C. J., L. G. Pedraza, et al. (2003). "The tuberous sclerosis complex (TSC) pathway and mechanism of size control." Biochem Soc Trans **31**(Pt 3): 584-6.

Potter, C. J. and T. Xu (2001). "Mechanisms of size control." Curr Opin Genet Dev **11**(3): 279-86.

Prevot, D., A. P. Morel, et al. (2001). "Relationships of the antiproliferative proteins BTG1 and BTG2 with CAF1, the human homolog of a component of the yeast CCR4 transcriptional complex: involvement in estrogen receptor alpha signaling pathway." J Biol Chem **276**(13): 9640-8.

Pyronnet, S. (2000). "Phosphorylation of the cap-binding protein eIF4E by the MAPK-activated protein kinase Mnk1." Biochem Pharmacol **60**(8): 1237-43.

Queralt, E. and J. C. Igual (2003). "Cell cycle activation of the Swi6p transcription factor is linked to nucleocytoplasmic shuttling." Mol Cell Biol **23**(9): 3126-40.

Radcliffe, P., J. Trevethick, et al. (1997). "Deregulation of CLN1 and CLN2 in the Saccharomyces cerevisiae whi2 mutant." Yeast **13**(8): 707-15.

Rajasekhar, V. K., A. Viale, et al. (2003). "Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes." Mol Cell **12**(4): 889-901.

Rayner, T. F., J. V Gray, et al. (2002). "Direct and novel regulation of cAMP-dependent protein kinase by Mck1p, a yeast glycogen synthase kinase-3." J Biol Chem **277**(19): 16814-22.

Rechsteiner, M. and S. W. Rogers (1996). "PEST sequences and regulation by proteolysis." Trends Biochem Sci **21**(7): 267-71.

Reed, S. I. (1980). "The selection of S. cerevisiae mutants defective in the start event of cell division." Genetics **95**(3): 561-77.

Reed, S. I., J. A. Hadwiger, et al. (1989). "Analysis of the Cdc28 protein kinase complex by dosage suppression." J Cell Sci Suppl **12**: 29-37.

Resnitzky, D., L. Hengst, et al. (1995). "Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27Kip1." Mol Cell Biol **15**(8): 4347-52.

Richardson, H. E., C. Wittenberg, et al. (1989). "An essential G1 function for cyclin-like proteins in yeast." Cell **59**(6): 1127-33.

Rintelen, F., H. Stocker, et al. (2001). "PDK1 regulates growth through Akt and S6K in Drosophila." Proc Natl Acad Sci U S A **98**(26): 15020-5.

Rogers, S., R. Wells, et al. (1986). "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis." Science **234**(4774): 364-8.

Rowley, A., G. C. Johnston, et al. (1993). "Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast Saccharomyces cerevisiae." Mol Cell Biol **13**(2): 1034-41.

Rowley, A., R. A. Singer, et al. (1991). "CDC68, a yeast gene that affects regulation of cell proliferation and transcription, encodes a protein with a highly acidic carboxyl terminus." Mol Cell Biol **11**(11): 5718-26.

Rupes, I. (2002). "Checking cell size in yeast." Trends Genet **18**(9): 479-85.

Russell, P. and P. Nurse (1986). "cdc25+ functions as an inducer in the mitotic control of fission yeast." Cell **45**(1): 145-53.

Russell, P. and P. Nurse (1987). "Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog." Cell **49**(4): 559-67.

Salama, S. R., K. B. Hendricks, et al. (1994). "G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover." Mol Cell Biol **14**(12): 7953-66.

Santalucia, T., M. Christmann, et al. (2003). "Hypertrophic agonists induce the binding of c-Fos to an AP-1 site in cardiac myocytes: implications for the expression of GLUT1." Cardiovasc Res **59**(3): 639-48.

Saul, D. J. and P. E. Sudbery (1985). "Molecular cloning of WHI2, a gene involved in the regulation of cell proliferation in Saccharomyces cerevisiae." J Gen Microbiol **131 (Pt 7)**: 1797-806.

Schaefer, J. B. and L. L. Breeden (2004). "RB from a bud's eye view." Cell **117**(7): 849-50.

Schalm, S. S., D. C. Fingar, et al. (2003). "TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function." Curr Biol **13**(10): 797-806.

