CELL SIZE CONTROL IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

A Dissertation Presented
By

DANIEL LYNN KEIFENHEIM

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Interdisciplinary Graduate Program

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The signatures of the Dissertation Defense Committee signify
completion and approval as to style and content of the Dissertation

Nick Rhind, Ph.D., Thesis Advisor

James Moseley, Ph.D., Member of Committee

Job Dekker, Ph.D., Member of Committee

Peter Pryciak, Ph.D., Member of Committee

Jennifer Benanti, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation
meets the requirements of the Dissertation Committee

Dannel McCollum, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences
signifies that the student has met all graduation requirements of the school

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
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Abstract

The coordination between cell growth and division is a highly regulated process that is intimately linked to the cell cycle. Efforts to identify an independent mechanism that measures cell size have been unsuccessful. Instead, we propose that size control is an intrinsic function of the basic cell cycle machinery.

My work shows that in the fission yeast *Schizosaccharomyces pombe* Cdc25 accumulates in a size dependent manner. This accumulation of Cdc25 occurs over a large range of cell sizes. Additionally, experiments with short pulses of cycloheximide have shown that Cdc25 is an inherently unstable protein that quickly returns to a size dependent equilibrium in the cell suggesting that Cdc25 concentration is dependent on size and not time. Thus, Cdc25 can act as a sizer for the cell. However, cells are still viable when Cdc25 is constitutively expressed suggesting that there is another sizer in the case that Cdc25 expression is compromised.

Cdc13 is a likely candidate due to the similar characteristics to Cdc25 and the ability to activate Cdc2. Cdc13 accumulates during the cell cycle in a manner similar to Cdc25. I show that in the absence of Cdc2 tyrosine phosphorylation, the cell size is sensitive to Cdc13 activity showing that Cdc13 accumulation can determine when cells enter mitosis. These results suggest a two sizer model where Cdc25 is the main sizer with Cdc13 acting as a backup sizer in the event of Cdc25 expression is compromised.

Additionally, in the absence of Cdc2 phosphorylation by the kinases Wee1 and Mik1, mitotic entry is regulated by the activity of Cdc2. In the absence of Cdc2 phosphorylation, this activity is regulated by binding of cyclins to Cdc2. Under these circumstances, the activity of Cdc13 can regulate mitotic entry provide further evidence that Cdc13 could be a sizer of the cell in the case where Cdc25 expression is compromised.

The results I present in this dissertation provide the groundwork for understanding how cells regulate size and how this size regulation affects cell cycle control in *S. pombe*. The results show how the intrinsic cell cycle

machinery can act as a sizer for the G2/M transition in *S. pombe*. Interestingly, this mitotic commitment pathway is well conserved suggesting a general solution for size control in eukaryotes at the G2/M transition. Understanding the mechanism of how protein concentration is regulated in a size dependent manner will give much needed insight into how cells control size. Elucidating the mechanism for size control will capitalize on decades of research and deepen our understanding of basic cell biology.

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Chapter 1: Introduction

Introduction

Throughout every cell cycle, a cell has to faithfully duplicate and divide its contents in order to successfully produce a subsequent generation. The coordination between growth and cell division is intimately linked to this process to ensure that the daughter cells will be a sufficient size to function and grow properly. Without size control, small variations in growth and timing of division would lead to a large distribution of cell sizes in a population, which could have a detrimental effect on the fitness of the cells due to either improper surface to volume ratio, which affects the ability of the cell to import enough nutrients, in large cells or mitotic catastrophe in small cells.

Cells, among the many organisms on earth, vary in size by several orders of magnitude. *Mycoplasma gallicepticum* is the smallest known cell with a diameter of 200 nm (Morowitz & Tourtellotte, 1962). On the other side of the cell spectrum, *Syringammina fragilissima* is the largest known single cell organism with a diameter of up to 20 cm (Barker & Brady, 1960). Form

follows function with cell size being crucial to survival of the organism. In single cell organisms, maintaining size homeostasis is critical. If the cell grows too large, the surface to volume ratio becomes unfavorable due to limited surface transport. If a cell tries to divide when it is too small, it can cause mitotic catastrophe when the cell cannot properly divide its contents. In multicellular organisms, cell size is crucial for function of the cell with the most extreme example being the difference in size between a sperm and egg cell.

