Cyclin-Dependent Kinase Regulation and Function During Meiosis

by

Thomas M. Carlile

B.S. Molecular and Cell Biology
Texas A&M University, 2003

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2010

Signature of the Author:_____
Department of Biology
15 March 2010

Certified by:_____
Angelika Amon
Professor of Biology
Thesis Supervisor

Accepted by:_____
Stephen P. Bell
Professor of Biology
Chair, Committee for Graduate Students

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Submitted to the Department of Biology
On March 15, 2010 in Partial Fulfillment of the
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ABSTRACT

Meiosis is the process by which haploid gametes are produced from a diploid progenitor cell. Accurate completion of the meiotic divisions requires a variety of modifications to the mitotic chromosome segregation machinery, which allow the reductional meiotic chromosome segregation program to occur. Oscillations in the activity of Cyclin-Dependent Kinases (CDKs) drive virtually every event in the mitotic cell cycle, including events such as cell cycle entry, DNA replication, and chromosome segregation. While much is known about the activity of CDKs, the regulation of CDK activity, and the mechanisms by which CDK activity promotes cell cycle events during vegetative growth in *Saccharomyces cerevisiae*, relatively little is known about the roles of CDKs during the meiotic divisions. This work examines CDK activity during meiosis, the regulation of CDK activity during meiosis, and mechanisms by which CDKs regulate proper meiotic chromosome segregation. First, a striking diversity in Clb-CDK activity is observed during meiosis, including the identification of Clb1-CDK, and Clb3-CDK as meiosis I and meiosis II specific Clb-CDKs respectively. Second, Clb3 protein is shown to be restricted to meiosis II by translational control mediated by the 5'UTR of the *CLB3* message. Finally, premature production of Clb3 results in the premature separation of sister-chromatids during meiosis I.

Thesis Supervisor: Angelika Amon
Title: Professor of Biology

Dedicated to Jadyn Damon

Acknowledgements:

There are many people who I would like to thank for making my time in graduate school such a great and memorable experience.

Foremost I would like to thank Angelika for being such a great advisor. Thanks for the open door, the willingness to talk, and all of your helpful suggestions. Finally, thanks for hurrying me along when needed, both in science and in life.

I would like to thank all members of the Amon lab past and present for making the lab such a great place to work. There are too many of you to name. Thanks to Andreas Hochwagen for being a great rotation mentor, and thanks to Adèle Marston for helping me get started on my project when I joined the lab. Thanks to everyone for helpful suggestions throughout my grad career, especially everyone in the meiosis group. Thanks to Matt, Elçin, and Luke for following up on various observations.

Thanks to all of my in lab friends for making the lab such a fun place to work, especially to those no longer in the lab, Gloria, Brendan, Vineet, and Brett, the lab isn't the same without you. Thanks to Matt, Jeremy, and Elçin for variously, home brewing, the gym, beer fests, and other craziness. Thanks to everyone else in lab for contributing to the environment in your own way: Leon, Ana, Chi, Alexi, Michelle, and Folkert. Thanks to Christian for all you've done for me.

I would like to thank my thesis committee, Frank Solomon and Steve Bell, for all of your interest and input over the years. I would also like to thank Iain Cheeseman and Monica Colaiacovo for participating in my thesis defense.

I'd like to thank my roommates over the years Susan Cohen, Kevin Wang, and Dave Harris for making my time outside of lab fun, and for the hours of *Law & Order SVU* that we watched.

Thanks to all of my family who have been so supportive during my time in grad school. Special thanks go to my parents, Tom and Janet, and my sister, Ashley. Thanks to my grandparents Woodrow and Catherine, and Bob and Dorothy. Thanks to Aunt Tammy, Uncle Roger, Chris and Mike. Finally, thanks to Uncle Bubba, Stephanie, Sara, Rebecca, and Austin.

Most of all I would like to thank my partner Jadyn for all of her love and support through the years. You are amazing.

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Chapter 1:

Introduction

The Significance of Meiosis

Evolutionary success relies on the ability of an organism to survive, reproduce, and pass on its genetic material to subsequent generations. An organism carrying an adaptive mutation will, on average, survive longer, and thus produce more offspring than an organism lacking such a mutation. There are two major reproductive strategies used to pass on genetic material, asexual reproduction and sexual reproduction.

In asexual reproduction, an organism reproduces clonally, resulting in the production of offspring that are nearly genetically identical to the parent. Many single celled organisms primarily reproduce clonally through successive rounds of cell division. In eukaryotes the ordered process of cell division is termed the mitotic cell cycle. During the cell cycle a cell will undergo DNA replication to produce two copies of its genome, will partition the two copies, and will then divide, forming two genetically identical daughter cells. In clonally reproducing populations genetic variation arises when an error occurs during DNA replication, during partitioning of genetic material, or due to environmental insult.

Sexual reproduction relies on the fusion of gametes produced by the parental organism(s) to produce offspring that are genetically distinct from the parent(s). Meiosis is the specialized cell division that produces gametes that are genetically distinct from the progenitor cell in two ways. First, progenitor cells are generally diploid, whereas gametes are generally haploid. This maintains the ploidy of the organism, by allowing the diploid state to be restored by fusion of two gametes. Second, genetic information is exchanged between homologous chromosomes, which along with the fusion of parental gametes,

allows for the production of new combinations of genetic material. While mutation can lead to the appearance of new alleles, this meiotic exchange of genetic material allows for the production of new combinations of alleles. Meiosis is thus a process central to the generation of genetic diversity, and its conservation reflects this fact. Below is an overview of differences between mitosis and meiosis, followed by a more detailed discussion of the meiotic program of *Saccharomyces cerevisiae*.

An Overview of Mitosis and Meiosis

The production of genetically identical daughter cells is accomplished by alternating rounds of genome duplication (S phase) and chromosome segregation (M phase), which are punctuated by G1 and G2, gap phases. Following G1, cells enter S phase and replicate their DNA, pass through G2 phase, and finally enter M phase. During M phase the genomic copies are segregated, after which cells divide, producing two daughter cells, both in G1. How does the cell accurately partition both copies of its genome? Physical linkages between sister-chromatids provide the key. These linkages (sister-chromatid cohesion) are provided by ring-shaped protein complexes called cohesins. Cohesins, along with attachment of sister-kinetochores to microtubules emanating from opposite poles of the mitotic spindle, a situation called bi-orientation, allow sister-chromatids to come under tension on the spindle (Figure 1A). This tension ensures that sisters are segregated towards opposite poles.

Meiosis results in the production of four genetically non-identical, haploid gametes. This reduction in ploidy is accomplished by carrying out two rounds of chromosome

segregation following a single round of DNA replication. The first meiotic division is reductional (homologs are segregated), while the second meiotic division is equational, (sister-chromatids are segregated) (Figure 1B). Though much of the chromosome segregation machinery is shared between mitosis and meiosis, there are four specializations that occur. First, homolog pairs are physically linked during meiosis I, through a process called recombination, in which genetic material is exchanged between homologs. Crossovers, or chiasmata, the physical manifestations of these sites of exchange, along with distal cohesion, provide these linkages, which allow homolog pairs to come under tension on the meiosis I spindle. However, in some organisms, such as male *Drosophila*, homologs are accurately segregated despite the absence of recombination. Second, sister-chromatids must remain linked until meiosis II. This is accomplished through stepwise loss of cohesion. Arm cohesion is lost during meiosis I, allowing segregation of homologs, and centromeric cohesion is lost during meiosis II, allowing segregation of sisters. Stepwise loss of cohesins requires that the mitotic cohesin subunit Scc1 be replaced by the meiosis-specific subunit Rec8. Third, sister-kinetochores must attach to spindle microtubules emanating from the same spindle pole during meiosis I, a process called co-orientation, ensuring that sisters travel to the same pole during this division. Finally, DNA replication must be suppressed between meiosis I and meiosis II (Marston and Amon, 2004).

Meiotic Entry

In *S. cerevisiae* meiotic entry reflects commitment to a developmental program leading to the formation of robust and stress-resistant spores. As such, it is a highly regulated

process controlled by multiple input signals. There are several requirements for meiotic commitment: the absence of glucose; the absence of a key nutrient, such as nitrogen; the presence of a non-fermentable carbon source; and cells must be diploid and respiration competent. These signals are integrated at the promoter of *IME1*, a transcription factor required for meiotic entry (Honigberg and Purnapatre, 2003; Kassir et al., 1988).

Figure 1

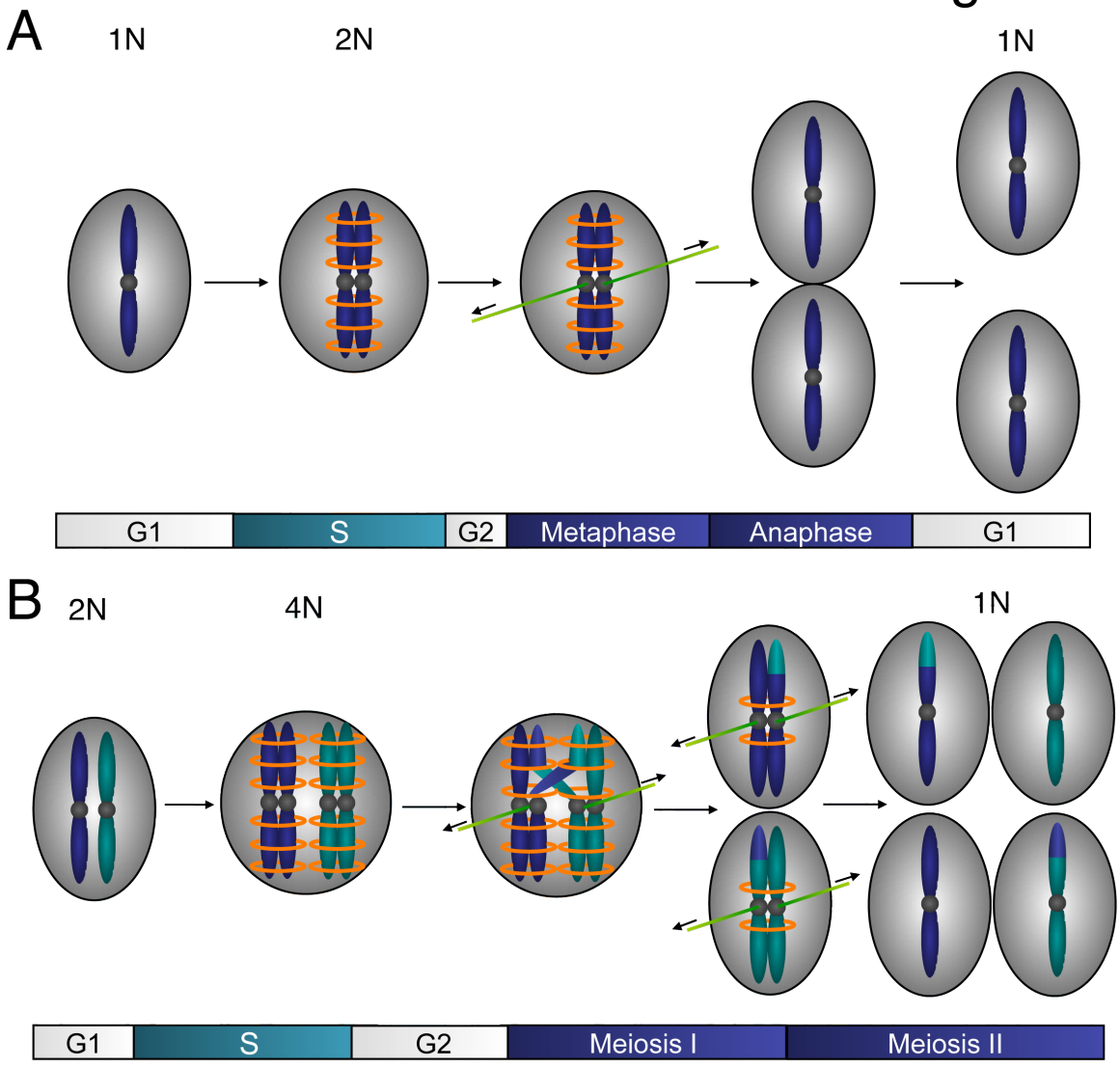

Figure 1: Mitotic Chromosome Segregation versus Meiotic Chromosome Segregation.

A) During S phase cohesins (orange rings) are loaded onto chromosomes, and physically link sister-chromatids. During metaphase sisters bi-orient on the spindle (green bars), and during anaphase sisters are pulled towards opposite poles.

B) During pre-meiotic S phase cohesins are loaded onto chromosomes. During meiotic prophase physical linkages between homologs are established through recombination. Homologous chromosomes are segregated away from each other during meiosis I, and sister chromatids are segregated away from each other during meiosis II.

The transcriptional repressor *RME1* prevents expression of *IME1* in haploid cells, thus restricting meiosis to diploid cells. This transcriptional repression is relieved in diploid cells through the action of the *MATa1* and *MATα2* gene products, which form a complex that represses *RME1* (Covitz et al., 1991). The *IME1* promoter also contains distinct regulatory sites for each of the nutritional stimuli above (Sagee et al., 1998). Ime1 activates transcription of early meiotic genes, which are involved in pre-meiotic DNA replication and recombination, including *IME2* a protein kinase required for entry into pre-meiotic S-phase (Chu et al., 1998; Primig et al., 2000). Interestingly, asynchrony in meiosis is predominantly due to variation in the timing of *IME1* expression, suggesting a populational bet-hedging strategy, preventing the entire population from committing to meiosis in response to transient environmental fluctuations (Nachman et al., 2007).

Though meiosis is well conserved, there is considerable divergence in the mechanisms regulating meiotic initiation. This is perhaps not surprising, given the varied environments and conditions under which meiosis is induced in eukaryotes. *S. cerevisiae*

evolved to undergo meiosis in response to nutrient-poor environmental conditions; however, other single-cellular organisms enter meiosis in response to a variety of environmental stimuli (Honigberg and Purnapatre, 2003). In multi-cellular organisms, the decision of a cell to undergo meiosis is largely controlled by stimuli from the surrounding tissues. In mouse for example, retinoic acid induces meiotic initiation through Stra8 (Anderson et al., 2008).

Pre-Meiotic DNA Replication

The general mechanisms regulating entry into both pre-mitotic and pre-meiotic S phase are similar, however the specific regulators used are different. In both mitosis and meiosis activation of Clb5, Clb6-Cyclin Dependent Kinases (CDKs) promotes entry into S phase. This activation occurs when the CDK inhibitor (CKI) Sic1 is phosphorylated and targeted for degradation. During G1 the accumulation of Cln-CDKs results in the phosphorylation of Sic1 and entry into pre-mitotic S phase. However, since Cln-CDKs prevent meiotic entry through repression of *IME1*, they cannot promote pre-meiotic S phase (Colomina et al., 1999). Instead the meiosis specific kinase Ime2 targets Sic1 for degradation (Dirick et al., 1998). Interestingly, in the absence of *CLB5* and *CLB6*, vegetative cells can initiate DNA replication due to the accumulation of other *CLBs*. However, in the absence of *CLB5* and *CLB6* meiotic cells cannot initiate DNA replication despite the accumulation of other *CLB* transcripts (Schwob and Nasmyth, 1993; Stuart and Witterberg, 1998; Dirick et al., 1998). The reason for these differences is not clear. *CLB1*, *CLB3*, and *CLB4* are targets of *NDT80*; perhaps cells are no longer competent to initiate DNA replication by the time *NDT80*-dependent transcription is activated.

The same mechanisms are used for both pre-mitotic and pre-meiotic replication initiation. Sequences at origins of replication are bound by the origin-recognition-complex, which recruits the replication factor Cdc6. Cdc6 then recruits the Mcm2-7 complex, the replicative helicase, to form the pre-replicative complex (pre-RC). Initiation of DNA replication depends upon phosphorylation of replication machinery components by both CDK and Dbf4-Dependent Kinase (DDK), a complex of the kinase Cdc7 and activating subunit Dbf4 (Bell and Duta, 2002). Pre-meiotic S phase depends on many of the same components as pre-mitotic S phase, such as *CDC6* (Ofir et al., 2004; Hochwagen et al., 2005). Interestingly, various studies have suggested that *CDC7* is not absolutely required for pre-meiotic DNA replication (Schild and Byers, 1978; Wan et al., 2006). However, the alleles used may be leaky, and thus may obscure a role for DDK in pre-meiotic DNA replication. DDK has also been shown to be required for a variety of post-replicative meiotic events, suggesting that DDK coordinates the ordered events of meiosis (Matos et al., 2008; Wan et al., 2008; Lo et al., 2008). The same origins of replication are used during both pre-mitotic and pre-meiotic DNA replication, and the rate of replication fork progression is the same (Collins and Newlon, 1994; Johnston et al., 1982). Despite these similarities in origin use and fork progression, pre-meiotic DNA replication takes two to three times as long as pre-mitotic DNA replication (Williamson et al., 1983). One explanation for this difference is based on the observation that passage through pre-meiotic DNA replication is required for later meiotic prophase events. For example, meiotic cohesins are loaded onto chromosomes during DNA replication, and are required for recombination (Klein et al., 1999). However, there is conflicting evidence as to

whether progression through S phase is required for double-strand break (DSB) formation (Borde et al., 2000; Hochwagen et al., 2005).

Meiotic Prophase

Perhaps one of the most striking differences between mitosis and meiosis is the occurrence of reciprocal recombination between homologs, which promotes their accurate disjunction at meiosis I and produces new combinations of alleles. Recombination occurs in concert and interdependently with the formation of a proteinaceous structure between homologs called the synaptonemal complex (SC). Below is a discussion of meiotic prophase events in *S. cerevisiae*.

Initiation of recombination occurs when the topoisomerase-like protein Spo11 catalyzes formation of DSBs in the genome (Keeney et al., 1997). Spo11 remains covalently linked to DNA, and its removal is dependent on Mre11, Rad50, and Xrs2 (Alani et al., 1990; Neale et al., 2005). Exonucleolytic resection of DSBs produces single-stranded 3' overhangs that are bound by Rad51 and Dmc1, which promote homolog-directed strand invasion and homology search (Bishop, 1994; Schwacha and Kleckner, 1997). DSBs are processed via two pathways, the first produces non-crossovers (NCOs), and the second produces crossovers (COs). The NCO pathway repairs DSBs via synthesis-dependent-strand-annealing, in which a resected end invades the homolog, and is ejected after a short patch of DNA synthesis. NCO formation does not lead to homolog linkage, and can sometimes be observed as gene conversion events. The CO pathway proceeds via stable invasion of the homolog by a resected end. Repair leads to the formation of a Double

Holliday Junction (DHJ), which is resolved as a CO (Figure 2). The presence of alternate DSB repair pathways is supported by *zmm* mutants, in which CO levels are reduced, despite high DSB and NCO levels (Bishop and Zickler, 2004; Börner et al., 2004).

During meiotic prophase chromosomes condense and pair, SCs form between homologs, and recombination occurs. These processes are intimately linked and are interdependent. SC consists of lateral elements (LEs), which include Hop1 and Red1, and form along individual chromosomes, joined by central elements (CEs), consisting of Zip1, that join the LEs along the length of a homolog pair. SC morphogenesis begins with the formation of LEs along the length of individual chromosomes during leptotene, and is dependent on meiotic cohesins (Klein et al., 1999). Interhomolog interactions that are destined to become COs mature into axial associations, and nucleate the formation of SC between homologs, which eventually forms along the entire length of the homolog pair (Page and Hawley, 2004; Zickler and Kleckner, 1999). The ZMM proteins Zip1, Zip2, Zip3, Mer3, and Msh5 are required for both processes, demonstrating that recombination and SC formation are intimately linked (Bishop and Zickler, 2004, Börner et al., 2004). Finally, upon exit from prophase DHJs are resolved into COs, and SC is disassembled.

Figure 2

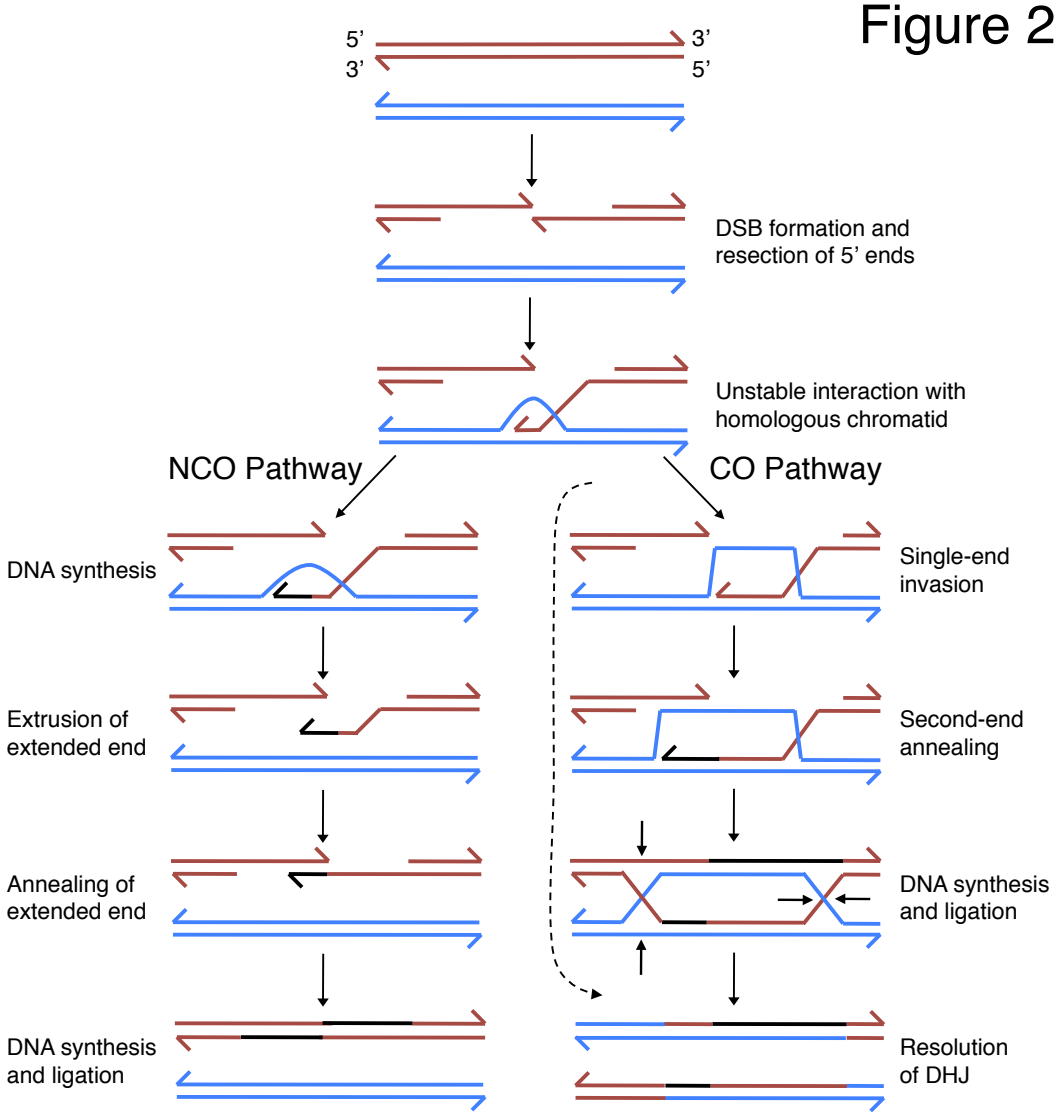

Figure 2: Meiotic Recombination

DSBs are processed via two pathways, which result in the formation of two types of products, NCOs and COs. In COs flanking sequences are exchanged, while flanking sequences in NCOs have the parental configuration. The major CO pathway proceeds through a DHJ intermediate, while the minor CO pathway (dashed arrow) may not. Adapted from (Bishop and Zickler, 2004).

Recombination involves the regulated introduction of damage to the genome. The pachytene checkpoint prevents meiotic progression until DSBs are repaired and synapsis is complete. Components of the pachytene checkpoint that sense and signal DNA damage are shared with the mitotic DNA damage checkpoint, including Rad24, Rad17, Mec3, Ddc1 and Mec1, but not Rad9, Rad53 or Chk1. Other proteins required for checkpoint function include the chromosomal proteins Red1 and Hop1, the silencing factors Sir2 and Pch2, the protein phosphatase Glc7, and the proline isomerase Fpr3. Checkpoint activation impinges upon two factors necessary for progression into the meiotic divisions, CDK (through the inhibitory kinase Swe1), and Ndt80 (Roeder and Bailis, 2000; Hochwagen et al., 2005).

Meiosis I Chromosome Segregation

Entry into the meiotic divisions requires the activity of the transcription factor Ndt80, which activates expression of meiotic middle genes allowing progression into the meiotic divisions. Key Ndt80 targets include the cyclins *CLB1*, *CLB3*, *CLB4*, and the polo-like kinase *CDC5* whose expression allows sufficient CDK activity to progress through both meiotic divisions (Chu et al., 1998; Chu and Herskowitz, 1998; Hepworth et al., 1998).

During meiosis I homologs are segregated away from each other, meaning sister-chromatids are co-segregated. Accurate homolog segregation requires linkages between homologs, which are conferred by recombination, and cohesion distal to crossovers. In cells that fail to undergo recombination homologs randomly segregate at meiosis I (Figure 3A; Keeney et al., 1997; Klein et al., 1999). During metaphase I the kinetochore-localized monopolin complex, consisting of the proteins Mam1, Lrs4, Csm1, and Hrr25,

mediates co-orientation of sister-kinetochores (Figure 3B; Tóth et al., 2000; Rabitsch et al., 2003; Petronczki et al., 2006). Monopolin is thought to act by limiting the attachment of spindle mictrotubules to one per pair of sister-kinetochores (Winey et al., 2005; Monje-Casas et al., 2007). Spo13, a meiosis specific protein, and Cdc5, the polo-like kinase, promote co-orientation by recruiting and maintaining monopolin at kinetochores (Lee and Amon, 2003; Katis et al., 2004b; Lee et al., 2004). The kinase Aurora B/Ipl1 destabilizes kinetochore-microtubule attachments that fail to generate tension on the meiosis I spindle, thus promoting attachment of homologs to opposite spindle poles (Monje-Casas et al., 2007). Once all homolog pairs are properly attached to the metaphase I spindle the anaphase promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin-ligase, with its specificity factor Cdc20 promotes anaphase entry. The key APC/C^{Cdc20} target is Pds1 (securin), an inhibitor of Esp1 (separase), the protease that cleaves the Rec8 subunit of cohesins. The Esp1-mediated removal of arm-cohesins distal to crossovers allows homolog segregation at the metaphase I to anaphase I transition (Buonomo et al., 2000). Accurate disjunction of sister-chromatids at meiosis II requires that centromeric-cohesins be retained until metaphase II. Protection of centromeric-cohesins is mediated by MEI-S332/Sgo1, which localizes to kinetochores and pericentric chromatin during meiosis I, and recruits protein phosphatase 2A, protecting centromeric-cohesins from removal, thus allowing sister-chromatids to remain linked until meiosis II (Figure 3C; Kerrebrock et al., 1995; Kitajima et al., 2004; Katis et al., 2004a; Marston et al., 2004; Kiburz et al., 2005; Riedel et al., 2006). These specializations ensure segregation of homologs at meiosis I, and linkage of sister-chromatids until meiosis II.

The Meiosis I to Meiosis II Transition

The transition from meiosis I to meiosis II is unique in that two consecutive rounds of chromosome segregation occur without intervening DNA replication. This transition is less well studied than the mechanisms governing meiotic chromosome segregation. However, it is clear that the cell must accomplish two seemingly contradictory tasks: the cell must disassemble its spindle, a process requiring low CDK activity, and it must prevent DNA re-replication, a process requiring high CDK activity. Work in *Xenopus* suggests that the cell performs a balancing act by inactivating CDKs enough to allow spindle disassembly, but preserving enough CDK activity to inhibit the reformation of pre-RCs (Furuno et al., 1994; Iwabuchi et al., 2000). The evidence for regulation of this transition in budding yeast by modulation of CDK activity is discussed below.

Meiosis II Chromosome Segregation

During meiosis II sister-chromatids are segregated away from each other, in this respect meiosis II is a mitosis-like division. Mam1 disappears from chromosomes at the metaphase I to anaphase I transition, allowing sister-chromatids to bi-orient on the meiosis II spindle (Toth et al., 2000). Aurora B/Ipl1 promotes proper attachment of sister-kinetochores to the meiosis II spindle by destabilizing kinetochore-microtubule attachments that fail to generate tension (Monje-Casas et al., 2007). Interestingly, Sgo1 is removed from chromosomes at the metaphase II to anaphase II transition, and may serve to help bias sister-chromatids towards bi-orientation (Katis et al., 2004a; Kiburz et al., 2008). Once all chromosomes are properly attached to the metaphase II spindle the

APC^{Cdc20} promotes Esp1-mediated cleavage of centromeric-cohesins allowing segregation of sister-chromatids.

Spore Formation and Morphogenesis

In budding yeast after completion of meiosis each of the four nuclei are packaged into spores, and remain enclosed in the mother cell, which matures into an ascus that holds them together in a tetrad. During meiosis II, the outer plaque of spindle pole bodies (SPBs) is modified. These modifications allow recruitment of vesicles that fuse to form double layered prospore membranes, which expand during meiosis II, and enclose each nucleus after the meiosis II division. After membrane closure, the spore wall is deposited between the layers of the prospore membrane. During this process the outer prospore membrane disappears, leaving the inner prospore membrane, which serves as the spore plasma membrane. Maturation of the ascus results in the formation of a mature tetrad (Neiman, 2005).

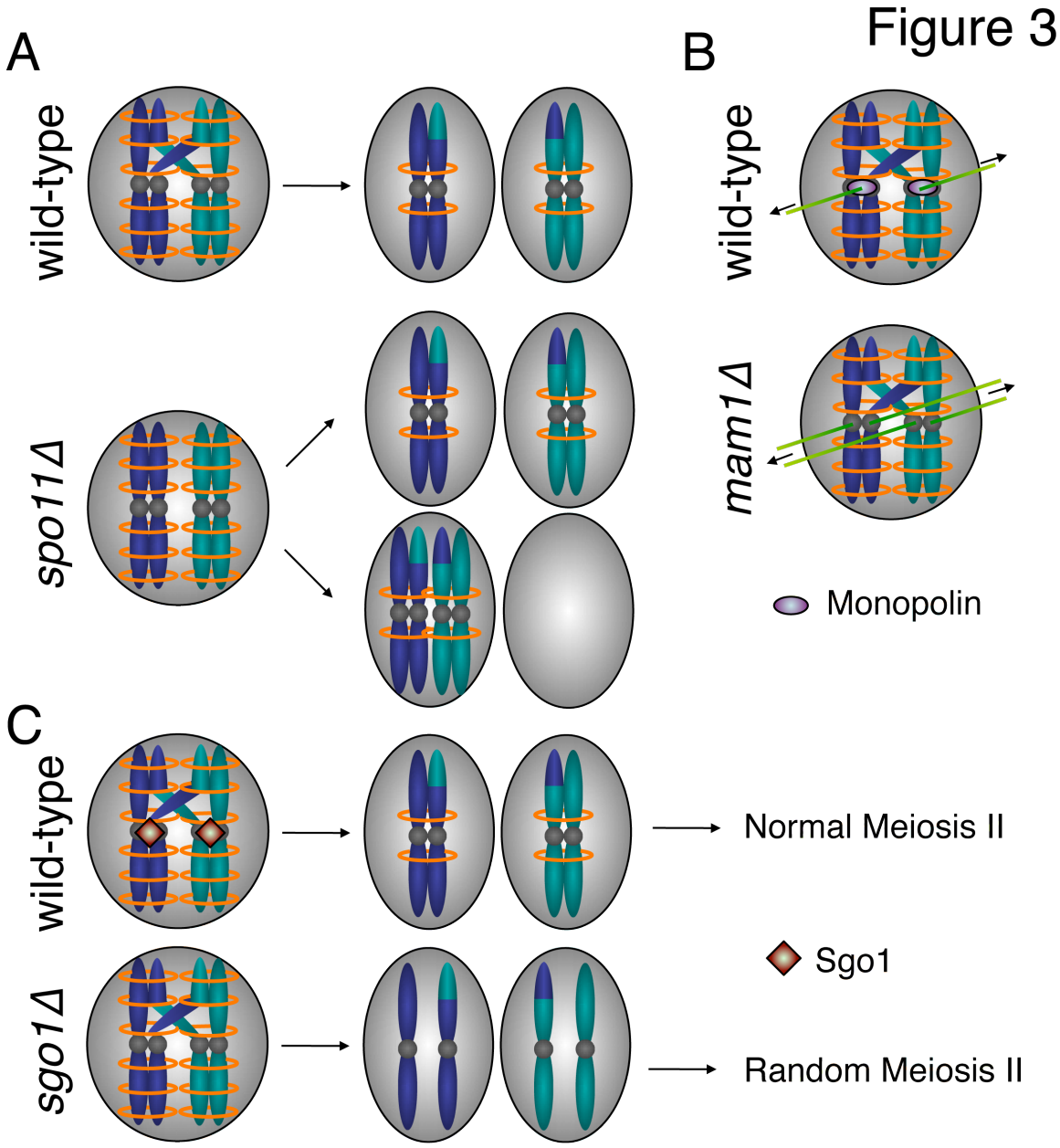

Figure 3: Specializations allowing Meiotic Chromosome segregation.

A) Linkages between homologs allow their accurate segregation at MI. In cells defective for recombination (*spo11*Δ) the absence of linkages causes random segregation at MI. B) The monopolin complex forces co-orientation of sisters during MI. In monopolin mutants (*mam1*Δ) sisters bi-orient during MI. C) Sgo1 protects centromeric cohesion during MI. In *sgo1*Δ mutants centromeric cohesion is lost during MI, and the absence of cohesion during MII causes the random segregation of sisters at this division.

Cyclin-Dependent Kinases

The mitotic cell cycle, at its heart, consists of alternating rounds of genome duplication and chromosome segregation. CDKs, the underlying oscillator, drive these events. CDK was identified as Maturation/Mitosis Promoting Factor (MPF), a protein that promoted mitosis in *Xenopus* oocyte extracts (Masui and Markert, 1971). Cyclins were discovered as proteins that cyclically appeared and disappeared with cell divisions in sea urchin embryos, suggesting their involvement in the cell cycle (Evans et al., 1983). A large body of subsequent work demonstrated that MPF was a kinase whose activity depended on cyclin binding (Jackson, 2008). Below is a discussion of the regulation of CDKs, and an overview of the events that they regulate during both mitosis and meiosis in *S. cerevisiae*.

Cyclin-Dependent Kinases in Budding Yeast

CDKs in budding yeast consist of a single CDK, Cdc28 (also called Cdk1), which is bound and activated by nine cyclin subunits (Hartwell et al., 1973; Reed et al., 1985; Richardson et al., 1989). There are three G1 cyclins, *CLN1* to *CLN3*, and six B-type cyclins, *CLB1* to *CLB6*, which can be grouped into three pairs according to similarity, timing of expression, and function: *CLB1* to *CLB2*, *CLB3* to *CLB4*, and *CLB5* to *CLB6*. In this text Clb5 and Clb6 will be referred to as S phase Clb-CDKs, and Clb1 to Clb4 will be referred to as M phase Clb-CDKs (Figure 4A). During meiosis Cdc28 is activated by all Clbs, except Clb2, and Cln-CDKs are replaced by a monomeric kinase, Ime2 (Figure 4B).

Activation of CDK during G1 and progression into the cell cycle depends on Cln1, Cln2, and Cln3 (Hadwiger et al., 1989; Cross, 1988; Nash et al., 1988). After entry into the cell cycle, activation of CDK depends on successive waves of *CLB* cyclins. The cyclins *CLB5* and *CLB6* are first expressed in late G1, and become active at the G1 to S transition (Schwob and Nasmyth, 1993). *CLB3* and *CLB4* appear near the beginning of S phase, and *CLB1* and *CLB2* appear during mitosis (Ghiara et al., 1991; Surana et al., 1991; Fitch et al., 1992; Richardson et al., 1992). Below is a discussion of CDK regulation, followed by a discussion of the regulation of mitotic and meiotic events.

Regulation of Cyclin-Dependent Kinases

As a key component of the cell cycle machinery CDK activity is highly regulated, especially given the relatively constant levels of Cdc28 present through the cell cycle. There are three major modes of regulation that take place: by the binding of cyclin subunits to Cdc28, which are themselves regulated at the levels of synthesis transcriptionally and translationally, and, predominantly, at the level of degradation; positive and negative regulation of Cdc28 by phosphorylation; and by binding of cyclin-Cdc28 complexes by CDK Inhibitors (CKIs).

The most striking level of regulation of CDK activity is its activation by cyclin binding (Figure 5b). The formation of the cyclin-CDK complex is largely regulated by the presence or absence of a particular cyclin. Structural work on mammalian CyclinA-Cdk2 reveals that cyclin binding activates CDK by contributing to the proper positioning of a catalytic glutamic acid residue, and by positioning the T-loop of CDK away from the

catalytic cleft (Pavletich, 1999). In addition to cyclin binding, full activation of Cdc28 requires phosphorylation on Thr169 of the T-loop by the CDK activating kinase (CAK), Cak1 (Figure 5a; Mendenhall and Hodge, 1998).

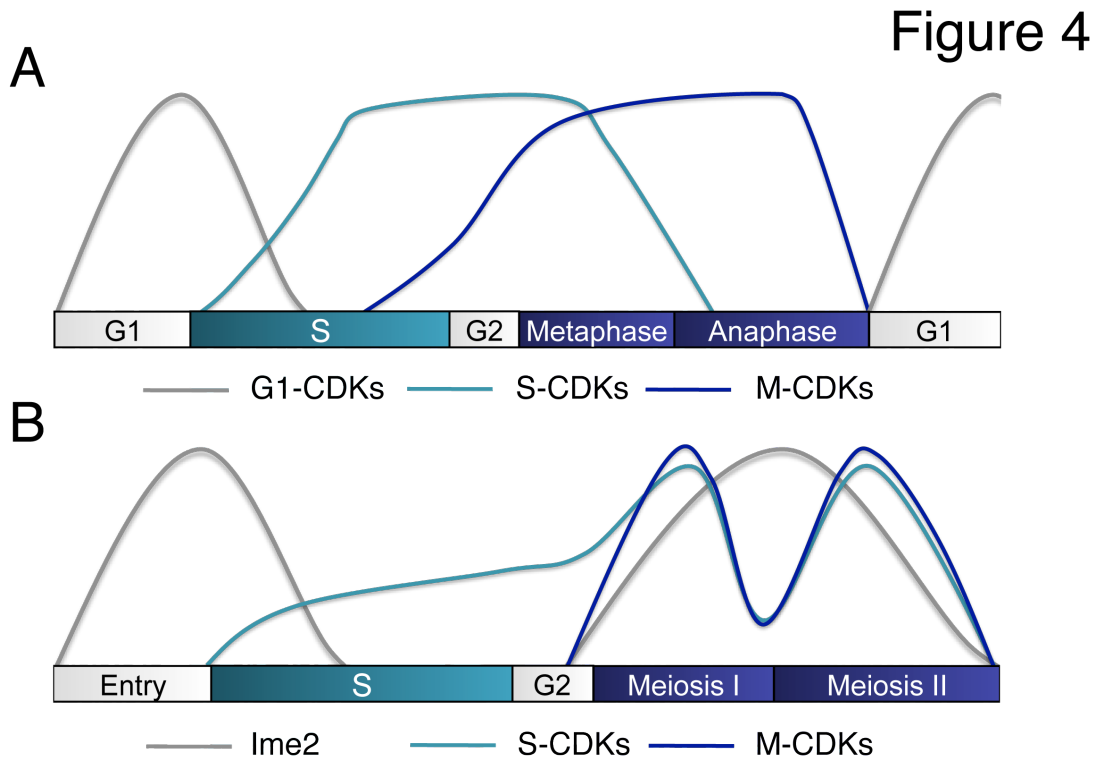

Figure 4: CDK Activity During Mitosis and Meiosis

A) G1 cyclins accumulate during G1 and lead to the G1 to S transition. S phase CDKs are activated in S phase, and persist until the metaphase-anaphase transition. Clb3 and Clb4 appear during S phase, and Clb1 and Clb2 appear during mitosis. M phase CDKs are inactivated during mitotic exit. B) Ime2 controls entry into pre-meiotic S phase, and has another peak of activity during the divisions. S phase CDKs appear during pre-meiotic S phase, and accumulate further during the divisions. The M phase CDKs Clb1, Clb3, and Clb4 appear during the divisions. Clb-CDK activity is thought to have two peaks during meiosis, one during M I and one during M II. Adapted from (Marston and Amon, 2004).

CDK is also negatively regulated by phosphorylation, and by the binding of CKIs. However, inhibition of CDK by phosphorylation plays a much more important role in cell cycle control in other organisms than it does in *S. cerevisiae*. Phosphorylation of residues corresponding to Thr18 and Tyr19 of Cdc28 exerts an inhibitory effect upon CDK. In S. *pombe* this regulates mitotic entry, is mediated by the kinase wee1+ (Swe1 in *S. cerevisiae*), and is reversed by the phosphatase cdc25+ (Mih1 in *S. cerevisiae*). In budding yeast Swe1 and Mih1 regulate CDK activity in response to defects in bud formation (Figure 5c; Mendenhall and Hodge, 1998). Binding of the two budding yeast CKIs, Sic1 and Far1, potently inhibit CDKs (Figure 5d). Far1 inhibits Cln-CDK complexes, preventing entry into the cell cycle in response to mating pheromone (Tyers and Futcher, 1993; Peter and Herskowitz, 1994; Jeoung et al., 1998). Sic1 inhibits entry into S-phase by restraining the activity of Clb-CDK complexes (Mendenhall, 1993).

Although the regulation of CDKs plays an important role in cell cycle control, in an unperturbed cell cycle the most striking regulation of kinase activity occurs at the level of synthesis and degradation of the cyclin subunits. Though the accumulation of *CLN3* transcript is not strongly periodic, Cln3 protein accumulation is regulated by a small upstream open reading frame (uORF) in the 5'UTR of the message, which negatively affects the translational efficiency of the message in nutrient poor conditions, thus linking cell growth to the cell cycle (Polymenis and Schmidt, 1997). For the rest of the cyclins, regulation of accumulation occurs at a transcriptional level, which is itself controlled by CDKs. The accumulation of early cyclin-CDKs activates expression of later cyclins, and

late cyclin-CDK activity inhibits expression of early cyclins (Figure 5e-f; Mendenhall and Hodge, 1998; Bloom and Cross, 2007).