Schmelzle, T. and M. N. Hall (2000). "TOR, a central controller of cell growth." Cell **103**(2): 253-62.

Schmidt, A., J. Kunz, et al. (1996). "TOR2 is required for organization of the actin cytoskeleton in yeast." Proc Natl Acad Sci U S A **93**(24): 13780-5.

Schneider, B. L., E. E. Patton, et al. (1998). "Yeast G1 cyclins are unstable in G1 phase." Nature **395**(6697): 86-9.

Schneider, B. L., Q. H. Yang, et al. (1996). "Linkage of replication to start by the Cdk inhibitor Sic1." Science **272**(5261): 560-2.

Schwob, E., T. Bohm, et al. (1994). "The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae." Cell **79**(2): 233-44.

Sears, R., F. Nuckolls, et al. (2000). "Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability." Genes Dev **14**(19): 2501-14.

Sekito, T., J. Thornton, et al. (2000). "Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p." Mol Biol Cell **11**(6): 2103-15.

Shamji, A. F., P. Nghiem, et al. (2003). "Integration of growth factor and nutrient signaling: implications for cancer biology." Mol Cell **12**(2): 271-80.

Sharp, L. (1926). An introduction to cytology. New York, McGraw-hill Book Company, Inc.

Sherr, C. J. (1996). "Cancer cell cycles." Science **274**(5293): 1672-7.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-12.

Shi, X., A. Finkelstein, et al. (1996). "Paf1p, an RNA polymerase II-associated factor in Saccharomyces cerevisiae, may have both positive and negative roles in transcription." Mol Cell Biol **16**(2): 669-76.

Shioi, T., P. M. Kang, et al. (2000). "The conserved phosphoinositide 3-kinase pathway determines heart size in mice." Embo J **19**(11): 2537-48.

Sidorova, J. and L. Breeden (1993). "Analysis of the SWI4/SWI6 protein complex, which directs G1/S-specific transcription in Saccharomyces cerevisiae." Mol Cell Biol **13**(2): 1069-77.

Sidorova, J. M. and L. L. Breeden (1997). "Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae." Genes Dev **11**(22): 3032-45.

Sidorova, J. M., G. E. Mikesell, et al. (1995). "Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization." Mol Biol Cell **6**(12): 1641-58.

Sillje, H. H., E. G. ter Schure, et al. (1997). "Effects of different carbon fluxes on G1 phase duration, cyclin expression, and reserve carbohydrate metabolism in Saccharomyces cerevisiae." J Bacteriol **179**(21): 6560-5.

Simanis, V. and P. Nurse (1986). "The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation." Cell **45**(2): 261-8.

Simic, R., D. L. Lindstrom, et al. (2003). "Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes." Embo J **22**(8): 1846-56.

Simon, I., J. Barnett, et al. (2001). "Serial regulation of transcriptional regulators in the yeast cell cycle." Cell **106**(6): 697-708.

Smith, A., M. P Ward, et al. (1998). "Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation." Embo J **17**(13): 3556-64.

Sonenberg, N. and A. C. Gingras (1998). "The mRNA 5' cap-binding protein eIF4E and control of cell growth." Curr Opin Cell Biol **10**(2): 268-75.

Spellman, P. T., G. Sherlock, et al. (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Squazzo, S. L., P. J. Costa, et al. (2002). "The Paf1 complex physically and functionally associates with transcription elongation factors in vivo." Embo J **21**(7): 1764-74.

Stevens, C. and N. B. La Thangue (2003). "E2F and cell cycle control: a double-edged sword." Arch Biochem Biophys **412**(2): 157-69.

Stocker, H. and E. Hafen (2000). "Genetic control of cell size." Curr Opin Genet Dev **10**(5): 529-35.

Stocker, H., T. Radimerski, et al. (2003). "Rheb is an essential regulator of S6K in controlling cell growth in Drosophila." Nat Cell Biol **5**(6): 559-65.

Stuart, D. and C. Wittenberg (1995). "CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells." Genes Dev **9**(22): 2780-94.

Sudbery, P. E., A. R. Goodey, et al. (1980). "Genes which control cell proliferation in the yeast Saccharomyces cerevisiae." Nature **288**(5789): 401-4.