Cell size has been studied for over 100 years. The first insights into cell size control in the early 1900s noted the strong correlation between cell size and ploidy (Wilson, 1925). Despite the ongoing study of cell size control, there is no determined molecular mechanism for how cells coordinate growth and division.

Confounding this problem is the study of a very similar yet separate problem of how cells regulate growth in response to environment. Many signalling pathways that monitor cellular environment affect cell size. These signalling pathways adjust the cell size in response to changes in the cellular environment. These two areas of study are often interlinked

due to intimate relation of growth and division with growth regulation dramatically influencing the desired cell size. I am going to focus on how cells measure and regulate size.

Timers and Sizers

Two general models for how cells regulate size are “timers” and “sizers”. In order to understand these models, one has to consider the growth rate of the cell. A cell could grow in a linear fashion where the cell increases in size at a constant rate. Alternatively, a cell could grow in an exponential fashion where growth rate is proportional to cell size. Determining which growth rate a cell follows is crucial to understanding which model cells use to control size. However, it is quite difficult to measure growth rate accurately enough to distinguish the two growth rates (Tzur, Kafri, LeBleu, Lahav, & Kirschner, 2009). Therefore, a consensus has not been reached on which model, timer or sizer, is used for cell size control.

The timer model postulates that a cell attempts to grow for a fixed amount of time before the cell tries to divide. This model requires a linear growth rate by default so that the growth rate of the cell is size independent. If the

growth rate of the cell was exponential, the cell would be unable to maintain a stable cell size since a small variations in cell size during the fixed amount of growth time would be amplified in the following generations. With a linear growth rate, cells could maintain an average size by simply adjusting the amount of time the cells grow. There would be a positive, linear relation between the birth size of the cell and the amount of size added during the cell cycle in a timer model (Figure 1.1). A timer model would be slow to react to a change in cell size since there is no active monitoring of cell size.

A sizer model postulates that cells actively monitor cell size and regulate progress through the cell cycle once the cell has fulfilled the size requirements. There would be a negative linear relation between the birth size of the cell and the amount of size added during the cell cycle in a sizer model (Figure 1.1). The sizer model is proposed for cells that have an exponential growth rate where a larger cell grows faster than a smaller cell. Due to the different growth rates of different size cells, small fluctuations in cell cycle length can lead to differences in cell size in future generations. Therefore, active size control is necessary in exponentially growing cells. The factors that control cell size have been termed a sizer.