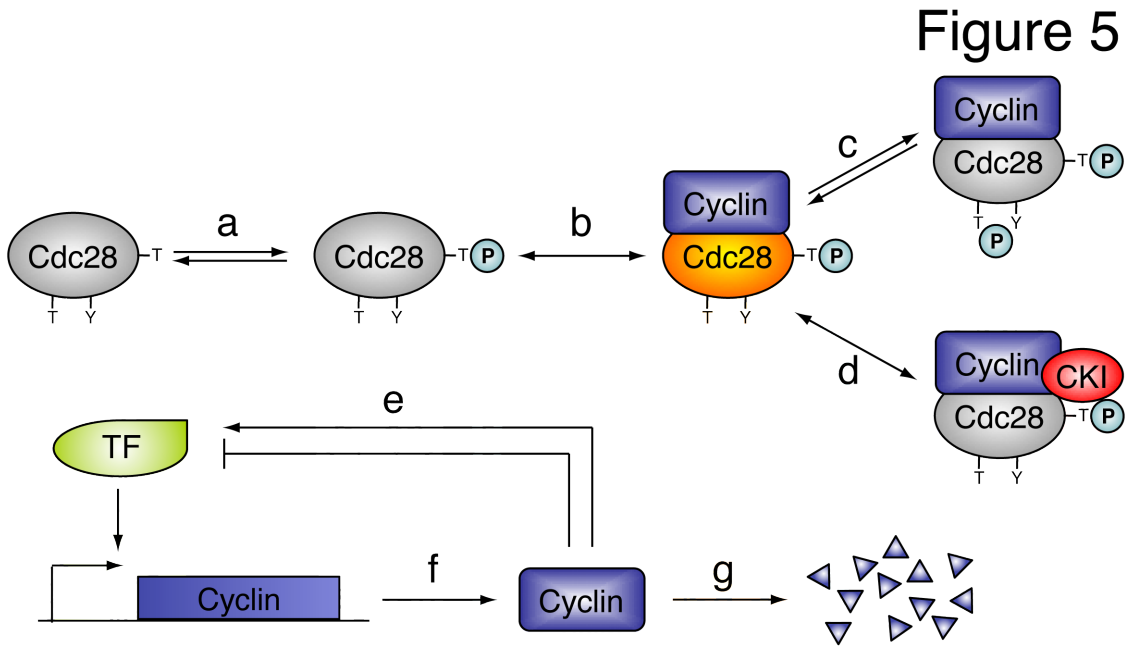

Figure 5: Regulation of Cyclin-CDKs

This figure illustrates the modes of regulation of Cyclin-CDKs. Inactive Cdc28 is represented in grey, active in orange. a) Phosphorylation Cdc28 on of Thr169 by Cak1 is required for activation, b) as is cyclin binding. CDKs are also negatively regulated in two ways: c) first inhibitory phosphorylation on Thr18 and Tyr19 is mediated by Swe1, and is reversed by Mih1, and d) second cyclin-CDK complexes are bound and inhibited by CKIs. Cyclin abundance is also regulated in three ways: e) both positive and negative signals act at the level of cyclin transcription, and are often regulated by CDKs f) the accumulation of protein is regulated for some, but not all cyclins, and g) cyclins are targeted for ubiquitin-mediated degradation by either the APC or the SCF, depending upon the cyclin. Adapted from (Morgan, 1995).

CDK inactivation is an important step in cell cycle control, and regulated degradation by ubiquitin-mediated proteolysis is largely how inactivation is accomplished (Figure 5g). There are two major E3 ligases, the APC/C and the SCF, each activated by different specificity factors. APC/C mediated degradation is modulated by specificity factor regulation, and SCF mediated degradation is modulated by substrate phosphorylation (generally by CDKs). SCF targets include Cln3, Cln1, Cln2, and Clb6. While SCF^{Grr1} targets Cln1 and Cln2, and SCF^{Cdc4} targets Clb6, the adaptor protein for Cln3 is not known. The E3 responsible for Clb4 degradation has not been identified; however, Clb1-Clb3, and Clb5 are APC/C targets. At the metaphase-anaphase transition the APC/C^{Cdc20} targets Clb5 and a subset of Clb2, and at exit from mitosis the APC/C^{Cdh1} targets Clb1, Clb3, and the remaining Clb2 (Barral et al., 1995; Yaglom et al., 1995; Irniger and Nasmyth, 1997; Skowyra et al., 1997; Shirayama et al., 1999; Schwab et al., 2001; Jackson et al., 2006). Given the importance of CDKs in proper timing of cell cycle it is not surprising that the degradation machinery itself is under tight control.

The Control of Mitotic Events by Cyclin-Dependent Kinases

The ordered progression of the cell cycle is driven by both increasing CDK activity and the sequential accumulation of different cyclin-CDK complexes. CDKs control major cell cycle events including DNA replication, SPB duplication, spindle formation, and chromosome segregation, as well as regulating other cellular events (Figure 6A).

G1 is characterized by low Clb-CDK activity, and the G1 to S transition is marked by activation of Clb-CDKs. Growth dependent accumulation of Cln3-CDKs results in the

phosphorylation and nuclear exit of Whi5, an inhibitor of G1 transcription, allowing *CLN1* and *CLN2* expression (Costanzo et al., 2004; de Bruin et al., 2004). Cln1, Cln2-CDKs phosphorylate the CKI, Sic1, during late G1, which targets it for SCF^{Cdc4} mediated degradation, thus allowing activation of Clb5, Clb6-CDKs and entry into the cell cycle (Mendenhall, 1993; Nash et al., 2001).

DNA replication is controlled by Clb-CDKs in two ways: in a direct fashion, and in a regulatory fashion. Most proximally, Clb5, Clb6-CDKs phosphorylate replication factors including Sld2, and Sld3, allowing recruitment of DNA polymerases to origins (Bloom and Cross, 2007; Tanaka et al., 2007; Zegerman and Diffley, 2007). Additionally, Clb-CDKs ensure that DNA replication occurs only once per cell cycle. Clb-CDK activity, through a variety of mechanisms, inhibits pre-RC formation, thus ensuring that pre-RCs form only in G1, limiting DNA replication to once per cell cycle (Bell and Dutta, 2002).

CDKs also regulate SPB duplication and separation, and mitotic spindle assembly. Phosphorylation of the SPB component Spc42, and Mps1, a kinase required for SPB duplication, by Cln1, Cln2-CDKs promotes SPB duplication during G1 (Jaspersen et al., 2004). Additionally, through mechanisms that are not clear, M and S phase Clb-CDKs promote SPB maturation and separation, and M phase Clb-CDKs, but not S phase Clb-CDKs, prevent SPB reduplication (Haase et al., 2001).

CDKs also regulate spindle formation, morphogenesis, and chromosome segregation. M phase Clb-CDKs control the association of Kar9 and Dyn1, proteins involved in spindle

orientation and positioning, with SPBs (Bloom and Cross, 2007). Clb-CDKs phosphorylate and inhibit the APC subunit Cdh1, thus preventing APC^{Cdh1} mediated proteolysis of the microtubule associated proteins Cin8, Kip1, and Ase1, the stabilization of which allows spindle assembly to occur (Crasta et al., 2006; Crasta et al, 2008). Additionally, M phase Clb-CDKs promote spindle elongation, and trigger anaphase through activation of the APC^{Cdc20} (Rahal and Amon, 2008).

Mitotic exit and entry into the next cell cycle requires inactivation of Clb-CDKs. During anaphase two networks, the Cdc14 Early Anaphase Release (FEAR) network and the Mitotic Exit Network (MEN), promote the release of the phosphatase Cdc14 from its inhibitor Cfi1/Net1. Once released Cdc14 dephosphorylates and stabilizes Sic1, and dephosphorylates and activates Cdh1, allowing Clb-CDK inactivation (Stegmeier and Amon, 2004). Ultimately accumulation of Clb-CDK activity triggers Clb destruction. M-phase Clb-CDKs trigger anaphase entry (which promotes MEN activation) and phosphorylate Cfi1/Net1, promoting the release of active Cdc14 (Stegmeier and Amon, 2004; Bloom and Cross, 2007).

The Control of Meiotic Events by Cyclin-Dependent Kinases

Compared to mitosis, there is a paucity of knowledge about how CDKs promote and regulate meiotic events. Though it is largely assumed that they promote analogous events by the same general mechanisms, and it has long been known that genes involved in mitosis, including *CDC28*, play important roles in meiosis (Shuster and Byers, 1989). One of the most obvious differences in CDK regulation between mitosis and meiosis

occurs at the level of cyclin expression. The *CLN* cyclins are not expressed during meiosis, and their functions are replaced by the protein kinase Ime2. The timing of early expression of *CLB5* and *CLB6* is similar to mitosis. However, regulation of expression of the M phase cyclins *CLB1*, *CLB2*, *CLB3*, and *CLB4* is quite different. *CLB2* is not expressed during meiosis, and *CLB1*, *CLB3*, and *CLB4* are expressed as cells enter the meiotic divisions in an *NDT80*-dependent manner. Additionally, *CLB5* and *CLB6* are also transcriptionally up-regulated in an *NDT80*-dependent fashion as cells enter the meiotic divisions (Figure 6B; Grandin and Reed, 1993; Chu et al., 1998; Primig et al., 2000).

While Ime2 is essential for entry into pre-meiotic S, Clb5, Clb6-CDKs also promote pre-meiotic DNA replication. Though their essential targets during meiosis are unknown, it seems likely that the mitotic and meiotic targets would be the same (Stuart and Witterberg, 1998; Dirick et al., 1998; Benjamin et al., 2003). Additionally, Clb5, Clb6-CDKs phosphorylate Mer2, a component of the recombination machinery, a step that is essential for DSB formation (Henderson et al., 2006).

Interestingly, SPB duplication occurs twice during meiosis, once prior to each division. However, little is known about how these duplication events are regulated with respect to meiotic CDK activity. Meiosis I SPBs resemble mitotic SPBs ultrastructurally; however, meiosis II SPBs have a structurally distinct outer plaque (Jaspersen and Winey, 2004; Neiman, 2005). The first round of meiotic SPB duplication presumably occurs concomitant with pre-meiotic DNA replication, and it seems likely that Ime2 might be required in analogy to the roles of Cln-CDKs. Little is known about the regulation of the

second round of SPB duplication. Some *IME2* mutants fail to undergo meiosis II, consistent with a role for Ime2 in promoting this duplication event (Benjamin et al., 2003). Additionally, it seems that down-regulation of CDK activity between meiosis I and meiosis II is required for the second round of SPB duplication to occur. FEAR network mutants, which fail to down-regulate CDKs between meiosis I and meiosis II, show a varied ability to undergo the second round of SPB duplication. *spo12*Δ mutant cells largely fail to form four SPBs, while the majority of *slk19*Δ mutant cells form four SPBs (Buonomo et al., 2003). These results suggest that CDK down-regulation during the transition may play a role in the second round of SPB duplication. However, further experiments are needed to fully elucidate the role of CDK down-regulation in the second round of SPB duplication. As in mitosis, both rounds of SPB separation require Clb-CDKs (Shuster and Byers, 1989).

CDKs are also key regulators of meiotic chromosome segregation, and spindle dynamics. The mechanisms by which CDKs promote and regulate these events in meiosis are unknown, however they are likely similar to those in mitosis. Clb-CDK activity is required for exit from the pachytene stage of meiosis and entry into the meiotic divisions (Shuster and Byers, 1989; Benjamin et al., 2003). Clb-CDK activity is also required for meiosis II, as *clb1*Δ *clb3*Δ *clb4*Δ undergo meiosis I but not meiosis II (Dahmann and Futcher, 1995). Ime2 activates *NDT80* transcription, and phosphorylates Ndt80, which promotes entry into meiosis I. Additionally, Ime2 is required for meiosis II (Benjamin et al., 20003). Thus, Clb-CDKs and Ime2 promote both meiotic divisions.

Figure 6

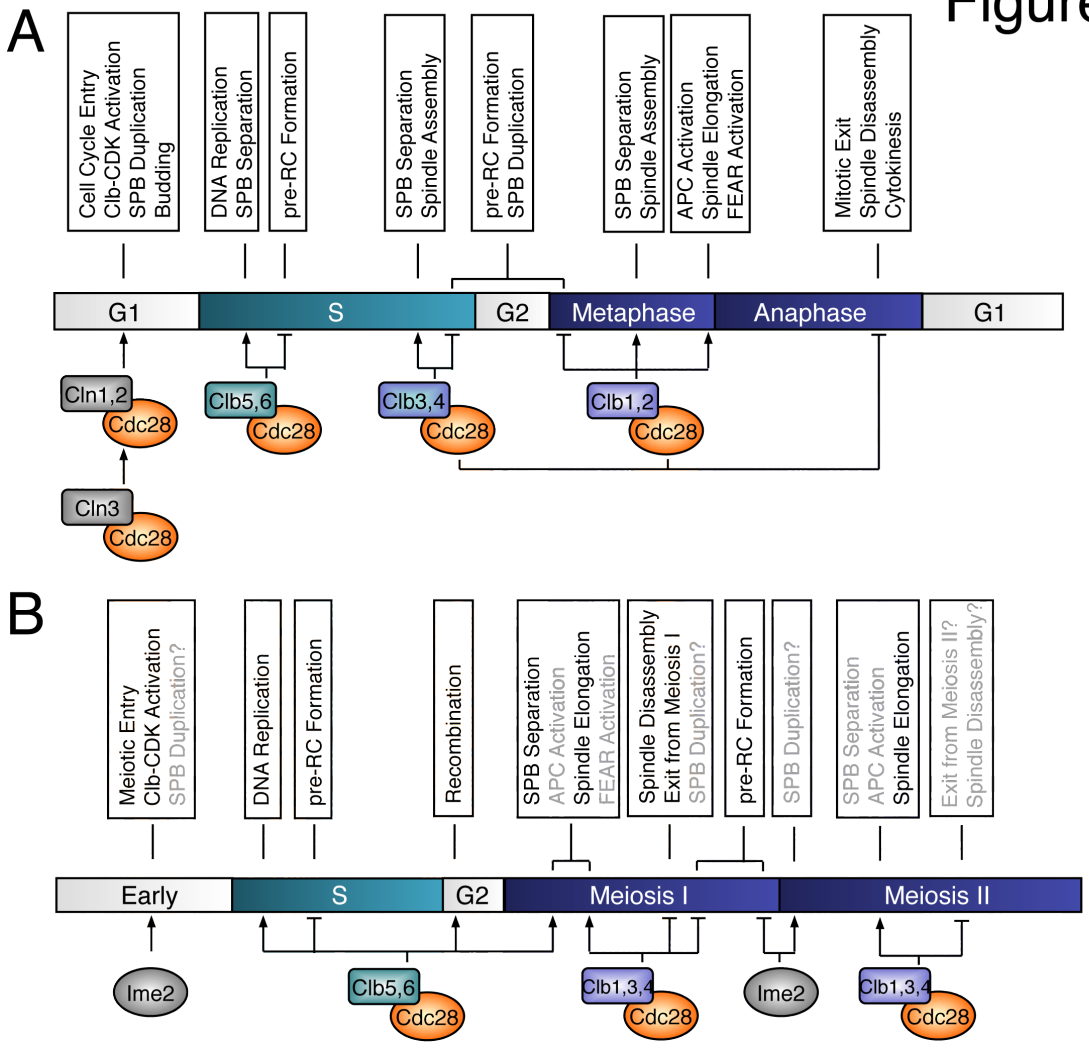

Figure 6: Regulation of Mitotic and Meiotic Events by CDKs

A) A schematic of the mitotic events that are controlled by different cyclin-CDK complexes. Events promoted by CDKs are indicated with arrows, and events inhibited by CDKs are indicated with T-arrows.

B) A schematic of the meiotic events that are controlled by different cyclin-CDK complexes. Events promoted by CDKs are indicated with arrows, and events inhibited by CDKs are indicated with T-arrows. Events indicated in grey are assumed to be regulated by CDKs based on analogy to mitosis, and those events that are more speculative are indicated by a "?".

Clb-CDKs also regulate the meiosis I to meiosis II transition. Several observations suggest that in *S. cerevisiae* CDKs play an important role in the meiosis I to meiosis II transition. First, *clb1*Δ*clb3*Δ*clb4*Δ mutants undergo meiosis I, but not meiosis II (Dahmann and Futcher, 1995). Second, meiotic expression of a non-degradable cyclin prevents meiosis I spindle disassembly (Marston et al., 2003). Finally, the FEAR network but not the MEN is required for this transition (Buonomo et al., 2003; Marston et al., 2003; Kamieniecki et al., 2005). Since the MEN is a more potent Cdc14 activator than the FEAR network this suggests that CDK activity may be only partially down-regulated between meiosis I and meiosis II, or may be inactivated for less time than is required for complete exit. Additionally, Ime2 is both required for the meiosis I to meiosis II transition, and seems to exert an inhibitory effect on Cdh1, and on pre-RC formation similar to that of CDK (Bolte et al., 2002; Benjamin et al., 2003; Holt et al., 2007). The full range of mechanisms by which CDK and Ime2 collaborate to prevent pre-RC reformation and DNA rereplication between meiosis I and meiosis II has yet to be determined.

Cyclin Specificity

There is strong evidence for differences in specificity between Cln-CDKs and Clb-CDKs, and there is also evidence for specialization of Clb-CDKs. There are four general mechanisms by which cyclin specificity could be achieved: differential timing of expression, differential subcellular localization, differential susceptibility to inhibition, and differences in intrinsic substrate specificity. The timing of cyclin expression can

explain much of the observed cyclin specificity, however it is clear this cannot explain all observed differences in Clb-CDK function.

Differential function of Clb2-CDKs and Clb5-CDKs is one of the most well studied examples of cyclin specificity. Deletion of both *CLB5* and *CLB6* leads to a delay in pre-mitotic DNA replication (Schwob and Nasmyth, 1993). One explanation for these results is that this replication delay is due to a delay in accumulation of sufficient Clb-CDK activity. However, replacing *CLB5* with *CLB2* allows early accumulation of Clb-CDKs, but fails to rescue the replication defect, indicating that Clb5-CDK has an intrinsic ability to promote DNA replication that Clb2-CDK lacks (Cross et al., 1999). Indeed, Clb5-CDKs are better able to phosphorylate substrates involved in DNA replication than are Clb2-CDKs, and this difference is conferred by a hydrophobic patch on Clb5 (Loog and Morgan, 2005). There are many other examples of cyclin specificity, for an excellent review see (Bloom and Cross, 2007).

Translational Regulation of Cyclins

In budding yeast *cyclin* transcription is periodic, but cyclin protein levels are regulated predominantly post-translationally by regulated proteolysis. However, there are examples of translational regulation of cyclins in budding yeast and higher eukaryotes. In yeast, *CLN3* translation is regulated by an uORF, which serves to link cell cycle entry with cell growth, and in higher eukaryotes *cyclin* translation is regulated during meiosis. The paragraphs below will discuss translation initiation, translational regulation in yeast, and regulation of *cyclin* translation during meiosis in higher eukaryotes.

Translational Regulation

Regulation of gene expression occurs predominantly at the level of transcription; however, translational regulation is employed in various circumstances, or for various genes to further control protein accumulation. This can allow for regulation in response to changes in the cell's translational capacity, and can allow for rapid changes in protein accumulation. Below is an overview of the process of translation, a discussion of known mechanisms of translational regulation in *S. cerevisiae*, followed by a discussion of translational regulation during meiosis in higher eukaryotes. One key difference between translational regulation in budding yeast and translational regulation in higher eukaryotes is that in budding yeast *cis* regulatory sequences are more commonly located in the 5'UTR of the message, while in higher eukaryotes *cis* regulatory sequences are more commonly located in the 3'UTR of the message.

Translational Initiation, Elongation and Termination

Translation can be divided into three phases: initiation, elongation, and termination. Initiation is a multistep process that involves binding of initiation factors (IFs) to the mRNA to be translated (Figure 7a), binding of IFs to the 40S ribosomal subunit to form the pre-initiation complex (PIC) (Figure 7b), assembly of the PIC and activated mRNA into a complex (Figure 7c), ribosomal scanning, recognition of the cognate start codon by the PIC, and 60S subunit joining. The PIC consists of the 40S ribosomal subunit, eIF1, eIF1A, eIF3, eIF5, and the ternary complex (TC) consisting of the initiator tRNA (Met-$tRNA_i^{Met}$)•eIF2•GTP. The m^7G cap of the mRNA is bound by eIF4F, a complex of eIF4A (DEAD-box helicase), eIF4E (cap-binding protein), and eIF4G. Binding of the cap by

eIF4F, and the poly(A) tail by poly(A) binding protein (PABP) facilitates eIF4B binding and mRNA circularization (Figure 7a). Interactions between eIF3/eIF5 in the PIC and mRNA bound eIF4G/eIF4B facilitate assembly of the PIC and mRNA into the 48S complex (Figure 7c). eIF1/eIF1A promote 5'UTR scanning, and proper start codon selection. Messages with structured 5'UTRs require the helicase activity of eIF4A for ATP dependent scanning (Figure 7d). When an AUG codon is encountered, scanning halts, and the TC hydrolyzes GTP, promoting dissociation of eIF2•GDP, Pi, eIF1, eIF3 and eIF5 (Figure 7e). eIF1A then recruits eIF5B•GTP, which hydrolyzes GTP, resulting in 60S subunit joining and release of eIF1A and eIF5B, leaving the Met-$tRNA_i^{Met}$ in the P site of the ribosome (Figure 7f; Soneneberg and Hinnebusch, 2009).

During elongation aminio-acyl tRNAs (aa-tRNAs) are delivered to the vacant A site of the ribosome complex consisting of aa-tRNA•eEF1A•GTP. Codon-anticodon base pairing activate GTP hydrolysis by eEF1A, allowing dissociation of eEF1A•GDP, leaving the aa-tRNA in the A site. The ribosomal peptidyl transferase activity then catalyzes transfer of the nascent peptide to the aa-tRNA in the A site. eEF2 then catalyzes ribosomal translocation along the mRNA in a GTP dependent manner, moving the tRNA formerly in the P site to the E site, and moving the aa-tRNA, now carrying the nascent peptide, into the P site. Release of the deacylated tRNA from the E site requires ATP hydrolysis by eEF3, a Fungi specific elongation factor. Recycling of eEF1A•GDP to eEF1A•GTP requires the GEF eEF1B. This process then repeats until a stop codon is encountered. In eukaryotes the release factor eRF1 seems to recognize all three stop codons, and is bound and stimulated by the GTPase eRF3. eRF1 stimulates peptidyl

transferase-catalyzed hybrolysis of the peptide-tRNA bond, allowing release of the peptide (Noble and Song, 2008). Following termination, ribosomes are recycled, a process poorly understood in eukaryotes.

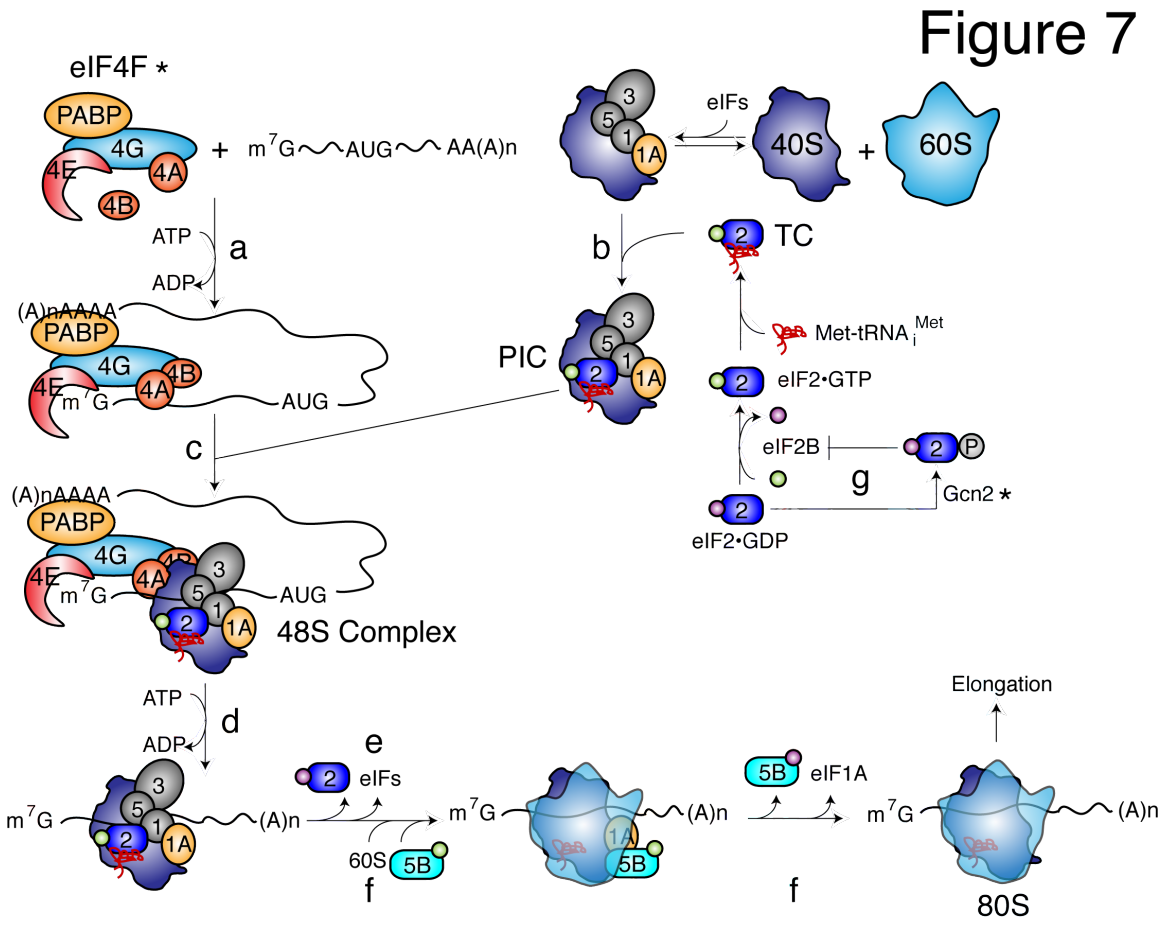

Figure 7: Translation Initiation

a) mRNA is activated and circularized by the binding of the eIF4F complex. b) eIFs and TC bind the 40S subunit to form the PIC. c) The activated mRNA and PIC assemble into the 48S complex, and d) begin scanning the 5'UTR until an AUG is recognized. e) AUG recognition causes the TC to hydrolyze GTP, promoting release of eIFs. f) Subsequently the 60S subunit and eIF5B•GTP are recruited, and GTP hydrolysis by eIF5B catalyzes 60S subunit joining. Steps regulated by nutrient availability are marked with an "*". Adapted from (Soneneberg and Hinnebusch, 2009).

Global Translational Regulation in Budding Yeast

In general, translational regulation occurs at the initiation step. There are two major types of regulation that occur, global regulation, and gene specific regulation, which are often linked. Translational activation of some mRNAs occurs during times of global translational down-regulation. One mode of global translational repression occurs at the level of TC formation. Recycling of eIF2•GDP to eIF2•GTP is catalyzed by the GEF eIF2B, and this step is a point of regulation of global translation. Gcn2 phosphorylates eIF2 in response to uncharged tRNAs (Garcia-Barrio et al., 2000). Phosphorylated eIF2 then acts as an inhibitor of the GEF activity of eIF2B, reducing eIF2•GTP levels, and therefore TC levels, acting to reduce global translation initiation (Figure 7g; Dever, 2002). The TOR kinases are master regulators of cell growth and proliferation, which serve as integrators of nutrient, energy, and stress signals. TOR activation promotes various biosynthetic processes, while TOR activity inhibits processes such as autophagy and protein degradation (Rhode et al., 2008). The TOR pathway regulates translational initiation in several ways, including by modulating the eIF2 pathway. TOR signaling promotes the stability of eIF4G, an eIF4F component, and positively regulates the activity of eIF4E by inhibiting Eap1, an eIF4E inhibitor (Berset et al., 1998; Cosentino et al., 2000). Thus, regulation of eIF activity at several levels serves as a mechanism to regulate translation globally in response to changes in nutrient availability.

P bodies are also involved in global translational repression. They are cytoplasmic foci that are the sites of mRNA decapping, deadenylation, and mRNA decay, they are involved in global translational repression in response to glucose limitation, and are

thought to act as sites of storage of non-translating mRNAs, which later reenter translation (Brengues et al., 2005; Coller and Parker, 2005). The fate of mRNAs that enter P bodies, either degradation or eventual release back into the translating pool of mRNAs, is thought to be mediated by factors associated with specific mRNAs. The mechanisms involving shuttling of specific mRNAs to and from P bodies are poorly understood, however they are thought to involve competition between the translation and P body aggregation (Parker and Sheth, 2007).

Gene Specific Translational Regulation in Budding Yeast

In nutrient-poor conditions global translation is down-regulated, however cellular response to nutrient deprivation requires translation of a subset of messages. Activation of translation of these messages is accomplished by a variety of mechanisms. Two examples of which are uORFs and internal ribosome entry sites (IRESs).

The transcription factor Gcn4 activates transcription of amino acid biosynthetic genes in response to amino acid starvation, and Gcn4 activity is controlled at the levels of transcription and translation. Translation of *GCN4* mRNA is controlled by four uORFs in the 5'UTR that sensitize it to TC levels. Ribosomes scan the *GCN4* 5'UTR, and encounter and translate uORF1. uORF1 associated sequences then promote resumption of scanning following translation. If nutrient levels are high, the TC concentration is high, and the scanning ribosome will bind TC prior to encountering one of the three downstream uORFs. Translation of one of the three downstream uORFs promotes dissociation of the ribosome, and thus prevents translation initiation at the *GCN4* AUG.

However, if nutrient levels are low, the TC concentration is low, and the scanning ribosome is less likely to bind TC prior to encountering one of the three downstream uORFS. Bypass of the downstream uORFs allows translation initiation at the *GCN4* AUG by ribosomes that bind TC between uORF4 and the start codon (Hinnebusch, 2005). Thus, uORFs provide a way to sensitize a specific message to the translational capacity of the cell. The *CLN3* message is also regulated by a uORF. However, this uORF modulates translation through a leaky scanning mechanism, and functions to reduce translational efficiency in nutrient poor conditions (Polymenis and Schmidt, 1997). During glucose deprivation cells down-regulate global translation, and undergo a developmental program leading to invasive growth. Invasive growth mRNAs contain IRESs in their 5'UTRs, consisting of unstructured poly(A) tracts. These poly(A) tracts recruit PABP (Pab1 in yeast) to the 5'UTRs. PABP then recruits eIF4G to promote cap-independent translation (Gilbert et al., 2007). Thus cells employ a variety of methods to up-regulate translation of specific messages when global translation is down-regulated.

Translational regulation is also employed to spatially restrict the accumulation of specific proteins. In budding yeast mRNAs encoding bud specific proteins are translationally repressed outside of the bud, in a process that is linked bud-directed transport of these mRNAs. The *ASH1* gene encodes a transcription factor that inhibits *HO* transcription, and localized translation of *ASH1* mRNA prevents *HO* transcription in the bud. The She2-She3-Myo4 complex transports the *ASH1* mRNA to the bud, along the actin cytoskeleton, and binding of Puf6 and Khd1 repress translation during transport. Repression is relieved when the *ASH1*-Khd1-Puf6 complex reaches the bud, and the

membrane-associated kinase Yck1 phosphorylates Khd1, allowing localized translation of *ASH1* (Paquin and Chartrand, 2008). Additionally, a recent analysis of a large set of RNA binding proteins (RBPs) shows widespread interaction of the transcriptome with RBPs, and binding of individual RBPs to functionally related mRNAs (Hogan et al., 2008). Thus, mRNA-RBP interactions may represent a widespread and underappreciated mechanism of regulation of gene expression.

Translational Regulation During Meiosis in Higher Eukaryotes

In many higher eukaryotes early embryonic development is driven by a series of extremely rapid cell divisions that utilize stored maternal mRNAs. As such, translational regulation, rather than transcription, is heavily utilized to control protein accumulation during these divisions. Additionally, oocytes undergo the first stages of meiosis, and then enter a prolonged arrest that can last for days to decades, depending upon the organism. Relief of this arrest is associated with translational activation of a variety of mRNAs required for meiotic progression. Such regulation has been observed in diverse organisms including *Xenopus*, *Drosophila*, and *C. elegans*, where *cyclin* mRNAs are common, conserved targets of translational activation (Hake and Richter, 1994; Stebbins-Boaz et al., 1999; Mendez and Richter, 2001; Sugimura and Lilly, 2006; Vardy and Orr-Weaver, 2007a; Biedermann et al., 2009). The specific mechanisms of translational regulation vary from transcript to transcript, and organism to organism. However, the general themes of translational regulation used are the same. The 3'UTRs of these messages are bound by RBPs, which in turn regulate the association of eIF4E and eIF4G with the message, and modulate poly(A) tail length (Vardy and Orr-Weaver, 2007b).

Conclusions

Active research into meiosis has led to insights into the mechanisms of meiotic chromosome segregation, and into the processes that are required for accurate segregation such as recombination, co-orientation of sisters during meiosis I, and protection of centromeric cohesion during meiosis I. However, comparatively little was known about how CDK activity was regulated during meiosis, and how meiotic events were regulated by CDKs. The following chapters will discuss the regulation of CDKs during meiosis in budding yeast, specifically through regulation of cyclin translation, and how this regulation of CDK activity affects meiotic progression.

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Chapter 2:

Meiosis I is Established through Division-Specific Translational Control of a Cyclin.

Reprinted from Cell Press:

Carlile, T.M., and Amon, A. (2008). Meiosis I is established through division-specific translational control of a cyclin. Cell *133*, 280-291.

Summary

In budding yeast key meiotic events such as DNA replication, recombination, and the meiotic divisions are controlled by Clb cyclin-dependent kinases (Clb-CDKs). Using a novel synchronization procedure, we have characterized the activity of these Clb-CDKs and observed a surprising diversity in their regulation during the meiotic divisions. Clb1-CDK activity is restricted to meiosis I, and Clb3-CDK activity to meiosis II through 5'UTR-mediated translational control of its transcript. The analysis of cells inappropriately producing Clb3-CDKs during meiosis I furthermore defines Clb3 as an inhibitor of the meiosis I chromosome segregation program. Our results demonstrate an essential role for Clb-CDK regulation in establishing the meiotic chromosome segregation pattern.

Introduction

Meiosis is a specialized cell division used by sexually reproducing organisms to produce haploid gametes from diploid progenitor cells. Pre-meiotic DNA replication is followed by two chromosome segregation phases, meiosis I and meiosis II. During meiosis I homologous chromosomes are segregated, while during meiosis II sister chromatids are split. As during the mitotic cell cycle, cyclin-dependent kinases (CDKs) promote progression through the meiotic program. Budding yeast contains six B-type cyclins, Clb1 – Clb6 (reviewed in Bloom and Cross, 2007). Clb5 and Clb6, in conjunction with the sole CDK, Cdc28, are essential for the initiation of pre-meiotic S-phase (Dirick et al., 1998; Stuart and Wittenberg, 1998) and the initiation of homologous recombination (Henderson et al., 2006). B-type cyclins and Cdc28 are also required for the two meiotic divisions (Benjamin et al., 2003). The major mitotic cyclin *CLB2* is not expressed during meiosis. Instead, *CLB1*, *CLB3* and *CLB4* promote progression through the meiotic divisions (Dahmann and Futcher, 1995). Deletion of any two of these three cyclins results in cells executing only a single meiotic division. During this single division, homologous chromosomes are segregated (Dahmann and Futcher, 1995), but meiosis II events such as loss of Sgo1, the sister chromatid cohesion control factor, from chromosomes occur (Kiburz et al., 2008).

Studies of Clb-CDKs during mitosis revealed that these kinases are regulated at multiple levels. Transcription of the *CLB* genes is periodic during the cell cycle, with their expression typically confined to those cell cycle stages when their activity is needed. In addition to transcriptional control, cell cycle regulated degradation of Clb cyclins is

essential for restricting Clb-CDK activities to the appropriate stages of the cell cycle (reviewed in Mendenhall and Hodge, 1998). At the metaphase – anaphase transition an ubiquitin ligase known as the Anaphase Promoting Complex or Cyclosome (APC/C), together with the specificity factor Cdc20, degrades Clb5 and a fraction of Clb2. During late anaphase another APC/C specificity factor called Cdh1 degrades Clb1, Clb3 and the remaining pool of Clb2. Down-regulation of Clb-CDK activity at the end of mitosis is primarily brought about by degradation of the Clb proteins, but the Clb-CDK inhibitor Sic1, which directly binds to the cyclin-CDK complex, helps restrain Clb-CDK activity during exit from mitosis and G1 (reviewed in Bloom and Cross, 2007).

CDK activity associated with the different Clb cyclins has been characterized extensively during the mitotic cell cycle. However, the asynchrony with which sporulating cultures of *S. cerevisiae* proceed through the meiotic divisions has prevented the detailed characterization of CDK activity during meiosis. We developed a novel synchronization method that produces meiotic cultures that proceed through the meiotic divisions with a high degree of synchrony, comparable to that of synchronized mitotic cultures. Using this synchronization method we characterized Clb1-, Clb3-, Clb4- and Clb5-CDK activity and observed a striking diversity in their regulation during meiosis. Clb1-CDK activity, but not Clb1 protein, is restricted to meiosis I. Clb3-CDK activity, on the other hand, is meiosis II-specific because Clb3 protein is not translated during meiosis I. This meiosis I-specific translational inhibition is mediated by the 5' untranslated region (UTR) of the RNA. Finally, we show that restricting Clb3 protein to meiosis II is essential for establishing the meiosis I chromosome segregation pattern. Our results demonstrate a

high degree of specialization of Clb-CDK regulation during meiosis and demonstrate an essential role for Clb-CDK regulation in establishing the meiotic chromosome segregation pattern.

Results

A method to generate synchronous meiotic cultures in budding yeast.

In budding yeast, the resolution with which meiotic events can be observed is limited by the relative asynchrony of meiotic cultures, much of which stems from variations in the timing of entry into the meiotic program (Nachman et al, 2007). We eliminated this source of asynchrony by developing conditions that arrest cells reversibly in prophase I, prior to the two meiotic divisions. *NDT80* is a transcription factor that is required for progression out of the pachytene stage of meiosis and into meiosis I (Xu et al., 1995; Chu et al., 1998). To arrest cells reversibly in pachytene, the *NDT80* open reading frame was placed under the control of the inducible *GAL1-10* promoter (*GAL-NDT80*; Benjamin et al., 2003). In cells producing a Gal4-estrogen receptor fusion protein (Gal4.ER), transcription from the *GAL1-10* promoter can be induced by the addition of estrogen to the medium (Picard et al., 1999; Benjamin et al., 2003; Figure 1A).

In the absence of β-estradiol *GAL4.ER GAL-NDT80* cells failed to undergo any meiotic divisions (Figure 1B, C). However, when 1 μM β-estradiol was added 5 hours after transfer into meiosis-inducing conditions (henceforth called the *GAL-NDT80* block), *GAL4.ER GAL-NDT80* cells underwent both meiotic divisions synchronously (Figure 1B, C). Cells initiated meiosis I one hour post release, and approximately 80% of cells had undergone this division one hour later (Figure 1B, left panel). Meiosis II occurred with a similarly high degree of synchrony (Figure 1B, right panel). In contrast, cultures of cells that expressed *NDT80* from its native promoter took approximately four hours to complete the first division and another four hours to complete meiosis II with the peaks

of meiosis I and meiosis II largely overlapping (Figure 1B). The high degree of synchrony of meiotic cultures that were blocked in pachytene and then released from the block was particularly evident when the percentages of cells in metaphase I, anaphase I, metaphase II and anaphase II were examined (Figure 1C). More than 40% of cells progressed through the different cell cycle stages simultaneously, whereas only 10% of cells with *NDT80* under its native promoter did (Figure 1C). This analysis also showed that cells spent longer periods of time in metaphase II and anaphase II, compared to their meiosis I counterparts (Figure 1C). Why progression through meiosis II takes longer than progression through meiosis I is at present unknown.

Tetrad analysis confirmed that the pachytene arrest caused by the depletion of *NDT80* and the release from the block did not interfere with meiotic progression. Sporulation efficiency and spore viability were high (96.5% and 95.5% respectively; Figure 1D). We conclude that modulating the production of Ndt80 can be used to generate meiotic cultures that progress through the meiotic divisions with a high degree of synchrony.

Figure 1

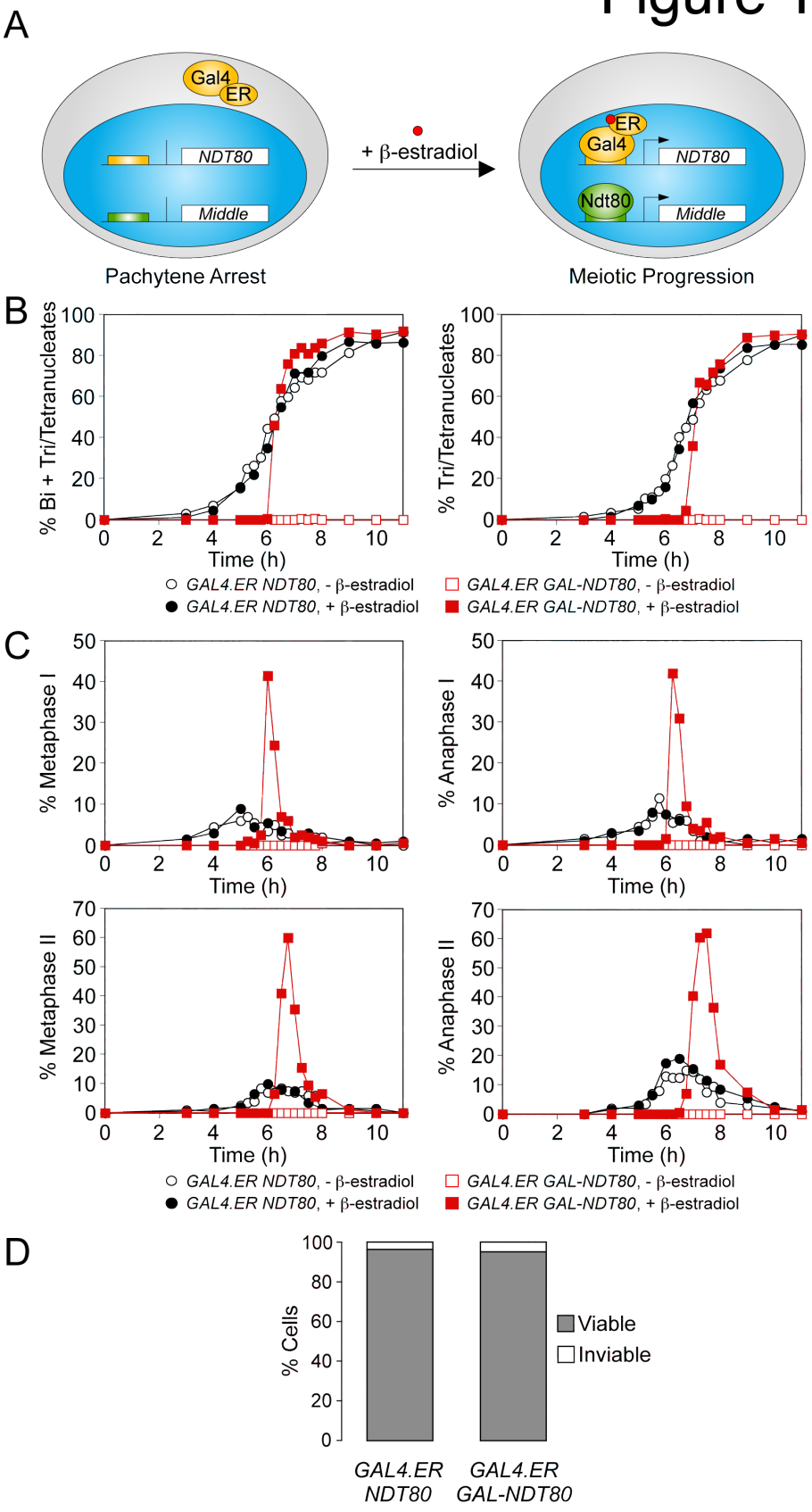
A
Gal4
ER
NDT80
Middle
+ β-estradiol
ER
Gal4
NDT80
Ndt80
Middle
Pachytene Arrest
Meiotic Progression
B
% Bi + Tri/Tetranucleates
% Tri/Tetranucleates
Time (h)
GAL4.ER NDT80, - β-estradiol
GAL4.ER NDT80, + β-estradiol
GAL4.ER GAL-NDT80, - β-estradiol
GAL4.ER GAL-NDT80, + β-estradiol
C
% Metaphase I
% Anaphase I
% Metaphase II
% Anaphase II
Time (h)
GAL4.ER NDT80, - β-estradiol
GAL4.ER NDT80, + β-estradiol
GAL4.ER GAL-NDT80, - β-estradiol
GAL4.ER GAL-NDT80, + β-estradiol
D
% Cells
Viable
Inviable
GAL4.ER NDT80
GAL4.ER GAL-NDT80

Figure 1: A meiotic block-release synchronization system

(A) A method for synchronizing meiotic cells using an inducible allele of *NDT80*. See text for details.