Sutton, A., J. Bucaria, et al. (2001). "Yeast ASF1 protein is required for cell cycle regulation of histone gene transcription." Genetics **158**(2): 587-96.

Sveiczer, A., B. Novak, et al. (1996). "The size control of fission yeast revisited." J Cell Sci **109**(Pt 12): 2947-57.

Swift, H. (1950). "The constancy of desoxyribose nucleic acid in plant nuclei." Proc Natl Acad Sci U S A **36**(11): 643-54.

Takuwa, N. and Y. Takuwa (1997). "Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts." Mol Cell Biol **17**(9): 5348-58.

Tee, A. R., D. C. Fingar, et al. (2002). "Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling." Proc Natl Acad Sci U S A **99**(21): 13571-6.

Terada, Y., T. Yamada, et al. (1997). "Overexpression of cell cycle inhibitors (p16INK4 and p21Cip1) and cyclin D1 using adenovirus vectors regulates proliferation of rat mesangial cells." J Am Soc Nephrol **8**(1): 51-60.

Thevelein, J. M. and J. H. de Winde (1999). "Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae." Mol Microbiol **33**(5): 904-18.

Tirone, F. (2001). "The gene PC3(TIS21/BTG2), prototype member of the PC3/BTG/TOB family: regulator in control of cell growth, differentiation, and DNA repair?" J Cell Physiol **187**(2): 155-65.

Topisirovic, I., B. Culjkovic, et al. (2003). "The proline-rich homeodomain protein, PRH, is a tissue-specific inhibitor of eIF4E-dependent cyclin D1 mRNA transport and growth." Embo J **22**(3): 689-703.

Tu, V C., J. J. Bahl, et al. (2003). "Distinct roles of p42/p44(ERK) and p38 MAPK in oxidant-induced AP-1 activation and cardiomyocyte hypertrophy." Cardiovasc Toxicol **3**(2): 119-33.

Tyers, M. (1996). "The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for Cln G1 cyclin function at Start." Proc Natl Acad Sci U S A **93**(15): 7772-6.

Tyers, M., G. Tokiwa, et al. (1993). "Comparison of the Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins." Embo J **12**(5): 1955-68.

Tyers, M., G. Tokiwa, et al. (1992). "The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation." Embo J **11**(5): 1773-84.

Tyson, C. B., P G. Lord, et al. (1979). "Dependency of size of Saccharomyces cerevisiae cells on growth rate." J Bacteriol **138**(1): 92-8.

Tyson, J. J. and O. Diekmann (1986). "Sloppy size control of the cell division cycle." J Theor Biol **118**(4): 405-26.

Ueda, T., R. Watanabe-Fukunaga, et al. (2004). "Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development." Mol Cell Biol **24**(15): 6539-49.

Verdu, J., M. A. Buratovich, et al. (1999). "Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB." Nat Cell Biol **1**(8): 500-6.

Verma, R., R. S. Annan, et al. (1997). "Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase." Science **278**(5337): 455-60.

Vermeulen, K., D. R. Van Bockstaele, et al. (2003). "The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer." Cell Prolif **36**(3): 131-49.

Viswanathan, P., J. Chen, et al. (2003). "Identification of multiple RNA features that influence CCR4 deadenylation activity." J Biol Chem **278**(17): 14949-55.

Walker, S. S., W. C. Shen, et al. (1997). "Yeast TAF(II)145 required for transcription of G1/S cyclin genes and regulated by the cellular growth state." Cell **90**(4): 607-14.

Wang, H., E. Gari, et al. (2004). "Recruitment of Cdc28 by Whi3 restricts nuclear accumulation of the G1 cyclin-Cdk complex to late G1." Embo J **23**(1): 180-90.

Wang, J., R. Tokarz, et al. (2002). "The expression of TGFbeta signal transducers in the hypodermis regulates body size in C. elegans." Development **129**(21): 4989-98.

Weeks, G. and G. B. Spiegelman (2003). "Roles played by Ras subfamily proteins in the cell and developmental biology of microorganisms." Cell Signal **15**(10): 901-9.

Weinmann, A. S., P. S. Yan, et al. (2002). "Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis." Genes Dev **16**(2): 235-44.