Figure 1.1 Timers vs. Sizers vs. Adders

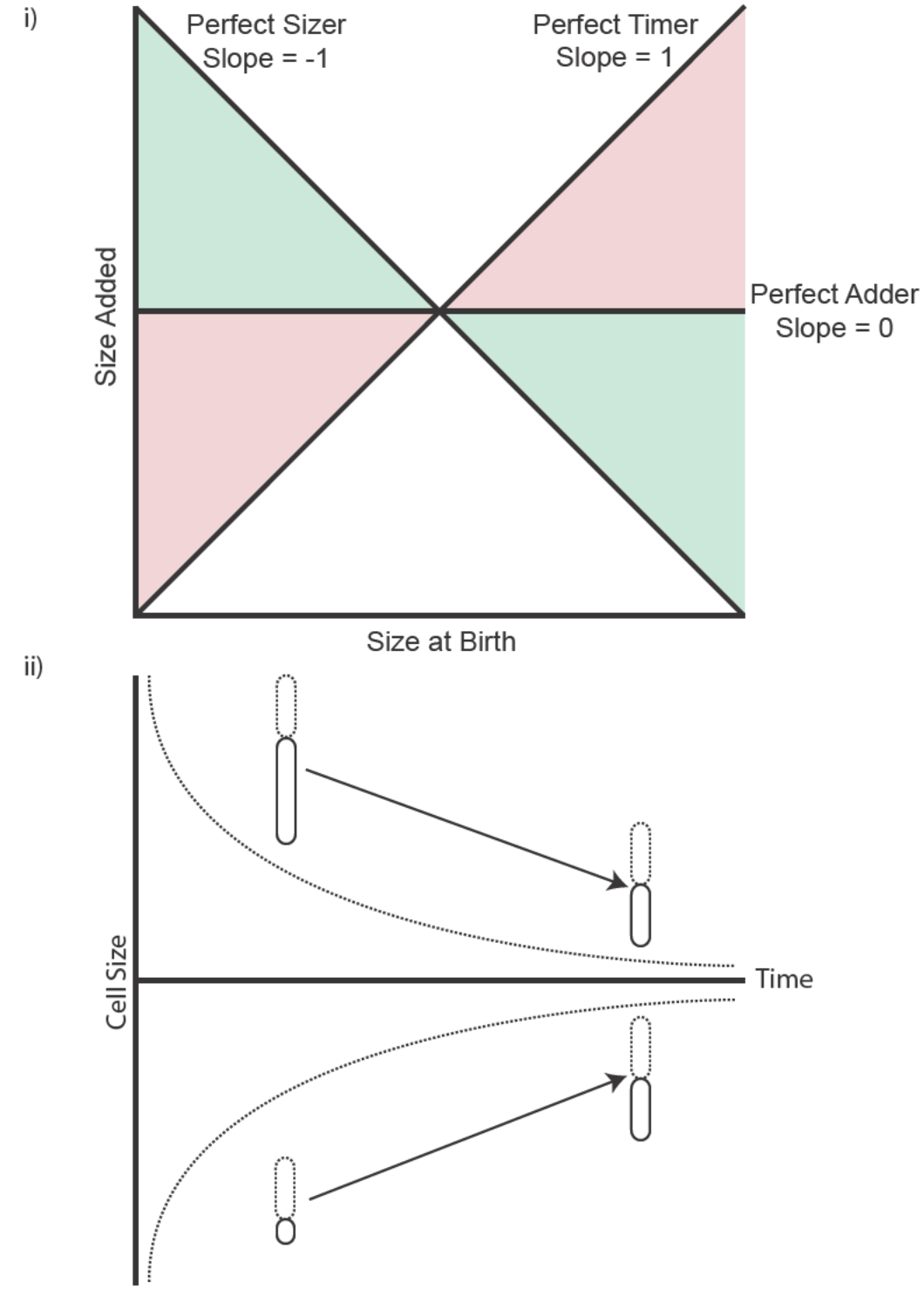


Figure 1.1 Timers vs. Sizers vs. Adders

Two diagrams to illustrate difference between timers, sizers, and adders. i) A graph of birth size versus size added during a given interval of a population of cells. A slope of 1 means the population size is controlled by a perfect timer where the amount of size added is positively correlated with the birth size due to the linear growth rate and fixed cell cycle time. A slope of -1 means the population size is controlled by a perfect sizer where cell size is actively monitored and the amount of size added is dictated by the size threshold which the cell has to surpass to progress through the cell cycle. A slope of 0 means the population size is controlled by a perfect adder since the amount of size added is independent of size. A curve that falls in the green zone is a combination of a sizer and adder. A curve that falls in the red zone is a combination of a timer and adder. ii) A diagram of the adder size control model. The x-axis represents time in generations. A large or small cell will passively approach the average size by adding a constant size represented by the dotted outline. The curves represent the return to size homeostasis over generations for a large or small cell. Both figures adapted from Jun and Taheri-Araghi (Jun & Taheri-Araghi, 2015).

A sizer model would react quickly to a change in cell size since there is active monitoring of the cell.

Size Control in Prokaryotes

Cell size control in bacteria was first postulated in a study that suggests that DNA replication begins at a constant mass (Donachie, 1968). This observation was further developed into a sizer + timer model where the cell grows for a set time after reaching a critical size threshold to trigger DNA replication (Cooper & Helmstetter, 1968).