(B and C) *GAL4.ER* (A14200; circles) and *GAL4.ER GAL-NDT80* strains (A14201; squares) were induced to sporulate at 30°C by transfer into SPO medium. After 5 hours either ethanol (open symbols) or 1μM β-estradiol was added (closed symbols). The percentages of bi- and tri- or tetranucleate cells ([B], left graph), of tri- or tetranucleate cells ([B], right graph), and of cells with metaphase I ([C], upper left graph), anaphase I ([C], upper right graph), metaphase II ([C], lower left graph) or anaphase II spindles ([C], lower right graph) were determined at the times indicated after transfer into SPO medium.

(D) *GAL4.ER* (A14200) and *GAL4.ER GAL-NDT80* strains (A14201) were grown as described in Figure 1B. Samples were taken after 28 hours (n=40 tetrads).

Clb1-CDK activity is restricted to meiosis I, Clb3-CDK activity to meiosis II.

To determine how Clb-CDK activity is controlled during the meiotic divisions we examined the expression and activity of four of these cyclins, *CLB1*, *CLB3*, *CLB4* and *CLB5* using the synchronization procedure described above. Consistent with the role of *CLB5* in pre-meiotic DNA-replication, both Clb5 protein and associated kinase activity were present upon release from the *NDT80* block, peaked during metaphase I, declined in anaphase I and peaked again as cells formed metaphase II spindles (Figure 2A, D; Figure 3C; Figure 4A). Comparison of Clb5 protein levels and Clb5-associated kinase activity showed that Clb5-CDK activity paralleled Clb5 protein levels (Figure 2D). We conclude that Clb5-CDK activity is regulated primarily at the level of Clb5 protein abundance and that Clb5 protein and associated kinase activity appear in two waves, one during meiosis I and one during meiosis II.

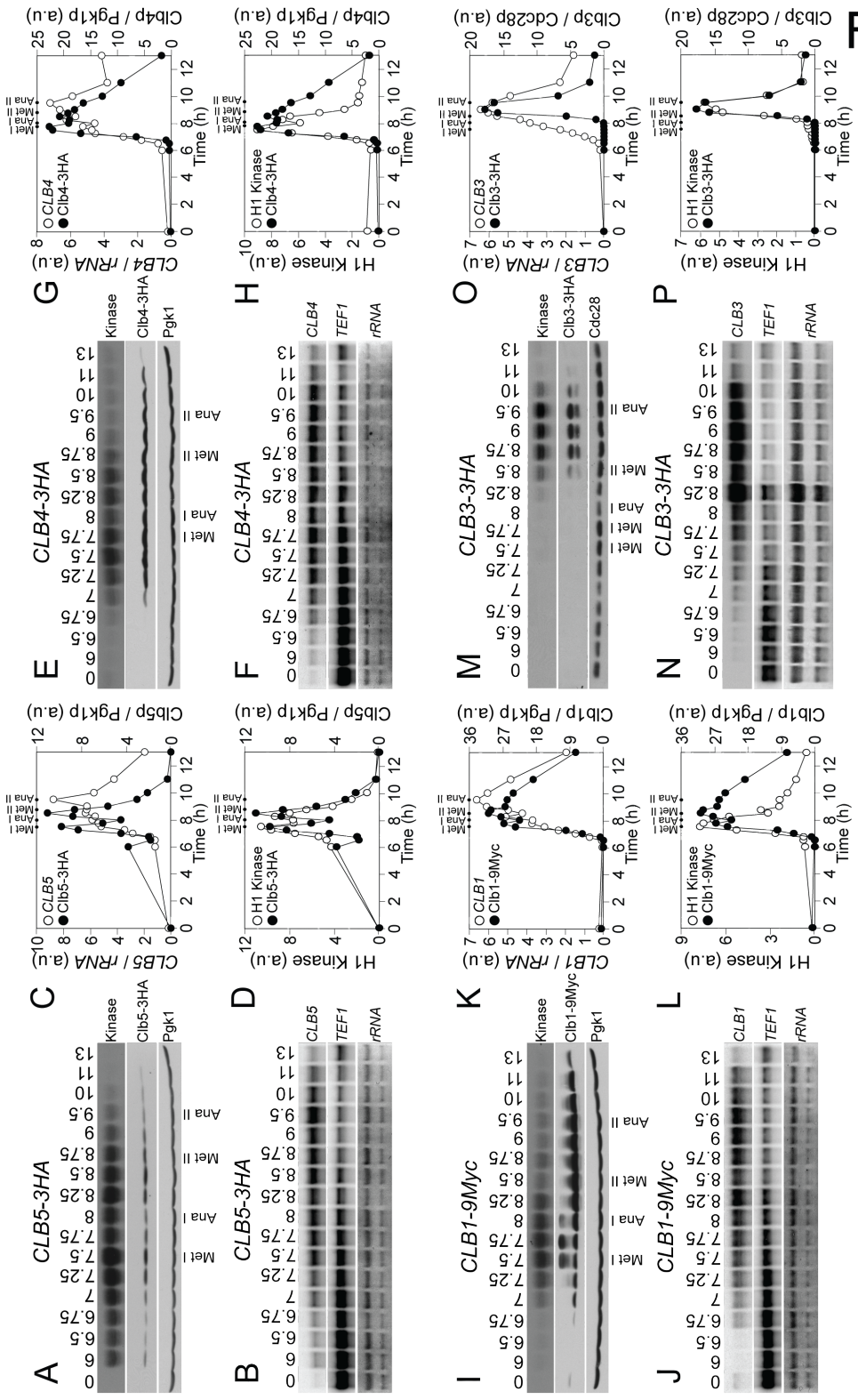
A
CLB5-3HA
Kinase
Clb5-3HA
Pgk1
Met I
Ana I
Met II
Ana II
B
CLB5-3HA
CLB5
TEF1
rRNA
C
CLB5 / rRNA (a.u)
Clb5-3HA
Clb5p / Pgk1p (a.u)
Time (h)
D
H1 Kinase (a.u)
Clb5-3HA
Clb5p / Pgk1p (a.u)
E
CLB4-3HA
F
CLB4-3HA
G
CLB4 / rRNA (a.u)
Clb4-3HA
Clb4p / Pgk1p (a.u)
H
H1 Kinase (a.u)
Clb4-3HA
Clb4p / Pgk1p (a.u)
I
CLB1-9Myc
J
CLB1-9Myc
K
CLB1 / rRNA (a.u)
Clb1-9Myc
Clb1p / Pgk1p (a.u)
L
H1 Kinase (a.u)
Clb1-9Myc
Clb1p / Pgk1p (a.u)
M
CLB3-3HA
Cdc28
N
CLB3-3HA
O
CLB3 / rRNA (a.u)
Clb3-3HA
Clb3p / Cdc28p (a.u)
P
H1 Kinase (a.u)
Clb3-3HA
Clb3p / Cdc28p (a.u)

Figure 2

Figure 2: Cyclin expression and activity during the meiotic divisions.

GAL4.ER GAL-NDT80 strains carrying tagged versions of cyclins *CLB5-3HA* (A15804), *CLB4-3HA* (A15090), *CLB1-9Myc* (A15591) and *CLB3-3HA* (A15802) were induced to sporulate at 30°C, and were released from the *GAL-NDT80* block at 6 hours.

(A, E, I and M) The amount of protein and associated Histone H1 kinase activity for the indicated cyclin ([A] Clb5-3HA, [E] Clb4-3HA, [I] Clb1-9Myc and [M] Clb3-3HA) is shown. Pgk1 or Cdc28 were used as loading controls. MetaI, AnaI, MetaII and AnaII signify the peaks of cells with metaphase I, anaphase I, metaphase II and anaphase II spindles, respectively (all time points are shown in Figure 4A – D).

(B, F, J, N) Transcript levels for the indicated cyclins ([B] *CLB5-3HA*, [F] *CLB4-3HA*, [J] *CLB1-9Myc* and [N] *CLB4-3HA*). *TEF1* and *rRNA* are shown as loading controls.

(C, G, K, O) Quantifications of cyclin transcript levels normalized to the *rRNA* (open symbols, left axis) and cyclin protein levels normalized to the loading control (closed symbols, right axis) for the indicated cyclins ([C] Clb5-3HA, [G] Clb4-3HA, [K] Clb1-9Myc and [O] Clb3-3HA).

(D, H, L, P) Quantifications of cyclin associated Histone H1 kinase activity (open symbols, left axis) and cyclin protein levels normalized to the loading control (closed symbols, right axis) for the indicated cyclins ([D] Clb5-3HA, [H] Clb4-3HA, [L] Clb1-9Myc and [P] Clb3-3HA).

Clb4-CDKs displayed a more complex pattern of regulation. *CLB4* RNA and protein accumulated as cells entered meiosis I and remained high throughout the two meiotic divisions (Figure 2E, F, G; Figure 4B). Comparison of Clb4 protein levels with Clb4-CDK activity showed that the two correlated well until metaphase I. Thereafter, the decline in Clb4-CDK activity was not paralleled by a decline in Clb4 protein. This was particularly evident during exit from meiosis II (Figure 2E, H; Figure 3A). The loss of Clb4-CDK activity was not due to a failure to immunoprecipitate Clb4 during late stages

of meiosis (data not shown) indicating that during exit from meiosis I and meiosis II posttranslational mechanisms other than degradation of Clb4 protein down-regulate Clb4-CDK activity.

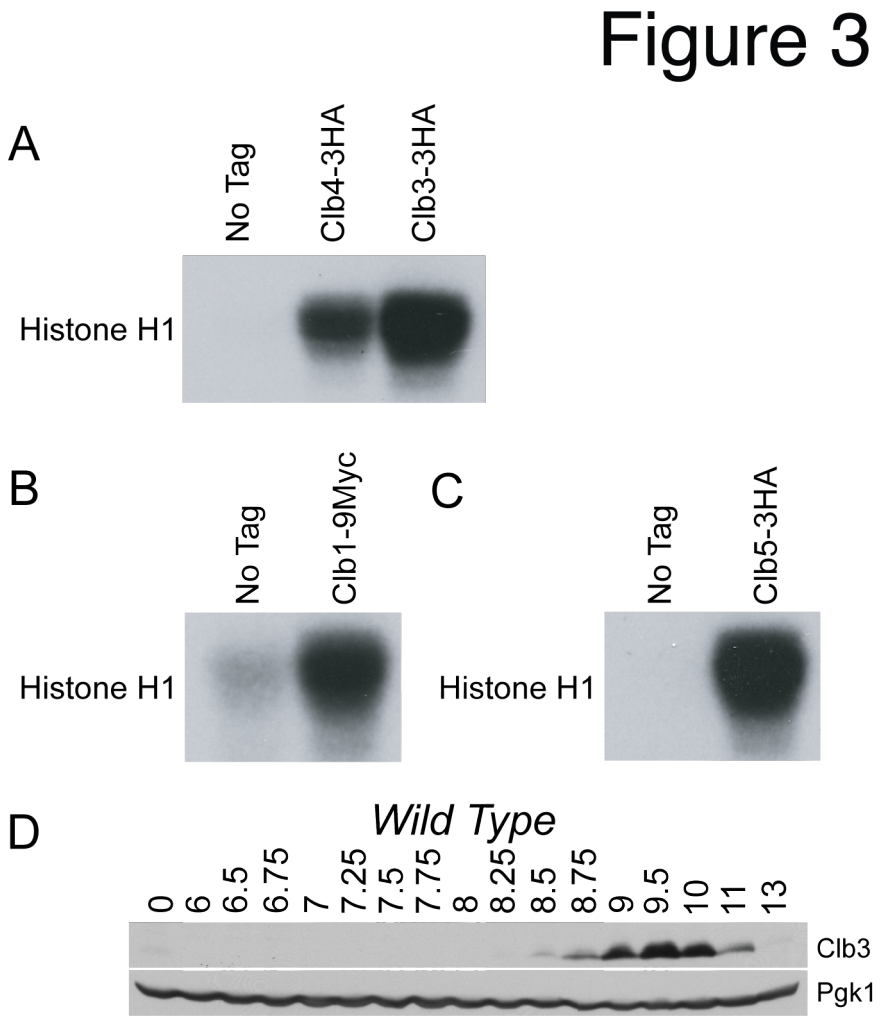

Figure 3: Kinase assay controls for tagged cyclins shown in Figure 2.

(A-C) Kinase assays were performed on samples from wild-type cycling cultures carrying the tagged cyclins shown: no tag ([A-C] A4841), *CLB4-3HA* ([A], A4736), *CLB3-3HA* ([A], A11955), *CLB1-9Myc* ([B], A7057) and *CLB5-3HA* ([C], A15109).

(D) A strain carrying *GAL4.ER GAL-NDT80 CLB1-9Myc* (A15591) was induced to sporulate at 30°C, and was released from the *GAL-NDT80* block at 6 hours with the addition of 1μM β-estradiol. Clb3 protein as detected by an anti Clb3-antibody (Santa

Cruz) is shown with Pgk1 as a loading control. Progression through meiosis for this experiment is shown in Figure 4C.

CLB1 mRNA and Clb1 protein levels rose during meiosis I and remained high until exit from meiosis II (Figure 2I, J, K; Figure 4C). A slower migrating form of Clb1 was only detected during meiosis I and correlated with Clb1-CDK activity (Figure 2I; Figure 3B, Figure 4E) suggesting that this form of Clb1 signifies active Clb1-CDK complexes. Quantification of Clb1 protein and associated kinase activity confirmed this result and demonstrated that Clb1-CDK was only active during meiosis I (Figure 2L; Figure 4E). The loss of Clb1-CDK activity during meiosis II was not due to an inability to immunoprecipitate Clb1 (data not shown), excluding the possibility that degradation of Clb1 in extracts or insolubility of the protein were responsible for the lack of Clb1-CDK activity during meiosis II. Our results show that during the meiotic divisions Clb1-CDK is a meiosis I-specific CDK. The finding that Clb1, which is an APC/C-Cdh1 substrate during the mitotic divisions (J. Simpson and M. Brandeis, personal communications), is not degraded during exit from meiosis I further indicates that APC/C-Cdh1 is inactive during this transition. The observation that Clb1-CDK activity is nevertheless low during meiosis II demonstrates that posttranslational mechanisms other than protein degradation inhibit Clb1-CDK activity during exit from meiosis I and during meiosis II.

Figure 4

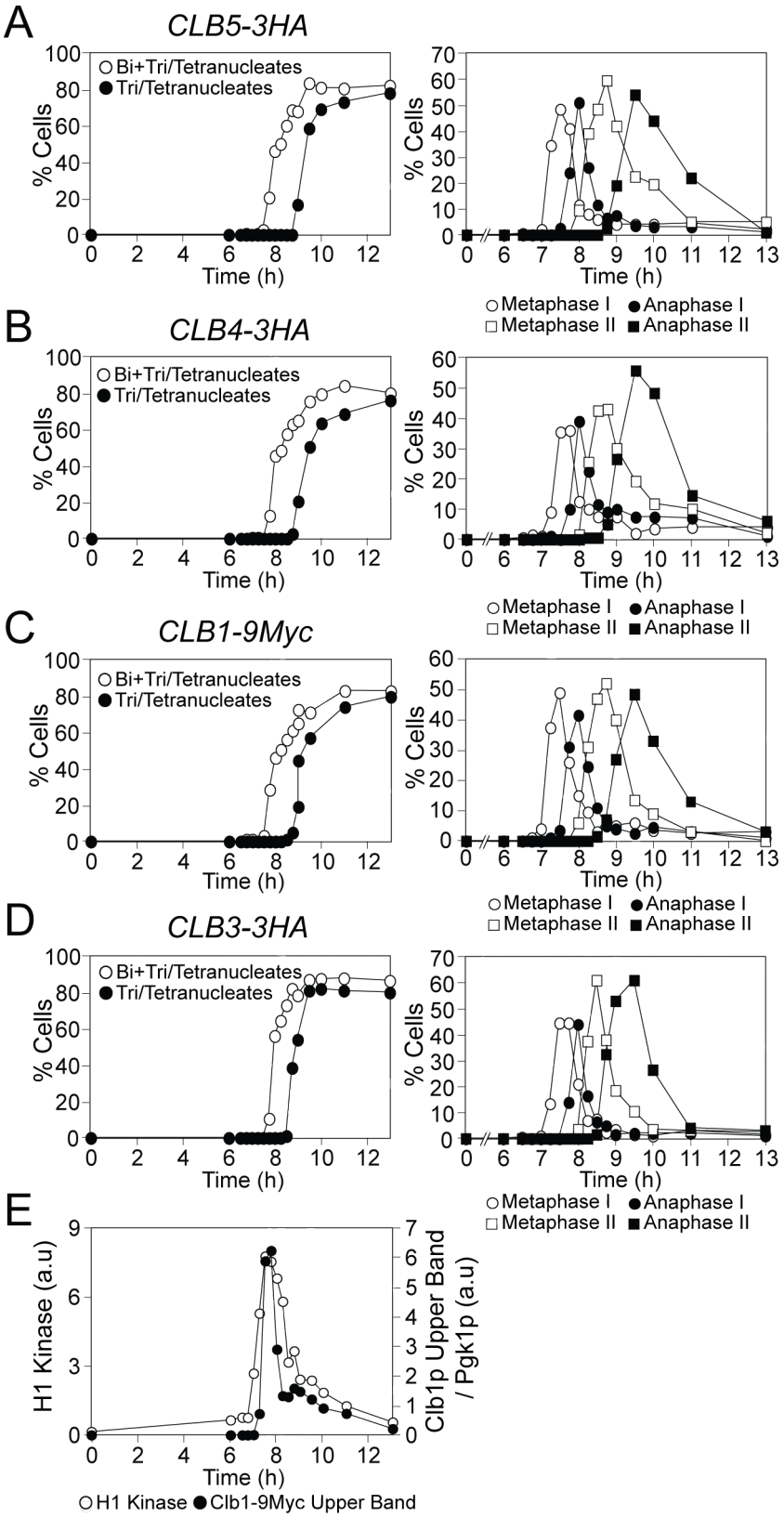
A
CLB5-3HA
Bi+Tri/Tetranucleates
Tri/Tetranucleates
% Cells
Time (h)
Metaphase I
Anaphase I
Metaphase II
Anaphase II
B
CLB4-3HA
C
CLB1-9Myc
D
CLB3-3HA
E
H1 Kinase (a.u)
Clb1p Upper Band / Pgk1p (a.u)
H1 Kinase
Clb1-9Myc Upper Band

Figure 4: Kinetics of meiotic divisions for strains shown in Figure 2.
(A-D) Meiotic progression was monitored in the strains shown in Figure 2, *CLB5-3HA* ([A], A15804), *CLB4-3HA* ([B], A15090), *CLB1-9Myc* ([C], A15591) and *CLB3-3HA* ([D], A15802). The percentages of bi- and tri- or tetranucleate cells (open symbols) or the percentages of tri- or tetranucleate cells (closed symbols) were determined at the times indicated after inoculation into SPO medium [A-D, left panels]. The percentages of cells with metaphase I (open circles), anaphase I (closed circles), metaphase II (open squares) or anaphase II spindles (closed squares) were determined at the times indicated after inoculation into SPO medium [A-D, right panels].
(E) Quantification of Clb1-9Myc associated Histone H1 kinase activity (open symbols, left axis) and levels of the slower migrating form of Clb1-9Myc normalized to the loading control (closed symbols, right axis).

The *CLB3* transcript accumulated somewhat later than that of *CLB1* and *CLB4* but nevertheless reached high levels during meiosis I and peaked during meiosis II (Figure 2N, O; Figure 4D). Interestingly, a 3HA tagged version of Clb3 protein and its associated kinase activity did not appear until the onset of meiosis II (Figure 2M, P; Figure 3A; Figure 4D). Untagged Clb3 also did not accumulate until the onset of meiosis II (Figure 3D; Figure 4C). This data shows that during the meiotic divisions Clb3 is a meiosis II-specific cyclin, and that post-transcriptional mechanisms restrict the protein to meiosis II.

Protein degradation is not responsible for restricting Clb3 to meiosis II.

Clb3 is an APC/C substrate (Zachariae et al., 1996), and in chicken cells APC/C-Cdh1 is active during G2 (Sudo et al., 2001). It was therefore possible that during the prolonged

pre-meiotic G2 arrest induced by the *NDT80* block APC/C-Cdh1 was activated leading to the degradation of Clb3, or that a novel-degradation pathway was responsible for keeping Clb3 protein levels low during meiosis I. To test this hypothesis we examined the effects of inhibiting the 26S proteasome and the APC/C on Clb3 levels during meiosis I.

Figure 5

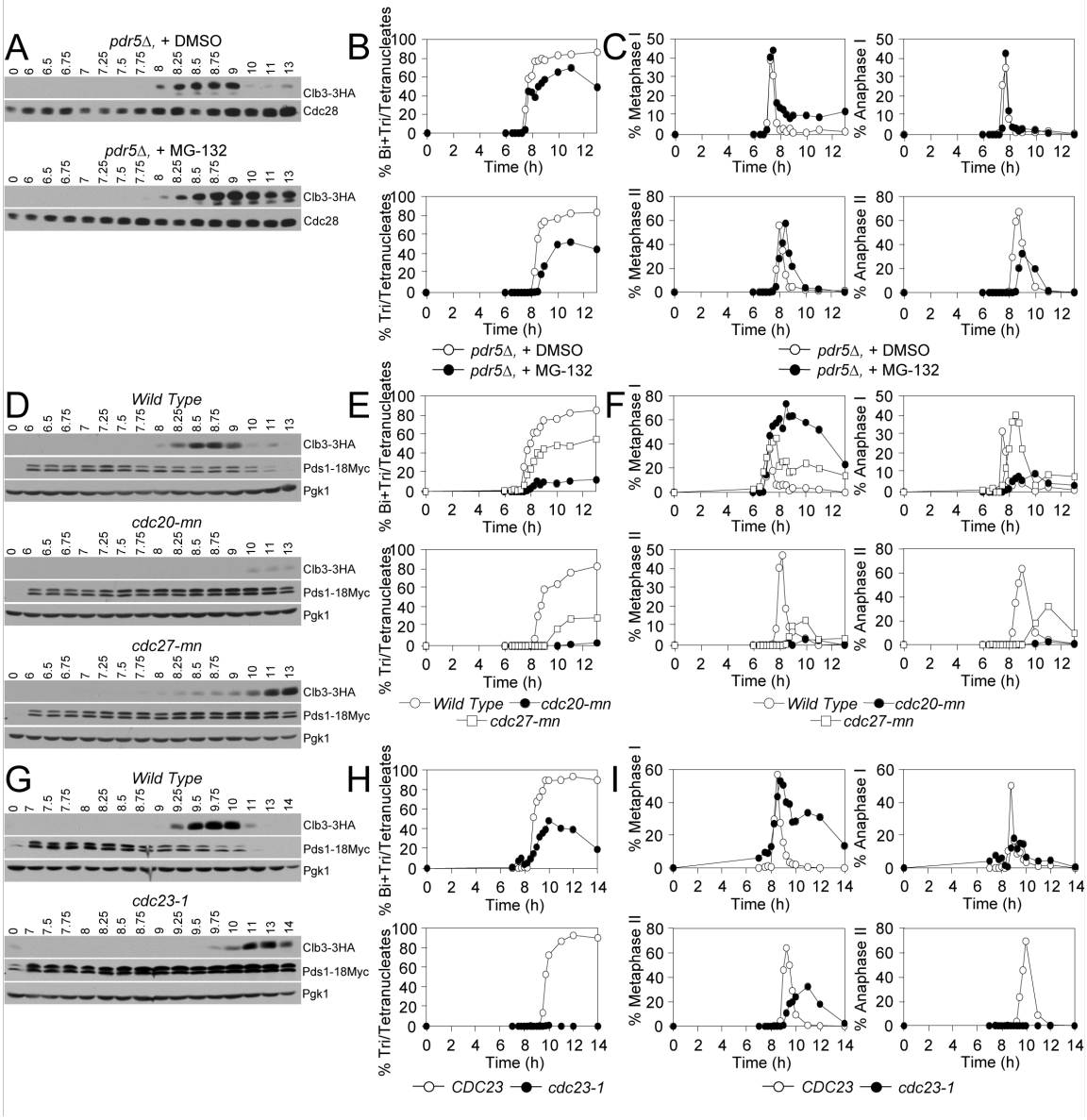

Figure 5: Clb3 does not accumulate during meiosis I in proteasome inhibited cells or in APC mutant cells.

(A-C) Duplicate cultures of *GAL4.ER GAL-NDT80 pdr5*Δ *CLB3-3HA* (A16011) were sporulated at 30°C, and were released from the *GAL-NDT80* block at 6 hours. Either DMSO (open symbols) or 20 μM MG-132 (closed symbols) was added at 6.5, 8.5 and 10.5 hours after transfer into SPO medium. Cells lacked the *PDR5* gene to prevent export of MG-132.

(D-F) Wild-type (A17946, open circles), P_{CLB2}-*CDC20* (A17855, closed circles) and P_{CLB2}-*3HA-CDC27* (A17856, open squares) cells carrying *GAL4.ER*, *GAL-NDT80*, *PDS1-18Myc* and *CLB3-3HA* alleles were sporulated at 30°C, and were released from the *GAL-NDT80* block at 6 hours.

(G-I) *CDC23* (A17946, open symbols) or *cdc23-1* (A17947, closed symbols) cells carrying *GAL4.ER*, *GAL-NDT80*, *PDS1-18Myc* and *CLB3-3HA* alleles were induced to sporulate at room temperature, were released from the *GAL-NDT80* block at 7 hours, and were shifted to 34°C at 8 hours to inactivate the *cdc23-1* allele.

(A) Western blots for Clb3-3HA.

(D, G) Western blots for Clb3-3HA and Pds1-18Myc.

(B, C, E, F, H, I) The percentages of bi- and tri- or tetranucleate cells ([B, E, H], upper graphs), of tri- or tetranucleate cells ([B, E, H], lower graphs), and of cells with metaphase I ([C, F, I], upper left graphs), anaphase I ([C, F, I], upper right graphs), metaphase II ([C, F, I], lower left graphs) or anaphase II spindles ([C, F, I], lower right graphs) were determined at the times indicated after transfer into SPO medium.

Addition of MG-132 to cells inhibits the proteasome. When added to meiotic cultures, 15% of cells arrested in metaphase I (Figure 5C) and progression through the meiotic divisions was somewhat hampered (Figure 5B) indicating that the proteasome was only partially inactivated by MG-132 in these experiments. This partial inhibition was nevertheless sufficient to prevent degradation of Clb3 during exit from meiosis II (Figure

5A). However, treatment with MG-132 did not lead to accumulation of Clb3 during meiosis I (Figure 5A) suggesting that proteasome-mediated protein degradation was not responsible for keeping Clb3 protein levels low during meiosis I.

Inactivation of the APC/C by depleting the APC/C component Cdc27 or the APC/C activator Cdc20, or by employing a temperature sensitive allele of the APC/C subunit Cdc23 (*cdc23-1*) did not allow Clb3 to accumulate during meiosis I. Meiotic depletions of Cdc20 and Cdc27 were achieved by placing these genes under the control of the mitosis specific *CLB2* promoter (Lee and Amon, 2003). Cdc20-depleted cells (*cdc20-mn* cells) arrested in metaphase I (Figure 5E, F), and Cdc27-depleted cells (*cdc27-mn* cells) were delayed in metaphase I (Figure 5E,F) indicating that Cdc27 depletion was not complete or that Cdc27 was not essential for the metaphase I – anaphase I transition. *cdc23-1* cells were delayed in metaphase I, but some cells completed meiosis I before arresting in metaphase II (Figure 5H,I). In *cdc20-mn*, *cdc27-mn* and *cdc23-1* cells Pds1-18Myc, an APC/C target whose degradation is required for anaphase I and anaphase II onset, was stabilized during exit from meiosis II indicating that APC/C function is impaired in these mutants (Salah and Nasmyth, 2000; Figure 5D and 5G). However, Clb3 did not accumulate during meiosis I in any of these strains (Figure 5D and 5G). In fact its accumulation was greatly delayed in the APC/C mutants that are defective in progressing through meiosis I further confirming that Clb3 is indeed a meiosis II-specific cyclin. Our results do not rule out a minor role for APC/proteasome dependent degradation in preventing Clb3 accumulation during meiosis I, but they indicate that

other posttranscriptional mechanisms are primarily responsible for preventing the accumulation of Clb3 protein during meiosis I.

The 5'UTR of *CLB3* is required to restrict Clb3 protein to meiosis II.

Although Clb1-, Clb3-, and Clb4-CDK activity all appeared to be regulated in an interesting manner during the meiotic divisions, we chose to further study Clb3 regulation because our results raised the possibility that translational control restricts Clb3 protein to meiosis II. This form of regulation would represent a novel mechanism of controlling Clb-CDKs in yeast.

To investigate the possibility that translational control confines Clb3 protein to meiosis II, we tested whether the UTRs of the mRNA mediate such regulation. Because the Clb3 protein employed in this study carries a tag at its C-terminus resulting in the disruption of the native 3'UTR of *CLB3* (Longtine et al., 1998) we examined the role of the 5'UTR in preventing Clb3 accumulation during meiosis I by replacing the promoter and 5'UTR of *CLB3* with that of the *GAL1-10* gene. Thus cells carrying the *GAL4.ER* fusion express *CLB3* upon addition of β-estradiol. Upon release from the *GAL-NDT80* block *GAL-CLB3* was expressed within 30 minutes (Figure 6B). The amount of *CLB3* RNA generated from the *GAL1-10* promoter was significantly lower than that produced by the native *CLB3* promoter. This was best seen when the amounts of *CLB3* RNA at the 7.5, 7.75 and 8 hour time points of the wild-type were compared to that of the 6.5, 6.75 and 7 hour time points of the *GAL-CLB3* carrying strain (Figure 6B). Clb3 protein however was undetectable in wild-type cells at the 7.5, 7.75 and 8 hour time points but the protein was highly

produced at the 6.5, 6.75 and 7 hour time points of the *GAL-CLB3* carrying strain (Figure 6A).

Quantification of the amount of Clb3 protein produced by *CLB3* mRNA demonstrated that the promoter and 5'UTR of *CLB3* affected Clb3 protein levels in at least two ways. First, it functions to restrict Clb3 protein to meiosis II. The ratio of Clb3 protein/*CLB3* mRNA was low during meiosis I but high during meiosis II (Figure 6E, left panel). Second, the 5'UTR prevents efficient translation. This was evident when we compared the amount of Clb3 protein generated per *CLB3* mRNA in wild-type cells with the amount of Clb3 protein generated from *CLB3* mRNA carrying the *GAL1-10* 5'UTR (Figure 6E, right panel). The amount of Clb3 protein produced per RNA was much higher in the *GAL-CLB3* expressing strains than in wild-type cells. Similar results were obtained when *CLB2* was controlled by the *CLB3* promoter and 5'UTR (Figure 7A). We conclude that the promoter and 5'UTR of *CLB3* are required for restricting translation of Clb3 protein to meiosis II.

Figure 6

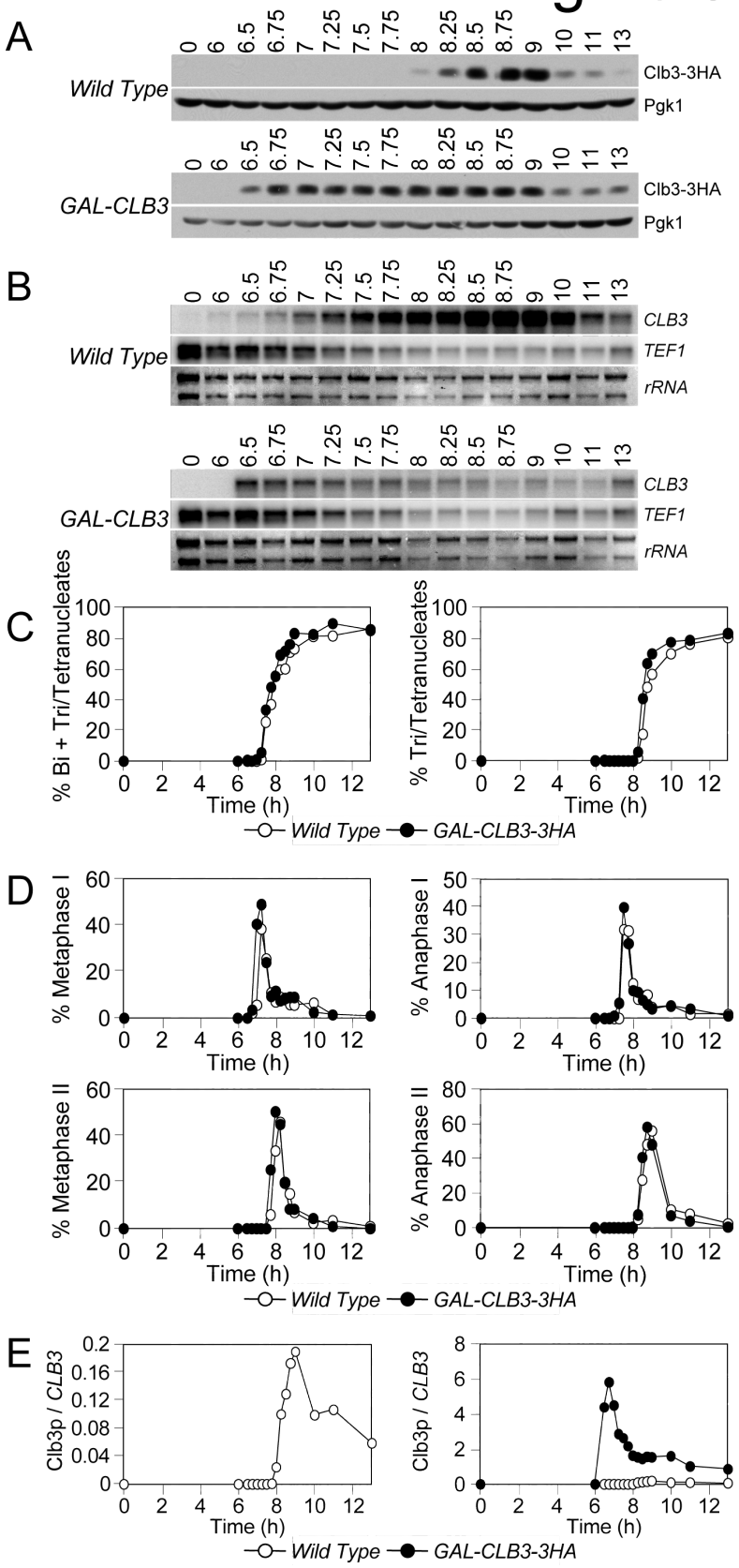
A
0 6 6.5 6.75 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 10 11 13
Wild Type
Clb3-3HA
Pgk1
GAL-CLB3
B
CLB3
TEF1
rRNA
C
% Bi + Tri/Tetranucleates
% Tri/Tetranucleates
Time (h)
Wild Type
GAL-CLB3-3HA
D
% Metaphase I
% Anaphase I
% Metaphase II
% Anaphase II
E
Clb3p / CLB3

Figure 6: The *CLB3* promoter and 5'UTR are required to prevent Clb3 accumulation during meiosis I.

CLB3-3HA (A15055, open symbols) and *GAL-CLB3-3HA* (A18095, closed symbols) cells carrying *GAL4.ER* and *GAL-NDT80* were sporulated at 30°C, and were released from the *GAL-NDT80* block at 6 hours by the addition of 1 μm β-estradiol.

(A) Western blots for Clb3-3HA protein.

(B) Northern blots for *CLB3*.

(C and D) The percentages of bi- and tri- or tetranucleate cells ([C], left graph), of tri- or tetranucleate cells ([C], right graph), of cells with metaphase I ([D], upper left graph), anaphase I ([D], upper right graph), metaphase II ([D], lower left graph) or anaphase II spindles ([D], lower right graph) were determined at the times indicated.

(E) Quantifications of the ratio of Clb3-3HA protein to *CLB3* mRNA ([Clb3p/Pgk1]/[*CLB3*/*TEF1*]). Quantifications of Clb3 protein/*CLB3* RNA for wild-type are shown in the left graph. The same data was then compared with the amount of Clb3protein/*CLB3* RNA generated from the *GAL1-10* promoter (right graph).

The 5'UTR of *CLB3* is sufficient to prevent accumulation of proteins during meiosis I.

To determine whether the *CLB3* promoter and 5'UTR were sufficient to restrict accumulation of proteins to meiosis II we replaced the open reading frame and 3'UTR of *CLB3* with that of *CLB2* and *ADH1*, respectively (see Experimental Procedures). As a control, we also placed *CLB2* fused to the *ADH1* 3'UTR under the control of the *CLB4* promoter. Expression of *CLB2* from the *CLB3* or *CLB4* promoters did not significantly interfere with progression through meiosis, although exit from meiosis II appeared slightly delayed in P_{CLB4}-*CLB2* strains (Figure 7C-D).

Figure 7

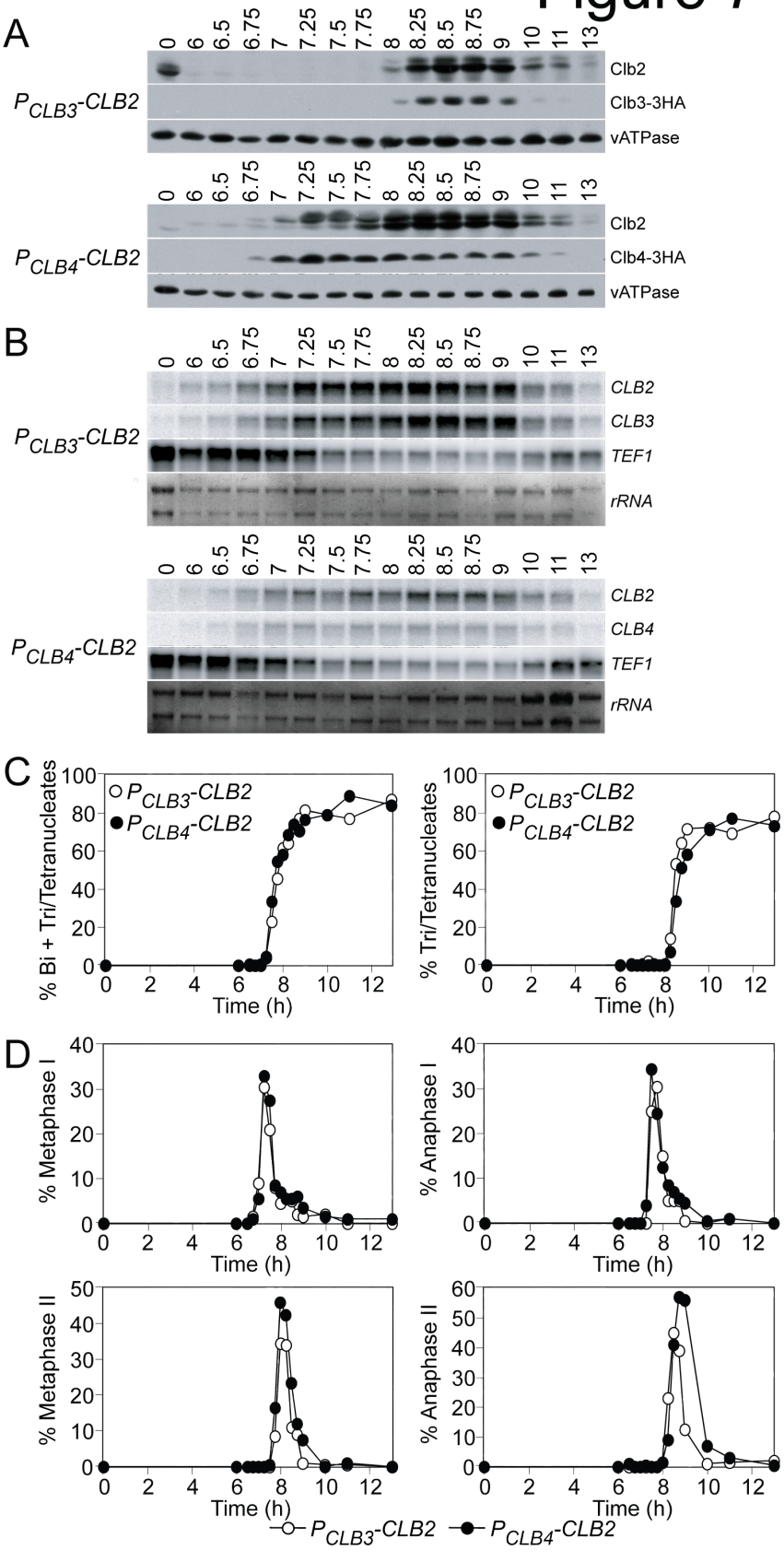
A
0 6 6.5 6.75 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 10 11 13
P_{CLB3}-CLB2
Clb2
Clb3-3HA
vATPase
P_{CLB4}-CLB2
Clb2
Clb4-3HA
vATPase
B
P_{CLB3}-CLB2
CLB2
CLB3
TEF1
rRNA
P_{CLB4}-CLB2
CLB2
CLB4
TEF1
rRNA
C
% Bi + Tri/Tetranucleates
% Tri/Tetranucleates
P_{CLB3}-CLB2
P_{CLB4}-CLB2
Time (h)
D
% Metaphase I
% Anaphase I
% Metaphase II
% Anaphase II
Time (h)
P_{CLB3}-CLB2
P_{CLB4}-CLB2

Figure 7: The *CLB3* promoter and 5'UTR are sufficient to prevent protein accumulation during meiosis I.

P_{CLB3}-*CLB2 CLB3-3HA* (A18574, open symbols) and P_{CLB4}-*CLB2 CLB4-3HA* (A18578, closed symbols) cells carrying *GAL4.ER* and *GAL-NDT80* alleles were cultured as described in Figure 4.

(A) Western blots for Clb2, Clb3-3HA and Clb4-3HA. vATPase is shown as a loading control.

(B) Northern blots for *CLB2*, *CLB3* and *CLB4.*

(C and D) The percentages of bi- and tri- or tetranucleate cells ([C], left graph), of tri- or tetranucleate cells ([C], right graph) and of cells with metaphase I ([D], upper left graph), anaphase I ([D], upper right graph), metaphase II ([D], lower left graph) or anaphase II spindles ([D], lower right graph) were determined at the times indicated times.

As expected, in both P_{CLB3}-*CLB2* and P_{CLB4}-*CLB2* strains *CLB2* transcript levels mirrored those of *CLB3* and *CLB4,* respectively (Figure 7B). However, despite the presence of *CLB2* transcript during meiosis I in P_{CLB3}-*CLB2* cells, Clb2 protein did not accumulate during meiosis I, but was, as Clb3, present only during meiosis II (Figure 7A). In P_{CLB4}-*CLB2* strains Clb2 appeared during meiosis I, indicating that Clb2 protein can accumulate during the first division (Figure 7A). Our results indicate that the promoter and 5'UTR of *CLB3* are sufficient to confine translation of proteins to meiosis II.