Westmoreland, T. J., J. R. Marks, et al. (2004). "Cell cycle progression in G1 and S phases is CCR4 dependent following ionizing radiation or replication stress in Saccharomyces cerevisiae." Eukaryot Cell **3**(2): 430-46.

Westmoreland, T. J., J. A. Olson, et al. (2003). "Dhh1 regulates the G1/S-checkpoint following DNA damage or BRCA1 expression in yeast." J Surg Res **113**(1): 62-73.

Wijnen, H. and B. Futcher (1999). "Genetic analysis of the shared role of CLN3 and BCK2 at the G(1)-S transition in Saccharomyces cerevisiae." Genetics **153**(3): 1131-43.

Wijnen, H., A. Landman, et al. (2002). "The G(1) cyclin Cln3 promotes cell cycle entry via the transcription factor Swi6." Mol Cell Biol **22**(12): 4402-18.

Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science **285**(5429): 901-6.

Wittenberg, C., K. Sugimoto, et al. (1990). "G1-specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase." Cell **62**(2): 225-37.

Woollard, A. and P. Nurse (1995). "G1 regulation and checkpoints operating around START in fission yeast." Bioessays **17**(6): 481-90.

Wu, L., A. Yee, et al. (1994). "Molecular cloning of the human CAK1 gene encoding a cyclin-dependent kinase-activating kinase." Oncogene **9**(7): 2089-96.

Wu, M., L. Newcomb, et al. (1999). "Regulation of gene expression by glucose in Saccharomyces cerevisiae: a role for ADA2 and ADA3/NGG1." J Bacteriol **181**(16): 4755-60.

Yaglom, J., M. H. Linskens, et al. (1995). "p34Cdc28-mediated control of Cln3 cyclin degradation." Mol Cell Biol **15**(2): 731-41.

Yen, A., J. Fried, et al. (1975). "The kinetic significance of cell size, I. Variation of cell cycle parameters with size measured at mitosis." Exp Cell Res **95**(2): 295-302.

Zetterberg, A. and D. Killander (1965). "Quantitative cytochemical studies on interphase growth. II. Derivation of synthesis curves from the distribution of DNA, RNA and mass values of individual mouse fibroblasts in vitro." Exp Cell Res **39**(1): 22-32.

Zhang, H., J. P. Stallock, et al. (2000). "Regulation of cellular growth by the Drosophila target of rapamycin dTOR." Genes Dev **14**(21): 2712-24.