Recent compelling evidence supports a different model where in each cell cycle the bacterial cell extends the cell size by a constant length, which was termed the “adder” model (Campos et al., 2014; Taheri-Araghi et al., 2015). The data show that regardless of birth size, the cell adds on a constant amount of cell volume. Over a few divisions the cell size would asymptotically approach size homeostasis, which correlates with the amount of cell volume added on during each cell cycle. For example, a cell that is larger than the normal cell size will add on a constant amount of cell volume that is less than double the birth size of the cell. After the

subsequent division the cell will be closer to the normal cell size than it was at birth. The cell will return to a normal size over a number of generations (Figure 1.1). This logic also follows for a cell that is smaller than the normal cell size. This adder model was shown to be true in gram positive and gram negative bacteria as well as symmetrically and asymmetrically dividing bacterial species. Despite these convincing observations, there is no mechanistic insight into how this type of control can be regulated.

Size Control in Eukaryotes

Nuclear to Cytoplasmic Ratio

A defining characteristic of size control in eukaryotes is the apparent universal correlation between cell size and genome copy number. Experiments in the early 1900s with sea urchins showed that cell size increased with ploidy (Wilson, 1925). From these observations, the idea of a karyoplasmic ratio developed where the ratio of nuclear content and cytoplasmic volume remain constant. Later, experiments with heteroploid salamander larvae showed the correlation between ploidy and cell size (Fankhauser, 1945). Fankhauser made the observations that cell size

increased with ploidy, however the size of the organs and salamander remained relatively unchanged. In other words, a diploid organism would have half the number of cells as a haploid organism but those cells would be twice as big. Similar observations have been made in a diverse array of other organisms such as yeast, plants, and mice (Kondorosi, Roudier, & Gendreau, 2000; Mortimer, 1958; Henery, Bard, & Kaufman, 1992).

The midblastula transition in metazoan embryonic transition provides further evidence of the importance of the nuclear to cytoplasmic ratio in regulating cell size. In fertilized embryos, there are a series of rapid, synchronous divisions. When the cells have reached a critical size, which is after a predictable number of divisions for a particular organism, the cell cycle lengthens and the subsequent divisions become more asynchronous. Altering the ploidy changes the number of synchronous divisions the embryo goes through in a predictable fashion (Newport & Kirschner, 1982; Masui & Wang, 1998; Collart, Allen, Bradshaw, Smith, & Zegerman, 2013).

In the nuclear to cytoplasmic ratio model, there is a nuclear component that measures the nuclear content of the cell and inhibits mitosis and a

cytoplasmic component that measures cytoplasmic content of the cell and induces mitosis. These components could be any measurable part of the cell, such as protein concentration or genome copy number. The nuclear component sets a size threshold which the cytoplasmic component has to overcome in a size dependent manner in order to trigger mitosis.

The nuclear to cytoplasmic ratio model has been directly tested by a number of striking experiments. In one set of experiments, multinucleated cells were subjected to UV irradiation, which destroyed a number of nuclei. Following UV treatment, the proceeding intermitotic periods were noticeably shorter (Devi, Guttes, & Guttes, 1968). In another complementary experiment, an amoeba was prevented from dividing by periodic amputation of the cytoplasm. The amoeba cell was prevented from reaching the size necessary to trigger mitosis and, in one case, the amoeba cell was prevented from dividing for 6 months (Prescott, 1956).

It is unknown how the nuclear to cytoplasmic ratio is determined. A couple of models have been proposed that could explain how cells measure the nuclear to cytoplasmic ratio (Fantes, Grant, Pritchard, Sudbery, & Wheals, 1975). A common theme among these proposed models is that either the

nuclear factor or the cytoplasmic factor is somehow influenced by the size of the cell and there are specific predictions about how the nuclear factor, cytoplasmic factor, and cell will respond to size perturbation.