To further define the sequences that bring about translational control to *CLB3* we determined the length of the *CLB3* 5'UTR. This analysis revealed a start site at 130 bp upstream of the ATG and additional transcriptional initiation between 130 bp and 156 bp upstream (Figure 8). Thus, the 5'UTR has a maximal length of 156 bases, which is in good agreement with a recent genome-wide analysis of the yeast transcriptome (David et

al., 2006). Importantly, the length of the 5'UTR was the same during vegetative growth, meiosis I and meiosis II (Figure 8) indicating that changes in 5'UTR length are not likely to be responsible for the differences in translation observed between meiosis I and meiosis II.

Figure 8

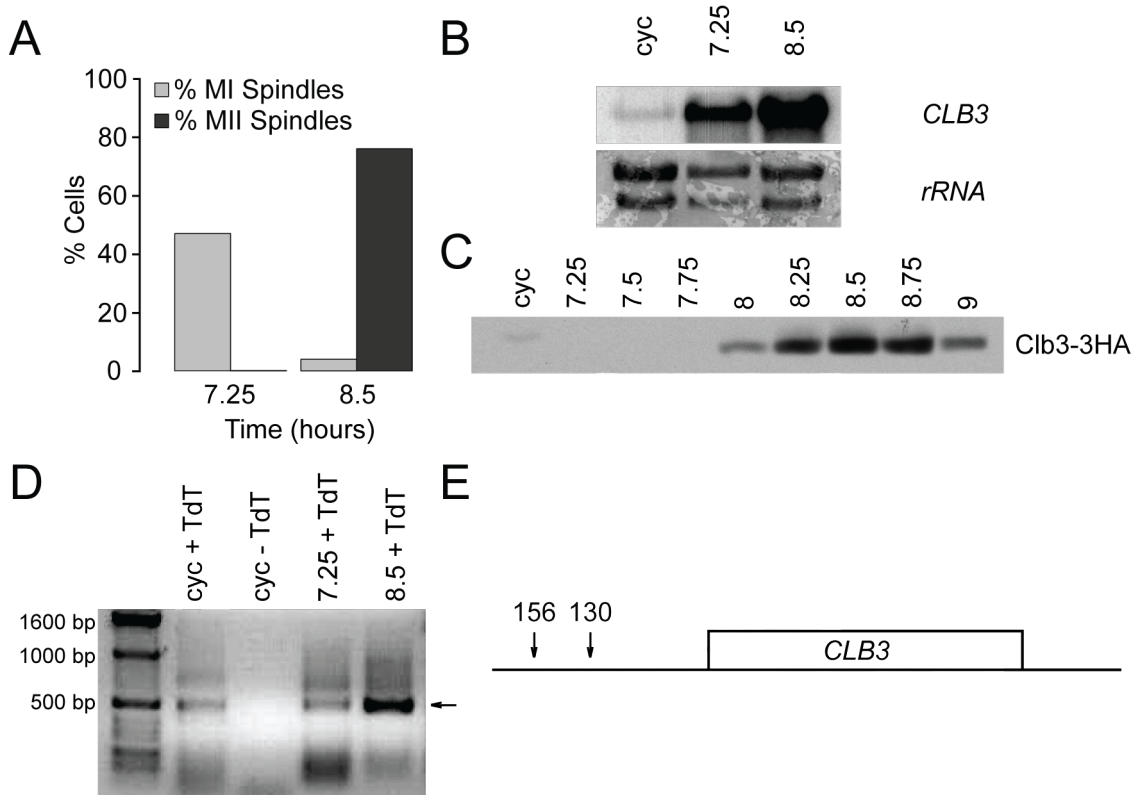

Figure 8: 5'RACE Analysis of *CLB3* transcript from cycling, meiosis I and meiosis II cells.

CLB3-3HA (A15055) cells carrying *GAL4.ER GAL-NDT80* alleles were sporulated at 30°C, and were released from the *GAL-NDT80* block at 6 hours with the addition of 1μM β-estradiol. Samples were also taken from cycling cultures of *CLB3-3HA* (A15055).

(A) The percentages of cells with either MI or MII spindles was determined at the times indicated after inoculation into SPO medium.

(B) Northern blot showing *CLB3*. *rRNA* is shown as a loading control.

(C) Western blot for Clb3-3HA. Samples were taken from cycling cells (cyc) or from meiotic cultures at the times indicated after inoculation into SPO medium.
(D) Products from 5'RACE analysis of *CLB3* transcript in cycling cells or at the indicated times after inoculation into SPO medium. Arrow indicates expected size of 5'RACE product.
(E) Schematic showing obtained lengths of the *CLB3 5'UTR*. Similar results were obtained for cycling, MI and MII samples.

To determine whether the *CLB3* 5'UTR is sufficient to restrict translation of proteins to meiosis II, we placed *CLB2* under the control of a fusion between the *GAL1-10* promoter and 153 bases of the 5'UTR of *CLB3* (*GAL-5'UTR*$_{CLB3}$*-CLB2*). Cells that carry *CLB2* under the control of the *GAL1-10* promoter and *GAL1 5'UTR (GAL-CLB2)* were used as a control. Expression of *CLB2* RNA was similar in the two strains (Figure 9B). In contrast, Clb2 protein accumulated during meiosis I in *GAL-CLB2* cells, but did not accumulate until meiosis II in *GAL-5'UTR*$_{CLB3}$*-CLB2* strains (Figure 9A). Furthermore, consistent with our analysis of Clb3 translation, Clb2 translation was significantly less efficient when the 5' UTR of *CLB3* was employed to drive *CLB2* expression (Figure 9A). Our results show that the *CLB3 5'UTR* is sufficient to prevent protein accumulation during meiosis I.

Figure 9

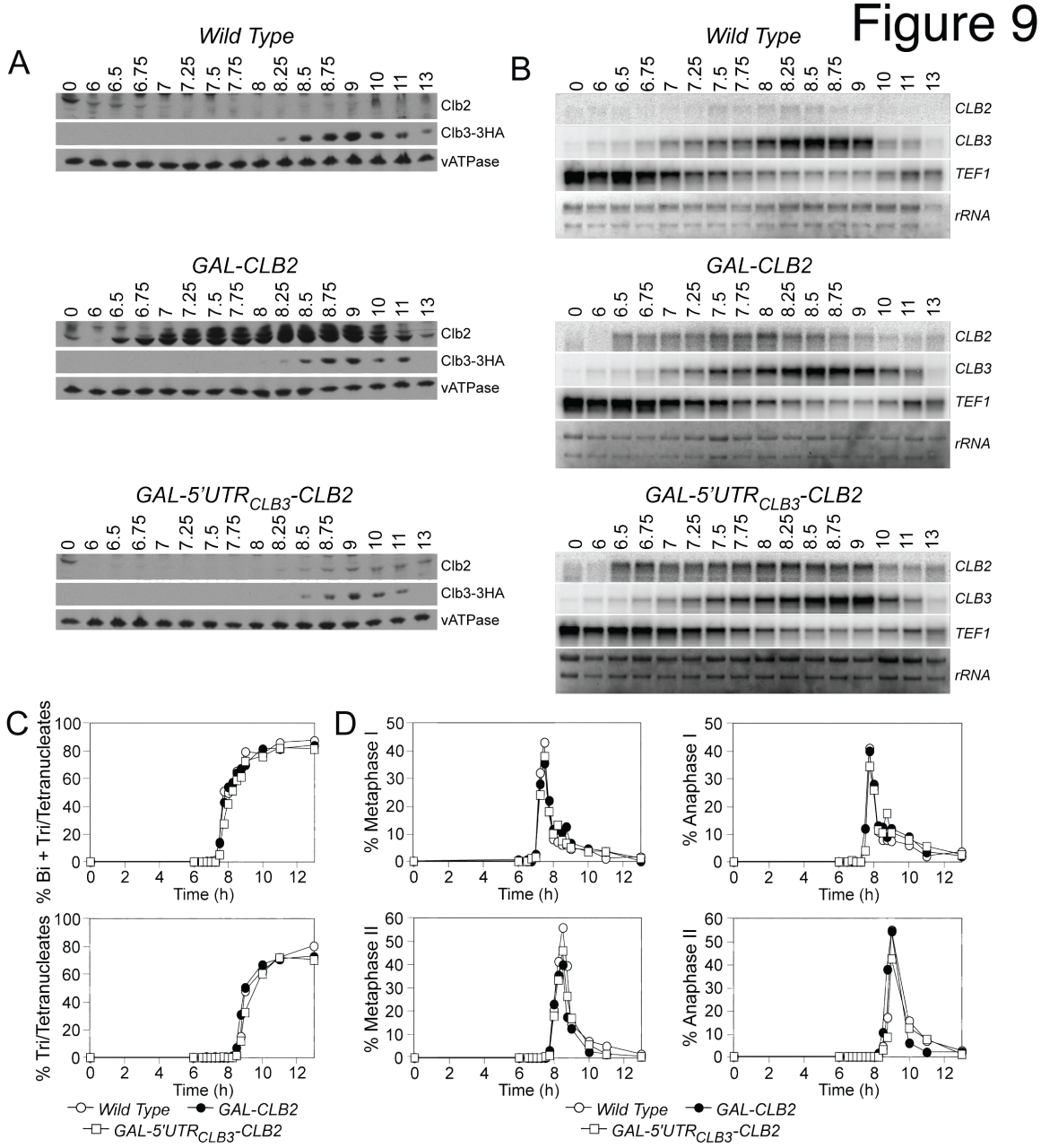

Figure 9: The *CLB3 5'UTR* is sufficient to prevent protein accumulation during meiosis I.

Wild-type (A15802, open circles), *GAL-CLB2* (A19060, closed circles) and *GAL-5'UTR$_{CLB3}$-CLB2* (A19026, open squares) cells carrying *GAL4.ER*, *GAL-NDT80* and *CLB3-3HA* alleles were cultured as described in Figure 6.

(A) Western blots for Clb2 and Clb3-3HA.

(B) Northern blots for *CLB2* and *CLB3*.
(C and D) The percentages of bi- and tri- or tetranucleate cells ([C], upper graph), of tri- or tetranucleate cells ([C], lower graph), and of cells with metaphase I ([D], upper left graph), anaphase I ([D], upper right graph), metaphase II ([D], lower left graph) or anaphase II spindles ([D], lower right graph) were determined at the times indicated.

Consequences of translating Clb3 during meiosis I.

To determine whether preventing Clb3 accumulation during meiosis I was important for the successful execution of this division we produced Clb3 during meiosis I by placing the gene under the control of the *GAL1-10* promoter. Importantly, the amount of Clb3 produced from this promoter during meiosis I was less than the amount of Clb3 that accumulates during meiosis II in wild-type cells (Figure 6A) indicating that the protein was not significantly overproduced when expressed from the *GAL1-10* promoter. To follow the fate of chromosomes during the meiotic divisions we integrated a tandem array of tetO sequences at *LEU2* on both copies of chromosome III (homozygous LEU2-GFP dots). These cells also expressed a tetR-GFP fusion, which binds to tetO, to visualize the repeats (Michaelis et al., 1997).

Wild-type and *GAL-CLB3* cells carrying *NDT80* under its native promoter were induced to enter meiosis. Three hours thereafter, when most cells had completed DNA replication, β-estradiol was added to induce expression of *CLB3* in the *GAL-CLB3* cells. In wild-type cells, homologous chromosomes segregated during meiosis I giving rise to binucleate cells with a LEU2-GFP dot in each nucleus (Figure 10A, left panel). In contrast, 17% of *GAL-CLB3* cells missegregated homologs to the same pole during meiosis I leading to

binucleate cells with a LEU2-GFP dot in only one of the two nuclei (Figure 10A, left panel). The distribution of LEU2-GFP dots was also abnormal in cells that had completed meiosis II (Figure 10A, right panel). Despite these segregation abnormalities, both meiotic nuclear divisions occurred in *GAL-CLB3* cells to the same extent as in wild-type cells (65% tetranuclate cells in *GAL-CLB3* cultures compared to 57% in wild-type cultures; n=200). Tetrad formation was however reduced (30.5% tetrads in *GAL-CLB3* cells compared to 59% in wild-type cells; n=200). Analysis of the spore viability revealed that in cells that were able to form spores chromosome segregation was little affected. 66% of spores of *GAL-CLB3* strains were viable compared to 93% of the wild-type (n=160).

Next we examined whether production of Clb3 during meiosis I affected sister chromatid segregation by analyzing the behavior of strains in which only one of the two homologs carried LEU2-GFP dots (heterozygous LEU2-GFP dots). In this situation, a wild-type meiosis I chromosome segregation pattern gives rise to binucleate cells with a LEU2-GFP dot in one of the two nuclei. In *GAL-CLB3* cells, 30% of binucleate cells contained a GFP dot in both nuclei (Figure 10B; left panel). This outcome was not due to increased recombination between the centromere and the *LEU2* locus because GFP dots at the centromere of chromosome V (CENV-GFP dots) behaved similarly (Figure 10B, right panel). Our results show that expression of Clb3 prior to and during meiosis I interferes with homolog disjunction and causes cells to segregate sister chromatids during the first nuclear division.

Are the effects of expressing Clb3 on meiosis I chromosome segregation specific to this cyclin or does any high Clb-CDK activity interfere with meiotic chromosome segregation? To address this question we examined the consequences of producing Clb2, another cyclin that is normally not expressed during meiosis I. Sister chromatid separation during the first meiotic division also occurred in cells expressing *CLB2* from the *GAL1-10* promoter but at a lower frequency (12% in *GAL-CLB2* cells compared to 51% in *GAL-CLB3* cells; Figure 10B, C). In contrast, replacing the promoter of a cyclin that is normally expressed during meiosis I (*CLB4*) with the *GAL1-10* promoter did not interfere with meiotic chromosome segregation (Figure 10C), sporulation efficiency and spore viability (94.5%; n=160). Our results show that expression of a cyclin that is normally expressed during meiosis I from the *GAL1-10* promoter (Clb4) does not affect meiosis I chromosome segregation. In contrast, expression of a cyclin that is not normally expressed during meiosis I such as Clb2 or Clb3, which likely results in an increase in overall Clb-CDK activity during meiosis I, interferes with meiotic chromosome segregation. Furthermore, it appears that Clb3 is a more potent inhibitor of meiosis I chromosome segregation than Clb2.

Production of Clb3 during meiosis I causes premature sister chromatid separation.

Production of Clb3 prior to and during meiosis I caused the appearance of bi-nucleate cells that had separated their sister chromatids. To determine whether this was due to premature sister chromatid separation during meiosis I we examined the behavior of heterozygous CENV-GFP dots in cells that were arrested in metaphase I, due to the depletion of Cdc20. During metaphase I kinetochores of sister chromatid pairs attach to

microtubules emanating from the same pole (co-orientation). Thus the sister kinetochores are not under tension and only one CENV-GFP dot is visible (Figure10D, Lee and Amon, 2003). In contrast, when sister chromatids are bi-oriented, their kinetochores attach to microtubules from opposite poles. The pulling force of the spindle then leads to the appearance of two CENV-GFP dots (Lee and Amon, 2003).

Production of Clb3 in Cdc20-depleted cells led to separation of sister chromatids as judged by the appearance of two GFP dots (Figure 10D). Interestingly, expression of Clb3 also suppressed the metaphase I arrest caused by the depletion of Cdc20. GFP-dots integrated at *URA3* also separated in *GAL-CLB3* cells and spindle elongation and the formation of binucleate cells occurred (Figure 10D-F). Our results show that production of Clb3 suppresses the need for high levels of Cdc20 in promoting entry into anaphase I and causes premature sister chromatid separation during meiosis I.

Figure 10

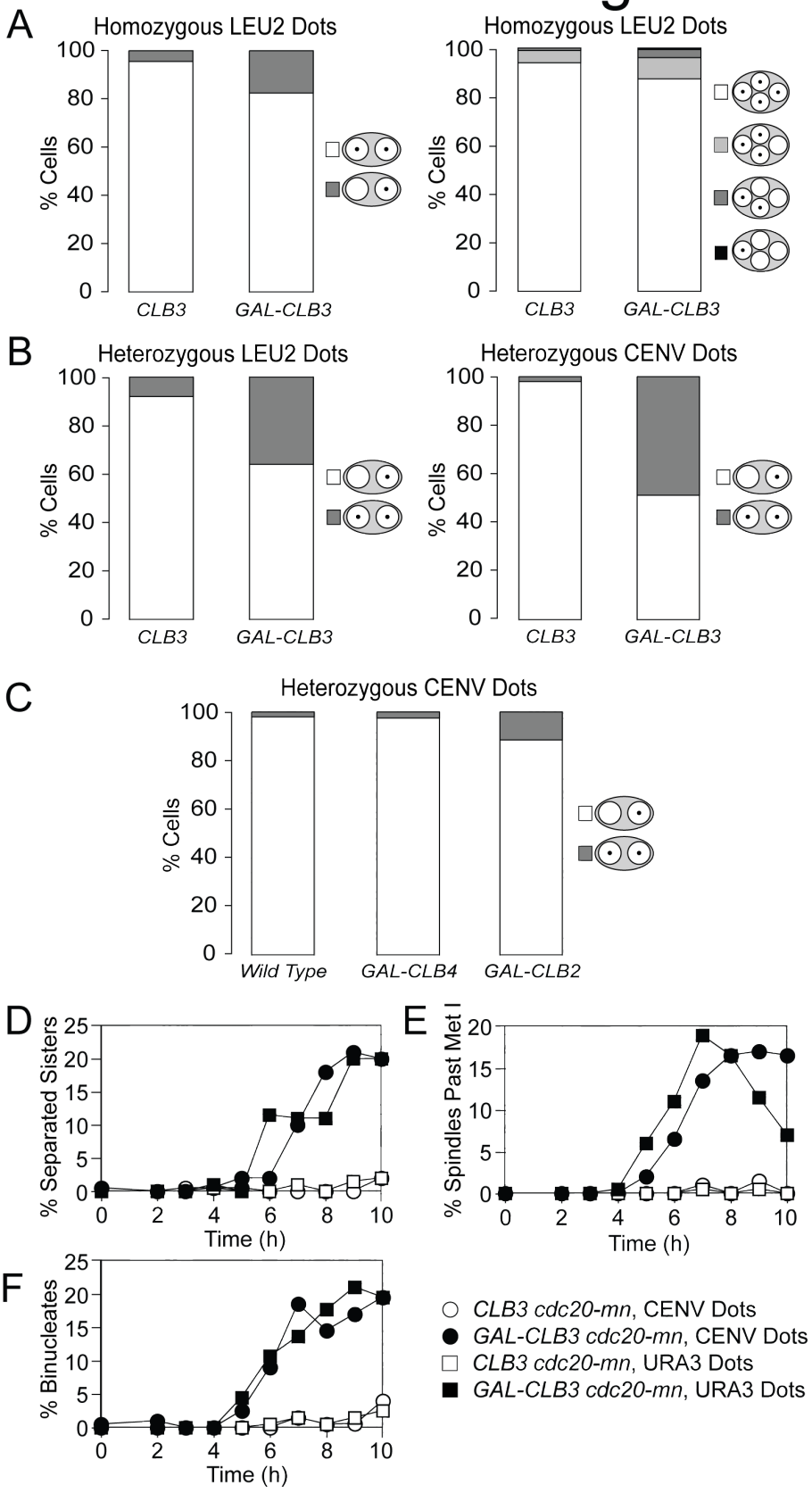
A
Homozygous LEU2 Dots
Homozygous LEU2 Dots
% Cells
CLB3
GAL-CLB3
B
Heterozygous LEU2 Dots
Heterozygous CENV Dots
C
Heterozygous CENV Dots
Wild Type
GAL-CLB4
GAL-CLB2
D
% Separated Sisters
Time (h)
E
% Spindles Past Met I
F
% Binucleates
CLB3 cdc20-mn, CENV Dots
GAL-CLB3 cdc20-mn, CENV Dots
CLB3 cdc20-mn, URA3 Dots
GAL-CLB3 cdc20-mn, URA3 Dots

Figure 10: Production of Clb3 or Clb2 during meiosis I causes premature sister chromatid separation.

The strains listed below were sporulated at 30°C. 1μM β-estradiol was added at 3 hrs after inoculation into SPO medium to induce *CLB3* expression.
(A) *CLB3-3HA* (A18655) and *GAL-CLB3-3HA* (A18656) strains carrying *GAL4.ER* and homozygous LEU2 dots were used. The percentage of binucleates with either one or two dots was determined at 7 hrs after inoculation into SPO ([A] left panel, n=200). The percentage of tetranucleates with one, two, three or four dots was determined at 12 hrs after inoculation into SPO ([A] right panel, n=200).
(B) *CLB3-3HA* (A18686) and *GAL-CLB3* (A18687) strains carrying *GAL4.ER* and heterozygous LEU2 dots, and *CLB3* (A19396) and *GAL-CLB3* (A19400) strains carrying *GAL4.ER* and heterozygous CENV dots were used. The percentage of binucleates with either one or two dots was determined at 8.5 hrs after transfer into SPO (n=200).
(C) *Wild Type* (A19397), *GAL-CLB4* (A19399) and *GAL-CLB2* (A19687) strains carrying *GAL4.ER* and heterozygous CENV dots were used. Binucleate cells were examined 7 hrs after transfer into SPO (n=200).
(D-F) *CLB3* (A19402, open circles) and *GAL-CLB3* (A19406, closed circles) strains carrying *cdc20-mn*, *GAL4.ER* and heterozygous CENV dots, and *CLB3* (A19408, open squares) and *GAL-CLB3* (A19410, closed squares) strains carrying *cdc20-mn*, *GAL4.ER* and heterozygous URA3 dots were used. The percentage of cells with separated GFP dots [D], spindles past metaphase I [E], and two DAPI masses [F] were determined at the times indicated.

Discussion

Clb-CDK control during meiosis.

We developed a method to produce budding yeast cultures that proceed through the meiotic divisions with a high degree of synchrony. The examination of Clb-CDKs using this system revealed a surprising diversity in the regulation of the different cyclin-CDK complexes that was not appreciated previously because of the poor synchrony of meiotic cultures generated by standard conditions. During vegetative growth, transcription and ubiquitin-dependent protein degradation are primarily responsible for controlling Clb-CDK activity (reviewed in Bloom and Cross, 2007). Clb6 is a substrate of the SCF ubiquitin ligase, and Clb5 and a fraction of Clb2 are degraded by APC/C-Cdc20 at the metaphase – anaphase transition. Clb1, Clb2 and Clb3 are degraded later during mitosis by APC/C-Cdh1. The ubiquitin ligase responsible for degrading Clb4 has not been identified. During meiosis only Clb5-CDK activity is regulated at the level of Clb5 protein abundance. Clb3-CDKs are regulated at the level of Clb3 translation. Clb1-CDKs and Clb4-CDKs are restricted to specific meiotic stages by posttranslational mechanisms other than protein degradation.

Which post-translational mechanisms could be responsible for down-regulating Clb4-CDKs after metaphase II and Clb1-CDKs during all of meiosis II? CDK inhibition by tyrosine 19 phosphorylation on Cdc28 or binding of the CDK inhibitor Sic1 could inhibit Clb4-CDKs during exit from meiosis II. One of the two pathways or both could also be responsible for preventing Clb1-CDKs from being active throughout meiosis II. This, however, would require a high degree of Clb-CDK specificity for these regulatory

mechanisms that has not been observed previously. We therefore believe it to be more likely that a novel Clb1-CDK specific mechanism exists that prevents this particular kinase from being active during meiosis II. Such a mechanism could involve a selective inhibitor of Clb1-CDKs or preventing the association of Clb1 with Cdc28. In this regard it is interesting to note that Clb1 protein is not degraded during exit from meiosis I but exported out of the nucleus (Buonomo et al., 2003; Marston et al., 2003). Perhaps this nuclear export of Clb1 is important for maintaining Clb1-CDKs inactive during meiosis II.

Translational control in meiosis.

Clb3 expression is restricted to meiosis II through translational control. In higher eukaryotes, translational control of B-type cyclins plays a key role in controlling meiotic progression. In *Xenopus*, progesterone mediated oocyte maturation relieves the translational inhibition of a number of mRNAs including that of *cyclin B1* by promoting their polyadenylation (Stebbins-Boaz et al., 1996). Translational control of *cyclin B* mRNA is also observed in *Drosophila*. There, the PAN GU kinase promotes translation of the *cyclin B* message during exit from meiosis II by antagonizing the translational repressor Pumilio (Vardy and Orr-Weaver, 2007). Translational control of Clb3 appears to be meiosis I specific. *CLB3* mRNA is translated during meiosis II and also during mitosis, because Clb3 protein accumulates as soon as cells enter the cell cycle (as judged by bud formation) and APC/C-Cdh1 activity is turned off (Figure 11). The simplest interpretation of this data is that *CLB3* translation is controlled by a meiosis I-specific translational repressor.

Figure 11

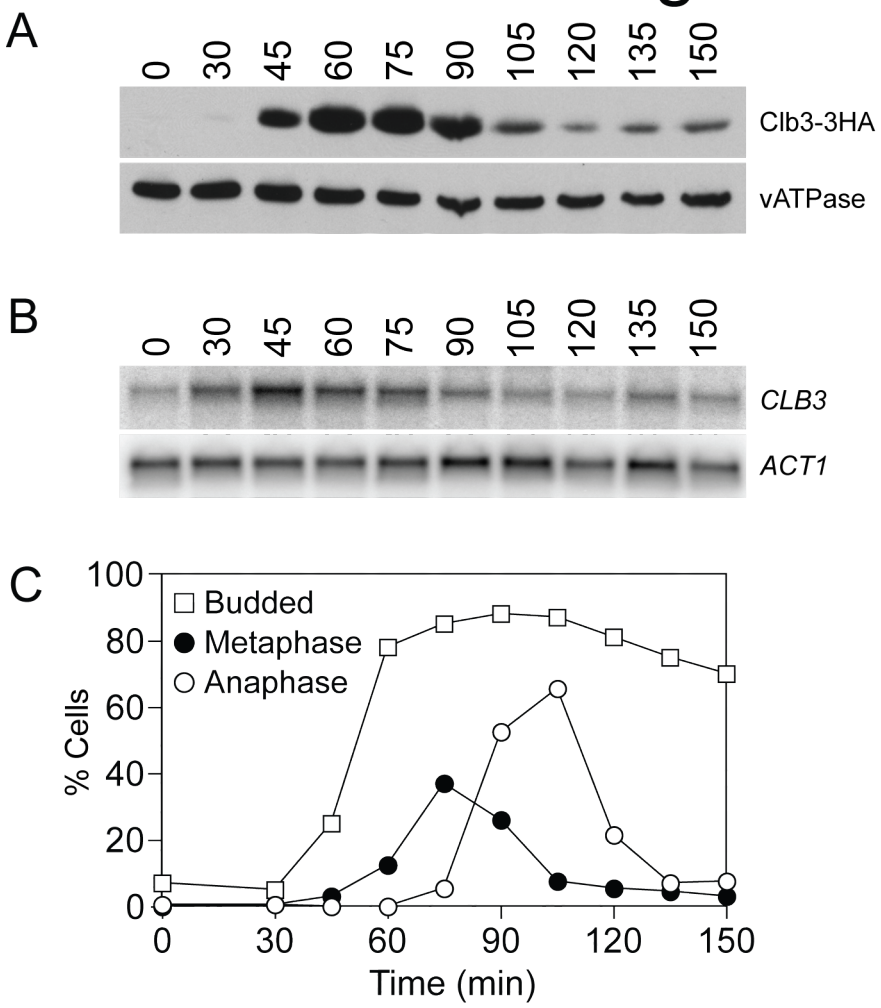

Figure 11: *CLB3* transcript and Clb3 protein levels during mitosis.

CLB3-3HA (A11955) cells were synchronized in G1 by treatment with 5 µg/mL alpha-factor. Cells were released from this arrest by washing and allowed to proceed through mitosis at room temperature. 10 µg/mL alpha-factor was added at 90 minutes to prevent a second cell cycle. Timepoints were taken at the times indicated.

(A) Western blot for Clb3-3HA. vATPase is shown as a loading control.

(B) Northern blot showing *CLB3*. *ACT1* is shown as a loading control.

(C) The percentages of budded (n=100), metaphase (n=200) and anaphase (n=200) cells were determined at the times indicated after release into the cell cycle.

The 5'UTR affects translation of Clb3 in at least two ways. The 5'UTR decreases translational efficiency and restricts translation to meiosis II. Whether these two effects are linked or are brought about by separate mechanisms remains to be determined. Translational control occurs in budding yeast, but to date has not been observed during meiosis. Small upstream open reading frames (uORFs) regulate the translation of the *GCN4* mRNA. These uORFs are thought to serve as a measure of the translational capacity of the cell, preventing translation of *GCN4* mRNA when the translational capacity is high (Hinnebusch, 2005). The 156 bp 5'UTR of *CLB3* does not contain uORFs (T. M. C. unpublished observations). It is therefore not likely that translation of *CLB3* is governed by such a mechanism. Processing bodies (P-bodies) are cytoplasmic foci that are sites of mRNA degradation and of storage of non-translating mRNAs, which can later reenter translation (reviewed in Parker and Sheth, 2007). *PAT1* and *DHH1* have been implicated in regulating mRNA stability and translation in P-bodies (Coller and Parker, 2005). Deletion of neither gene allowed Clb3 protein to accumulate during meiosis I (T. M. C., unpublished observations) indicating that this pathway was not responsible for controlling Clb3 translation. Identifying the mechanisms that prevent translation of Clb3 during meiosis I and determining whether other RNAs are regulated in this manner will be an important question in the future. Furthermore we note that the 156 bp *CLB3* 5'UTR sequence will prove useful in studying the effects of expressing genes specifically during meiosis II.

Importance of translational control of Clb3.

During meiosis I, homologous chromosomes rather than sister chromatids segregate from each other. For this unusual chromosome segregation to occur several meiosis specific events must take place (reviewed in Marston and Amon, 2004). First, reciprocal recombination between homologs generates linkages between them, which ensure that homologs are accurately aligned on the metaphase I spindle. Second, mediated by the monopolin complex, sister kinetochores attach to microtubules emanating from the same pole (co-orientation) to facilitate sister chromatid co-segregation during anaphase I. Lastly, cohesin complexes that hold sister chromatids together are lost in a step-wise manner. Loss of arm cohesion allows for the segregation of homologs during meiosis I. Retention of centromeric cohesion ensures that sister chromatids properly align on the meiosis II spindle. Inhibition of Clb3 production during meiosis I is critical for establishing the meiosis I chromosome segregation pattern. In cells producing Clb3 during meiosis I, sister kinetochore co-orientation and the step-wise loss of cohesion appear to be disrupted. Clb3 could interfere with sister kinetochore co-orientation by preventing the association of the monopolin complex with kinetochores. How could Clb3 interfere with the step-wise loss of cohesion? During meiosis I, Sgo1 associates with kinetochores where it prevents loss of cohesins (reviewed in Ishiguro and Watanabe, 2007). Clb3 could inhibit the association of Sgo1 with kinetochores or prevent the protein from recruiting the protein phosphatase PP2A, which renders cohesins resistant to removal.

Figure 12

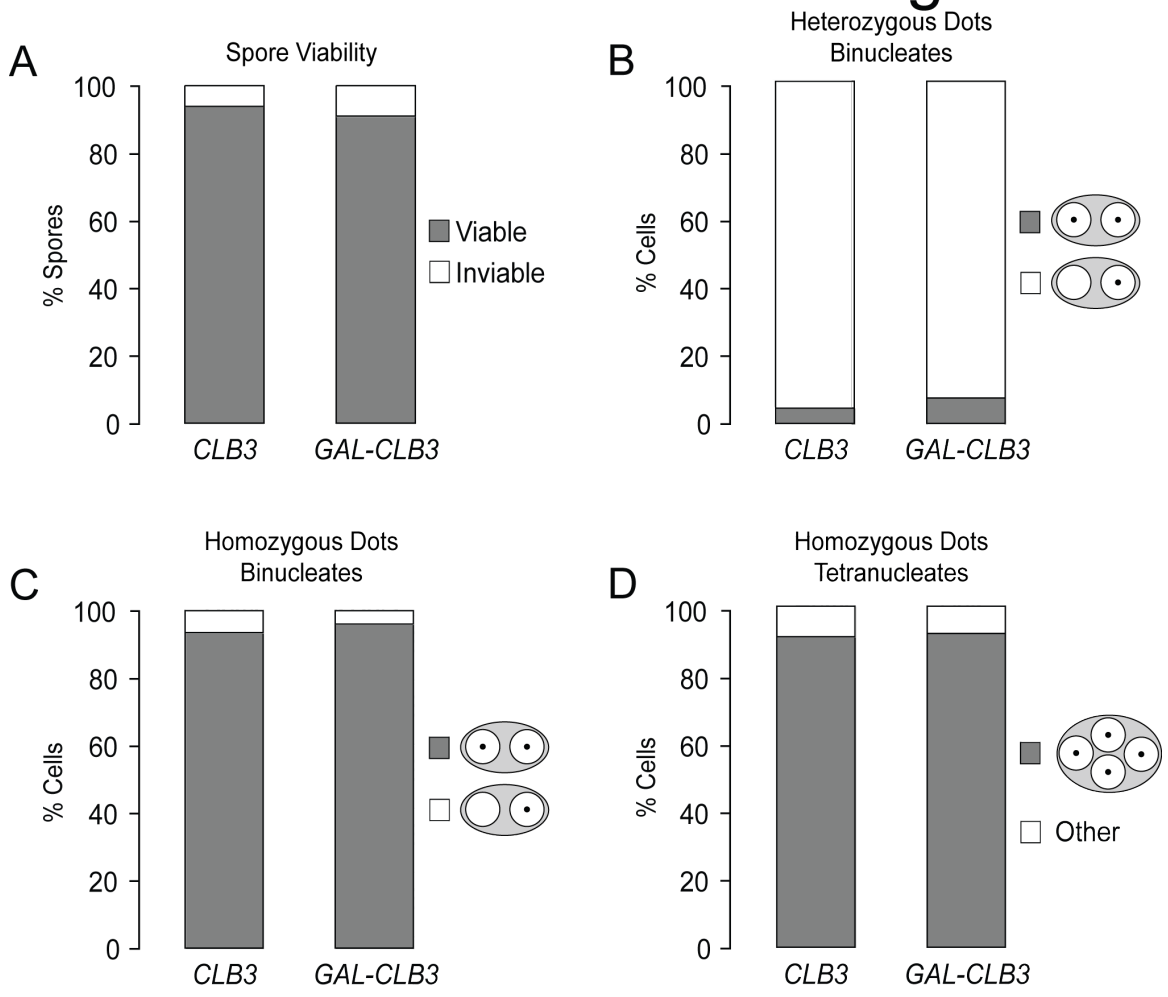

Figure 12: Spore Viability and GFP dot segregation in *GAL4.ER GAL-NDT80 GAL-CLB3* strains.

(A) *CLB3-3HA* (A15055) and *GAL-CLB3-3HA* (A18095) cells carrying *GAL4.ER*, *GAL-NDT80* were induced to sporulate at 30°C. After 6 hours in SPO medium 1μM β-estradiol was added. Samples were taken after 24 hours and tetrads were dissected (n=44).

(B) *CLB3-3HA* (A18185) and *GAL-CLB3-3HA* (A18206) cells carrying *GAL4.ER*, *GAL-NDT80* and heterozygous *LEU2* GFP-dots were induced to sporulate at 30°C. After 6 hours in SPO medium 1μM β-estradiol was added. Sister-chromatid separation was scored at 7.75 hours after inoculation into SPO (n=200).

(C and D) *CLB3-3HA* (A18186) and *GAL-CLB3-3HA* (A18207) cells carrying *GAL4.ER*, *GAL-NDT80* and homozygous *LEU2* GFP-dots were induced to sporulate at 30°C. After 6 hours in SPO medium 1μM β-estradiol was added. Chromosome separation was scored at 7.75 hours after inoculation into SPO (n=200) for binucleates (C) and at 13 hours for tetranucleates (n=100).

The phenotype of cells expressing *CLB3* during meiosis I is reminiscent of that of cells lacking the meiosis I specific gene *SPO13*. In both, *GAL-CLB3* cells and *spo13*Δ cells, sister chromatids separate during meiosis I. In both strains, the metaphase I arrest brought about by Cdc20 depletion is suppressed (Shonn et al., 2002; Katis et al., 2004) and delaying progression through early meiosis suppresses premature sister chromatid separation (McCarroll and Esposito, 1994; Figure 12). *SPO13* is, however, not required for regulating *CLB3* translation. In cells lacking *SPO13* translation of *CLB3* did not occur prematurely (Figure 13). Whether Clb3-CDK activity inhibits Spo13 function remains to be determined.

Figure 13

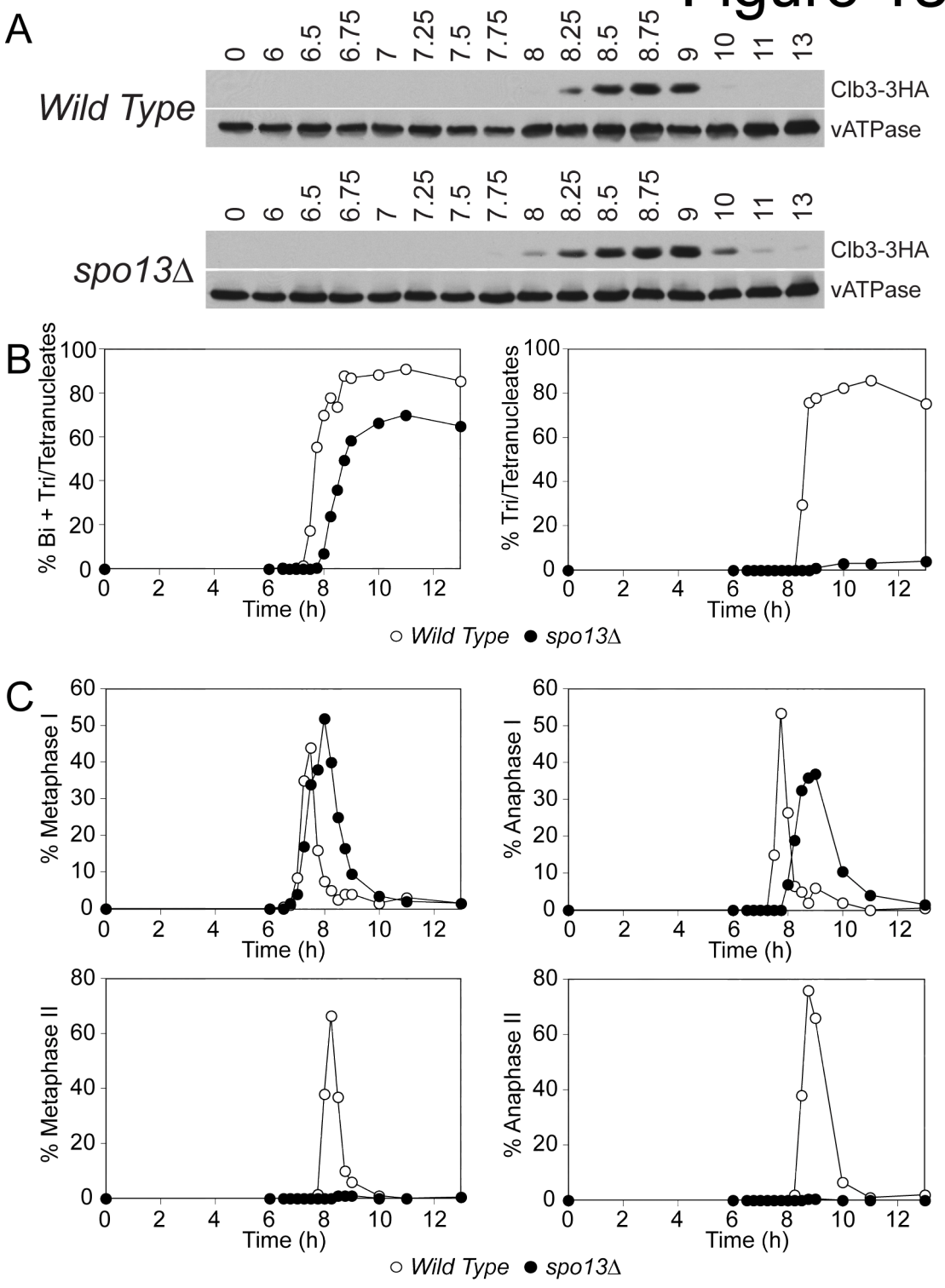
A
0 6 6.5 6.75 7 7.25 7.5 7.75 8 8.25 8.5 8.75 9 10 11 13
Wild Type
Clb3-3HA
vATPase
spo13Δ
B
% Bi + Tri/Tetranucleates
% Tri/Tetranucleates
Time (h)
○ Wild Type ● spo13Δ
C
% Metaphase I
% Anaphase I
% Metaphase II
% Anaphase II
Time (h)
○ Wild Type ● spo13Δ

Figure 13: *SPO13* is not required for translational repression of *CLB3*.

Wild-type (A15505, open circles) and *spo13*Δ (A19503, closed circles) cells carrying *GAL4.ER*, *GAL-NDT80* and *CLB3-3HA* alleles were sporulated at 30°C, and were released from the *GAL-NDT80* block at 6 hours with the addition of 1μM β-estradiol.

(A) Western blots for Clb3-3HA. vATPase is shown as a loading control.

(B and C) The percentages of bi- and tri- or tetranucleate cells ([B], left graph) or the percentages of tri- or tetranucleate cells ([B], right graph), and the percentages of cells with metaphase I ([C], upper left graph), anaphase I ([C], upper right graph), metaphase II ([C], lower left graph) or anaphase II spindles ([C], lower right graph) were determined at the times indicated after inoculation into SPO medium.

Clb-CDK specificity during meiosis.

Is Clb3 unique amongst the Clb cyclins in repressing meiosis I chromosome segregation? Consistent with this idea is the observation that Clb2 is not as effective in suppressing the meiosis I chromosome segregation pattern as Clb3. Several other observations, however argue against this notion. First, to date no CDK substrate has been identified that is phosphorylated exclusively by one Clb-CDK subtype (Loog and Morgan, 2005). Second, deletion of individual *CLB* genes does not interfere with progression through meiosis (Dahmann and Futcher, 1995). Finally, expression of *CLB4* from the *GAL1-10* promoter instead of its native promoter, which is not expected to substantially increase overall Clb-CDK activity, does not interfere with meiotic chromosome segregation. On the other hand, moderate expression of Clb2 during meiosis I, which is expected to elevate overall Clb-CDK activity, causes some premature sister chromatid separation. Increasing Clb-CDK activity levels even further through overexpression of a stabilized version of Clb2, leads to more than half of sister chromatids segregating during the first nuclear division,

which could not solely be explained by meiosis II events occurring on the anaphase I spindle (Marston et al., 2003). We therefore favor the idea that high Clb-CDK levels interfere with the meiosis I chromosome segregation pattern but will not exclude the possibility that some substrate-specificity exists among Clb1, Clb2, Clb3 and Clb4 causing Clb3 to be better at inhibiting meiosis I than other Clbs.