Appendix A. List of Petite Mutants

ORF	Mean Size	ORF	Mean Size	ORF	Mean Size	ORF	Mean Size
YMR089C	24.2	YDR194C	28.59	YMR035W	32.53	YLL033W	35.42
YER077C	25.31	YDL107W	29.03	YLR439W	32.56	YDR114C	35.44
YKL155C	25.6	YLR139C	29.03	YGR255C	32.59	YDR405W	35.46
YHR011W	25.76	YLL006W	29.19	YBL090W	32.59	YER017C	35.57
YMR150C	26.1	YGR171C	29.2	YOL023W	32.61	YDL176W	35.60
YGR150C	26.11	YKR006C	29.33	YNR036C	32.68	YJL063C	35.61
YIL093C	26.16	YMR066W	29.34	YDR332W	32.69	YER153C	35.61
YDR065W	26.31	YJR113C	29.45	YML061C	32.72	YPL215W	35.78
YLR202C	26.36	YOR065W	29.45	YEL050C	32.86	YNR042W	35.81
YHR091C	26.57	YPR047W	29.47	YAL039C	32.95	YMR021C	35.84
YMR256C	26.65	YFL036W	29.62	YMR257C	32.95	YDR079W	35.92
YLR201C	26.69	YMR193W	29.65	YEL024W	33.01	YDR529C	35.93
YDR204W	26.74	YMR286W	29.65	YBL100C	33.03	YNR045W	35.93
YGR102C	26.88	YMR158W	29.72	YBL080C	33.16	YPL078C	35.96
YJL023C	26.97	YDR231C	30.01	YGL129C	33.18	YKL134C	37.14
YGR220C	26.98	YMR228W	30.04	YCR028C-A	33.21	YKL080W	37.19
YMR097C	27.14	YDR175C	30.12	YGR062C	33.34	YDR337W	37.21
YLL009C	27.15	YDR350C	30.22	YKL170W	33.35	YHR008C	37.29
YOL095C	27.17	YLR295C	30.23	YNL003C	33.39	YOR125C	37.29
YOR330C	27.22	YMR188C	30.24	YER058W	33.44	YLR382C	37.50
YPR123C	27.27	YGR215W	30.29	YDR462W	33.51	YPL148C	37.94
YJL022W	27.39	YDR322W	30.34	YBR251W	33.54	YNL315C	37.94
YHR051W	27.5	YGL218W	30.42	YPR099C	33.56	YMR293C	38.51
YMR098C	27.55	YER050C	30.47	YHR038W	33.56	YLL027W	39.63
YKL016C	27.78	YBR268W	30.49	YNR037C	33.58	YIL047C	40.26
YGR222W	27.82	YLR149C	30.57	YMR282C	33.67	YFR019W	40.39
YPL132W	27.85	YKL169C	30.7	YCR024C	33.74	YBL045C	41.64
YOL009C	27.89	YBL038W	30.74	YLR260W	33.76	YJL096W	42.25
YIR021W	27.91	YHL038C	30.92	YBL099W	33.77	YLR304C	42.45
YDR115W	27.95	YDR298C	31.17	YJL180C	33.78	YPR100W	43.45
YNL252C	28	YML110C	31.35	YGL218W	33.95	YGR165W	43.96
YPR124W	28.01	YDR296W	31.53	YJL102W	34.50	YBR037C	44.15
YDR230W	28.02	YMR064W	31.53	YOR150W	34.57	YIL067C	44.3
YMR151W	28.18	YGR101W	31.54	YJR034W	34.58	YLR038C	45.31
YDR268W	28.26	YKL138C	31.56	YPL005W	34.58	YJL046W	46.31
YLR203C	28.32	YLR439W	31.59	YHR147C	34.66	YBR282W	46.99
YDR197W	28.33	YPL013C	31.73	YDL069C	34.73	YLR239C	47.16
YBL007C	28.34	YMR287C	31.76	YMR267W	34.85	YJR077C	47.96
YLL018C-A	28.37	YGR219W	31.77	YKL003C	34.87	YNL064C	48.75
YLR369W	28.4	YNL005C	31.84	YDL068W	34.89	YCR084C	52.75
YDR237W	28.47	YJL166W	31.90	YGL107C	34.89	YIL125W	53.60
YPR116W	28.47	YJL209W	31.96	YDL067C	34.91	YOR158W	66.2
YHR168W	28.53	YML129C	32.13	YDL202W	35.18	YGR180C	67.13
YGR076C	28.55	YDR377W	32.41	YNR041C	35.28	YBR112C	74.09
YLR312W-A	28.56	YDL198C	32.53	YDR347W	35.36	YBL093C	95.3
YPR166C	28.58						