One model that has been proposed is the ratio of nuclear volume to cytoplasm volume as a way to monitor the nuclear to cytoplasmic ratio. In this model, a protein that induces mitosis increases in amount as the cell size increases (as most proteins do to maintain a constant concentration). This protein would localize to the nucleus of a constant size and therefore would increase in concentration as the cell got bigger. This protein would trigger mitosis once it reached a critical threshold. This model depends on the assumption that nuclear volume scales with genome count. However, it has been shown that nuclear size actually scales with cytoplasm rather than genome count (Jorgensen et al., 2007; Neumann & Nurse, 2007), ruling out this class of models.

Another model that has support is the concentration model. This model has two variants. In one variant, the inducer of mitosis increases in concentration during the intermitotic period until it reaches a threshold to trigger mitosis. This increase in concentration would be size dependent.

The threshold could either be set by another protein, whose activity the inducer would have to overcome, or binding sites that titrate away the inducer and at some point become saturated, allowing the inducer to trigger mitosis.

A second variant of the model involves a production of a fixed amount of inhibitor early in the cell cycle. As the cell grows the inhibitor is diluted until it reaches a threshold to trigger mitosis (Fantes et al., 1975). The fixed amount of inhibitor produced would be size dependent. Similar to the above variant, the threshold could either be set by another protein, whose activity the inhibitor represses and dilution relieves the repression, or the inhibitor is a competitive inhibitor of binding sites necessary to trigger mitosis and dilution relieves the inhibition.

The nuclear to cytoplasmic ratio has a great effect on the size of the cell. However, there are many ways cell size can vary based on environment, and cells can vary in size within a multicellular organism. Therefore, the nuclear to cytoplasmic ratio should be viewed as a constraint that can set a baseline for cell size which can be altered based on cell type and environment.

Growth, Division, and Size Threshold

To regulate cell size, eukaryotic cells have tied cell size to cell cycle progression. In order for cells to progress through the cell cycle, they must grow. Inhibiting growth, by removal of nutrients or growth factors, can block progression through the cell cycle with the cells typically arresting in G1 (Pardee, 1974; Prescott, 1976; Yanagida, Ikai, Shimanuki, & Sajiki, 2011; Conway, Grunwald, & Heideman, 2012). Conversely, arresting the cell cycle does not have an effect on cell growth; cells will continue to grow when they are blocked from progressing through the cell cycle (Mitchison & Creanor, 1971; Johnston, Pringle, & Hartwell, 1977; Weigmann, Cohen, & Lehner, 1997; Neufeld, de la Cruz, Johnston, & Edgar, 1998; Conlon, Dunn, Mudge, & Raff, 2001).

To regulate cell size during continuous growth, size checkpoints have been proposed to regulate the progression through the cell cycle. The checkpoints occur at either the G1/S phase transition and/or the G2/M phase transition. These size thresholds are believed to set a minimum size necessary for the cell to pass the checkpoint. If cells are too small, they will delay progression through the cell cycle until the cell has met the

minimum size requirement. If the cell is larger than the minimum size, then the cell will progress through the cell cycle with no delay. The evidence for size thresholds suggest they are well conserved with a number of published results in a variety of organisms, from yeast to mammals, supporting the size checkpoint hypothesis (Killander & Zetterberg, 1965; Frazier, 1973; Nurse, 1975; Johnston et al., 1977; Worthington, Salamone, & Nachtwey, 1976; Donnan & John, 1983; Wang, Hayden, & Masui, 2000; Dolznig, Grebien, Sauer, Beug, & Müllner, 2004).

Size thresholds as a method for size control mean that growth rate will not have any affect on a cell's ability to regulate cell size. Larger cells grow faster than smaller cells and therefore cell growth is generally exponential. Larger cells that have a higher growth rate than smaller cells will meet the minimum size requirements and progress through the cell cycle faster compared to smaller cells (Figure 1.2). A cell that is larger than the minimum size threshold at the beginning of the cell cycle will pass through the cell cycle in a minimal amount of time and therefore not have time to double its size. At division, the daughter cells will be smaller than the initial size of the parent cell. A small cell will arrest at the size checkpoint, and