Aside from the issue of Clb-CDK specificity our studies raise the following question. Why do cells restrict different Clb-CDKs to different stages of meiosis and employ such diverse strategies to accomplish this? We propose that the answer may lie in the unique feature of meiosis that is the occurrence of two consecutive chromosome divisions. One division immediately following another one requires a balancing act between the necessity to down-regulate Clb-CDKs to bring about exit from meiosis I and ensuring that a sufficient amount of Clb-CDKs are present to execute the second meiotic division. Employing APC/C-mediated protein degradation to bring about Clb-CDK down-regulation during exit from meiosis I as is done during mitosis would necessitate the re-synthesis of Clb cyclins prior to entry into meiosis II, which may be a lengthy undertaking under the extreme nutrient-limiting conditions of meiosis. It may thus be advantageous for the cell to inhibit APC/C-Cdh1 during exit from meiosis I. Indeed, our data and that of others on Clb1 protein abundance (Marston et al., 2003; Buonomo et al., 2003) indicate that this is the case. Instead, other mechanisms have evolved that down-regulate Clb1-CDKs to promote exit from meiosis I. Following exit from meiosis I, maintaining a pool of *CLB3* RNA ready for translation may ensure quick entry into

meiosis II. We suggest that the strategies governing progression through two consecutive meiotic divisions required novel ways of regulating Clb-CDKs.

Acknowledgments

We are grateful to Kirsten Benjamin for strains and Fred Cross for the anti-Clb2 antibody. We thank Roy Parker for helpful discussions and Steve Bell, Frank Solomon, Andreas Hochwagen and members of the Amon lab for their critical reading of this manuscript. This research was supported by National Institutes of Health grant GM62207 to A.A. A.A. is also an Investigator of the Howard Hughes Medical Institute.

Experimental Procedures

Strains and Plasmids

All strains are SK1 derivatives and are described in Table 1. *GAL-NDT80* and *GAL4.ER* constructs are described in Benjamin et al. (2003). *CLB3-3HA*, *CLB4-3HA, CLB5-3HA*, *pdr5*Δ, P_{CLB2}*-CDC20*, P_{CLB2}*-3HA-CDC27*, *GAL-CLB3-3HA* and *GAL-CLB4-3HA* were constructed using the PCR-based method described in Longtine et al. (1998). Endogenous *CLB3* and *CLB4* were replaced with *CLB2* using the PCR-based method described in Longtine et al. (1998). The *cdc23-1* allele was integrated at the *CDC23* locus in SK1. *GAL-5'UTR*$_{CLB3}$*-CLB2* was created by cloning 153 base pairs of the *CLB3 5'UTR* into a *GAL-CLB2* plasmid, and integrating at the *CLB2* locus.

Table 1: SK1 Derivatives

Strain Number	Relevant Genotype
A4841	MATa
A4736	MATa *CLB4-3HA::KANMX6*
A7057	MATa *CLB1-9Myc::TRP1*
A11955	MATa *CLB3-3HA::KANMX6*
A15109	MATa *CLB5-3HA::KANMX6*
A14200	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3*
A14201	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1*
A15055	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6*
A15090	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB4-3HA::KANMX6/CLB4-3HA::KANMX6*
A15591	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB1-9Myc::TRP1/+*
A15802	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-*

	3HA::KANMX6/+
A15804	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB5-3HA::KANMX6/+*
A16011	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 pdr5::TRP1/pdr5::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6*
A17855	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 PDS1-18MYC::LEU2/+*
A17856	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 cdc27:: pCLB2-3HA-CDC27::KanMX6/pCLB2-3HA-CDC27::KanMX6 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 PDS1-18MYC::LEU2/+*
A17946	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 PDS1-18MYC::LEU2/+*
A17947	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 cdc23-1/cdc23-1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 PDS1-18MYC::LEU2/+*
A18095	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6*
A18185	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A18186	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/leu2::tetR-GFP::LEU2::TetO-HIS3*
A18206	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A18207	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::tetR-GFP::LEU2::TetO-HIS3/leu2::tetR-GFP::LEU2::TetO-HIS3*
A18485	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 clb4::pGAL-CLB4-*

	3HA::KANMX6::HIS3MX6/clb4::pGAL-CLB4-3HA::KANMX6::HIS3MX6
A18486	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6*
A18574	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/clb3::pCLB3-CLB2::HIS3MX6*
A18578	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB4-3HA::KANMX6/clb4::pCLB4-CLB2::HIS3MX6*
A18655	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/leu2::tetR-GFP::LEU2::TetO-HIS3*
A18656	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/ clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::tetR-GFP::LEU2::TetO-HIS3/leu2::tetR-GFP::LEU2::TetO-HIS3*
A18686	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A18687	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A19026	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/+ CLB2::GAL-5'UTR(CLB3)-CLB2::TRP1/CLB2::GAL-5'UTR(CLB3)-CLB2::TRP1*
A19060	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/+ CLB2::GAL-CLB2::TRP1(2 copies)/+*
A19396	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/+ leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*
A19397	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*
A19399	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 clb4::pGAL-CLB4-3HA::KANMX6::HIS3MX6/clb4::pGAL-CLB4-3HA::KANMX6::HIS3MX6 leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*
A19400	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/+ clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*

A19402	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/+ cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6 leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*
A19406	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/+ cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*
A19408	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::TETOx224::URA3 cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6 leu2::pURA3-TetR-GFP::LEU2/+*
A19410	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::TETOx224::URA3 cdc20::pCLB2-CDC20::KanMX6/cdc20::pCLB2-CDC20::KanMX6 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::pURA3-TetR-GFP::LEU2/+*
A19503	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 spo13::hisG/spo13::hisG*
A19687	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB2::GAL-CLB2::TRP1(2 copies)/+ leu2::pURA3-TetR-GFP::LEU2/+ CENV::TetOx224::HIS3/+*

Sporulation Conditions

Strains were grown to saturation in YPD, diluted in YPA (1% yeast extract, 2% bactopeptone, 1% potassium acetate) to OD_{600} = 0.3, and grown overnight. Cells were resuspended in sporulation medium (0.3% potassium acetate [pH 7], 0.02% raffinose) to OD_{600} = 1.9 and sporulated at 30°C. Meiotic cultures synchronized using the *GAL-NDT80 GAL4.ER* system were prepared for sporulation as follows. Strains were grown on YPG (3% glycerol) plates at room temperature for 12 hours, were then transferred to YPD4 (YPD+4% glucose) plates for 24 hours, were then grown to saturation in YPD (24 hrs at room temperature), diluted in YPA (1% yeast extract, 2% bactopeptone, 1% potassium acetate) to OD_{600} = 0.3 and grown overnight. Cells were pelleted, washed with water and resuspended in sporulation medium (0.3% potassium acetate [pH 7], 0.02% raffinose) to a final OD_{600} =1.9. Following inoculation into sporulation medium,

GAL4.ER GAL-NDT80 strains were allowed to progress into the *GAL-NDT80* block at 30°C for 6 hours (unless otherwise noted; Fig1 [5h], Fig5G-I [7h]) in the absence of β-estradiol. After 6 hours, β-estradiol was added to a final concentration of 1 μM (5mM stock in ethanol, Sigma E2758-1G) in order to induce expression of *NDT80* from the *GAL1-10* promoter by activating the Gal4.ER fusion protein. Cells were then allowed to proceed through the meiotic divisions at 30°C. Time points were taken for a further 7 hours (13 hours total) by which time most cells had completed both meiotic divisions.

Western Blot Analysis

Samples were prepared as described in Moll et al. (1991), immunoblots as in Cohen-Fix et al. (1996). Antibody concentrations are described in Monje-Casas et al. (2007), except anti-Pgk1 (Molecular Probes) was used at 1:5000. Rabbit anti-Clb2 was used at a concentration of 1:2000, rabbit anti-Cdc28 at 1:1000 and rabbit anti-Clb3 (Santa Cruz, sc-7167) at 1:500. Sheep anti-mouse conjugated to HRP (GE Healthcare) was used as a secondary antibody at 1:5000.

Immunofluorescence

Indirect immunofluorescence was performed as described in Visintin et al. (1999). Rat anti-tubulin antibodies (Oxford Biotechnology) were used at a dilution of 1:50, and anti-rat-FITC antibodies (Jackson) were used at a dilution of 1:100. Spindle morphologies were classified as in Lee et al. (2003). Metaphase I cells were defined as cells with a single DAPI mass spanned by a short, thick, bipolar, meiotic spindle (approximately 2-3 μm in length). Anaphase I cells were defined as cells with two distinct (though not

always separated) DAPI masses, and a single long spindle that spans both DAPI masses. Metaphase II cells were defined as cells with two separate DAPI masses with each spanned by a bipolar, short, thick, meiotic spindle. Anaphase II cells were defined as cells with four distinct (though not always separated) DAPI masses with two long spindles.

Histone H1 IP Kinase Assays

Histone H1 kinase assays were performed as described in Hochwagen et al. (2005). 25 mls of meiotic culture were pelleted, transferred to a 2 ml tube and snap frozen in liquid nitrogen for later processing. Cells were broken with glass beads in 200 µL NP40 Lysis Buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40) containing 1mM DTT and protease and phosphatase inhibitors (60 mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 15 mM p-nitrophenylphosphate, 0.095U/ml aprotinin, 1mM pefablock, 1X complete protease inhibitors (Roche 11873580001, 50X solution = 1 tablet in 1mL H_2O). After breaking, extracts were cleared twice by centrifugation and protein concentration was determined by Bradford assay. Immunoprecipitations were performed in 50 µl extract (500 µg total protein). Clb1-9Myc was immunoprecipitated with an anti-Myc antibody (Covance) at a concentration of 1:50; Clb3-3HA, Clb4-3HA and Clb5-3HA were immunoprecipitated with an anti-HA.11 antibody (Covance) at a concentration of 1:50. Extracts were incubated at 4°C with antibody for 1.5 hours, followed by addition of 20 µL of Protein G Sepharose (Pierce, 20398) and additional incubation with rotation at 4°C with for 2 hours. Beads were washed 4 times with NP40 buffer followed by two washes with 25 mM MOPS (morpholinepropanesulfonic acid [pH 7.0]). For the

kinase reaction, beads were incubated with 6 µL buffer HBII (25 mM MOPS, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 1mM PMSF, 0.02 mg/mL leupeptin, 0.04 U/mL aproteinin, 0.1mM soudium orthovanadate, 15 mM p-nitrophenylphosphate) for 15 min at room temperature, followed by the addition of 10 µL kinase reaction mixture (25 mM MOPS, 2 mg/ml histone H1, 0.2 mM ATP) containing 50 nCi [g-^{32}P]ATP. Kinase reactions were allowed to proceed for 15 minutes at room temperature before they were stopped by the addition of 10 µL 3X SDS loading buffer. Kinase samples were separated on a 15% SDS-PAGE gels, fixed in 10% methanol-10% acetic acid for 30 minutes, dried, and analyzed by autoradiography.

Other Techniques

Total RNA was isolated as described in Cross and Tinkelenberg (1991). Northern blots were performed as described in Hochwagen et al. (2005). GFP-tagged chromosomes were fixed for visualization as described in Monje-Casas et al. (2007). 200 cells were counted for each time point unless otherwise noted. Criteria for classifications of spindle morphologies are described in the Supplemental Online Material. Quantification of immunoblots, Northern blots and kinase assays were performed using NIH ImageQuant. To synchronize mitotic cultures in G1, a-factor was added to a concentration of 5 µg/ml. When fully arrested, cells were washed and resuspended in pheromone free media. Total RNA prepared for 5'RACE analysis as described in Collart and Oliviero (1993). 5'RACE was performed using the 5'RACE System Version 2.0 (Invitrogen, 18374-058).

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Chapter 3:

Examination of the Mechanisms of *CLB3* Translational Regulation

The experiments in Figure 8 were performed in collaboration with Ana Oromendia. The method for construction of the UTR deletion strains was developed by Matt Miller.

Introduction

In the course of characterizing Clb-CDK activity during the meiotic divisions, we observed that the cyclin Clb3 is restricted to meiosis II as is its associated kinase activity, while *CLB3* transcript is present during both meiosis I and meiosis II. We subsequently demonstrated that the absence of Clb3 protein during meiosis I was not due to rapid protein turnover, but rather was due to regulation of translation mediated by the *CLB3* 5'UTR (Carlile and Amon, 2008). While our experiments implicated the 5'UTR of *CLB3* in translational regulation, they did not address the mechanism by which this translational regulation occurs. The experiments presented in this chapter are aimed at elucidating the mechanism by which translation of *CLB3* is controlled during meiosis.

There are several known mechanisms of gene specific translational control in yeast. These include the regulation of translation of individual messages by the presence of small upstream open reading frames (uORFs), by the presence of internal ribosome entry sites (IRESs), and by the binding of RNA binding proteins (RBPs) to regulatory elements within the message. Small uORFs in the 5'UTRs of cellular messages are often used to modulate the translational efficiency of individual messages in response to changes in nutrient availability, which impacts the overall translational capacity of the cell. uORFs regulate the translation of many cellular messages, including the *GCN4* and *CLN3* transcripts. Translation of *GCN4* is regulated by the presence of four uORFs. As discussed in Chapter 1, these uORFs sensitize translation of the *GCN4* message to the levels of ternary complex (TC), which reflect the nutritional status of the environment. In nutrient rich environments TC levels are high, which negatively affect *GCN4* translation,

and in nutrient poor environments TC levels are low, which positively affect *GCN4* translation (Hinnebusch, 2005). However, the *CLN3* uORF regulates *CLN3* translation by leaky scanning of the uORF AUG, and reduces translational efficiency in nutrient poor conditions (Polymenis and Schmidt, 1997). In budding yeast cellular IRESs have been demonstrated to control translation of cellular messages required for invasive growth. These IRESs are located in the 5'UTRs of messages, and consist of short poly(A) tracts. These tracts are bound by Poly(A) Binding Protein (PABP), which is then thought to recruit the initiation factor eIF4G, promoting cap-independent translation (Gilbert et al., 2007). Additionally, the translation of many messages is regulated by the binding of RBPs, which can modulate translation in response to a variety of factors. Indeed, a genome wide study suggests that much of the yeast transcriptome may be bound by a variety of RBPs, which themselves seem to bind sets of functionally related mRNAs. This suggests that translational regulation may represent a widespread and underappreciated mode of regulation of gene expression in budding yeast (Hogan et al., 2008). Newly developed techniques that allow global profiling of translation have identified translational initiation at non-cognate start codons as a common mechanism of translational control. These techniques also have the potential to identify previously unknown examples of translational regulation in response to a variety of conditions (Ingolia et al., 2009).

To further characterize the role of the *CLB3* 5'UTR in translational regulation we made a series of deletions of the 5'UTR to determine which regions were important for regulation, and additionally determined the chromosome segregation phenotype of these

mutants. Additionally, to determine the mechanism by which *CLB3* translation was modulated, we first took a candidate based approach. We examined the possibility of translational regulation by both uORFs and IRESs. We then examined the roles of several RBPs known to be expressed during meiosis in budding yeast. Additionally, we performed a yeast three-hybrid (Y3H) assay to identify proteins that interact with the *CLB3* 5'UTR, and therefore may be candidate translational regulators.

Results

Deletion analysis of the *CLB3* 5'UTR

In order to determine which regions of the 156 base *CLB3* 5'UTR were involved in translational regulation, a series of deletions of the endogenous 5'UTR were made using a two-step transformation protocol (see Experimental Procedures). First, three 50 base pair deletions were made in the endogenous *CLB3* 5'UTR, which spanned from -150 to -101 (Δ-150-101) bases upstream of the start codon, from -100 to -51 (Δ-100-51) bases, and from -50 bases to -1 (Δ-50-1) bases. The consequences of these deletions were examined in strains synchronously progressing through the meiotic divisions. Analysis of spindle and nuclear morphology revealed that none of these deletions affected either the kinetics of the meiotic divisions, or the efficiency of the meiotic divisions (Figure 1A; data not shown). This is consistent with our previous finding that premature accumulation of Clb3 protein in *GAL-CLB3* strains does not interfere with meiotic progression in this strain background (Carlile and Amon, 2008). Clb3 was restricted to meiosis II in wild type strains, as expected, and was also restricted to meiosis II in strains carrying the deletion Δ-150-101, indicating that this region of the 5'UTR is not absolutely required for translational regulation (Figure 1B). However, strains carrying either the Δ-100-51 deletion or the Δ-50-1 deletion allowed Clb3 accumulation during meiosis I (Figure 1B). In these strains Clb3 levels during meiosis I were lower than in meiosis II, which is consistent with the presence of lower levels of *CLB3* transcript during meiosis I than in meiosis II (Figure 1B; Carlile and Amon 2008). These data indicate that the maximum region of the *CLB3* 5'UTR required to prevent Clb3 accumulation spans from -100 to -1 bases upstream of the start codon.

Figure 1

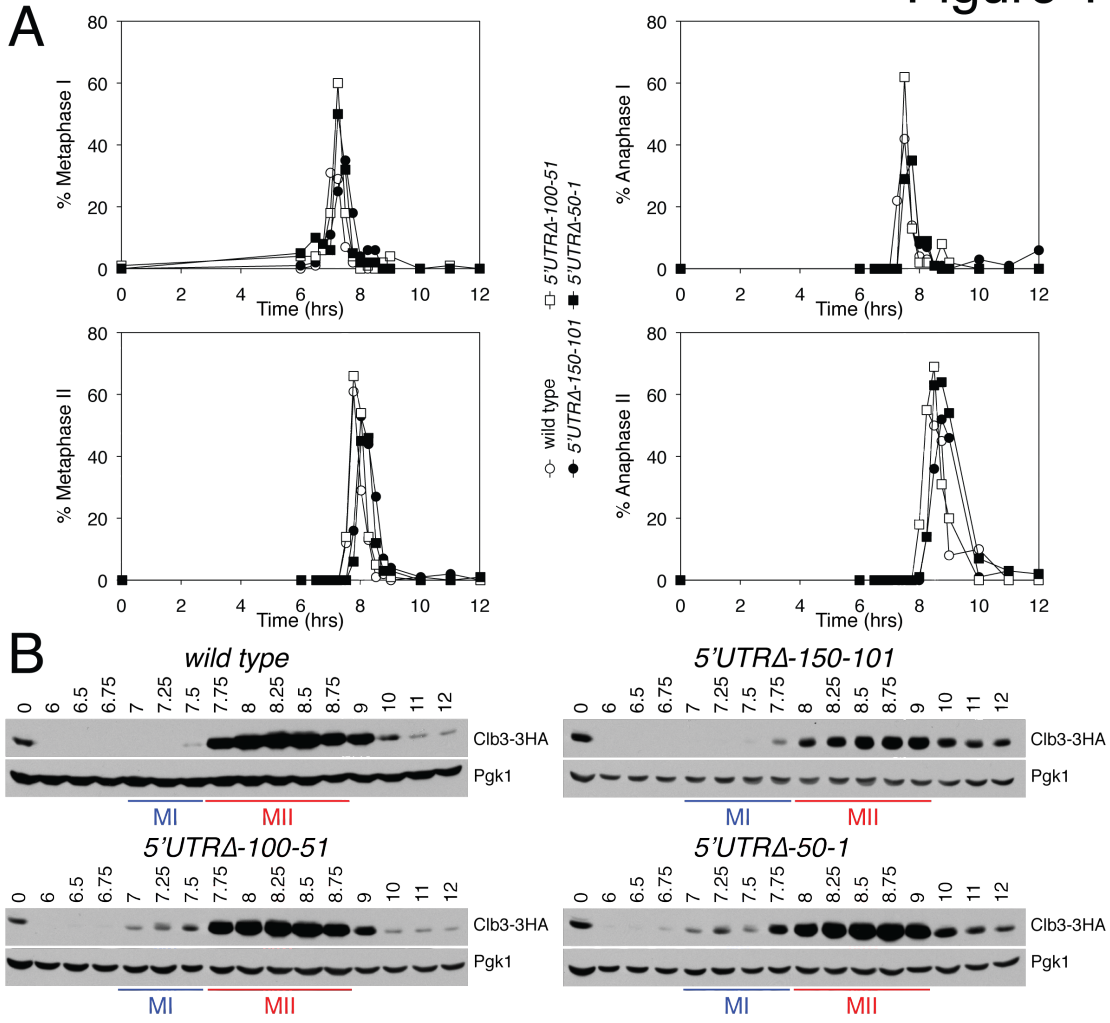


Figure 1: Analysis of 50 base deletions of the *CLB3* 5'UTR.

GAL4.ER, GAL-NDT80 strains with *CLB3-3HA* (A15055, open circles), *5'UTRΔ-150-101-CLB3-3HA* (A22596, closed circles), *5'UTRΔ-100-51-CLB3-3HA* (A22597, open squares), and *5'UTRΔ-50-1-CLB3-3HA* (A22598, closed squares), were induced to sporulate at 30°C by transfer into SPO medium. At 6 hours 1μM β-estradiol was added. A) The percentages of cells with metaphase I spindles (top left), anaphase I spindles (top right), metaphase II spindles (bottom left), or anaphase II spindles (bottom right) were determined at the times indicated (n=100).

C) Western blots for Clb3-3HA. Samples were taken at the times indicated after inoculation into SPO medium. Pgk1 is used as a loading control. Times when cells were in meiosis I are indicated with a blue line, times when cells were in meiosis II are indicated with a red line.

We wished to further narrow down the region of the *CLB3* 5'UTR required for translational control. The experiments above defined the maximum region required for translational regulation as spanning from -100 to -1 bases upstream of the start codon. Therefore four 25 base deletions were made that spanned from -100 to -76 (Δ-100-76) bases upstream of the start codon, from -75 to -51 (Δ-75-51) bases, from -50 to -26 (Δ-50-26) bases, and from -25 to -1 (Δ-25-1) bases. Additionally, a 50 base deletion in the middle of this region spanning -75 to -26 (Δ-75-26) bases was made. As above, Clb3 levels were examined in synchronous meiotic timecourses. Again, deletion of portions of the *CLB3* 5'UTR did not affect either the kinetics of the meiotic divisions, or the efficiency of the meiotic divisions (Figure 2A; data not shown). In wild type strains and in strains carrying the deletion Δ-25-1, Clb3 was restricted to meiosis II. This indicates that this region of the 5'UTR is not required for translational regulation (Figure 2B). However, strains with deletions spanning Δ-100-76, Δ-75-51, Δ-50-26, or Δ-75-26 bases allowed Clb3 accumulation during meiosis I (Figure 2B). Again, Clb3 levels during meiosis I were lower than in meiosis II, likely due to lower *CLB3* transcript levels during meiosis I (Figure 2B; Carlile and Amon 2008). These data indicate that the maximum region of the *CLB3* 5'UTR that is required for translational regulation spans from -100 to

-26 bases upstream of the start codon (Figure 3). There are however, limitations of the analysis presented here (Figure 1,2), which will be discussed in detail below.

Figure 2

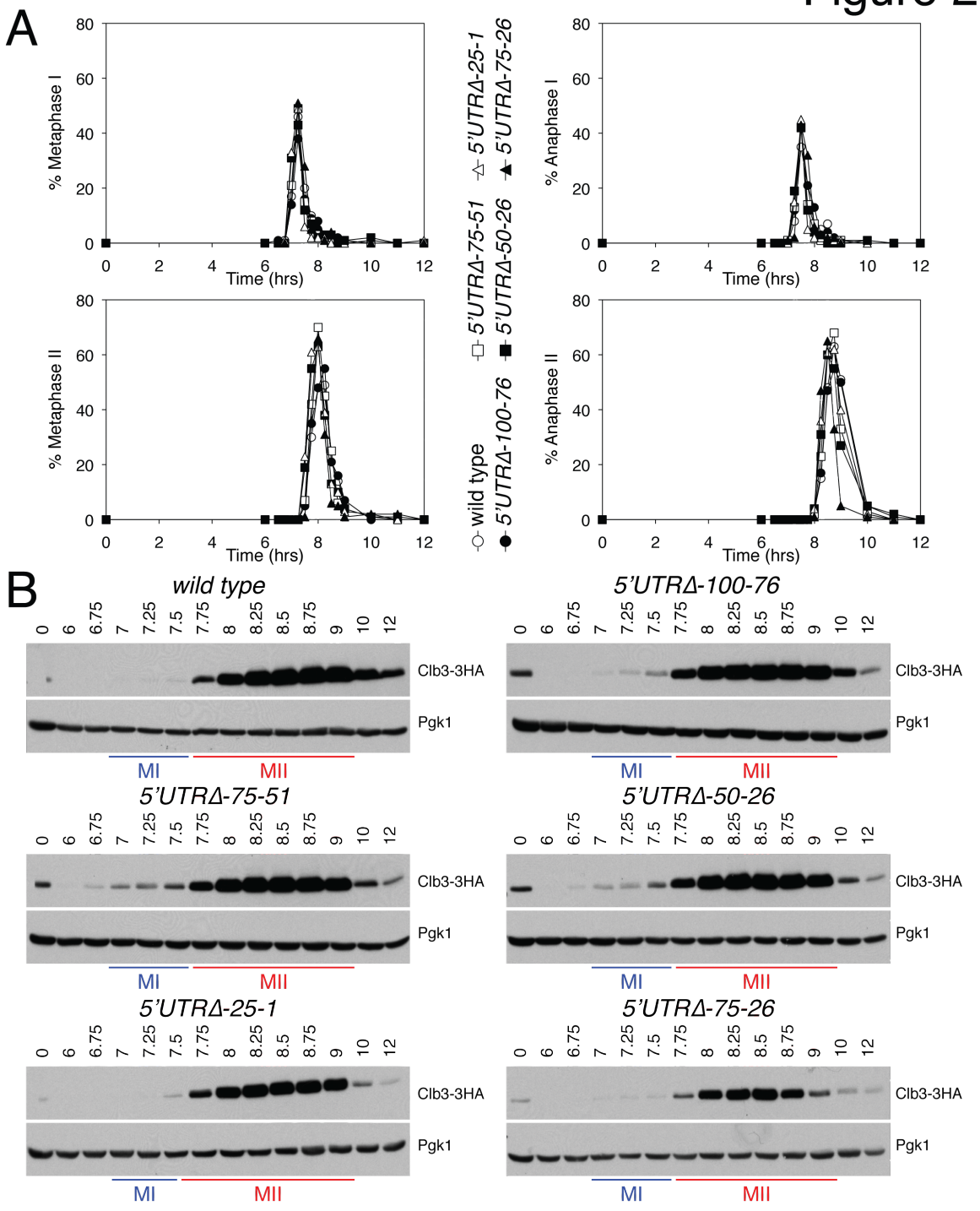


Figure 2: Analysis of 25 base deletions of the *CLB3* 5'UTR.

GAL4.ER, GAL-NDT80 strains with the alleles *CLB3-3HA* (A15055, open circles), *5'UTR*Δ*-100-76-CLB3-3HA* (A23342, closed circles), *5'UTR*Δ*-75-51-CLB3-3HA* (A23343, open squares), *5'UTR*Δ*-50-26-CLB3-3HA* (A23344, closed squares), *5'UTR*Δ*-25-1-CLB3-3HA* (A23345, open triangles), and *5'UTR*Δ*-75-26-CLB3-3HA* (A23346, closed triangles) were induced to sporulate at 30°C by transfer into SPO medium. After 6 hours 1μM β-estradiol was added.

A) The percentages of cells with metaphase I spindles (top left), anaphase I spindles (top right), metaphase II spindles (bottom left), or anaphase II spindles (bottom right) were determined at the times indicated (n=100).

C) Western blots for Clb3-3HA. Samples were taken at the times indicated after inoculation into SPO medium. Pgk1 is used as a loading control. Times when cells were in meiosis I are indicated with a blue line, times when cells were in meiosis II are indicated with a red line.

Figure 3

A

CLB3 5'UTR 153 bases

-153 UUUUGAUCCG GUCGAAAGAA AACGAUCAGA AAUUUUUAGC
UGCUUGCUUA GAAGAAAAGA AAAGAAAAUU GCAGAGAAAA
AGCUAUUUAU UGUUUCUGUC CUUCGCUUUA AAACAUAGAG
AUAUUUUGUC CUUUUUAUCA UUGCUUAUUA UAAAUG -1

B

Strain	Regulation
wt	+
Δ-150-101	+
Δ-100-51	-
Δ-50-1	-
Δ-100-76	-
Δ-75-51	-
Δ-75-26	-
Δ-50-26	-
Δ -25-1	+

Figure 3: Region of the *CLB3* 5'UTR required for translational regulation.

A) *153* bases of the CLB3 5'UTR. The maximum region required for translational regulation is highlighted in red.

B) A summary of the deletions characterized in this analysis. A + indicates that translational regulation is intact, and a – indicates that translational regulation is abolished.

Characterization of the phenotypes of CLB3 5'UTRΔ strains

We wished to determine if early accumulation of Clb3 in the 5'UTRΔ strains was sufficient to cause the premature sister-chromatid segregation (PSCS) phenotype previously observed in *GAL-CLB3* strains (Carlile and Amon, 2008). To follow sister-chromatid segregation during meiosis we used a system in which a tandem array of tetO sequences was integrated at the *LEU2* locus on one copy of chromosome III (heterozygous LEU2-GFP dots). These cells also expressed a tetR-GFP fusion, which binds to tetO, allowing visualization of the repeats (Michaelis et al., 1997). In wild type cells sister chromatids are co-segregated at meiosis I leading to the appearance of binucleate cells in which only one of the two nuclei contains a GFP dot (Figure 4A). When sister-chromatids are segregated away from each other at meiosis I, as is the case in *GAL-CLB3* strains, this leads to the appearance of binucleate cells with a GFP dot in each nucleus (Figure 4A). In 5'UTRΔ cells, the majority of binucleate cells have GFP dot in only one of the two nuclei, indicating that sister-chromatids are cosegregated at meiosis I (Figure 4A). In order to determine if there were other phenotypes associated with premature accumulation of Clb3 owing to disruption of the *CLB3* 5'UTR, spore viability was determined. However, there were no significant reductions in spore viability

in 5'UTRΔ strains (Figure 4B). Together these data indicate that either the levels of Clb3 that accumulate during meiosis I in some of the 5'UTRΔ strains are insufficient to cause PSCS or a decrease in spore viability, or that the PSCS observed in *GAL-CLB3* strains is a result of accumulation of Clb3 prior to meiosis I.

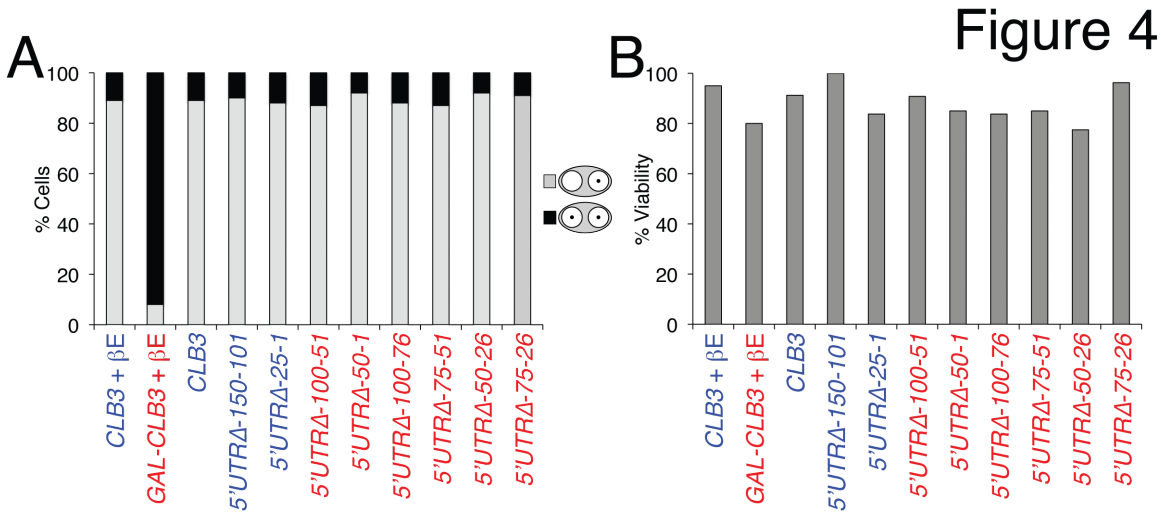


Figure 4: Premature accumulation of Clb3 in UTRΔ strains does not cause PSCS.
GAL4.ER, heterozygous *LEU2* GFP-dots strains with the alleles *CLB3-3HA* (A18686), *GAL-CLB3-3HA* (A18687), *5'UTRΔ-150-101-CLB3-3HA* (A24641), *5'UTRΔ-25-1-CLB3-3HA* (A24647)*5'UTRΔ-100-51-CLB3-3HA* (A24642), *5'UTRΔ-50-1-CLB3-3HA* (A24643),*5'UTRΔ-100-76-CLB3-3HA* (A24644), *5'UTRΔ-75-51-CLB3-3HA* (A24645), *5'UTRΔ-50-26-CLB3-3HA* (A24646), and *5'UTRΔ-75-26-CLB3-3HA* (A24648) were induced to sporulate at 30°C by transfer into SPO medium. After 3 hours 1μM β-estradiol was added to cultures labels +βE.
A) Sister-chromatid separation was scored at 7 hours after inoculation into SPO (n=100).
B) Samples were taken after 48 hours and tetrads were dissected (n=80 spores).

We reasoned that if the low Clb3 levels present during meiosis I were a result of only a partial relief of translational repression, then deletion of a larger portion of the *CLB3* 5'UTR might allow further accumulation of Clb3 during meiosis I. To do this we deleted bases -153 to -1 of the 156 base 5'UTR. We examined heterozygous LEU2-GFP dot segregation in these strains, spore viability, and protein levels. *5'UTR*Δ*-153-1* strains cosegregated sister-chromatids at meiosis I, to a similar extent as wild type, and showed no decrease in spore viability (Figure 5A-B). However, Clb3 protein levels were significantly lower in 5'UTRΔ-153-1 strains than in wild type, which indicates that when the entire *CLB3* 5'UTR is deleted either *CLB3* transcript is unstable, or that *CLB3* transcript is inefficiently translated (Figure 5C).

Together the above data suggest that 75 bases of the *CLB3* 5'UTR from -75 to -26 bases upstream of the start codon represent the maximum region of the *CLB3* 5'UTR that is likely required for translational regulation of *CLB3*, and that elimination of translational regulation by deletion of portions of the 5'UTR allows some Clb3 to accumulate during meiosis I, but that this premature accumulation is insufficient to promote PSCS.

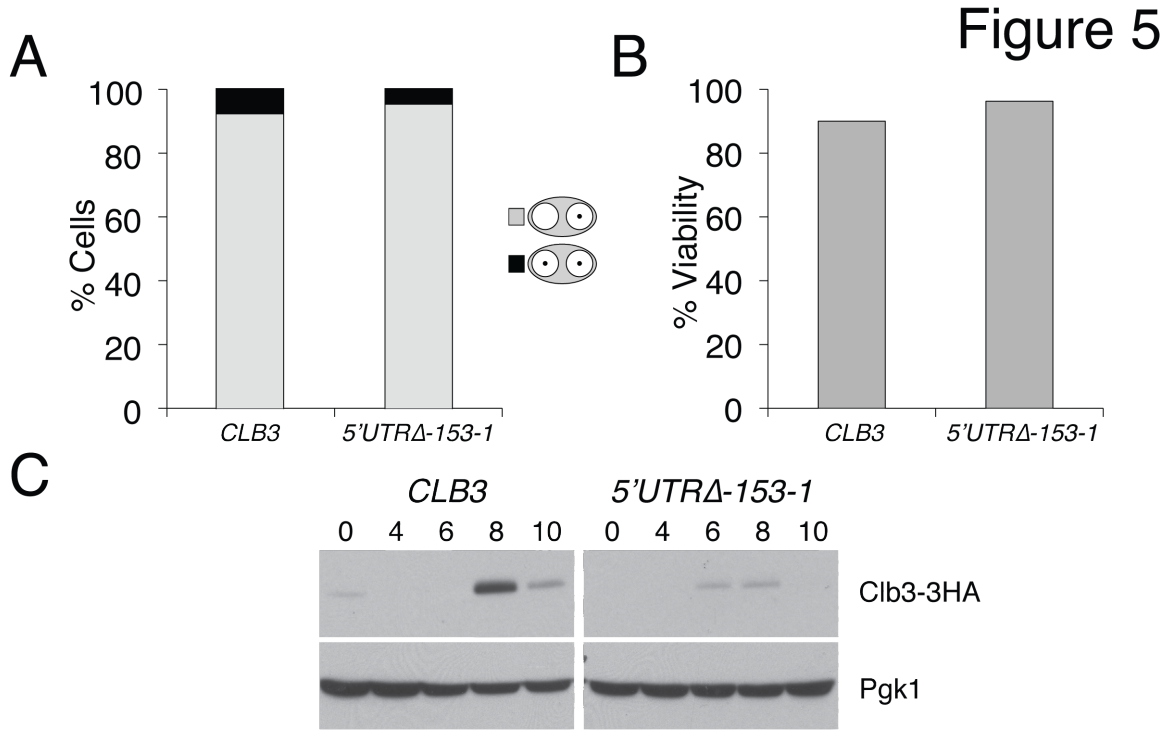


Figure 5: Chromosome segregation, spore viability, and Clb3 levels in strains with the entire *CLB3* 5'UTR deleted.

GAL4.ER, heterozygous *LEU2* GFP-dots strains with the alleles *CLB3-3HA* (A18686), and *5'UTR*Δ*-153-1-CLB3-3HA* (A24971) were induced to sporulate at 30°C by transfer into SPO medium.

A) Sister-chromatid separation was scored at 7 hours after inoculation into SPO (n=100).

B) Samples were taken after 48 hours and tetrads were dissected (n=80 spores).

C) Western blots for Clb3-3HA. Pgk1 is used as a loading control.

CLB3 translation is not regulated by a uORF

We first sought to determine if *CLB3* translation was regulated by a uORF, or by differential transcriptional start sites. To do this we performed 5'RACE analysis of the *CLB3* transcript from cycling, meiosis I, and meiosis II samples. This analysis revealed a maximal 5' UTR length of 156 bases, and no differences in 5'UTR length between cycling, meiosis I, and meiosis II cells (Carlile and Amon, 2008). This length is in good agreement with a genome wide study of the yeast transcriptome, which assigned a 5'UTR length of 153 bases (David et al., 2006). Importantly this analysis allows us to rule out regulation of *CLB3* translation by uORFs with canonical start codons, and of regulation by alternate transcriptional start sites. However, a recent genome-wide *in vivo* analysis of yeast translation has revealed uORFs with non-AUG start codons (Ingolia et al., 2009). Thus, it is still formally possible that *CLB3* translation is regulated by a uORF with a non-cognate start codon, and examination of the *CLB3* 5'UTR reveals several possible non-cognate start codons, which will be further addressed in the discussion.

CLB3 translation is not regulated by an IRES

Next we sought to determine if *CLB3* translation is regulated by an IRES in the 5'UTR of the message. The *CLB3* 5'UTR has a poly(A) tract, which is characteristic of yeast IRESs (Gilbert et al., 2007; Figure 6A). To determine if this poly(A) tract could support cap-independent translation we employed an *in vivo* IRES reporter system. In this system firefly luciferase (F-luc) mRNAs are *in vitro* transcribed, and are electroporated into spheroplasted yeast cells. Translation of the reporter mRNAs can then be measured using luciferase assays. A reporter mRNA with a physiological cap (m7GpppG) and an

unstructured 5'UTR is translated efficiently, while a reporter mRNA with a non-physiological cap (ApppG) and a stable stem-loop in the 5'UTR is translated very inefficiently (Figure 6B,C). However, if a 5'UTR containing an IRES sequence is inserted downstream of the stem-loop this allows cap independent translation (Gilbert et al., 2007; Figure 6B). Using this assay we tested the IRES activity of the *CLB3* 5'UTR, the reverse complement of the *CLB3* 5'UTR, and the 5'UTR of *NCE102*, which contains a known IRES. While the *NCE102* 5'UTR supported cap-independent translation, neither the *CLB3* 5'UTR nor its reverse complement supported cap-independent translation *in vivo* (Figure 1C). These results indicate that the *CLB3* 5'UTR does not contain an IRES sequence that can support cap-independent translation in mitotic cells. However, it is still formally possible that the *CLB3* 5'UTR can support cap-independent translation in meiotic cells, which will be addressed further in the discussion.

Figure 6

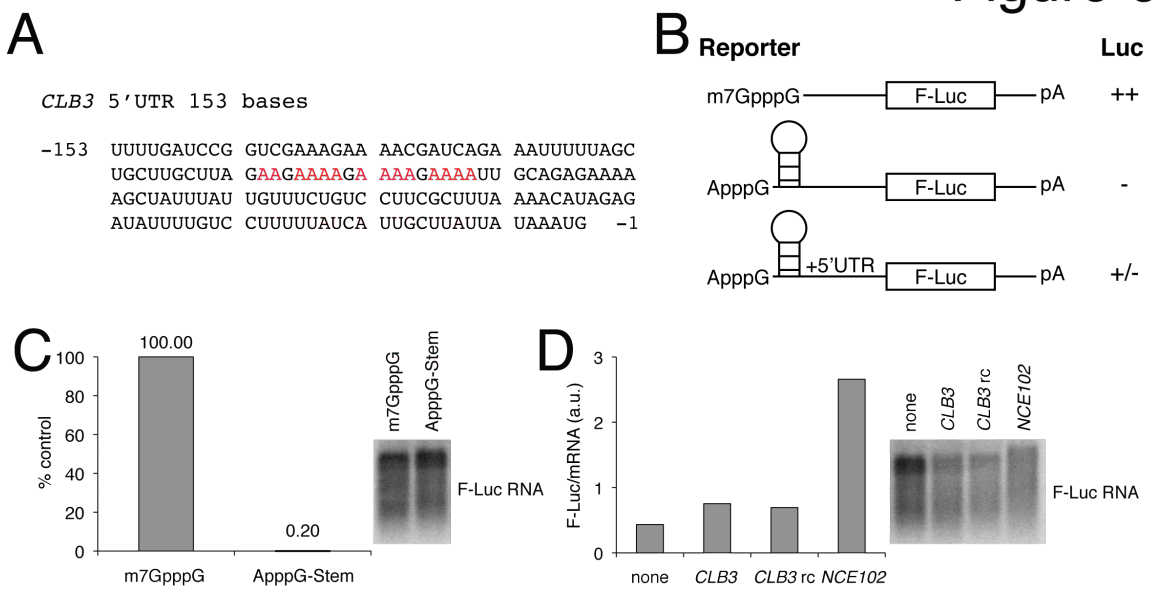


Figure 6: The CLB3 5'UTR does not support cap-indeendent translation.

Wild type W303 cells (A2587) were grown to mid-log phase, were spheroplasted, and were electroporated with *in vitro* transcribed F-luc reporter RNAs.

A) The *CLB3* 5'UTR. A potential poly(A) tract is indicated in red.

B) The *in vitro* transcribed firefly luciferase reporters used in this experiment. A reporter with a physiological cap and an unstructured 5'UTR is translated efficiently (top). A reporter with a cap analog and a stable stem-loop in the 5'UTR is not translated (middle). Addition of an endogenous 5'UTR to the reporter with cap analog and stable stem loop will be translated if the 5'UTR contains an IRES sequence. Adapted from (Gilbert et al., 2007).

C) F-luc activity normalized to F-luc RNA levels expressed as a percentage of control for control constructs ([B], top and middle).

D) F-luc activity normalized to F-luc RNA levels expressed in arbitrary units for control ApppG reporter ([B], middle), and ApppG reporters containing the *CLB3* 5'UTR, the *CLB3* 5'UTR reverse complement, and the *NCE102* 5'UTR.