Appendix B. List of Mutants with Growth Defects

ORF	Mean Size	ORF	Mean Size	ORF	Mean Size	ORF	Mean Size
YOR305W	19.79	YPL183W-A	36.78	YER014C-A	46.89	YGL234W	52.95
YOR187W	23.09	YGL135W	36.80	YER032W	47.05	YER156C	52.95
YOR147W	26.93	YJR121W	36.83	YLR350W	47.05	YPL268W	53.60
YNL177C	27.29	YIL018W	36.99	YOR157C	47.06	YPR163C	53.89
YOR200W	27.79	YDR518W	37.23	YDR137W	47.25	YER052C	54.51
YPL172C	27.9	YIL097W	37.75	YPR060C	47.29	YOR073W	54.81
YPL097W	28.59	YBR004C	37.87	YJL138C	47.52	YOR349W	54.99
YLR091W	28.71	YFL016C	38.60	YPR072W	47.74	YGL095C	55.02
YOR211C	28.72	YAL012W	38.75	YNR074C	48.05	YOR224C	55.39
YCR003W	28.73	YBL021C	38.79	YGL081W	48.16	YBR078W	55.50
YNL184C	29.21	YKR085C	38.85	YGL071W	48.19	YDL045W-A	55.51
YOR205C	29.42	YJR012C	38.93	YJR139C	48.22	YNL331C	55.79
YPL040C	29.53	YNL251C	39.53	YHL031C	48.26	YGL163C	56.36
YHR120W	29.56	YJL195C	39.88	YMR300C	48.26	YBR035C	56.43
YLR069C	29.74	YJL003W	40.08	YFR030W	48.32	YGL206C	56.78
YLR067C	30.08	YOR358W	40.88	YDL051W	48.42	YLR148W	57.18
YLR337C	30.11	YNL297C	40.97	YOR160W	48.44	YPL031C	57.74
YPL208W	30.96	YGL026C	41.36	YKL211C	48.54	YPL045W	57.94
YOR201C	31.1	YJL201W	41.39	YIL094C	48.58	YJL075C	58.82
YGR229C	31.13	YGL236C	41.46	YER010C	48.75	YNL280C	59.22
YPL173W	31.38	YPR067W	41.51	YOR104W	48.76	YMR038C	59.42
YPL118W	31.74	YGL064C	42.10	YER068C-A	48.81	YGR204W	59.73
YGL251C	31.83	YER068W	42.27	YML022W	49.00	YMR091C	59.81
YOR179C	31.86	YCR064C	42.36	YDR007W	49.09	YOL076W	59.85
YDR281C	32.06	YER061C	42.62	YBL033C	49.42	YBR171W	59.92
YJR122W	32.53	YGR155W	42.95	YOR340C	49.49	YGR262C	60.03
YDR375C	32.94	YCR044C	43.22	YDL119C	49.74	YOR331C	60.25
YPL104W	32.99	YLR406C	43.29	YJR104C	49.76	YPL042C	60.33
YER087W	33.11	YAL044C	43.49	YNR068C	49.78	YLR396C	60.66
YOR241W	33.12	YDL056W	43.82	YJL088W	49.89	YCR002C	61.75
YLR270W	33.18	YOL127W	44.13	YLR250W	49.91	YEL072W	61.93
YGL143C	33.33	YJR120W	44.15	YCR053W	49.97	YNL246W	62.74
YBR002C	33.38	YNL262W	44.16	YOR260W	50.19	YDR532C	65.80
YKR001C	33.40	YNL055C	44.27	YNR050C	50.26	YOL086C	66.48
YPL262W	33.54	YOR299W	44.38	YOR184W	50.46	YLR399C	67.83
YGR257C	33.69	YDR531W	44.49	YDR234W	50.60	YEL036C	69.13
YIL039W	33.75	YJL202C	44.52	YLR234W	50.78	YKL204W	69.43
YJL007C	33.78	YLR342W	44.84	YER090W	50.82	YER016W	69.70
YGL223C	34.09	YOL081W	45.03	YLR182W	50.91	YOR026W	70.66
YIL015C-A	34.45	YPL050C	45.27	YLR372W	51.06	YHR191C	76.50
YOL033W	34.45	YDR372C	45.58	YER069W	51.08	YLR226W	79.04
YBR163W	34.65	YNL296W	45.58	YLR338W	51.20	YDR369C	80.73
YDL044C	34.76	YLR303W	45.60	YIR034C	51.46	YKL114C	82.32
YNL081C	35.07	YLR244C	45.61	YDR127W	51.65	YAL047C	83.73
YBR003W	35.08	YHR007C	45.65	YPL024W	51.76	YDR359C	87.44
YPL029W	35.13	YNL147W	45.74	YOR319W	51.80	YOR198C	87.63
YAL010C	35.17	YLR027C	45.82	YER163C	51.83	YML013C-A	87.92
YER154W	35.25	YLR240W	46.02	YHR025W	51.95	YKL054C	89.71
YGL038C	35.54	YPL188W	46.60	YER091C	52.13	YLR435W	92.71
YER014W	35.59	YJL090C	46.62	YGR061C	52.19	YGR167W	93.10
YNL073W	35.94	YIR004W	46.70	YMR211W	52.49	YER054C	NA
YIL103W	36.06	YMR135W-A	46.70	YDR408C	52.50	YER070W	NA
YPL271W	36.06	YDR158W	46.75	YER086W	52.55	YFR019W	NA
YCR046C	36.21	YOR278W	46.82	YLR412W	52.68	YOL143C	NA