Figure 1.2 Size at Birth Will Determine How Long Cells Spend in G1

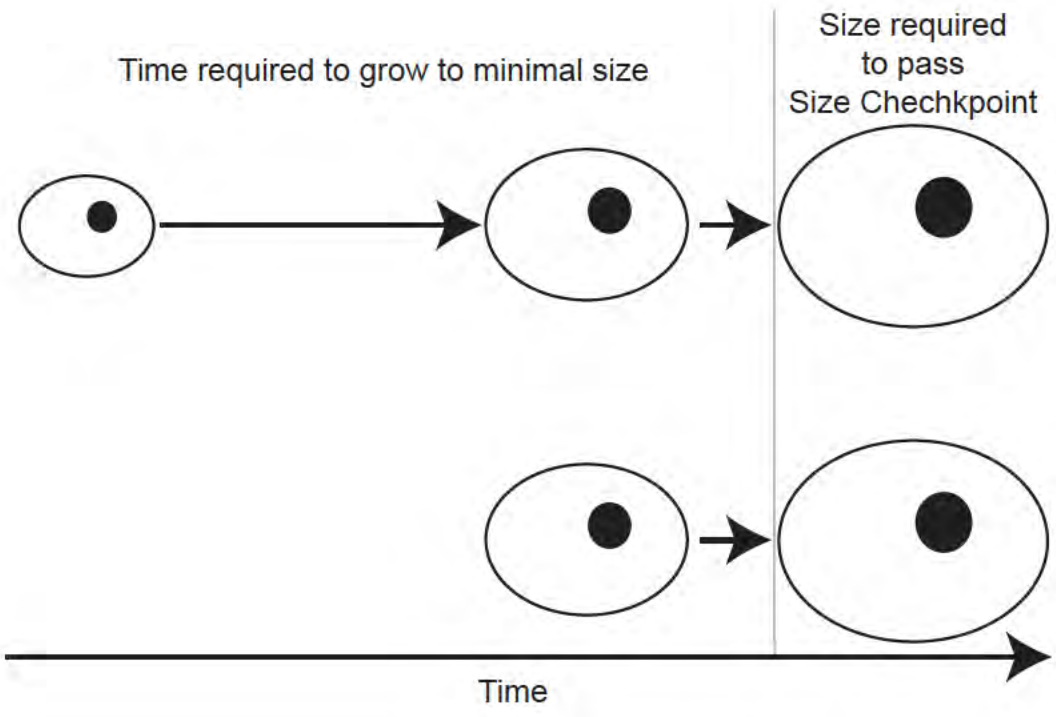


Figure 1.2 Size at Birth Will Determine How Long Cells Spend in G1

Cells will need a different amount of time to grow to the minimal size required to pass the size checkpoint. The cell in the top of the figure is born at a smaller size and needs more time than the cell in the bottom of the figure to reach the critical size to pass the checkpoint.

therefore will have sufficient time to grow to a size where the cell can go through a successful division.

The exact mechanism of size regulation is still unknown. However, there has been some insight generated from various model organisms into possible mechanisms for size control.

Size Control in Yeast

In the budding yeast *Saccharomyces cerevisiae* the main size checkpoint is at the G1/S transition, termed Start (Johnston et al., 1977; Hartwell & Unger, 1977). There is some evidence for a size checkpoint at the G2/M transition (Harvey & Kellogg, 2003); however, later studies suggest the morphogenesis checkpoint was responsible for the results (McNulty & Lew, 2005; Howell & Lew, 2012).

The size checkpoint at Start was first proposed when it was noted that small cells spend more time in G1 than large cells (Johnston et al., 1977). Budding yeast divide asymmetrically, producing a larger mother cell and a smaller daughter cell. The daughter cell must grow to a critical size before

it can become a mother cell and bud a daughter cell. Mother cells fulfill the size requirements and therefore bud daughter cells at regular intervals (Hartwell & Unger, 1977). Due to the asymmetric division, the size checkpoint is primarily invoked during the first passage of G1 by the daughter cell as it transitions to a mother cell (Lord & Wheals, 1983). The asymmetric distribution of the transcription factors Ace2 and Ash1 to the daughter cell can regulate the transcription of the G1 cyclin Cln3 (Weiss et al., 2002; Laabs et al., 2003; Di Talia et al., 2009).