Deletion of RBPs that are candidate modulators of *CLB3* translation

Given that in higher eukaryotes the translation of *cyclin* mRNAs is modulated by the binding of RBPs (Vardy and Orr-Weaver, 2007), we hypothesized that the *CLB3* 5'UTR may be bound by an RBP that modulates *CLB3* translation during meiosis. *A priori* there are two possible modes of translational regulation, repression during meiosis I, or activation during meiosis II. If an RBP encodes a translational repressor, then its deletion should lead to the accumulation of Clb3 protein during meiosis I. However, if an RBP encodes a translational activator, then its deletion should reduce or eliminate Clb3 translation during meiosis II. We reasoned that an RBP involved in translational regulation of *CLB3* would be non-essential, and would be expressed during meiosis. To

test this hypothesis we examined the effect of deletion of meiotically expressed RBPs on Clb3 protein levels in meiotic cells. A list of meiotically expressed RBP genes was compiled by pooling lists of genes obtained from meiotic expression data with a list of genes deleted in a screen that identified monopolin (Primig et al., 2000; Tóth et al., 2000). PROSITE was then used to scan the amino acid sequences of these genes for known RNA interaction motifs (Sigrist et al., 2002). This approach led to a list of genes that included: *YGR250C*, *HRB1*, *YDR374C*, *RRT5*, *MIP6*, *PES4*, *YLL032C*, *CBC2*, *SLF1*, *RIM4*, *TMA64*, and *PBP2*. These genes encode proteins that represent a broad range of functions including Poly(A) binding, nuclear export, splicing, ribosome interactors, and proteins of unknown function (Figure 7A). Samples were taken at early (7.25, and 7.5 hours), and late (8.75, and 9 hours) timepoints for each of these strains, which correspond to meiosis I and meiosis II respectively for most strains (Figure 7B). Most RBP deletions examined had no effect on Clb3 protein accumulation, indicating that the RBPs that they encode are not required for Clb3 translational control (Figure 7C). The deletion of *YGR250C*, *CBC2*, and *RIM4* reduced the levels of Clb3 protein during late timepoints. However, these effects are likely not due an effect on *CLB3* translation, as these strains failed to undergo the meiotic divisions efficiently, reducing the percentage of cells in meiosis II at late timepoints (Figure 7B,C).

Figure 7

A

ORF	Source	Motif	Description
YGR250C	Cluster 1	RRM	Cytoplasmic protein , interacts with PABP
HRB1	Cluster 2	RRM	Poly(A) BP, involved in nuclear export
YDR374C	Cluster 4	YTH	Hypothetical ORF
RRT5	Cluster 5a	RRM	Putative protein of unknown function
MIP6	Cluster 5a	RRM	Protein involved in nuclear export
PES4	Cluster 5a	RRM	Poly(A) BP
YLL032C	Cluster 5b	KH	Unknown function, may interact with ribosomes
CBC2	Cluster 5b	RRM	Involved in splicing
SLF1	Cluster 5b	HTH_LA	Proposed translational regulator, eIF4E iteractor
RIM4	Monopolin	RRM	RBP Required for meiotic gene expression
TMA64	Monopolin	PUA, SUI1	Unknown function, associates with ribosomes
PBP2	Monopolin	KH	RBP involved in regulation of telomeres

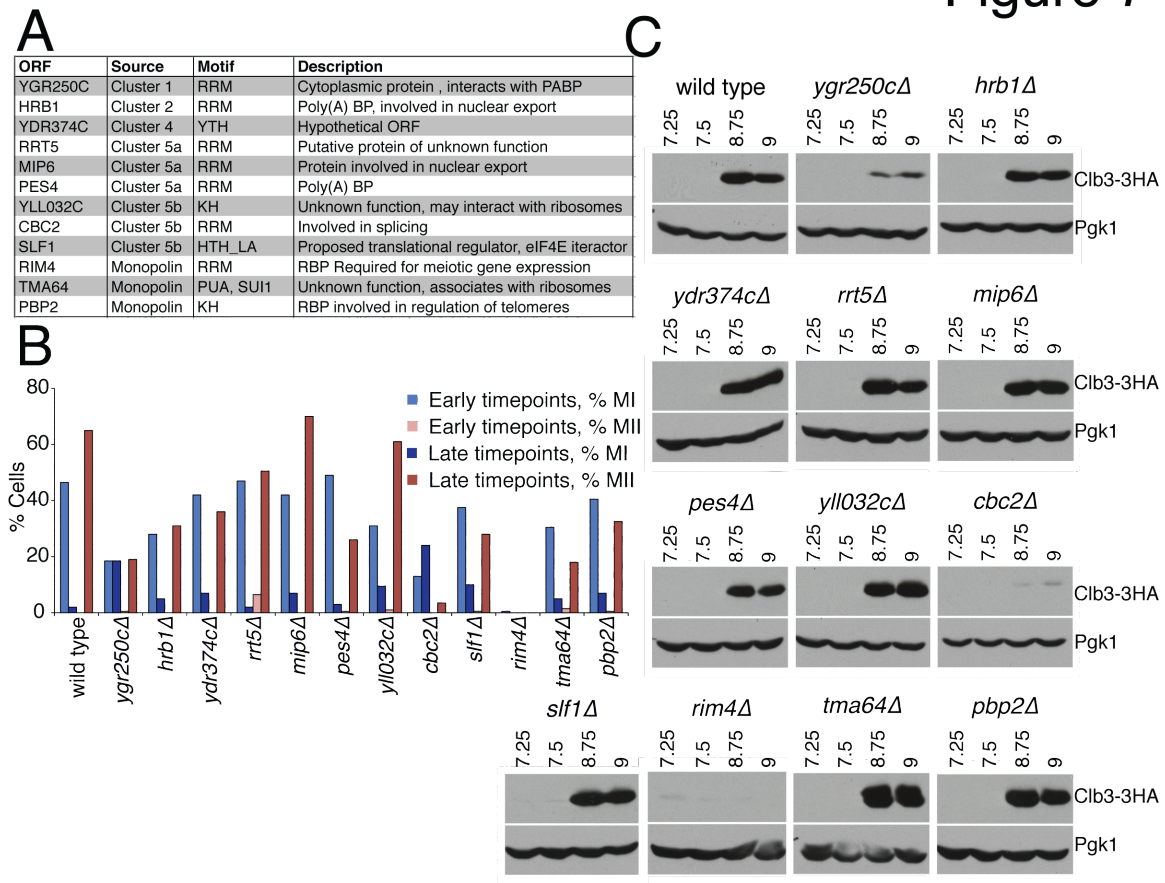


Figure 7: Candidate RBPs are not involved in *CLB3* translational regulation.

GAL4.ER, GAL-NDT80, CLB3-3HA (A15055) strains with the deletions *ygr250c*Δ (A20531), *hrb1*Δ (A20532), *ydr374c*Δ (A20533), *rrt5*Δ (A20534), *mip6*Δ (A20535), *pes4*Δ (A20536), *yll032c*Δ (A20537), *cbc2*Δ (A20538), *slf1*Δ (A20539), *rim4*Δ (A20540), *tma64*Δ (A20541), and *pbp2*Δ (A20542) were induced to sporulate at 30°C by transfer into SPO medium. After 6 1μM β-estradiol was added.

A) Genes in Clusters 1-5b were taken from (Primig et al., 2000), and "Monopolin" were taken from (Tóth et al., 2000). Motifs are: RNA Recognition Motif (RRM), YT521-B homology (YTH), (hnRNP) K homology (KH), La-type Helix-Turn-Helix (HTH_LA), PseudoUridine synthase (PUA), and SUI1 homolgy (SUI1).

B) The percentages of cells with MI spindles were counted and averaged for early (7.25, and 7.5 hours, Early timepoints %MI, light blue bars), and late timepoints (8.75, and 9 hours, Late timepoints %MI, dark blue bars). The percentages of cells with MII spindles

were counted and averaged for early (7.25, and 7.5 hours, Early timepoints %MII, light red bars), and late timepoints (8.75, and 9 hours, Late timepoints %MII, dark red bars).
C) Western blots for Clb3-3HA. Samples were taken at the times indicated after inoculation into SPO medium. Pgk1 is used as a loading control.

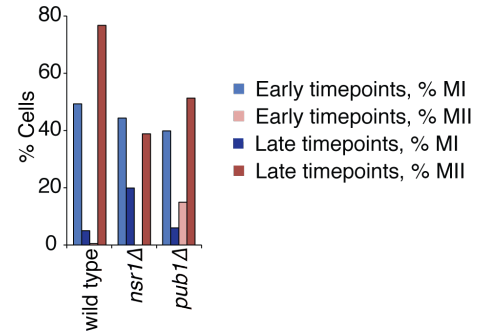
In *S. pombe* the Spo5 protein is an RBP required for normal meiotic progression, and its deletion affects multiple meiotic processes including recombination, spore formation, and timely occurrence of meiosis I (Kasama et al., 2006). We therefore searched for Spo5 homologs in the *S. cerevisiae* genome. This analysis revealed the genes *PAB1*, *PES4*, *MIP6*, *NSR1*, and *PUB1* to be Spo5 homologs. *PAB1* was not included in this analysis as it encodes the budding yeast PABP, which is essential, and *PES4* and *MIP6* were analyzed above (Figure 7). Therefore Clb3 levels during meiosis were examined in *nsr1*Δ and *pub1*Δ mutants. *NSR1* is a gene involved in pre-mRNA processing and ribosome biogenesis, and *PUB1* encodes a Poly(A) BP involved in mRNA stability (Figure 8A). As above, samples were taken at early (7.25, and 7.5 hours), and late (8.75, and 9 hours) timepoints, which correspond to meiosis I and meiosis II respectively (Figure 8B). Deletion of either *NSR1* or *PUB1* failed to alter Clb3 accumulation during meiosis, either in the timing or the levels of accumulation (Figure 8C). This indicates that neither *NSR1* nor *PUB1* are required for *CLB3* translational regulation during meiosis.

Figure 8

A

ORF	Source	Domain	Description
NSR1	Spo5	RRM	pre-rRNA processing, ribosome biogenesis
PUB1	Spo5	RRM	Poly(A) BP, involved in mRNA stability

B



C

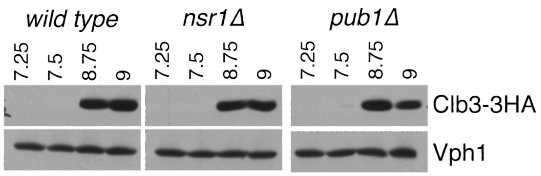


Figure 8: Candidate RBPs are not involved in *CLB3* translational regulation.

GAL4.ER, GAL-NDT80, CLB3-3HA (A15055) strains with the deletions *nsr1*Δ (A21494), and *pub1*Δ (A21495) were induced to sporulate at 30°C by transfer into SPO medium. After 6 1μM β-estradiol was added.

A) Genes in homologous to *S. pombe* Spo5.

B) The percentages of cells with MI spindles were counted and averaged for early (7.25, and 7.5 hours, Early timepoints %MI, light blue bars), and late timepoints (8.75, and 9 hours, Late timepoints %MI, dark blue bars). The percentages of cells with MII spindles were counted and averaged for early (7.25, and 7.5 hours, Early timepoints %MII, light red bars), and late timepoints (8.75, and 9 hours, Late timepoints %MII, dark red bars).

C) Western blots for Clb3-3HA. Samples were taken at the times indicated after inoculation into SPO medium. Vph1 is used as a loading control.

These data indicate that none of the genes examined play a role in regulating *CLB3* translation. There are several explanations as to why this approach may have missed a translational repressor of *CLB3*. First, it is possible that our list was not comprehensive, and that the relevant *RBP* is expressed during both mitotic growth and meiosis, but is not strongly up-regulated during meiosis. Second, it is possible that the regulator of *CLB3*

translation does not contain a recognizable RNA binding motif. Third, the regulator may be an essential gene with a non-essential function of modulating *CLB3* translation during meiosis. Finally, redundant RBPs may regulate *CLB3* translation during meiosis. In all of the latter cases the genes involved would have been omitted from our analysis, and in the former case the gene or genes involved would not have been identified as regulators.

Identification of candidate translational regulators by Yeast Three-Hybrid

The Y3H system identifies RNA-protein interactions by a method analogous to the Yeast Two-Hybrid system. In both systems interaction between bait and prey constructs drives expression of a reporter gene. In the Y3H system the bait consists of an RNA-protein complex consisting of a LexA-MS2 fusion protein, which binds a fusion RNA consisting of three MS2 hairpins and an RNA of interest. Binding of the LexA-MS2 fusion protein to a LexA operator sequence tethers the fusion RNA upstream of *HIS3* and *LacZ* reporter genes. The prey consists of a protein or library of proteins fused to a transcriptional activation domain (AD). Binding of the bait RNA by a prey protein brings the AD in close proximity to the reporter genes, and activates their transcription (Figure 1). The Y3H system also has a colony color assay to eliminate false positives due to RNA independent transcriptional activation. The plasmid from which the fusion RNA is expressed is marked with both *URA3* and *ADE2*, and the Y3H is performed under conditions in which this plasmid is not directly selected for. Thus, the plasmid is only maintained if *HIS3* expression depends on expression of the fusion RNA. Therefore RNA dependent *HIS3* expression leads to the formation of white colonies (*ADE2*+), and RNA

independent expression leads to the formation of red colonies (*ADE2*-) (Kraemer et al., 2000).

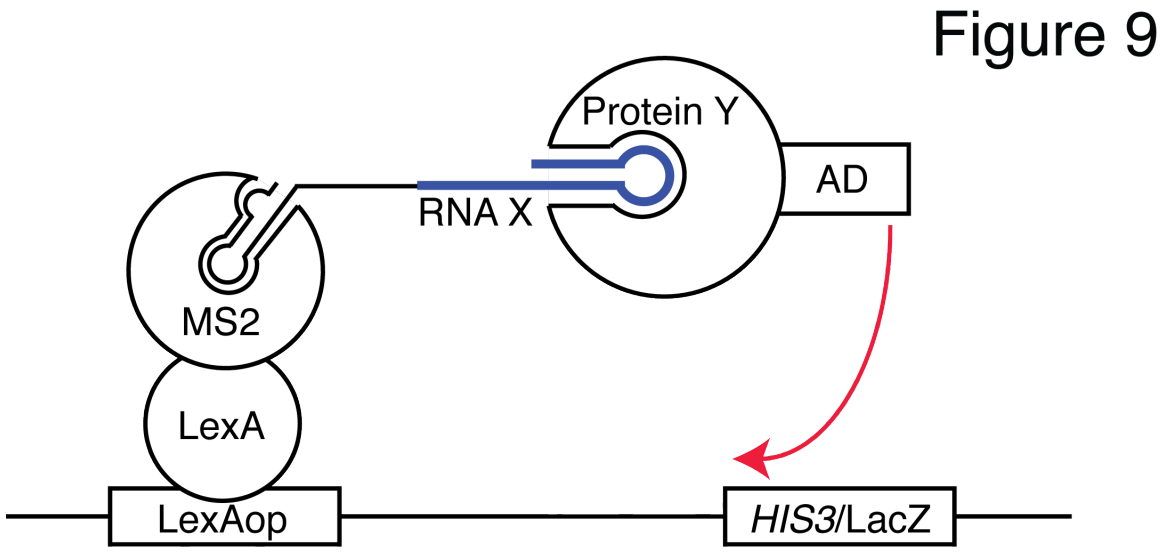


Figure 9: The Yeast Three-Hybrid System

A schematic of the Yeast Three-Hybrid system. "RNA X" consists of MS2 hairpins and an RNA of interest (blue). See text for details.

To attempt to identify proteins that interact with the *CLB3* 5'UTR, and potentially regulate *CLB3* translation, we performed a Y3H screen with the full-length *CLB3* 5'UTR fused to MS2 hairpins, and libraries of each reading frame of the yeast genome (C1, C2, C3) fused to the *GAL4* AD (James et al., 1996). The Y3H assay was performed as described in Experimental Procedures. A total of 676 colonies that exhibited RNA dependent *HIS3* expression were picked (392 from C1, 100 from C2, and 184 from C3). However, when expression of the LacZ reporter was tested for RNA dependence this number was reduced to 99 (57 from C1, 14 from C2, and 28 from C3). Library inserts

were PCRed from these isolates, and their identities were determined by sequencing or Southern blot analysis. Of these clones, 44 contained in frame fusions, which represented 9 different genes *RIM15* (18 hits)*, RPM2* (11 hits), *PTK2* (5 hits), *MSB1* (3 hits), *SAP185* (3 hits), *CCT5* (1 hit), *ENA1* (1 hit), *KAP123* (1 hit), and *VPS3* (1 hit) (Figure 10A). We chose to focus our analysis on the two top hits, *RIM15* and *RPM2*, which are both transcriptionally induced during meiosis. Quantitative LacZ assays demonstrated that both Rim15-GAD (Gal4 Activation Domain) and Rpm2-GAD fusions activated *LacZ* reporter transcription to a higher extent in the presence of *MS2-CLB3 5'UTR* RNA than in the presence of negative control RNA (Figure 10B). However, compared to known interactions assayed by Y3H these differences are relatively small, though they do suggest some specific interaction of the GAD fusions with the *CLB3* 5'UTR.

Figure 10

A

Gene	Hits	Description
RIM15	18	Kinase involved in signal transduction of establishment of stationary phase
RPM2	11	Protein subunit of mitochondrial RNase P
PTK2	5	S/T protein kinase involved in regulation of ion transport across membrane
MSB1	3	Positive regulator of 1,3-beta-glucan synthesis and the Pkc1p-MAPK pathway
SAP185	3	Forms a complex with the Sit4p phosphatase and is required for its function
CCT5	1	Subunit of chaperonin Cct ring, required for the assembly of actin and tubulin
ENA1	1	P-type ATPase sodium pump, involved in Na+ efflux to allow salt tolerance
KAP123	1	Karyopherin beta, nuclear import of ribosomal proteins, histone H3, and H4
VPS3	1	Protein required for the sorting and processing of soluble vacuolar proteins

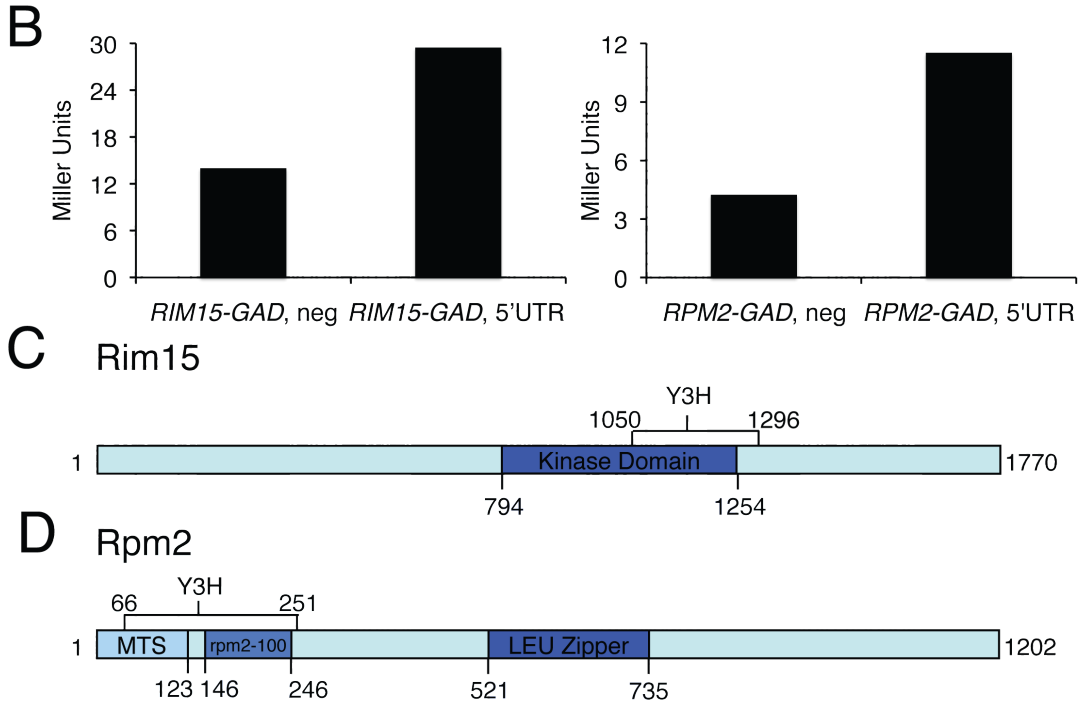


Figure 10: *RIM15* and *RPM2* are candidate RBPs for the *CLB3* 5'UTR

A) Candidate genes identified by Y3H, and the number of hits for each.

B) ONPG assays performed for *RIM15-AD* (left) and *RPM2-AD* (right) fusions in strains expressing *MS2 hairpins* alone, or *MS2-CLB3 5'UTR*.

C, D) Schematic diagrams of Rim15 and Rpm2 proteins, indicating the minimal interacting domains, and relevant domain structure. MTS is thought to be a mitochondrial targeting sequence that is cleaved upon import into the mitochondria. rpm2-100 indicates the amino acids that are deleted in the *rpm2-100* mutant.

Characterization of Yeast 3-Hybrid Hits *RIM15* and *RPM2*

RIM15 encodes a 1770 amino acid serine/threonine kinase involved in stationary phase entry. Rim15 kinase activity promotes entry into G0 and expression of a variety of early meiotic genes including *IME1*, *IME2*, *HOP1*, and *SPO13*, and is negatively regulated by nutrient sensing pathways including the TOR pathway, the protein kinase A pathway, and the phosphate sensing Pho80-Pho85 pathway (Vidan and Mitchell, 1997; Roosen et al., 2005; Wanke et al., 2005). The Rim15 Y3H interacting domain spans amino acids 1050 to 1296, which overlaps with the kinase domain that spans amino acids 794 to 1254 (Figure 10C).

RPM2 is a nuclear gene that encodes the protein subunit of mitochondrial RNase P, which consists of Rpm2 and *RPM1*, a mitochondrially encoded RNA. Mitochondrial RNase P cleaves 5' leader sequences from mitochondrially encoded tRNAs, and Rpm2 is also required for the maturation of *RPM1* RNA (Morales et al., 1992; Stribinskis et al., 2001b). In addition to its role in mitochondrial RNA processing Rpm2 has a variety of other functions. Rpm2 is involved in maintenance of the mitochondrial genome, as well as in mitochondrial biogenesis (Stribinskis et al., 2001a). It also transcriptionally activates nuclear genes, likely through its leucine zipper domain (Stribinskis et al., 2005; Figure 10D). There are also hints that Rpm2 may play a role in translational regulation. *rpm2-100* mutants, which retain wild type RNase P activity, show altered translation rates for mitochondrial proteins. Additionally, Rpm2 interacts with the P body component Dcp2 by yeast two-hybrid assay, and localizes to P bodies when expressed at endogenous levels. Additionally, *RPM2* interacts with P body components genetically. Specifically,

DHH1 is a high-copy suppressor of the temperature-sensitive growth of *rpm2-100* strains, and over-expression of *RPM2* inhibits P body disassembly (Stribinskis and Ramos, 2007). These data suggest that Rpm2 has the ability to regulate translation independent of its RNase P activity. The Rpm2 Y3H interacting domain spans amino acids 66 to 251, which overlaps with the mitochondrial targeting sequence (MTS), and with the region deleted in the *rpm2-100* allele (amino acids 146 to 246) (Figure 10D).

We first sought to examine Rim15 and Rpm2 protein levels during meiosis to determine if either was present during meiosis. Strains carrying *RIM15-TAP* and *RPM2-TAP* alleles were induced to undergo meiosis, and westerns blots were used to assess Rim15-TAP and Rpm2-TAP levels. This analysis revealed that both Rim15 and Rpm2 are present throughout meiosis (Figure 11A-D). Interestingly a slower migrating form of Rpm2 appeared as cells entered meiosis II (Figure 11D). This slower migrating form could represent a post-translational modification of Rpm2 that occurs or is stabilized during meiosis II, or it could represent full length Rpm2 that has not had its MTS cleaved, and is therefore presumably localized to either the cytoplasm or nucleus, but not the mitochondria. We reasoned that this form of Rpm2 might be the active form with respect to regulation of *CLB3* translation, and that its appearance in samples taken from cycling cells and cells in meiosis II might indicate that it acts as a translational activator during both vegetative growth and meiosis II. If Rpm2 serves as a translational activator during mitosis, then in cycling cells Clb3 levels should be reduced in *RPM2* mutants. Clb3 levels were compared in cycling cells in strains carrying *RPM2* or *rpm2-100* alleles in both the SK1 and W303 backgrounds. However no differences in Clb3 levels were seen (data not

shown), indicating that either Rpm2 does not act as a translational activator during vegetative growth, or that the *rpm2-100* allele is still capable of modulating *CLB3* translation.

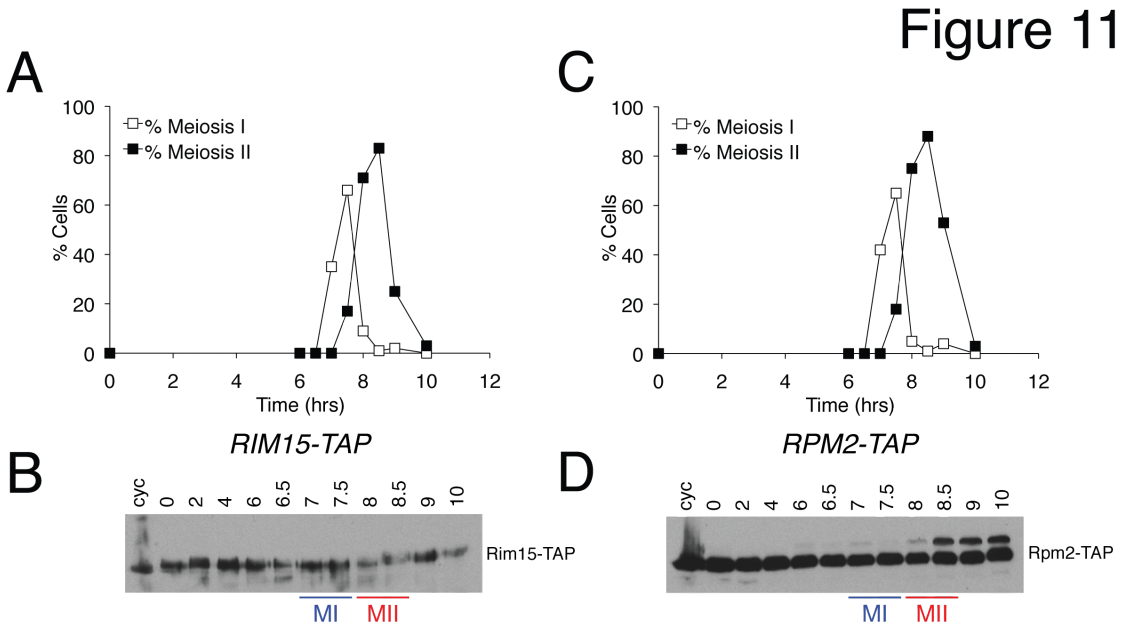


Figure 11: Rim15 and Rpm2 levels during meiosis

GAL4.ER, GAL-NDT80 strains with the tags *RIM15-TAP* (A23949), and *RPM2-TAP* (A23950) were induced to sporulate at 30°C by transfer into SPO medium. After 6 hours 1μM β-estradiol was added.

A,C) The percentages of cells with meiosis I spindles (open squares) or meiosis II spindles (closed squares) were determined at the times indicated (n=100).

C) Western blots for Rim15-TAP and Rpm2-TAP. Samples were taken at the times indicated after inoculation into SPO medium. Lanes labeled "cyc" were taken from exponentially growing cultures.

We wished to analyze the consequences of disruption of the functions of both *RIM15* and *RPM2* on translational regulation mediated by the *CLB3* 5'UTR. If either protein acted as a translational repressor during meiosis, then *RIM15* or *RPM2* mutant cells would be expected to accumulate Clb3 during meiosis I. Conversely, if either protein acted as a translational activator, then *RIM15* or *RPM2* mutant cells would be expected to show lower levels of Clb3 during meiosis II. However, we were unable to delete *RIM15* in the SK1 strain background, and the *rpm2-100* allele failed to sporulate in the background used for synchronous meioses. This prevented our analysis of the roles of *RIM15* and *RPM2* in translational control of *CLB3*.

Analysis of association of *CLB3* transcript with Rim15 and Rpm2.

The identification of Rim15 and Rpm2 by Y3H assay suggests that these proteins bind the *CLB3* transcript. Additionally, the pattern of association of these proteins with *CLB3* transcript might suggest their potential mode of translational regulation. A translational repressor would be expected to associate with *CLB3* transcript during meiosis I, and a translational activator would be expected to associate with *CLB3* transcript during meiosis II, and possibly during vegetative growth.

To determine if Rim15 and Rpm2 bind the *CLB3* transcript, TAP-tagged versions of these proteins were immunoprecipitated (IPed) from exponentially growing, meiosis I (7.5 hours), and meiosis II (9 hours) cells (Figure 12A). Additionally, samples taken from strains lacking TAP-tagged versions of either protein were included in this analysis. RNA was isolated from total lysates and from IPed material, and *CLB3* levels were assessed by

RT-PCR (Gerber et al., 2004). Additionally, the levels of *TUP1* mRNA, a negative control that should not interact with either Rim15-TAP or Rpm2-TAP were analyzed as well. The fold enrichment of a transcript in IPed samples was calculated as the ratio of IPed transcript to total transcript. A fold enrichment value greater than one is interpreted as an interaction between an mRNA and the protein being IPed. The fold enrichment of *CLB3* mRNA was first determined for samples from strains lacking either TAP-tagged protein to determine the level of background enrichment, which was 0.7 fold, 0.1 fold, and 0.2 fold for cycling, meiosis I, and meiosis II samples respectively, and the fold enrichment values for *TUP1* mRNA were 0.2 fold, 0 fold, and 0.1 fold respectively (Figure 12B). These values were then used to normalize the fold enrichments for cycling, meiosis I, and meiosis II samples, giving a normalized fold enrichment values of 1.0 for all no tag samples (Figure 12C). For Rim15-TAP IPs the fold enrichment values for *CLB3* were 0.8 fold, 1.2 fold, and 0.1 fold for cycling, meiosis I, and meiosis II samples respectively and the fold enrichment values for *TUP1* mRNA were 0.2 fold, 0.2 fold, and 0 fold respectively (Figure 12B). For Rim15-TAP the normalized fold enrichment values for *CLB3* were 1.2 fold, 13.2 fold, and 0.5 fold respectively, and for *TUP1* were 1.2 fold, 10.2 fold, and 0.3 fold respectively (Figure 12C). For Rpm2-TAP IPs the fold enrichment values for *CLB3* were 0.3 fold, 0.5 fold, and 0 fold for cycling, meiosis I, and meiosis II samples respectively and the fold enrichment values for *TUP1* mRNA were 0.1 fold, 0.1 fold, and 0 fold respectively (Figure 12B). For Rpm2-TAP the normalized fold enrichment values for *CLB3* were 0.4 fold, 5.5 fold, and 0.1 fold respectively, and for *TUP1* were 0.4 fold, 4.9 fold, and 0.1 fold respectively (Figure 12C). Importantly the fold enrichment values observed for *CLB3* for Rim15-TAP and Rpm2-TAP IPs were not

significantly above one, and additionally the normalized fold enrichment values calculated for *CLB3* and *TUP1* are similar. Together these data suggest that neither Rim15, nor Rpm2 binds to *CLB3* mRNA in vegetative, meiosis I, or meiosis II cells. However, this experiment has only been performed once, and should be repeated with further controls to demonstrate that Rim15 and Rpm2 are being efficiently IPed. Additionally, for Rpm2-TAP sample, the levels of *RPM1* RNA could be analyzed as *RPM1* is known to interact with Rpm2.

Figure 12

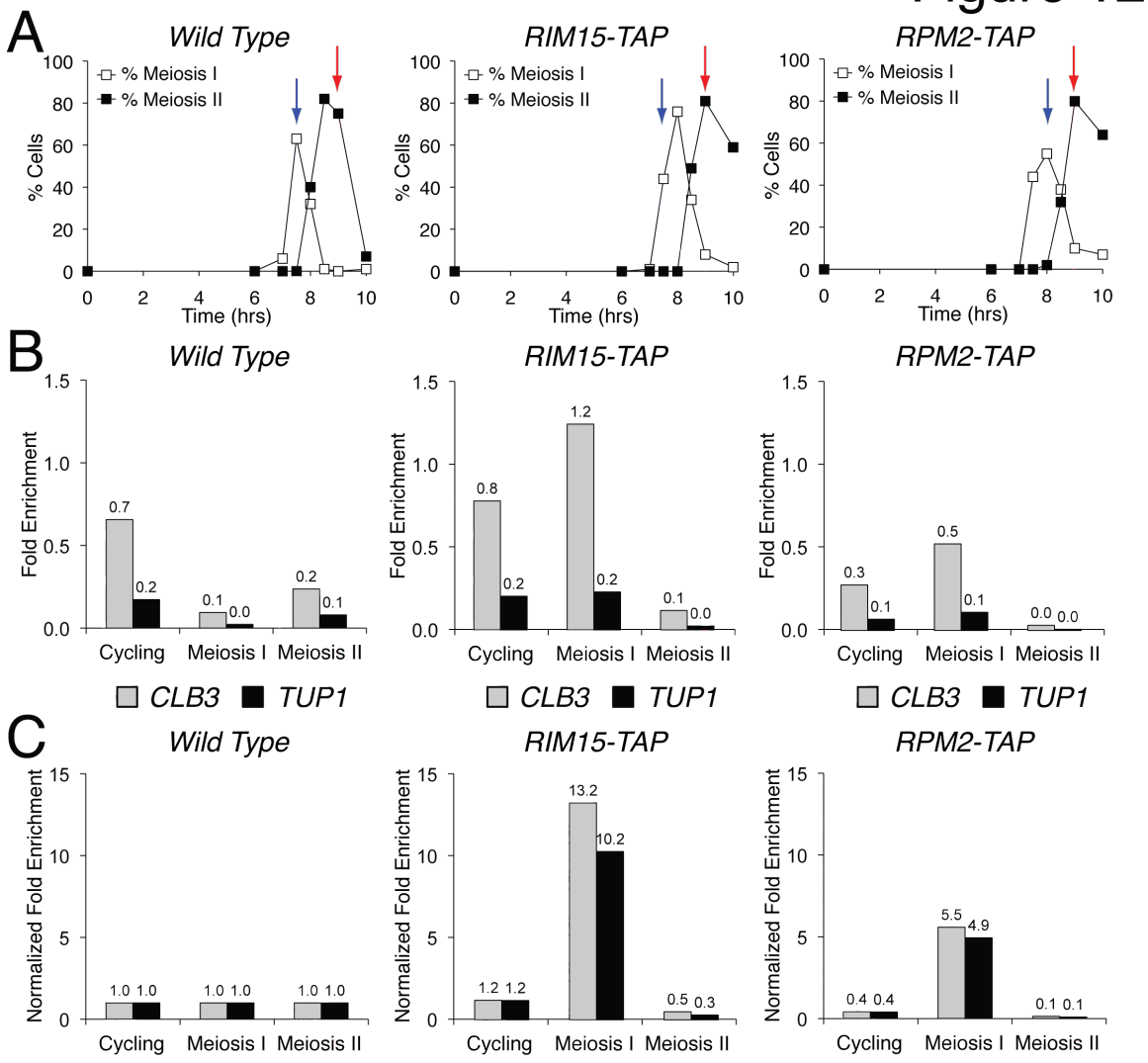


Figure 12: Association Rim15 and Rpm2 with *CLB3* transcript.

GAL4.ER, GAL-NDT80 (A14201, left graphs) strains with the tags *RIM15-TAP* (A23949, middle graphs), and *RPM2-TAP* (A23950, right graphs) were induced to sporulate at 30°C by transfer into SPO medium. After 6 hours 1μM β-estradiol was added. Meiosis I samples were taken at 7.5 hours and meiosis II samples were taken at 9 hours. For cycling samples these strains were inoculated into YPD and were grown to mid-log phase before harvesting.

A) The percentages of cells with meiosis I spindles (open squares) or meiosis II spindles (closed squares) were determined at the times indicated (n=100). Blue arrows indicate when the meiosis I timepoints were taken, and red arrows indicate when meiosis II timepoints were taken.

B,C) Cells were lysed and the tagged proteins were IPed. RNA was isolated from either total lysate or IPed samples, and the levels of *CLB3* transcript were determined by RT-qPCR. Grey bars represent values for *CLB3*, and black bars represent values for *TUP1*. The fold enrichment [B] is calculated as the ratio of *CLB3* transcript in IPed samples to *CLB3* transcript in total lysate. The normalized fold enrichment [C] is calculated as the ratio of the fold enrichment (in [B]) to the fold enrichment for each type of sample (cycling, meiosis I, meiosis II) in wild type (no tag). Numbers above the bars represent fold enrichment [B], or normalized fold enrichment [C].

Discussion

Control of *CLB3* translation in *cis*

In order to gain insight into the mechanisms of *CLB3* translational regulation we have also constructed a series of deletions of the *CLB3* 5'UTR in order to determine which regions are important for translational regulation. This analysis has revealed that the region of the 5'UTR spanning from -75 to -26 bases upstream of the start codon represents the maximal region required to prevent Clb3 accumulation during meiosis I, as deletions outside of this region do not allow Clb3 accumulation during meiosis I, and deletions inside of this region allow Clb3 accumulation during meiosis I.

The observation that Clb3 levels were lower in the 5'UTRΔ strains in meiosis I than in meiosis II can be explained in several ways. First, the lower levels of Clb3 present in meiosis I reflect the lower *CLB3* transcript levels present in meiosis I. Second, deletion of portions of the *CLB3* 5'UTR may reduce the stability of *CLB3* transcript, thus lowering the maximum possible levels of Clb3 during meiosis I. Finally, the 5'UTRΔs constructed may only partially relieve translational regulation of *CLB3*, preventing either all *CLB3* mRNA molecules from entering translation, or allowing only a partial increase in the translational efficiency of *CLB3* during meiosis I. To distinguish between these two latter possibilities the distribution of *CLB3* transcripts in meiosis I polysomes could be examined, which would show if a subset of *CLB3* transcripts were not entering translation. However, in the experiments above *CLB3* transcript levels were not examined, and the levels of Clb3 that accumulate during meiosis I in these strains are low. Therefore it is difficult to draw firm conclusions regarding how these deletions

affect *CLB3* translation. The fact that some Clb3 accumulates during meiosis I in some 5'UTRΔ strains suggests that translational regulation of *CLB3* is abolished in these strains. However, disruption of the 5'UTR may affect the stability of the message, the efficiency with which it is translated, or both. Therefore, given that *CLB3* transcript levels are lower in meiosis I than in meiosis II, and without data pertaining to *CLB3* transcript levels in these strains, or a direct comparison of Clb3 protein levels with *CLB3* transcript levels it is difficult to fully assess the effects of disruption of portions of the 5'UTR on *CLB3* translation. Repeating these experiments with analysis of both Clb3 protein levels and *CLB3* transcript levels would give some insight into how these 5'UTRΔs affect *CLB3* translation. However, to fully control for differences in *CLB3* transcript levels between meiosis I and meiosis II the effects of the 5'UTRΔs should be examined in an isolated context that is independent of transcript levels. We have previously demonstrated that the *CLB3* 5'UTR is sufficient to prevent protein accumulation during meiosis I. Therefore, examining Clb2 protein levels in strains in which *CLB2* has been placed under the control of the *GAL1-10* promoter, and the *CLB3* 5'UTR, or the *CLB3* 5'UTRΔs examined above would control for differences in transcript levels between meiosis I and meiosis II, and would allow direct assessment of the effects of deletion of portions of the *CLB3* 5'UTR on translation.

What sequence elements in this region are involved in translational control? It is possible that it is solely the primary sequence of this region that recruits an RBP, to prevent translation. It is also possible that this region of the 5'UTR contains some secondary or tertiary structure that is either bound by an RBP, or is stabilized by an RBP. We have

examined this region to determine if there are secondary structures that may represent possible structural elements bound by RBPs. However, m-fold gives only two predicted structures for this region, each with relatively low predicted stabilities (Zucker, 2003). Additionally, we looked for evolutionarily conserved secondary structures in this region of the *CLB3* 5'UTR between *S. cerevisiae*, *S. paradoxus*, *S. bayanus*, *S. mikatae*, and *S. kudriavzevii* using FOLDALIGN (Havgaard et al., 2005). Comparison of *S. cerevisiae* with each of the other yeasts yielded a potentially conserved structure, but there was no structure conserved between *S. cerevisiae* and all four other yeasts. However, it is difficult to judge the relevance of these conserved predicted structures in the absence of data regarding the translational status of the *CLB3* homologs during meiosis in the other yeasts.

Redundancy in promoting accurate chromosome segregation during meiosis

Why does premature accumulation of Clb3 in *GAL-CLB3* strains promote PSCS, while premature accumulation of Clb3 in 5'UTRΔ strains fails to promote PSCS (Carlile and Amon, 2008; Figures 4-5)? There are two possible explanations. First, the levels of Clb3 that accumulate during meiosis I in some 5'UTRΔ strains are insufficient to cause PSCS or a decrease in spore viability. This may be because *CLB3* mRNA levels are lower during meiosis I than in meiosis II. It may also reflect an incomplete alleviation of translational repression of *CLB3* during meiosis I. Second, the PSCS observed in *GAL-CLB3* strains might be a result of accumulation of Clb3 prior to meiosis I. If the *GAL-CLB3* phenotype is due to accumulation of Clb3 during meiosis I, then the low *CLB3* transcript levels might serve as a redundant mechanism to prevent enough Clb3 from

accumulating to promote PSCS during meiosis I, which would explain why no PSCS is observed in our 5'UTRΔ strains. However, *CLB3* is an *NDT80* target, and its levels are likely to be low prior to meiosis I. Thus, the PSCS phenotype observed for *GAL-CLB3* strains might be due to accumulation of *CLB3* transcript and Clb3 protein prior to meiosis I. We propose that Clb3 is a potent inhibitor of the meiosis I chromosome segregation program, and that there exist redundant mechanisms (transcriptional and translational) to prevent excess Clb3 accumulation during meiosis I, and PSCS. This potential redundancy is perhaps not surprising, given that the existence of redundant regulatory mechanisms is a common theme in cell cycle control. However, whether the PSCS phenotype seen in *GAL-CLB3* cells is due to Clb3 accumulation prior to or during meiosis I, it is interesting that overexpressed *CLB3* (*CLB3-UP*) promotes PSCS, while other overexpressed *CLBs* (*CLB-UP*) fail to promote PSCS. This phenotype seems to be specific to *CLB3*, as total CDK activity is higher in other *CLB-UP* strains than in *CLB3-UP* strains (Carlile and Amon, 2008; E. Ünal, personal communication).

Translational control of *CLB3* during meiosis

We have examined the roles of several known mechanisms of translational regulation in budding yeast in *CLB3* translation. These mechanisms include the regulation of translation by uORFs, by IRESs, and by the binding of messages by RBPs. Our data rule out the use of canonical uORFs in regulation, as the first AUG in the *CLB3* transcript is that of the *CLB3* ORF. However it is still formally possible that *CLB3* translation is regulated by a non-canonical uORF (Ingolia et al., 2009), and there are several potential uORFs with non-cognate start codons present in the *CLB3* 5'UTR. Indeed, genome wide

translational profiling during meiosis has revealed the presence of at least one uORF with a non-cognate start codon in the *CLB3* 5'UTR. However, this uORF is translated during both meiosis I and meiosis II (G. Brar, personal communication). How might such a uORF or uORFs regulate translation? One could imagine a situation in which translation of a single uORF prevents resumption of scanning, and thus prevents *CLB3* translation. If this were the case, then one would expect that the uORF would only be translated during meiosis I, or that there is an increased frequency of leaky scanning of the uORF during meiosis I. If *CLB3* translation were regulated by two or more uORFs then one might expect that *CLB3* translation might be regulated in a manner analogous to that of *GCN4*. In this situation translation of the first uORF would allow resumption of scanning downstream, and whether ribosomes reinitiated at the next downstream uORF or at the *CLB3* ORF would depend on TC levels in the cell. Such a mode of regulation would predict that TC levels would be lower during meiosis II than in meiosis I, and that these lower TC levels would allow bypass of downstream uORFs. To determine if *CLB3* translation is regulated by a non-canonical uORF or uORFs the non-cognate start codons of these observed uORFs should be mutated, and the effects on *CLB3* translation examined.

It was tempting to speculate that *CLB3* translation might be regulated by an IRES, as IRES dependent translation is up-regulated in nutrient poor conditions (Gilbert et al., 20007), and meiosis in *S. cerevisiae* only occurs upon nutrient limitation. However, our data indicate that translation of *CLB3* is likely not regulated by an IRES. Our analysis was performed in cycling cells in the W303 strain background using an *in vivo* reporter

system. Therefore this analysis does not definitively rule out regulation of translation by IRESs during meiosis in the SK1 strain background. The *in vivo* system requires that cells be spheroplasted, and subsequently recovered, which would be technically difficult to do at discreet timepoints in meiosis. To determine if the *CLB3* 5'UTR contains an IRES capable of promoting *CLB3* translation during meiosis II a similar assay could be performed using translational extracts derived from either meiosis I or meiosis II samples. If *CLB3* translation were regulated by an IRES during meiosis, then one would expect IRES reporter RNAs containing the *CLB3* 5'UTR to be translated in meiosis II extracts, but not meiosis I extracts. However, it may be technically difficult to perform these experiments, as SK1 may be infected with the L-A virus, which interferes with translation in these extracts (Iizuka and Sarnow, 1997).

To attempt to determine the mechanism of *CLB3* translational regulation we took a candidate gene based approach, in which we compiled a list of meiotically expressed RBPs. To do this we pooled a list of non-essential genes obtained from meiotic expression data with a list of genes deleted in a screen that identified monopolin, and subsequently used PROSITE to scan the amino acid sequences of these genes for known RNA interaction motifs (Primig et al., 2000; Tóth et al., 2000; Sigrist et al., 2002). This yielded a list of twelve genes, which represent a broad range of functions. We then examined the effects of deletion of each of these genes on Clb3 levels during meiosis. However, none of the genes examined seemed to have either a positive or negative effect on *CLB3* translation. There are several reasons that our analysis may have omitted or missed proteins required for *CLB3* translational control. First, there may be redundant

mechanisms of regulation, thus deletion of a single RBP that binds the *CLB3* 5'UTR may not be sufficient to relieve translational repression. Second, the true regulator of *CLB3* may be an essential gene, might lack a known RNA binding motif, or may not be strongly transcriptionally up-regulated during meiosis. Genes in this category would be omitted in our analysis. Indeed, an analysis of proteins that did not contain known RNA binding motifs revealed that they reproducibly associated with certain mRNAs (Hogan et al., 2008). Finally, it is possible that *CLB3* translation is not regulated by the binding of a protein, but may be regulated by some other mechanism, such as the association of a small non-coding RNA, or perhaps through an increased ribosome-intrinsic ability to translate the *CLB3* message during meiosis II.

The roles of Rim15 and Rpm2 in *CLB3* translational control

In order to identify proteins that interact with the *CLB3* 5'UTR, and thus represent potential translational regulators, we performed a Y3H assay with the *CLB3* 5'UTR. This assay revealed nine potential candidate regulators. However, we chose to limit our analysis to the top two hits, *RIM15* and *RPM2* since *RIM15* is involved in meiotic entry, and *RPM2* has been shown to interact genetically and physically with P bodies, which are known to play a role in translational regulation. Rim15 is a serine/threonine kinase involved in regulating meiotic entry in response to nutrient signals (Vidan and Mitchell, 1997; Roosen et al., 2005; Wanke et al., 2005), and Rpm2 is the protein subunit of mitochondrial RNase P, which cleaves the 5' leader sequences of mitochondrial tRNAs, and has several other functions in addition to its role in tRNA processing (Morales et al.,

1992; Stribinskis et al., 2001a; Stribinskis et al., 2001b; Stribinskis et al., 2005; Stribinskis and Ramos, 2007).

To attempt to verify that Rim15 and Rpm2 associate with the *CLB3* mRNA TAP-tagged versions of Rim15 and Rpm2 were IPed samples taken from mitotically growing, meiosis I, and meiosis II cells, and the association of *CLB3* mRNA was assessed by RT-PCR (Gerber et al., 2004). However, *CLB3* transcript was not significantly enriched in Rim15-TAP or Rpm2-TAP IPs taken from meiotic samples. This suggests that neither Rim15 nor Rpm2 physically interact with the *CLB3* transcript during meiosis, further suggesting that neither protein likely regulates *CLB3* translation during meiosis. However, these experiments were only performed once, and should be repeated with further controls to demonstrate that both proteins are being efficiently IPed.

We were unable to determine if Clb3 protein accumulated during meiosis I in *RIM15* or *RPM2* mutants, and were thus unable to assess the effects of either mutant on *CLB3* translation. However, in the absence of data demonstrating a direct interaction between either Rim15 or Rpm2, and *CLB3* mRNA the importance of these experiments is lessened. If a direct interaction of these proteins with *CLB3* mRNA can be demonstrated, then a role in *CLB3* translational regulation could be established using immunofluorescence (IF). Clb3 levels can be assessed during meiosis I and meiosis II by IF in *rim15*Δ mutants, provided that *rim15*Δ strains can be obtained, and in *rpm2-100* mutants, given that these mutants seem to sporulate relatively well outside of the *GAL-NDT80* background. In wild-type meiosis I cells Clb3 should not be observed by IF, but

should be observed in wild-type meiosis II cells. However, if either protein acts as a translational repressor during meiosis I, then Clb3 would be expected to be observed by IF in mutant meiosis I cells. Conversely, if either protein acts as a translational activator during meiosis II, then Clb3 would not be expected to be observed by IF in mutant meiosis II cells.

The Mechanism of Translational Control of *CLB3*

Our data suggest that Rim15 and Rpm2 do not bind to the *CLB3* mRNA, and therefore are likely not translational regulators of *CLB3*. However, we still favor the hypothesis that *CLB3* translational regulation is mediated by the binding of an RBP to the *CLB3* 5'UTR. How might such an RBP or RBPs modulate *CLB3* translation? If such an RBP were a translational repressor, it could bind to the *CLB3* 5'UTR during meiosis I and prevent translation in a variety of ways. First, binding might prevent the association of eIFs with the *CLB3* message, thus preventing translational initiation. Second, binding might prevent the ribosome from scanning through to the start codon. Finally, binding might stabilize a secondary structure in the 5'UTR that inhibits scanning or some other aspect of translational initiation. If *CLB3* translational regulation occurs through activation of translation during meiosis II (and possibly in vegetatively growing cells), an RBP might bind the *CLB3* 5'UTR and promote translation. First, such an RBP might promote the recruitment of eIFs to the message. Second, RBP binding might destabilize a secondary structure in the 5'UTR that prevents scanning. Finally, RBP binding might promote some other aspect of translational initiation. Additionally, it is possible that

either an activating RBP or a repressive RBP may regulate other transcripts in a similar manner, and such experiments are discussed further in Chapter 4.

How might translational repression be restricted to meiosis I, or translational activation be restricted to meiosis II? One possibility is that the translational regulators, presumably RBPs, might be present only during meiosis I (if a repressor), or only during meiosis II (if an activator). Another possibility is that such regulators might be present in both meiosis I, meiosis II, and the mitotic cell cycle, but may be inhibited during meiosis II and mitosis (for a repressor), or activated during meiosis II and possibly mitosis (for an activator). Such stage specific activation or inhibition could occur through a variety of mechanisms, including post-translational modifications, or through differential regulation of subcellular localization. For example, a slower migrating form of Rpm2 is present at high levels in vegetatively growing cells, and during meiosis II. This might represent a post-translational modification of Rpm2, or might represent Rpm2 that has not had its MTS cleaved. Such a form of Rpm2 would presumably be localized in the nucleus or cytoplasm. If Rpm2 is a regulator of *CLB3* translation, then this data might hint that Rpm2 is an activator of *CLB3* translation.

Experimental Procedures

Strains

All strains are SK1 derivatives and are described in Table 1, except A2587 (W303), and strains used for the Y3H assay, which are described in Table 2. *CLB3-3HA*, *RIM15-TAP*, *RPM2-TAP*, and all candidate RBP deletions were constructed using the PCR-based method described in (Longtine et al. 1998). *CLB3* 5'UTR deletions were made by a two step transformation procedure. First, *URA3* was PCRed from *K. lactis* genomic DNA with 40 bases of flanking homology for each desired deletion. This PCR fragment was then transformed into a *CLB3-3HA* tagged strain with selection on –URA. The resulting strains have deletions of the desired regions of the *CLB3* 5'UTR marked with *URA3*. Second, complimentary primers pairs were designed with 40 bases of flanking homology to the 5'UTR sequences on either side of the integrated *URA3* gene. These primers were then annealed, resulting in an 80 bp fragment of DNA with homology to the genomic sequences on either side of the integrated *URA3* gene. Annealed primers were then transformed into the *5'UTRΔ::URA3* strains with selection on 5'FOA. URA- colonies obtained in the second transformation step were then screened by PCR and sequencing to verify that the *K. lactis URA3* gene had been removed by the second transformation, and that no mutations were introduced into the 5'UTR during transformation.

Table 1: Strains

Strain Number	Relevant Genotype
A2587*	MATa (W303)
A14201	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1*
A15055	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-*

	GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6
A20531	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 ygr250cΔ::HIS3MX6/ygr250cΔ::HIS3MX6*
A20532	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 hrb1Δ::HIS3MX6/hrb1Δ::HIS3MX6*
A20533	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 ydr374cΔ::HIS3MX6/ydr374cΔ::HIS3MX6*
A20534	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 rrt5Δ::HIS3MX6/rrt5Δ::HIS3MX6*
A20535	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 mip6Δ::HIS3MX6/mip6Δ::HIS3MX6*
A20536	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 pes4Δ::HIS3MX6/pes4cΔ::HIS3MX6*
A20537	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 yll032cΔ::HIS3MX6/yll032ccΔ::HIS3MX6*
A20538	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 cbc2Δ::HIS3MX6/cbc2Δ::HIS3MX6*
A20539	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 slf1Δ::HIS3MX6/slf1Δ::HIS3MX6*
A20540	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 rim4Δ::HIS3MX6/rim4Δ::HIS3MX6*
A20541	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 tma64Δ::HIS3MX6/*

	tma64cΔ::HIS3MX6
A20542	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 pbp2Δ::HIS3MX6/ pbp2Δ::HIS3MX6*
A21494	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 nsr1Δ::HIS3MX6/ nsr1Δ::HIS3MX6*
A21495	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 pub1Δ::HIS3MX6/ pub1Δ::HIS3MX6*
A23949	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 RIM15-TAP::HIS3MX6/ RIM15-TAP::HIS3MX6*
A23950	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 RPM2-TAP::HIS3MX6/ RPM2-TAP::HIS3MX6*
A22596	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-150-101-CLB3-3HA::KANMX6/5'UTRΔ-150-101-CLB3-3HA::KANMX6*
A22597	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-100-51-CLB3-3HA::KANMX6/5'UTRΔ-100-51-CLB3-3HA::KANMX6*
A22598	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-50-1-CLB3-3HA::KANMX6/5'UTRΔ-50-1-CLB3-3HA::KANMX6*
A23342	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-100-76-CLB3-3HA::KANMX6/5'UTRΔ-100-76-CLB3-3HA::KANMX6*
A23343	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-75-51-CLB3-3HA::KANMX6/5'UTRΔ-75-51-CLB3-3HA::KANMX6*
A23344	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 URA3 5'UTRΔ-50-26-CLB3-3HA::KANMX6/5'UTRΔ-50-26-CLB3-3HA::KANMX6*
A23345	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1*

	5'UTRΔ-25-1-CLB3-3HA::KANMX6/5'UTRΔ-25-1-CLB3-3HA::KANMX6
A23346	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 5'UTRΔ-75-26-CLB3-3HA::KANMX6/5'UTRΔ-75-26-CLB3-3HA::KANMX6*
A18686	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 CLB3-3HA::KANMX6/CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A18687	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6/clb3::pGAL-CLB3-3HA::KANMX6::HIS3MX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24641	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-150-101-CLB3-3HA::KANMX6/5'UTRΔ-150-101-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24642	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-100-51-CLB3-3HA::KANMX6/5'UTRΔ-100-51-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24643	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-50-1-CLB3-3HA::KANMX6/5'UTRΔ-50-1-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24644	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-100-76-CLB3-3HA::KANMX6/5'UTRΔ-100-76-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24645	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-75-51-CLB3-3HA::KANMX6/5'UTRΔ-75-51-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24646	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-50-26-CLB3-3HA::KANMX6/5'UTRΔ-50-26-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24647	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-25-1-CLB3-3HA::KANMX6/5'UTRΔ-25-1-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24648	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-75-26-CLB3-3HA::KANMX6/5'UTRΔ-75-26-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*
A24971	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 5'UTRΔ-153-1-CLB3-3HA::KANMX6/5'UTRΔ-153-1-CLB3-3HA::KANMX6 leu2::tetR-GFP::LEU2::TetO-HIS3/+*

IRES Assays

IRES assays were performed as described in (Gilbert et al., 2007). Transcripts were prepared by *in vitro* transcription from Ecl136II (Fermentas) cut reporter plasmid using T7 RNA Polymerase in the presence of 7mG(ppp)G cap (NEB), or A(ppp)G cap analog (NEB). RNAs were extracted using phenol-chloroform, ethanol precipitated, and resuspended in DEPC treated water. Cells were grown to mid-log phase, and were harvested and spheroplasted. 2 µg of RNA was electroporated into cells (800C, 25 µF, 1000 Ω), which were recovered for 1 hr in YPD-1M Sorbitol. F-luc assays were performed using a Dual-Luciferase Reporter Assay System as per manufacturers instructions (Promega).

Other Methods

Sporulation conditions for *GAL-NDT80* strains are as described in (Carlile and Amon, 2008). Indirect immunofluorescence, GFP-Dot sample preparation, spindle counts, and western blots were performed as described in (Carlile and Amon, 2008). Mouse anti-Pgk1 (Molecular Probes) was used at a 1:5000 dilution. Mouse anti-Vph1 (Molecular Probes) was used at a 1:2000 dilution. Mouse anti-HA (HA.11, Covance) was used at a 1:1000 dilutions. Peroxidase anti-peroxidase (for TAP) was used at a 1:200 dilution. Sheep anti-mouse conjugated to HRP (GE Healthcare) was used as a secondary antibody at a 1:5000 dilution.

Library Amplification

1 mL aliquots of the C1, C2, and C3 libraries in *E. coli* were inoculated into 1 L LB^{Amp}. When the cultures reached an OD_{600} of 0.7 20 mL samples of each library were pelleted, resuspended in 10 mL 15% glycerol, and stored at -80ºC in 1 mL aliquots. When the cultures reached an OD_{600} of 1.0 cells were pelleted and stored at -20ºC overnight. Plasmid DNA was isolated by MAXI prep, and was titered by transformation into *E. coli* strain DH5α with 10-fold serial dilutions. A volume of each library sufficient to give approximately 10-fold library coverage was transformed into A20544, and was plated on selective media. The plates were incubated for one week at 30ºC, and were harvested with sterile scrapers. Cells were resuspended in 15% glycerol, divided into 1 mL aliquots and were stored at -80ºC.

Yeast Three-Hybrid Screen

The *CLB3* 5'UTR was cloned into pIIIA/MS2.2 to yield pA1702. This plasmid was transformed into the Y3H strain background to yield A21180. The *GAL4* AD libraries C1, C2 and C3 were transformed into A20544. A21180 was mated with aliquots of each library in A20544. Briefly, A21180 was grown to saturation in SC-URA, and aliquots of each library were grown to an OD_{600} of ~2.0 in SC-LEU. 75 OD units of each library in A20544 was then mixed with 50 OD units A21180. Cells were pelleted and then resuspended in 100 mL YPD. This mating mix was incubated at 30ºC overnight with shaking at a low rpm. 50 mL of each mating was then pelleted and resuspended in 0.5 L SC-URA-LEU, and was then grown for 10 hours at 30ºC. Cells were pelleted and

resuspended in 10 mL 15% glycerol. Serial dilutions of the matings were plated on SC-URA-LEU and YPD. The enrichment for diploids = (# colonies on SC-URA-LEU) /(# colonies on YPD) (at least ~6%).

The matings were then plated on –HIS, -LEU, Low Ade, 3-AT (from 1 to 25 mM) plates. White colonies were picked onto SC-URA-LEU from 10, 15, and 25 mM 3-AT plates beginning at day 4 and ending at day 13. Colonies were replica plates to 5-FOA to lose the bait plasmid, and a secondary screen was then performed using LacZ filter assays. The identities of inserts for colonies that exhibited RNA dependence for both HIS3 and LacZ reporters were determined by sequencing, and Southern blot analysis of library derived PCR fragments. All Y3H strains are described in Table 2.

Quantitative ONPG Assays

Cells were grown to mid-log phase in SC-URA-LEU. Cells were resuspended in 150 µL Breakage Buffer (0.1 M Tris [pH 8.0], 20% Glycerol, 1 mM β-mercaptoethanol, 2 mM PMSF), and were lysed by vortexing with glass beads, and clarified by centrifugation. Protein concentration was determined by Bradford assay. Equal amounts of protein were brought up to a final volume of 100 µL, to which 900 µL of Z Buffer (40mM $Na_2HPO_4 \cdot 7H_2O$, 60mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4$, 50 mM β-mercaptoethanol, [pH7.0]) was added. Samples were then pre-incubated at 28ºC, followed by addition of 200 µL ONPG solution (4mg/mL *o*-nitrophenyl-β-D-galacto-pyranoside in Z buffer). Samples were incubated at 28ºC until they turned a pale yellow color. Reactions were stopped with the addition of 500 µL 1M Na_2CO_3, and the OD_{420}

was measured. Miller units were calculated as ([OD420]x1.7)/(0.0045x[time in min]x[vol extract in mL]x[protein concentration in μg/μL]).

Table 2: Y3H Strains

Strain Number	Relevant Genotype
A20543	MATa, LYS2::(LexAop)-HIS3 ura3::(LexAop)-LacZ LexA-MS2coat::TRP1
A20544	MATα, LYS2::(LexAop)-HIS3 ura3::(LexAop)-LacZ LexA-MS2coat::TRP1
A21180	MATa, LYS2::(LexAop)-HIS3 ura3::(LexAop)-LacZ LexA-MS2coat::TRP1 [2μ - 5'UTRCLB3-MS2Stems::URA3::ADE2]

RNA Co-immunoprecipitation

RNA Co-IPs were performed as in (Gerber et al., 2004). Briefly, cells were harvested and washed twice in Buffer A (20 mm Tris [pH 8.0], 140 mM KCl, 1.8 mM $MgCl_2$, 0.1% NP-40, 0.02 mg/mL heparin). Cells were lysed by vortexing in Buffer B (Buffer A supplemented with 0.5 mM DTT, 1mM PMSF, 0.5μg/mL Leupeptin, 0.8 μg/mL Pepstatin, 0.2 mg/mL heparin) supplemented with 100 U/mL RNasin (Promega) and 20 U/mL DNase I (NEB). Extracts were clarified by centrifugation, and equal amounts of protein were IPed with Rabbit IgG-Agarose beads (pre-equilibrated in Buffer A) (Sigma) at 4ºC for 2 hours. Beads were washed once in Buffer B, followed by three washes in Buffer C (20 mm Tris [pH 8.0], 140 mM KCl, 1.8 mM $MgCl_2$, 10% glycerol, 0.5 mM DTT, 0.01% NP-40) supplemented with 10 U/mL RNasin. Beads were then resuspended in 1.5X bed volume Buffer C, and TEV-Protease (Invitrogen) was added to 0.15 U/μL. The TEV reactions were carried out at 15ºC for 2 hours. Eluates were collected, and, along with total RNA samples, were phenol:chloroform extracted, and ethanol

precipitated (IPs) or LiCl precipitated (total RNA). RT reactions were perfomed using SuperScript III (Invitrogen), and qPCRs were performed on a CFX96 Real-Time System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Primers used for *CLB3* detection by qPCR are TC89_CLB3_RT1F (5' - CGACGGAGAAAGCGAAGAGGATGAAG - 3') and TC90_CLB3_RT1R (5' - TCGGCAACCATGACCACATCGTAC -3').

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Chapter 4:

Discussion and Future Directions

Key Conclusions

In *S. cerevisiae* major mitotic and meiotic events are controlled by the activity of cyclin-dependent kinases (CDKs). Although CDK activity and regulation are well characterized in mitosis, they have, until now, remained poorly characterized in meiosis. The work described in this thesis has focused on the characterization of CDK activity, and on the regulation of CDKs during the meiotic divisions. First, a system was developed to synchronize cells during the meiotic divisions, and this system was then used to characterize CDK activity during the meiotic divisions. This analysis revealed a striking diversity in the regulation of Clb-CDK activities during meiosis. Each of the Clb-CDKs examined displayed a unique pattern of kinase activity. Next, the regulation of Clb3-CDKs was further studied in detail. Clb3 protein and Clb3-CDK activity were found to be restricted to meiosis II, though *CLB3* transcript was present during both meiosis I and meiosis II. Clb3 was then shown to be restricted to meiosis II through translational regulation mediated by the *CLB3* 5'UTR. Furthermore, premature accumulation of Clb3 was shown to cause premature sister-chromatid segregation (PSCS). Finally, experiments were performed to ascertain the mechanism of translational regulation of *CLB3*. Rim15 and Rpm2 were identified as potential interactors with the *CLB3* 5'UTR by yeast three-hybrid assay (Y3H), but subsequent experiments suggested that they do not bind the *CLB3* 5'UTR, and thus likely do not represent potential translational regulators. The potential implications of this work, and future directions are discussed below.

Synchronization of meiotic cells in *S. cerevisiae*

In budding yeast the events of mitosis are better understood than the events of meiosis. One reason for this disparity is the availability of more powerful tools to study mitosis. Budding yeast cells can be induced to undergo the mitotic cell cycle synchronously using a variety of synchronization techniques, which allow cells to be uniformly arrested in a particular cell cycle stage. Cells can then be released from the arrest allowing synchronous cell cycle progression. The power of these techniques lies in the fact that they allow one to enrich for cells in a particular phase of the cell cycle, which allows cell cycle events to be studied using population based assays. Synchronization techniques can reveal periodic accumulation of cell cycle regulated proteins, and cell cycle regulated transcripts. For example it was the synchronous early mitotic divisions in sea urchin embryos that allowed the identification of cyclins as cell cycle regulated proteins (Evans et al., 1983).

The synchrony of sporulating cultures of *S. cerevisiae* is poor, and the lack of analogous synchronization protocols for meiosis has limited the study of meiosis in budding yeast. Even in the strain background SK1, which sporulates efficiently and relatively synchronously, meiosis I and meiosis II events cannot be distinguished using population based assays. For example, an early study aimed at comparing CDK activity in mitosis and meiosis demonstrated clear differences in the accumulation of Clb3 and Clb4 protein and kinase activity compared to Clb1 and Clb2 protein and kinase activity during mitosis, but was unable to detect differential regulation of cyclins between meiosis I and meiosis II (Grandin and Reed, 1993).

To date, the study of events of the meiotic divisions in budding yeast has largely relied on single cell based cytological assays such as immunofluorescence of whole cells and of spread nuclei, and the GFP-dot system to follow meiotic chromosome segregation (Straight et al., 1996; Michaelis et al., 1997). The use of these techniques has allowed elucidation of many of the key events and specializations that occur to establish the meiotic chromosome segregation program (Klein et al., 1999; Tóth et al., 2000; Lee and Amon, 2003; Rabitsch et al., 2003; Katis et al., 2004a). However, a meiotic synchronization system would greatly enhance the study of meiosis by allowing the resolution of meiosis I and meiosis II events using population based assays, and to study processes that are not readily amenable to study at the single cell level.

Ndt80 is a transcription factor that promotes transcription of meiotic middle genes, and is required for progression out of the pachytene stage of meiotic prophase and into the meiotic divisions (Chu et al., 1998; Chu and Herskowitz, 1998; Primig et al., 2000). To reversibly arrest cells after meiotic entry, pre-meiotic DNA replication, and recombination, but before the meiotic divisions, an inducible allele of *NDT80* was employed. *NDT80* was placed under the control of the inducible *GAL1-10* promoter, and its transcription is driven using a Gal4-Estrogen receptor (Gal4.ER) fusion protein that activates transcription from the *GAL1-10* promoter in response to β-estradiol (Picard, 1999; Benjamin et al., 2003). Cells can be arrested in pachytene in the absence of β-estradiol, and can be released from this block with the addition of β-estradiol. This system allows cells to progress through the meiotic divisions far more synchronously

than do wild type strains, and allows the resolution of meiosis I and meiosis II events using population based assays.

The use of the *GAL-NDT80 GAL4.ER* system to characterize meiotic CDK activity revealed differences in the activity of different Clb-CDKs during meiosis I and meiosis II. For example, Clb1-CDK activity is restricted to meiosis I and Clb3-CDK activity is restricted to meiosis II (Carlile and Amon, 2008). These experiments revealed unexpected levels of CDK regulation during meiosis, which would have been difficult to uncover without the use of population based assays enabled by the synchronization of meiotic cells. Therefore, the *GAL-NDT80 GAL4.ER* system could be employed to study a variety of meiotic events including regulation of the meiosis I to meiosis II transition, regulation of differential events governing exit from meiosis I and exit from meiosis II, to gain further insight into the regulation of meiotic chromosome segregation, and should prove to be a valuable tool in the study of meiosis in budding yeast. Additionally, the development of earlier synchronization techniques might provide additional insights into early meiotic events. Earlier synchronization techniques could be achieved by placing other meiotic regulatory genes under inducible promoters, including *IME1* or *IME2*. For example, reversible inactivation of an inhibitor sensitive allele of *CDC7* allows cells to be synchronized prior to meiotic recombination (Wan et al., 2006).

Cyclin-dependent kinase activity in meiosis

During vegetative growth the activity of Clb-CDK complexes is controlled by *CLB* transcription, by the binding and inhibition of Clb-CDKs by CDK inhibitors (CKIs), and

by the ubiquitin-mediated proteolysis of Clbs, of which the latter plays the most important role (Bloom and Cross, 2007). However, the characterization of Clb-CDK activity during the meiotic divisions revealed a striking diversity in the patterns of activity of different Clb-CDKs, and regulation of Clb-CDKs at previously unknown levels.

There were two main patterns of *CLB* expression during the meiotic divisions. For *CLB5*, *CLB4*, and *CLB1* transcript reaches peak levels during the meiosis I, and remains high during meiosis II. However, *CLB3* transcript appears during meiosis I, but reaches peak levels during meiosis II (Figure 1). Several patterns of Clb-CDK activity were observed during the meiotic divisions. Clb5-CDK activity closely mirrored Clb5 protein levels, which appeared in two waves, one centered at meiosis I, and one centered at meiosis II. Clb1-CDK activity was found to be restricted to meiosis I, though Clb1 protein was present during meiosis II. Clb4-CDKs were found to be active during meiosis I and early meiosis II, though Clb4 was present during late meiosis II. Finally, Clb3-CDK activity and Clb3 protein were restricted to meiosis II, though *CLB3* transcript was present during meiosis I (Figure 1). Thus, none of the Clb-CDKs examined had identical patterns of meiotic CDK activity.

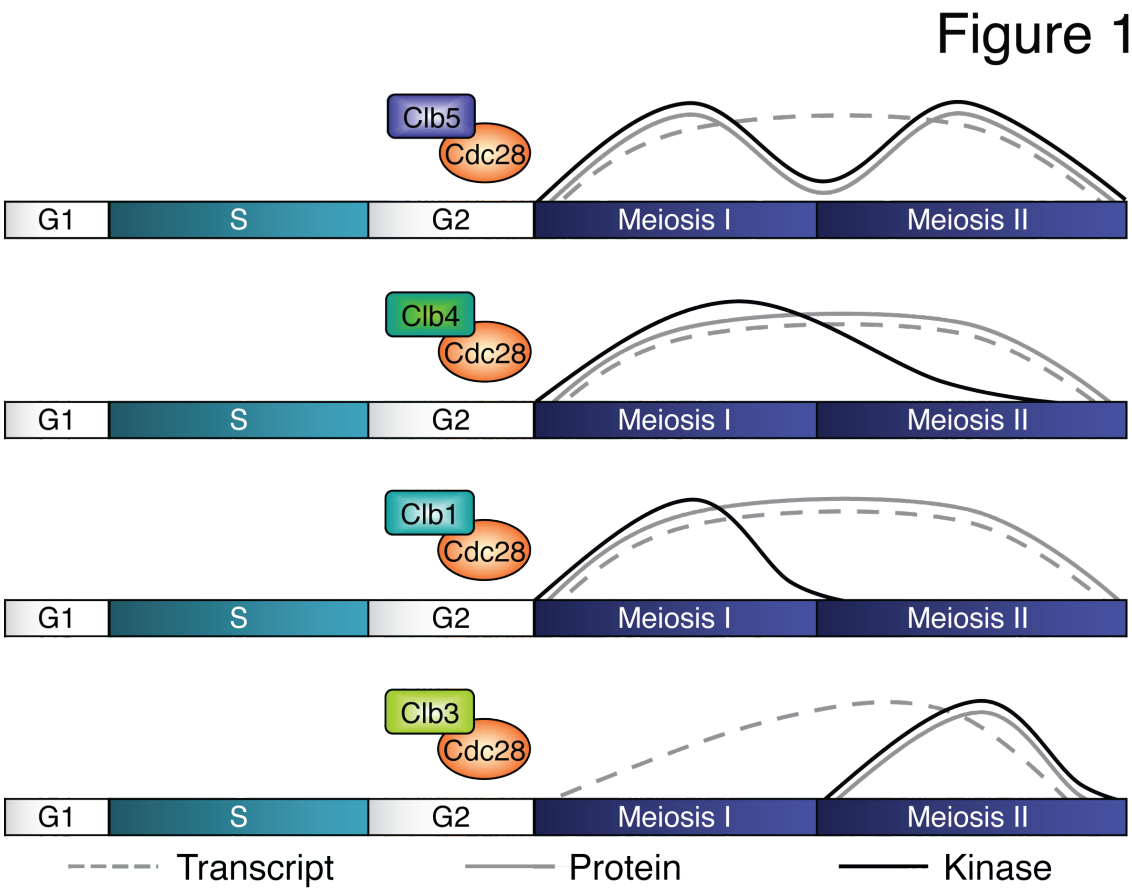

Figure 1: *CLB* expression, Clb protein, and Clb-CDK activity during the meiotic divisions.

CLB transcript levels are indicated with grey dashed lines. Clb protein levels are indicated by solid grey lines. Clb-CDK activities are indicated by solid black lines. The relevant Clb-CDK complexes are indicated to the left of the meiotic divisions. See the text for discussion.

Why does the cell display such a diverse pattern of meiotic CDK? One possibility is that the two sequential chromosome segregation phases that occur during meiosis require fine-scale control of CDK activity, especially during the meiosis I to meiosis II transition. Another possibility is that the activity of specific Clb-CDK complexes must be confined

to specific times during meiosis. Premature production of Clb3, for example, causes PSCS. It is therefore possible that inactivation of Clb1-CDKs or Clb4-CDKs during meiosis II and late meiosis II respectively may be required for accurate completion of some aspects of the meiotic program. Additionally, over-expression of non-degradable Clb2 during meiosis prevents the meiosis I to meiosis II transition from occurring (Marston et al., 2003). Therefore, it is tempting to speculate that inactivation of Clb1 after meiosis I may play a role in promoting this transition. The identification of the mechanisms by which Clb1-CDKs and Clb4-CDKs are regulated during meiosis might provide a method to test the effects of activation of these CDKs during meiosis II and late meiosis II respectively.

How are these various patterns of CDK activity established? Clb5 is an Anaphase Promoting Complex $(APC)^{Cdc20}$ substrate, and the activation of the APC^{Cdc20} promotes the metaphase I to anaphase I transition. Therefore it seems likely that the dip in Clb5 protein and Clb5-CDK activity between meiosis I and meiosis II is due to an increase in the rate of Clb5 degradation compared to Clb5 synthesis. How are the activities of Clb1-CDKs and Clb4-CDKs regulated? It seems likely that they are either specifically activated during meiosis I, or are specifically inactivated during meiosis II or late meiosis II respectively. It is possible that both are inhibited by either tyrosine 19 phosphorylation or by the binding of Sic1. It would therefore be interesting to examine Clb1-CDK and Clb4-CDK activities during meiosis in *sic1*Δ or *swe1*Δ strains. However, *sic1*Δ strains fail to undergo meiosis in the *GAL-NDT80* background, complicating analysis of the role of Sic1 in the regulation of these Clb-CDKs (M. Miller, personal communication). It is

also possible that Clb1-CDKs and Clb4-CDKs may be bound by either novel activators during meiosis I, or by novel inhibitors during meiosis II. Proteins that differentially co-purify with Clb1 in meiosis I and meiosis II could be identified by mass spectrometry, and would represent potential regulators of Clb1-CDK activity, and perhaps Clb4-CDK activity.

The analysis of CDK activity presented in Chapter 2 included the cyclins Clb1, Clb3, Clb4, and Clb5. However, it omitted the cyclin Clb6, the meiosis specific kinase Ime2, and bulk CDK activity. It would therefore be interesting to further characterize the activity of CDKs during meiosis. In vegetative cells Clb6 is targeted for degradation by the SCF early in the cell cycle. Therefore, Clb6-CDKs may exhibit a unique pattern of activity during meiosis. Additionally, the characterization of bulk CDK activity during meiosis would give insights into how the meiosis I to meiosis II transition is regulated, and into differences between meiosis I and meiosis II in total CDK activity. Additionally, the meiosis-specific kinase, Ime2, functions in place of the Cln-CDKs in early meiosis, and has a role in promoting the second meiotic division (Benjamin et al., 2003). Characterization of Ime2-associated kinase activity during meiosis would also yield insights into how it is regulated during the meiotic divisions, and into how late meiotic events are regulated by Ime2. As with the characterization of Clb1, Clb3, Clb4 and Clb5-CDK activities, characterization of Clb6-CDK activity, bulk CDK activity and Ime2 activity is likely to suggest additional avenues of investigation of the regulation and roles of these proteins during meiosis.

The regulation of chromosome segregation by Clb3

How does early accumulation of Clb3-CDKs promote PSCS? The data presented in Chapter 2, and subsequent experiments have shown that Clb3 is able to specifically promote PSCS. Only *GAL-CLB3* strains, but not other *GAL-CLB* strains show this phenotype. The simplest explanation is that Clb3-CDKs, but not other Clb-CDKs, are able to specifically phosphorylate some factor required for the establishment of the meiosis I chromosome segregation pattern, and such phosphorylation interferes with the activity of these regulators. There are three main modifications to the mitotic chromosome segregation machinery that occur during meiosis that establish the unique meiosis I chromosome segregation pattern, and in principle premature accumulation of Clb3 could interfere with any of them. They are the linkage of homologous chromosomes through recombination, the co-orientation of sister-chromatids during meiosis I, and the protection of centromeric cohesins during meiosis I (Marston and Amon, 2004). Subsequent experiments have shown that in *GAL-CLB3* strains recombination is unaffected, but that the localization of Mam1 to kinetochores is compromised, and centromeric cohesion is not protected at meiosis I, though Sgo1 localization seems normal (E. Ünal, personal communication). This suggests that monopolin function is compromised due to improper localization, and that Sgo1 may be unable to protect centromeric cohesins despite its proper localization in *GAL-CLB3* strains. Together these results indicate that premature accumulation of Clb3 interferes with the function of monopolin and the function of Sgo1, and that the inability of cells to co-orient sisters and protect centromeric cohesion is the reason that *GAL-CLB3* strains improperly segregate sisters during meiosis I. Additionally, more sensitive assays could be performed that

might allow characterization of subtle defects not readily apparent using cytological methods. For example, the use of chromatin immunoprecipitation could be employed to examine the association of Sgo1 with DNA. Such an analysis might reveal defects in the association of Sgo1 with pericentric chromatin, or may reveal quantitative defects in the association of Sgo1 with DNA.

It is currently unclear how the functions of monopolin and Sgo1 are compromised in *GAL-CLB3* strains. It seems likely that premature accumulation of Clb3-CDKs leads to the phosphorylation of a key substrate or set of substrates, and that the phosphorylated forms of these proteins interfere with the meiosis I chromosome segregation machinery. Many known regulators of meiotic chromosome segregation are phospho-proteins. It may therefore be informative to compare their phosphorylation status in wild type and *GAL-CLB3* cells, or between *GAL-CLB3* and another *GAL-CLB* strain. An increase in phosphorylation of a given protein in *GAL-CLB3* strains would suggest that it may be a target of Clb3, and could then be further investigated.

The meiosis specific factor *SPO13* coordinates the meiosis I chromosome segregation program by promoting protection of centromeric cohesion through Sgo1 localization and through co-orientation of sister-kinetochores at meiosis I (Lee et al., 2002; Shonn et al., 2002; Katis et al., 2004b; Lee et al., 2004). *GAL-CLB3* strains and *spo13*Δ strains share a variety of phenotypes including separation of sister-chromatids during meiosis I, suppression of the metaphase I arrest brought about by Cdc20 depletion, and suppression of PSCS by delaying progression through early meiosis (McCarroll and Esposito, 1994;

Shonn et al., 2002; Katis et al., 2004b; Carlile and Amon, 2008). Therefore, Spo13 may be phosphorylated and inhibited by Clb3 in *GAL-CLB3* strains. Such inhibition would explain the common phenotypes exhibited by both *spo13*Δ and *GAL-CLB3* mutants. Our preliminary data suggests that Spo13 may be hyper-phosphorylated in *GAL-CLB3* strains (data not shown). However, these experiments need to be repeated with other *GAL-CLB* strains as controls.

When does Clb3 function to promote PSCS? In *GAL-CLB3* strains PSCS is only observed when experiments are performed in an unsynchronized background, when the timing of *CLB3* expression cannot be precisely matched to entry into meiosis I. It is therefore possible that Clb3 exerts its function in promoting PSCS either prior to or during meiosis I. Additionally, the Clb3 that accumulates during meiosis I in some *CLB3* 5'UTRΔ strains is insufficient to cause PSCS. There are two possible explanations for these data. First, the levels of Clb3 that in 5'UTRΔ strains during meiosis I are insufficient to cause PSCS. If this is the case, then the lower *CLB3* transcript levels present during meiosis I may be a redundant mechanism to prevent large amounts of Clb3 from accumulating during meiosis I. Second, the PSCS observed in *GAL-CLB3* strains might result from accumulation of Clb3 prior to meiosis I. Since *CLB3* is an *NDT80* target *CLB3* transcript levels are likely to be low prior to meiosis I, thus the *GAL-CLB3* phenotype might be a result of both inappropriate transcription of *CLB3* and accumulation of Clb3 prior to meiosis I. However, when *CLB3* expression is induced prior to meiosis I in cells synchronized using the *GAL-NDT80* system the levels of PSCS observed are dramatically lower than in unsynchronized strains (E. Ünal, personal

communication). These data indicate that delaying cells prior to the meiotic divisions suppresses the PSCS phenotype associated with premature accumulation of Clb3, which might explain why no PSCS phenotype is observed in *GAL-CLB3 GAL-NDT80* strains. In order to determine if Clb3 acts during, or prior to meiosis I *CLB3* could be placed under the *CLB1* promoter, which would allow high levels of Clb3 protein to be produced specifically during meiosis I in unsynchronized meioses.

The regulation of meiotic events by cyclin dependent kinases

While a great deal is known about how CDKs control mitotic cell cycle events, much less is known about how they control meiotic events, and though it is largely assumed that analogous events are regulated in similar manners there is a relative paucity of studies directly demonstrating roles for CDKs in meiotic events. For example, it was only relatively recently that a direct role for CDKs in initiation of recombination was demonstrated (Henderson et al., 2006). While there are many meiotic events where the assumption that CDKs function analogously to the events of mitosis, such as DNA replication, there are several events unique to meiosis whose regulation by CDKs is either poorly characterized, or is almost entirely uncharacterized.

For example, very little is known about how the duplication and separation of spindle pole bodies (SPBs) is regulated by CDKs during meiosis in budding yeast. In meiosis SPBs are duplicated twice, once prior to the meiotic divisions and once prior to meiosis II, and the second round of SPB duplication results in SPBs with modified outer plaques (Jaspersen and Winey, 2004). However, little is known about how either the first or

second round of SPB duplication is regulated during meiosis by either the G1-CDK-like kinase Ime2, or by Clb-CDKs. In mitosis Cln-CDKs promote SPB duplication, however it is not known if Ime2 promotes meiotic SPB duplication in an analogous manner. Additionally, it is not known how the down-regulaton of CDKs between meiosis I and meiosis II affects the second round of meiotic SPB duplication.

How CDKs are regulated during the meiosis I to meiosis II transition, and how CDKs regulate this transition is also poorly understood. It is thought that CDKs must be only partially down-regulated between meiosis I and meiosis II (Marston et al., 2003; Kamienieki et al., 2005). The combined use of the *GAL-NDT80 GAL4.ER* synchronization system in concert with inhibitor sensitive alleles of both *CDC28* and *IME2* could be used to greatly enhance our understanding of the regulation of this transition by these two kinases (Bishop et al., 2000; Benjamin et al., 2003). This approach has been used to examine the role of CDKs in the meiosis I to meiosis II transition, and these preliminary data are presented in Appendix A.

Translational regulation of *CLB3* during meiosis

How is *CLB3* translation regulated during meiosis? *A priori* there are two possible modes of regulation. First, the *CLB3* 5'UTR may be bound by a translational repressor during meiosis I. Second, the *CLB3* 5'UTR may be bound by a translational activator during meiosis II, and possibly in vegetatively growing cells. The use of a Y3H screen to identify proteins that potentially interact with the *CLB3* 5'UTR yielded two candidate translational regulators, Rim15 and Rpm2, and subsequent preliminary experiments have

suggested that they may not bind *CLB3* transcript, and thus may not be regulators of *CLB3* translation. However, these experiments have only been performed once, and as such must be repeated. Additionally, we have been unable to examine the consequences of mutation of either *RIM15* or *RPM2* on *CLB3* translation, because we have been unable to obtain deletions of *RIM15* in the SK1 strain background, and because *rpm2-100* strains fail to sporulate in synchronous meiotic timecourses. A detailed description of experiments that could be performed to analyze the effects of these mutants on *CLB3* translation is outlined in the discussion of Chapter 3.