It has been proposed that Cln3 acts as a protein synthesis rate sizer whose abundance is a function of the total protein production rate in the cell. Cln3 has several predicted characteristics of a protein synthesis rate sizer: Cln3 is a rate-limiting, dose-dependent activator of Start (Nash, Tokiwa, Anand, Erickson, & Futcher, 1988; Cross, 1988). Cln3 weakly oscillates throughout the cell cycle and it is constitutively active while the cell is growing (Tyers, Tokiwa, Nash, & Futcher, 1992), and Cln3 has a short half-life and is therefore sensitive to protein production rates (Tyers, Tokiwa, & Futcher, 1993). These properties allow Cln3 to react to changes in protein synthesis rate, which is a good surrogate for cell size as the cell increases in size. However, since the abundance is proportional to the

increase in cell size, the concentration of Cln3 appears to be constant in the cell (Tyers et al., 1992). Therefore, it is unclear how Cln3 can produce a size dependent signal necessary to measure cell size when the concentration during the cell cycle does not change.

Cln3 activates Start by targeting Cdc28, the main cyclin dependent kinase in budding yeast, to phosphorylate the inhibitor Whi5. Phosphorylation of Whi5 triggers its dissociation from SBF and export out of the nucleus (de Bruin, McDonald, Kalashnikova, Yates, & Wittenberg, 2004; Costanzo et al., 2004). SBF is a transcription factor that drives expression of about 200 genes involved in DNA replication and cell cycle progression (Futcher, 2002). The cyclins Cln1 and Cln2 are expressed following SBF activation and provide a positive feedback loop to fully expel the remainder of Whi5 from the nucleus (Skotheim, Di Talia, Siggia, & Cross, 2008) (Figure 1.3). Recent results show that Whi5 decreases in concentration during G1 as the cell increases in size (Jan Skotheim, personal communication). This result suggests a concentration model where Whi5 is produced in a pulse early in the cell cycle and dilution down to a critical threshold dictates when the cell passes Start. Whi5 is proposed to be produced at the same

Figure 1.3 Start Pathway in *S. cerevisiae*

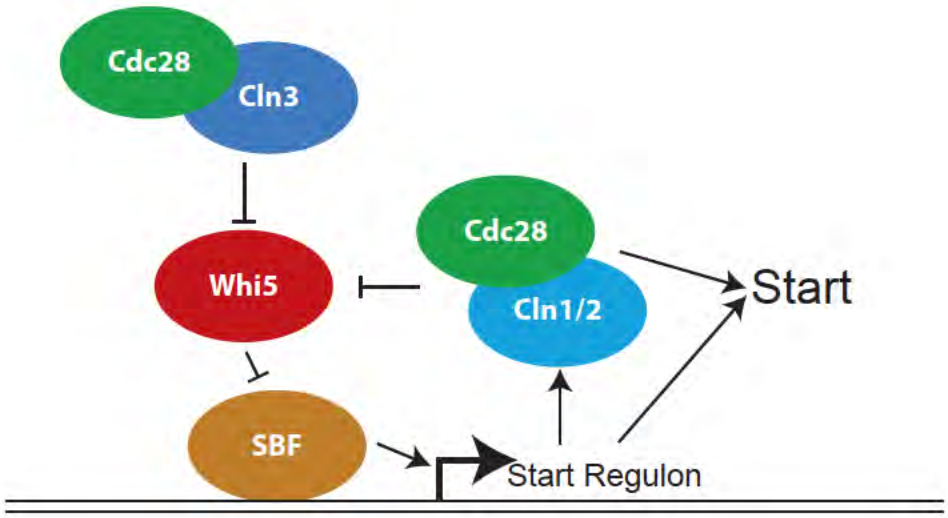


Figure 1.3 Start Pathway in *S. cerevisiae*

A diagram of the *cerevisiae* Start pathway. The Cdc28-Cln3 complex phosphorylates the inhibitor Whi5. Phosphorylation of Whi5 causes dissociation from SBF and export from the nucleus. SBF is a transcription factor that regulates transcription of the regulon for passage of Start. Cln1 and Cln2 are in this regulon and, upon expression, bind to Cdc28 to further inhibit Whi5 and trigger Start.

amount regardless of cell size. Therefore, small cells would have a higher concentration of Whi5 to start, and need to increase in size to sufficiently dilute Whi5. This dilution of Whi5 could be the model that accounts for the conundrum of the constant concentration of Cln3.