A variety of approaches can be taken to identify translational regulators of *CLB3*. First, proteins that interact with the *CLB3* 5'UTR could be identified by a purification strategy in which a *CLB3 5'UTR-MS2 hairpin* fusion RNA is bound to an MS2-MBP fusion protein. Purification of the MS2-MBP fusion protein should also result in the purification of the fusion RNA. Proteins that co-purify with the RNA could then be identified by mass spectrometry (Figure 2A). This approach has the advantage of identifying proteins that directly interact with the *CLB3* 5'UTR, and would be able to identify both positive and negative regulators of *CLB3* translation.

Second, a screen of the *S. cerevisiae* deletion collection could be performed to identify potential translational regulators. The deletion collection is a set of strains deleted for all non-essential yeast genes, which through a series of selection steps can have given mutations crossed in for the purposes of screening (Giaever et al., 2002). This screen would exploit the fluorescence of spores under UV light, and a meiosis I arrest. The

meiosis I arrest would be accomplished using a meiotic null allele of *CDC20* (*cdc20-mn*) (Lee and Amon, 2003). *cdc20-mn* cells do not progress past meiosis I, and form monads instead of tetrads. The fluorescence of spores under UV light is the result of production of the spore wall compound dityrosine, the production of which is dependent on the gene *DIT1* (Briza et al., 1994). We have observed that the pathway for dityrosine formation is intact in *cdc20-mn* cells, as they fluoresce under UV light (data not shown). *DIT1* will be placed under the control of the *HOP1* promoter to ensure its timely expression during meiosis I, and the *CLB3* 5'UTR to prevent *DIT1* translation during meiosis I. *cdc20-mn pHOP1-5'UTR$_{CLB3}$-DIT1* cells should not progress past meiosis I, therefore translation of the *DIT1* message should not occur, and these cells would not be expected to fluoresce under UV light. However, if a negative regulator of *CLB3* translation is deleted, then in these *cdc20-mn pHOP1-5'UTR$_{CLB3}$-DIT1 rbp*Δ cells *DIT1* translation should occur during meiosis I, and cells should fluoresce under UV light (Figure 2B). This approach has the advantage of being able to identify both direct and indirect regulators of *CLB3* translation, but has the disadvantage that it would only be able to identify negative regulators of *CLB3* translation.

Figure 2

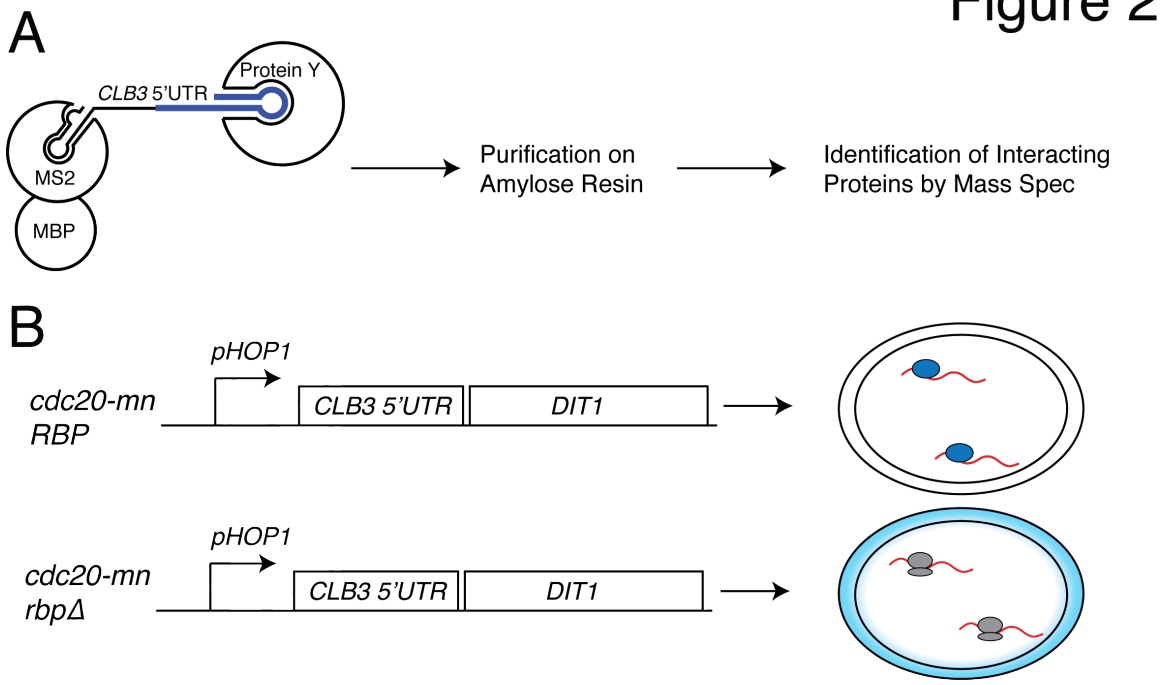

Figure 1: Strategies for the identification of translational regulators of *CLB3*

A) An approach to identify proteins that interact with the *CLB3* 5'UTR (blue) by mass spec. Purification of the *CLB3 5'UTR-MS2* hairpin fusion RNA is accomplished through the binding of an MS2-MBP fusion protein. This protein can be purified on amylose resin, and proteins that co-purify with the RNA can then be identified by mass spec.

B) A deletion collection screen to identify regulators of *CLB3* translation. See the text for a detailed description. The *DIT1* transcript is illustrated in red, and RBP that acts as a translational repressor is indicated in dark blue, ribosomes are illustrated in grey, and dityrosine fluorescence is indicated with light blue. In the top cell *DIT1* transcript is bound by an RBP that acts as a translational repressor, therefore Dit1 is not produced and cells do no fluoresce under UV light. In the bottom cell the RBP is not present, and *DIT1* transcript is translated. The production of Dit1 in these cells leads to fluorescence under UV light (light blue glow).

How would an RBP or translational regulator identified by either of the above techniques be validated? Several additional experiments could be performed to demonstrate that an RBP is a direct regulator of *CLB3* translation. First, it must be demonstrated that *CLB3* translation is altered in *rbp*Δ cells. This could be assessed by examining Clb3 levels in *RBP* mutant strains, or by examining Clb2 levels in *GAL-5'UTR$_{CLB3}$-CLB2* strains. If the RBP was an activator of *CLB3* translation, then Clb3 or Clb2 levels would be reduced during meiosis II in *RBP* mutant strains, and if the RBP was a repressor of *CLB3* translation, then Clb3 or Clb2 would accumulate during meiosis I in *RBP* mutant strains. These experiments could be used to assess the effects of both direct and indirect translational regulators. Additionally, for direct regulators of *CLB3* translation (RBPs), these proteins must be demonstrated to bind to *CLB3* transcript. These experiments were described and performed for Rim15 and Rpm2 in Chapter 3. However, the association of an RBP with the *CLB3* message should also be analyzed using CLIP (crosslinking and immunoprecipitation) (Jensen and Darnell, 2008). In CLIP, cells are crosslinked *in vivo*, and proteins of interest are subsequently immunopurified and RNase treated. Protected RNA fragments are then identified by sequencing. This method has the advantages of controlling for the formation of RNA protein complexes after lysis, gives positional information about the sequences bound by the RBPs, and can potentially lead to the identification of other targets that are translationally regulated by an RBP.

How does some yet unidentified regulator of *CLB3* translation function? Given that in eukaryotes most regulation of translation occurs at the level of initiation, it seems likely that *CLB3* translation would be regulated at this level as well (Sonenberg and

Hinnebusch, 2009). How might a translational repressor prevent translation of *CLB3* during meiosis I? First, binding of such a repressor might prevent the association of any of a number of eIFs with the *CLB3* message, preventing translational initiation. Second, binding of such a repressor might provide a block to scanning ribosomes, thus preventing them from reaching the start codon, and initiating translation. In principle any step of initiation may be regulated, including the binding of eIF4F to the message, polyadenylation of the message, binding of the pre-initiation complex to the message, and scanning of the 5'UTR of the message by the ribosome. However, given that much of the regulation of polyadenylation is mediated by the 3'UTR of the message, this seems unlikely for *CLB3*. In higher eukaryotes a common theme in translational regulation during meiosis and embryogenesis involves the binding of mRNAs by RBPs, which then either prevent the association of eIF4G with the message, or prevent the association of both eIF4E and eIF4G (Vardy and Orr-Weaver, 2007b). Therefore, it may be informative to assess the association of various eIFs, especially eIF4E and eIF4G, with the *CLB3* message during meiosis I to gain insight into the mechanism of *CLB3* translational regulation. The association or lack of association of a given eIF with the *CLB3* message would help to determine which step of translational initiation is regulated.

It may also be interesting to determine the roles of P bodies in *CLB3* translational regulation. P bodies are cytoplasmic foci that are sites of mRNA decapping, deadenylation, and decay. Additionally, they are involved in global translational repression, and are thought to act as sites of storage of non-translating mRNAs, which later reenter translation (Brengues et al., 2005; Coller and Parker, 2005). Potential

translational regulation of *CLB3* by P bodies might be mediated by shuttling of the transcript into P bodies during meiosis I and out of P bodies during meiosis II. Interestingly, *RPM2* has been shown to interact with P bodies by yeast two-hybrid, and genetically (Stribinskis and Ramos, 2007). Therefore, if Rpm2 is a translational regulator of *CLB3* it may act by modulating the association of the message with P bodies. It would therefore be interesting to assess the function of P bodies in *CLB3* translation in strains deleted for the P body components *DHH1* and *PAT1*. Additionally, it might be informative to examine the localization of *CLB3* transcript, and to determine if it co-localizes with P bodies.

Conservation and significance

The control of the mitotic cell cycle by CDKs is evolutionarily well conserved, and though less well studied, it seems that the control of meiotic events by CDKs is as well. Our characterization of CDK activity during meiosis has revealed diverse regulation of different Clb-CDKs. We have proposed that this diversity in regulation reflects a need to finely control CDK activity during the meiotic divisions. Therefore, differential regulation of cyclin-CDK complexes during meiosis could be a common and evolutionarily conserved phenomenon. Indeed, studies in *Xenopus* suggest that there may be differential regulation of cyclin-CDKs during meiosis in this organism (Hochegger et al., 2001). Additionally, preliminary studies in *Xenopus* and budding yeast hint that coordination of the meiosis I to meiosis II transition by down-regulation of CDKs may be evolutionarily conserved (Iwabuchi et al., 2003; Buonomo et al., 2003; Marston et al., 2003). Further studies of the roles of CDKs in meiosis in budding yeast, facilitated by the

GAL-NDT80 GAL4.ER system, may therefore give insight into how CDKs control meiotic events in higher eukaryotes.

In higher eukaryotes the translation of *cyclins* and other mRNAs is highly regulated during both meiosis and embryogenesis, and has been well studied in *Xenopus*, *Drosophila*, and *C. elegans*. For example, during meiosis in *Xenopus* the proteins CPEB and Maskin collaborate to repress translation of *cyclin B1* (Hake and Richter, 1994; Stebbins-Boaz et al., 1999). In *Drosophila* both the *cyclin A* and *cyclin B* mRNAs are translationally repressed by the RBPs Bruno and PUMILIO respectively (Sugimura and Lilly, 2006; Vardy and Orr-Weaver, 2007a). Finally, in *C. elegans* translational repression of *cyclin E* during meiosis by the RBP GLD-1 prevents premature entry of germ cells into mitosis (Biedermann et al., 2009). Our work demonstrates that the translational regulation of *cyclins* is not confined to metazoans, but also occurs in budding yeast, and may additionally suggest that translational regulation of *cyclins* has the potential to affect chromosome segregation during meiosis in other organisms. We observed that translational regulation of *CLB3* is mediated by the 5'UTR of the message. However, the translational regulation of *cyclins* observed in higher eukaryotes is largely mediated by the binding of regulatory proteins to the 3'UTRs of these messages. Though there are differences in the mode of translational regulation during meiosis in yeast (5'UTR mediated) and higher eukaryotes (3'UTR mediated), there may be some conservation in the mechanisms by which the binding of these RBPs affects *cyclin* translation.

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Appendix A:

Regulation of the Meiosis I to Meiosis II Transition by Cyclin-Dependent Kinases

Introduction

Alternating rounds of DNA replication (S phase) and chromosome segregation (M phase) characterize the mitotic cell cycle, and ensure that each daughter cell has the same ploidy as the parental cell. Unlike mitosis, the meiotic program is reductional, meaning that the ploidy of the meiotic products is half that of the progenitor cell. This reduction is accomplished by carrying out two consecutive M phases following a single round of DNA replication. The mechanisms ensuring accurate segregation during meiosis I and meiosis II are well characterized. However, the mechanisms governing the meiosis I to meiosis II transition in budding yeast are poorly understood.

This transition is unique in that two consecutive M phases occur without intervening DNA replication. Between meiosis I and meiosis II the cell must accomplish two seemingly contradictory tasks: it must disassemble the spindle, a process requiring low cyclin-dependent kinase (CDK) activity, and it must prevent DNA replication, a process requiring high CDK activity. CDKs promote spindle stability by restraining APC^{Cdh1} mediated proteolysis of microtubule-associated proteins (MAPs), including Ase1, Cin8 and Kip1. Thus, CDK inactivation during mitotic exit activates the APC^{Cdh1}, allowing MAP proteolysis. For example, deletion of *ASE1* leads to premature spindle disassembly, and expression of a stabilized version delays spindle disassembly (Juang et al., 1997; Crasta et al., 2006).

The cell uses oscillations in CDK activity to ensure that DNA replication occurs once per cell cycle. Licensing of DNA replication is limited to low-CDK states, while initiation of

DNA replication is limited to high-CDK states. Licensing requires the formation of pre-replicative complexes (pre-RCs) at origins of replication during G1. The origin recognition complex (ORC), binds origins and during G1 recruits the pre-RC components Cdc6 and Cdt1, which in turn recruit the Mcm2-7 complex, the putative replicative helicase. Activation of Clb-CDKs upon cell cycle entry initiates DNA replication, and inhibits pre-RC reformation. Not surprisingly, Clb-CDKs inhibit pre-RC formation through multiple, redundant mechanisms. First, Clb-CDKS phosphorylate Cdc6 targeting it for SCF^{Cdc4} mediated proteolysis. Second, Clb-CDKs promote nuclear export of Mcms. Finally, Clb-CDKs phosphorylate ORC subunits, inhibiting ORC activity (Nguyen et al., 2001; Bell and Dutta, 2002).

How does the cell accomplish these two tasks, one requiring low CDKs and one requiring high CDKs, during the same transition? Work in *Xenopus* oocyte extracts suggests that the cell only partially inactivates CDKs between meiosis I and meiosis II; inactivating CDKs enough to allow spindle disassembly, but preserving enough CDK activity to inhibit the pre-RCs formation (Furuno et al., 1994; Iwabuchi et al., 2000). There are also several lines of evidence that suggest that Clb-CDKs are important regulators of the meiosis I to meiosis II transition in *S. cerevisiae*. First, *clb1*Δ*clb3*Δ*clb4*Δ mutants arrest after meiosis I, and fail to undergo meiosis II (Dahmann and Futcher, 1995). Second, expression of a non-degradable cyclin during meiosis prevents meiosis I spindle disassembly (Marston et al., 2003). Third, the Cdc14 Early Anaphase Release Network (FEAR) but not the Mitotic Exit Network (MEN) is required for meiosis I spindle disassembly (Buonomo et al., 2003; Marston et al., 2003; Kamieniecki et al., 2005). In

mitosis both the FEAR Network and the MEN activate the phosphatase Cdc14 by promoting its release from the nucleolus. The MEN acts in late anaphase and promotes sustained release of Cdc14 from the nucleolus, which drives full Clb-CDK inactivation and mitotic exit. However, the FEAR Network acts in early anaphase and promotes a transient release of Cdc14 that is not capable of driving mitotic exit (Stegmeier and Amon, 2004). The requirement for the FEAR Network but not the MEN suggests that complete inactivation of Clb-CDKs by the MEN is restrained during the meiosis I to meiosis II transition. The CDK-like kinase Ime2 is also required for the meiosis I to meiosis II transition, and seems to exert an inhibitory effect on Cdh1, and on pre-RC formation similar to that of CDK (Bolte et al., 2002; Benjamin et al., 2003; Holt et al., 2007). The full range of mechanisms restraining DNA re-replication during the meiosis I to meiosis II is unclear. Here experiments are presented that were designed to investigate the role of CDKs in properly programming this transition.

Preliminary Results

To examine the role of CDKs in the meiosis I to meiosis II transition the *cdc28-as1* allele was used in conjunction with the *GAL-NDT80* synchronization system (Carlile and Amon, 2008). In the *cdc28-as1* allele a bulky hydrophobic amino acid in the ATP binding pocket has been mutated to glycine (F88G), rendering the kinase sensitive to inhibition by cell-permeable ATP analogs carrying a bulky, aromatic side-chain (1-NM-PP1; Bishop et al., 2000). It has been previously reported that CDK activity is required for both meiotic divisions (Shuster and Byers, 1989; Grandin and Reed, 1993; Dahmann and Futcher, 1995; Benjamin et al., 2003). Therefore, we first sought to determine if these results could be recapitulated with the *cdc28-as1* allele. To determine if CDKs are required for the first meiotic division *GAL-NDT80 GAL4.ER cdc28-as1* cells were either mock treated with DMSO or with 5 μM 1-NM-PP1 5 hours after inoculation into sporulation media (SPO). Cells were then released from the *GAL-NDT80* arrest at 6 hours with the addition of 1 μM β-estradiol. Mock treated cells formed both meiosis I and meiosis II spindles, and underwent both meiotic divisions (Figure 1A,B). However, cells treated with 1-NM-PP1 failed to form meiosis I or meiosis II spindles, and failed to undergo either meiotic division (Figure 1A,B). To determine if CDKs are required for the second meiotic division *GAL-NDT80 GAL4.ER cdc28-as1* cells were released from the *GAL-NDT80* block at 5 hours, and were subsequently either mock treated or treated with 5 μM 1-NM-PP1 at 6.5 hours, after completion of meiosis I. As before mock treated cells underwent both meiotic divisions (Figure 1C,D), whereas inhibitor treated cells underwent the first meiotic division, but failed to undergo the second (Figure 1C,D). These results confirm that CDK activity is required for both meiotic divisions.

Figure 1

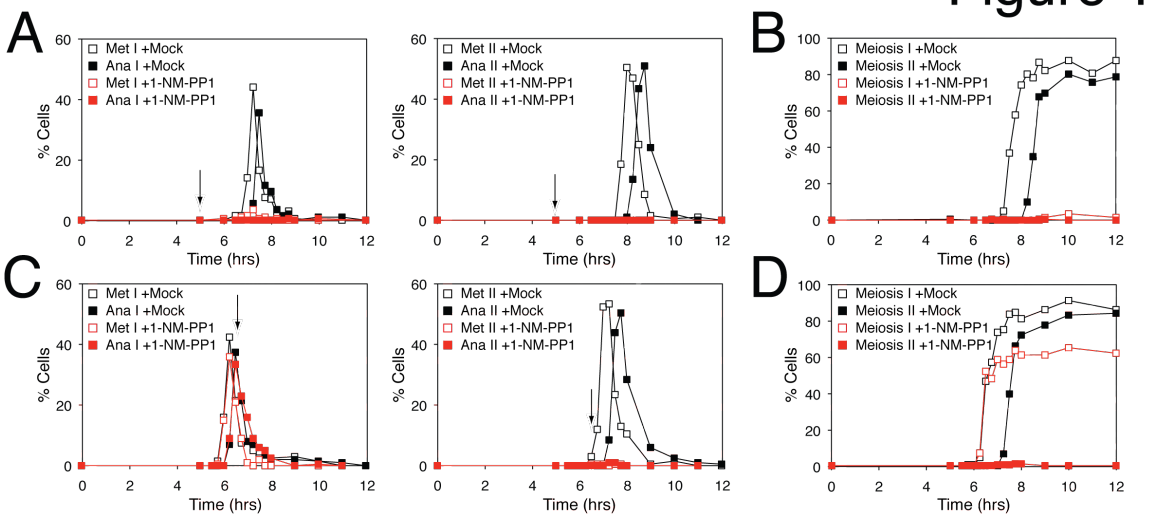

Figure 1: CDK activity is required for both meiotic divisions

GAL4.ER GAL-NDT80 cdc28-as1 (A14737) strains were induced to sporulate at 30°C by transfer into SPO medium, and were treated as described below.

(A,B) After 5 hours cells were treated with either DMSO (black symbols), or 5 µM 1-NM-PP1 (red symbols). After 6 hours 1µM β-estradiol was added.

(C,D) After 5 hours 1µM β-estradiol was added. After 6.5 hours were treated with either DMSO (black symbols), or 5 µM 1-NM-PP1 (red symbols).

(A-D) The percentages of bi- and tri- or tetranucleate cells ([B,D], open symbols), of tri- or tetranucleate cells ([B,D], closed symbols), and of cells with metaphase I ([A,C], open symbols, left graphs), anaphase I ([A,C], closed symbols, left graphs), metaphase II ([A,C], open symbols, right graphs) or anaphase II spindles ([A,C], closed symbols right graphs) were determined at the times indicated after transfer into SPO medium, n=200. Arrows [A,C] indicate timing of addition of DMSO or 1-NM-PP1.

We next wished to test the hypothesis that CDKs are only partially down-regulated between meiosis I and meiosis II, and that the low level of CDK activity preserved

between the two divisions is important for meiotic progression. To do this we wished to treat *cdc28-as1* cells with inhibitor just prior to the meiosis I to meiosis II transition, and subsequently wash out inhibitor as untreated cells were entering meiosis II. By treating cells with inhibitor in this manner we are able to transiently inhibit CDKs only during the meiosis I to meiosis II transition, and to leave CDK activity unaffected during the rest of meiosis. If preserving a modest amount of CDK activity between meiosis I and meiosis II is important for accurately coordinating the meiosis I to meiosis II transition, then in inhibitor treated cells defects in meiotic progression after inhibitor washout might be expected. However, first the efficiency of inhibitor removal was tested in cells treated prior to the meiotic divisions. *cdc28-as1* cells were treated with 5 μM 1-NM-PP1 five hours after inoculation into SPO, and were then either released from the *GAL-NDT80* block in the presence of inhibitor (Figure 1A,B), or were washed to remove inhibitor prior to addition of β-estradiol (Figure 2 C,D). Cells exposed to 5μM inhibitor for the course of the experiment failed to undergo either meiotic division (Figure 1A,B). Cells washed to remove inhibitor underwent meiosis I, but did so with a delay and reduced efficiency, and largely failed to undergo meiosis II (Figure 2C,D). Cells were also treated as above, but with 0.5 μM 1-NM-PP1. Cells exposed to 0.5 μM inhibitor for the course of the experiment also failed to undergo either meiotic division (Figure 2A,B). However, cells washed to remove inhibitor underwent both meiotic divisions efficiently (Figure 2E,F). Indicating that the second meiotic division is more sensitive to inhibition of CDKs than the first, that 0.5 μM 1-NM-PP1 inhibits both meiotic divisions (and data not shown) and that 0.5 μM 1-NM-PP1, but not 5 μM 1-NM-PP1 can be efficiently washed out of meiotic cells.

Figure 2

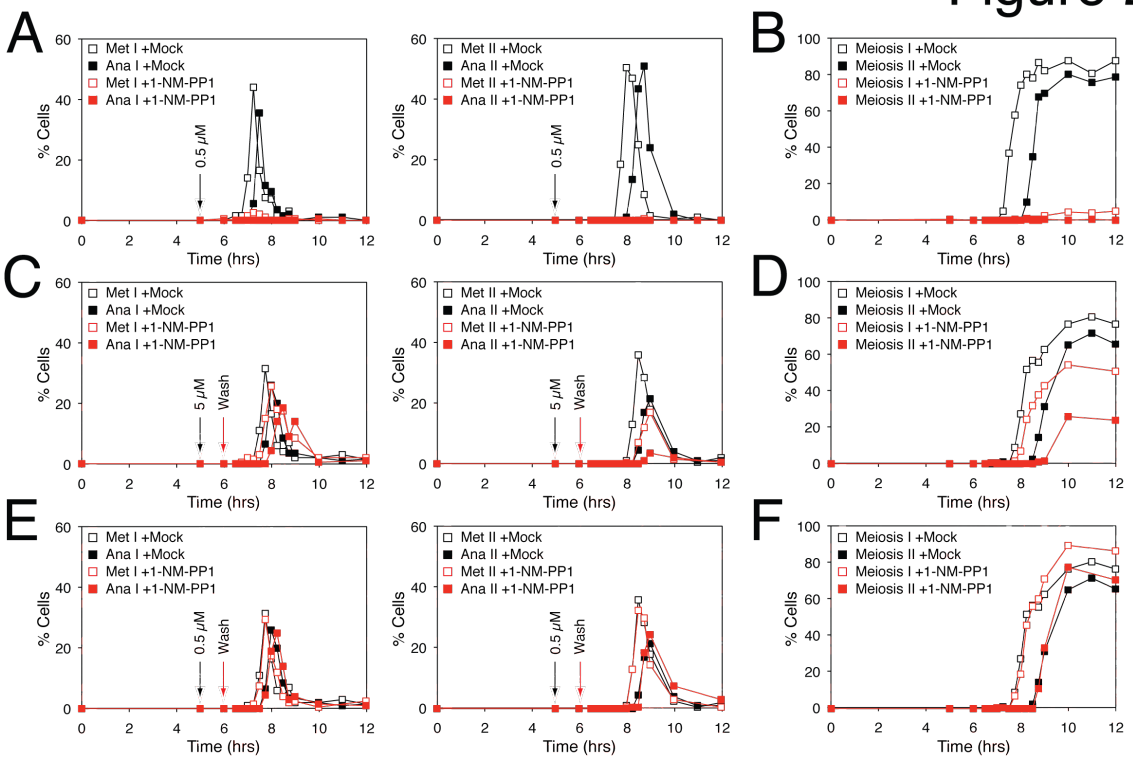

Figure 2: 0.5 µM 1-NM-PP1 can be washed out of meiotic cells efficiently.

GAL4.ER GAL-NDT80 cdc28-as1 (A14737) strains were induced to sporulate at 30°C by transfer into SPO medium. After 5 hours cells were treated with either DMSO (black symbols), 5 µM 1-NM-PP1 ([C,D], red symbols), or 0.5 µM 1-NM-PP1 ([A,B,E,F], red symbols). After 6 hours 1µM β-estradiol was added [A,B], or cells were washed with 10 volumes SPO +DMSO, followed by addition of 1µM β-estradiol. The percentages of bi- and tri- or tetranucleate cells ([B,D,F], open symbols), of tri- or tetranucleate cells ([B,D,F], closed symbols), and of cells with metaphase I ([A,C,E], open symbols, left graphs), anaphase I ([A,C,E], closed symbols, left graphs), metaphase II ([A,C,E], open symbols, right graphs) or anaphase II spindles ([A,C,E], closed symbols, right graphs) were determined at the times indicated after transfer into SPO medium, n=200. Black arrows [A,C,E] indicate timing of addition of DMSO or 1-NM-PP1, red arrows [C,E] indicate timing of washes.

We next sought to determine the effect of transient inactivation of CDKs during the meiosis I to meiosis II transition on meiotic progression. To do this *cdc28-as1* strains were treated either with DMSO or with 0.5 μM 1-NM-PP1 at 6.5 hours, the anaphase I peak, and DMSO or inhibitor was washed out at 7.25 hours, the metaphase II peak in untreated cells. Mock treated cells underwent both meiotic divisions efficiently. However, cells treated with inhibitor between 6.5 and 7.25 hours underwent meiosis I, but failed to undergo the meiosis II (Figure 3A,B). This suggests that a low level of CDK activity must be preserved between the meiotic divisions.

Figure 3

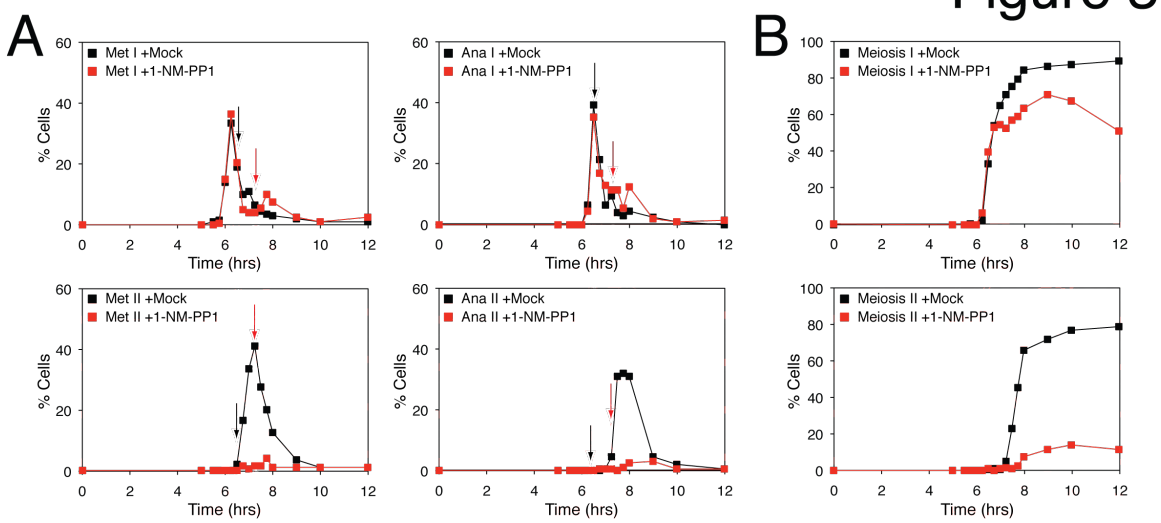

Figure 3: Inhibition of CDKs during the meiosis I to meiosis II transition prevents the second meiotic division.

GAL4.ER GAL-NDT80 cdc28-as1 (A14737) strains were induced to sporulate at 30°C by transfer into SPO medium. After 5 hours cells 1μM β-estradiol was added. Cells were treated with either DMSO (black symbols), or 0.5 μM 1-NM-PP1 (red symbols) at 6.5 hours. At 7.25 hours cells were washed with 10 volumes SPO media + DMSO + 1 μM β-estradiol. The percentages of bi- and tri- or tetranucleate cells ([B], upper graph), of tri- or tetranucleate cells ([B], lower graph), and of cells with metaphase I ([A], top left

graph), anaphase I ([A] top right graph), metaphase II ([A], lower left graph) or anaphase II spindles ([A], lower right graph) were determined at the times indicated after transfer into SPO medium, n=200. Black arrows indicate addition of DMSO or 1-NM-PP1, red arrows indicate timing of washes.

To determine if there was a minimum period of CDK inactivation during the meiosis I to meiosis II transition required to prevent the second meiotic division. To do this *cdc28-as1* strains were treated either with DMSO or with 0.5 μM 1-NM-PP1 at 7.5 hours, the anaphase I peak. The cultures were then split and washed either 0 minutes, 15 minutes, 30 minutes, or 45 minutes after addition of DMSO or 1-NM-PP1. The washes had no effect on meiotic progression in mock treated cultures (Figure 4A-I). Additionally, treatment of cells with inhibitor, followed by an immediate wash had no effect on meiotic progression (Figure 4A,B,I). Treatment of cells with 1-NM-PP1 for either 15 or 30 minutes had a modest effect on the percentage of cells undergoing the second meiotic division (untreated ~80%; 15 and 30 min ~50%) (Figure 4C-F,I). However, treatment of cells with 1-NM-PP1 for 45 minutes severely reduced the percentage of cells able to undergo the second meiotic division (untreated ~80%; 45 ~20%). These results suggest that in addition to being sensitive to levels of CDK activity between meiosis I and meiosis II, cells are also sensitive to the timing of CDK inactivation between meiosis I and meiosis II.

Figure 4

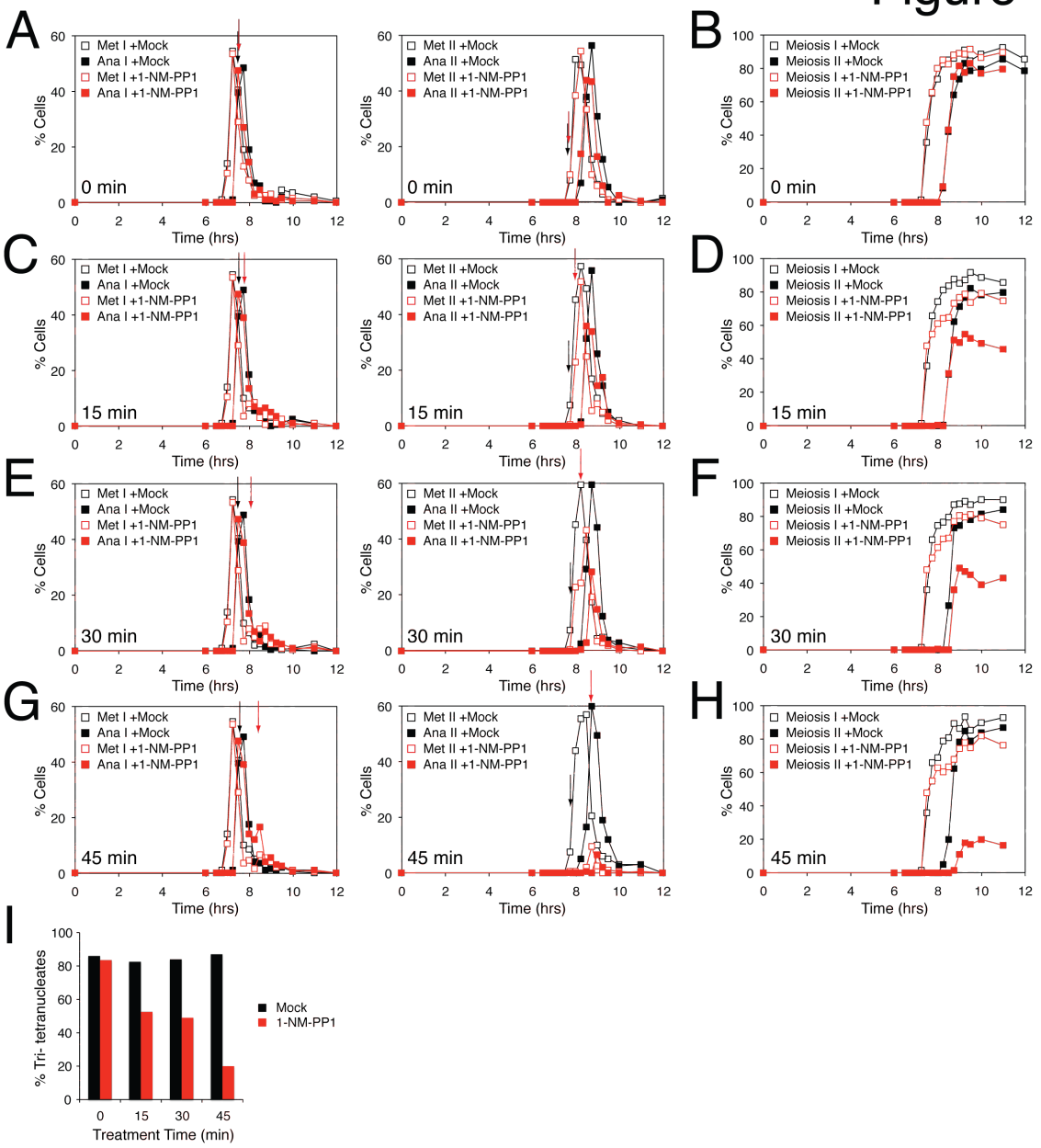

Figure 4: Timing of inhibition of CDKs during the meiosis I to meiosis II transition.

GAL4.ER GAL-NDT80 cdc28-as1 (A14737) strains were induced to sporulate at 30°C by transfer into SPO medium. After 6 hours 1μM β-estradiol was added. After 7.5 hours cells were treated with either DMSO (black symbols), or 0.5 μM 1-NM-PP1 (red symbols). After 7.5 ([A,B], 0 min), 7.75 ([C,D], 15 min), 8 ([E,F], 30 min), or 8.25

([G,H], 45 min) hours portions of the culture were taken and were washed with 10 volumes SPO +DMSO.

A-H) The percentages of bi- and tri- or tetranucleate cells ([B,D,F,H], open symbols), of tri- or tetranucleate cells ([B,D,F,H], closed symbols), and of cells with metaphase I ([A,C,E,G], open symbols, left graphs), anaphase I ([A,C,E,G], closed symbols, left graphs), metaphase II ([A,C,E], open symbols, right graphs) or anaphase II spindles ([A,C,E,G], closed symbols, right graphs) were determined at the times indicated after transfer into SPO medium, n=200. Black arrows [A,C,E,G] indicate timing of addition of DMSO or 1-NM-PP1, red arrows [A,C,E,G] indicate timing of washes. Times in the bottom left corners indicate treatment length.

I) The maximum percentages of tri- or tetranucleate cells are plotted for mock treated (black), and inhibitor treated (red) cells for the treatment lengths indicated.

Discussion

We have examined to role of CDKs in the meiosis I to meiosis II transition using highly synchronous meiotic cultures, and an allele of *CDC28* that can be reversibly inhibited. These results have shown that transient inhibition of CDKs during the meiosis I to meiosis II transition prevents the second meiotic division, suggesting that preservation of some CDK activity during this period is required for proper meiotic progression. Additionally, experiments examining the effects of modulating the timing of CDK inhibition during this period revealed that CDKs must be inactivated for between 30 and 45 minutes to fully prevent the second meiotic division from occurring. This indicates that the timing of CDK inactivation between meiosis I and meiosis II may play a role in meiotic progression.

Why does the length of time of CDK inhibition between meiosis I and meiosis II affect meiotic progression? The APC^{Cdh1} is inhibited by Clb-CDK activity during both mitosis and meiosis (Zachariae et al., 1998; Holt et al., 2007). It is possible that reducing CDK activity below a certain threshold, for a certain amount of time may allow reversal of CDK mediated APC^{Cdh1} inhibition; APC^{Cdh1} could then target the Clbs for proteolysis, reducing Clb-CDK activity enough to promote exit from meiosis, thus preventing meiosis II. This hypothesis predicts that Clb protein levels will be lower in cells treated with inhibitor for 45 minutes compared to cells treated with inhibitor for 30 minutes, and cells not treated with inhibitor. It also predicts that deletion of *CDH1* may be able to suppress the failure to undergo meiosis II. However, this interpretation may be complicated

because Ime2 also inhibits Cdh1, is thought to be active during the meiosis I to meiosis II transition, and is required for the second meiotic division (Bolte et al., 2002; Benjamin et al., 2003; Holt et al., 2007). This hypothesis would be consistent if CDK activity is required to maintain Ime2 activity, however there is currently no evidence that this is the case. Alternatively, inhibition of CDKs during the transition might prevent the second meiotic division through mechanisms other than Cdh1 activation. Perhaps CDK activity, but not Ime2 activity can prevent premature exit from meiosis.

How is DNA re-replication prevented between meiosis I and meiosis II? Our results show a role for preservation of CDK activity between the divisions, which suggests that residual CDK activity may play a role in preventing DNA re-replication. However, we observed no evidence of DNA re-replication by flow cytometry in inhibitor treated cultures. How Clb-CDKs restrain pre-RC formation during meiosis has not been studied extensively, though CDKs prevent nuclear accumulation of Mcms during meiosis (Holt et al., 2007). We have also seen that Cdc6 is absent during the meiotic divisions, suggesting that Clb-CDKs target it for degradation (M. Miller, unpublished data). It therefore seems likely that CDKs may also phosphorylate ORC during meiosis, preventing pre-RC assembly. Additionally, Ime2 seems to regulate pre-RC formation in a manner analogous to Clb-CDKs; it can phosphorylate Cdc6, ORC, and Mcms, and its activity has been shown to prevent nuclear accumulation of Mcms (Holt et al., 2007). Thus Clb-CDKs and Ime2 seem to collaborate to restrain pre-RC formation between meiosis I and meiosis II. Restraint of APC^{Cdh1} mediated proteolysis of Clbs by Ime2 could further inhibit pre-RC formation, and preserve sufficient Clb-CDK activity to promote the second division.

Analysis of *cdc28-as1 ime2-as1* double mutants could thus prove to be a valuable tool to dissect how pre-RC formation is inhibited between meiosis I and meiosis II.

How are other events of the meiosis I to meiosis II transition regulated? During this transition the meiosis I spindle is disassembled, and the second round of spindle pole body (SPB) duplication occurs. As in mitosis, high levels of CDK activity during the meiosis I to meiosis II transition inhibit meiosis I spindle disassembly (Buonomo et al., 2003; Marston et al., 2003). This suggests that there is some similarity in the mechanisms by which CDKs regulate spindle assembly and disassembly in both mitosis and meiosis. However, the APC^{Cdh1} is a regulator of spindle disassembly during mitosis, and is likely inhibited during the meiosis I to meiosis II transition. Therefore how CDK down-regulation promotes meiosis I spindle disassembly may differ from how CDK down-regulation promotes mitotic spindle disassembly. Interestingly, when *cdc28-as1* cells were treated with inhibitor at the anaphase peak, anaphase spindles appeared to persist, albeit with some thinning of the spindle and breakdown in the spindle midzone. This may suggest that spindle stability is not fully dependent on CDK activity during the meiosis I to meiosis II transition, which is consistent with reports that mitotic anaphase spindles are more stable than pre-anaphase spindles, and may additionally suggest that the spindle midzone is most sensitive to loss of CDK activity (Saunders and Hoyt 1992). Additionally, these spindles remain relatively stable over the course of treatment with inhibitor, suggesting that CDK down-regulation alone is not sufficient for full spindle disassembly. The chaperone Cdc48 has been shown to regulate spindle disassembly during exit from mitosis in budding yeast, by binding and facilitating proteolysis of

spindle associated proteins (Cao et al., 2003). Perhaps Cdc48 plays a role in modulating the association of MAPs with the spindle independent of its role in promoting their degradation. How CDKs play a role in SPB duplication during meiosis has also not been studied extensively. During mitosis Cln-CDKs promote SPB duplication during G1 (Jaspersen and Winey, 2004). Therefore it seems likely that Ime2 promotes both rounds of SPB duplication during meiosis. Additionally, different FEAR Network mutants show different abilities to undergo the second round of SPB duplication. *spo12*Δ mutant cells largely fail to form four SPBs, while the majority of *slk19*Δ mutant cells form four SPBs (Buonomo et al., 2003). These results suggest that down-regulation of CDKs plays a role in the second round of SPB duplication, but that additional experiments may be needed to fully elucidate the role of CDK down-regulation in this process.

Experimental Procedures

Strains

All strains are SK1 derivatives and are described in Table 1. The *cdc28-as1* allele was obtained from (Benjamin et al., 2003).

Table 1: SK1 Derivatives

Strain Number	Relevant Genotype
A14737	MATa/α *ura3::pGPD1-GAL4(848).ER::URA3/ura3::pGPD1-GAL4(848).ER::URA3 GAL-NDT80::TRP1/GAL-NDT80::TRP1 cdc28-as1/cdc28-as1*

Meiosis Conditions

Sporulation conditions for *GAL-NDT80 GAL4.ER* strains are described in (Carlile and Amon, 2008). 1-NM-PP1 was used from a stock of 5 mM in DMSO. To remove inhibitor cells were washed with 10 volumes SPO medium +DMSO (+β-estradiol if cells were washed after GAL-NDT80 release) on a Konte filtration apparatus, and were then resuspended in SPO medium +DMSO +β-estradiol. Indirect immunofluorescence was performed, and spindles were counted as described in (Carlile and Amon, 2008).

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