In the fission yeast Schizosaccharomyces pombe, the first clues into how fission yeast regulate cell size came from the discovery of the kinase Wee1 (Nurse, 1975; Nurse & Thuriaux, 1977). Mutants of *wee1* revealed that fission yeast have two size checkpoints, one at the G1/S boundary and one at the G2/M boundary. When the cells are small, as in a *wee1* mutant, the cells invoke the G1/S size checkpoint. When wild-type cells are growing in nutrient rich conditions, the size of newly divided cells are sufficient to pass the G1/S size checkpoint and therefore there is little to no G1 phase in exponentially growing cells. In exponentially growing cells the main size checkpoint is at the G2/M boundary (Fantes & Nurse, 1977).

Wee1 is a kinase that is integral in the G2/M transition (Russell & Nurse, 1987). In fission yeast, mitosis is triggered by the cyclin dependent kinase Cdc2 in complex with the B-type cyclin Cdc13. The Cdc2-Cdc13 complex is inhibited by phosphorylation on Tyrosine 15 by Wee1 (Gould & Nurse,

1989; Featherstone & Russell, 1991; Aligue, Wu, & Russell, 1997; Kim & Ferrell, 2007). The inhibitory phosphorylation is removed by the protein phosphatase Cdc25 (Russell & Nurse, 1986; Millar, McGowan, Lenaers, Jones, & Russell, 1991). There are feedback loops that provide a switch-like entry into mitosis. Active Cdc2-Cdc13 complex inhibits Wee1 and hyper-activates Cdc25 by phosphorylation (Pomerening, Sontag, & Ferrell, 2003; Pomerening, Kim, & Ferrell, 2005; Lu, Domingo-Sananes, Huzarska, Novak, & Gould, 2012) (Figure 1.4). The feedback loops succinctly activate the Cdc2-Cdc13 complex to drive the cell into mitosis. This pathway is well conserved among eukaryotic cells.

One intriguing model for cell size control in *S. pombe* is the Pom1 gradient as a ruler for cell length (Moseley, Mayeux, Paoletti, & Nurse, 2009; Martin & Berthelot-Grosjean, 2009). *S. pombe* is a rod shaped cell that grows by increasing its length. Pom1 is an upstream activator of Wee1 that is localized to the tips of the cell. There is a gradient of Pom1 that extends from the cell tips and decreases towards the center of the cell. When the cell is small, the Pom1 concentration is high at the center of the cell and Wee1 is active. However, as the cell grows in length and the tips of the

Figure 1.4 Mitotic Commitment Pathway in *S. pombe*

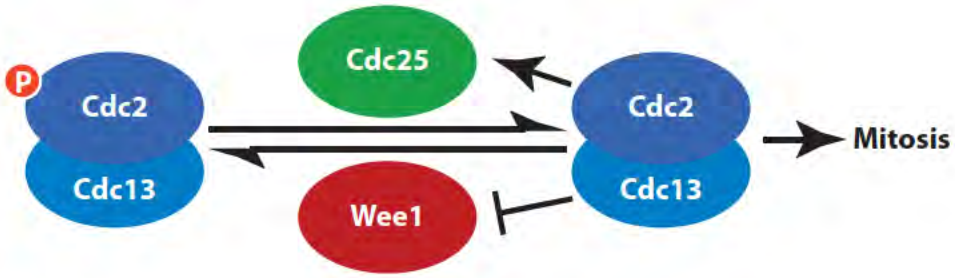


Figure 1.4 Mitotic Commitment Pathway in *S. pombe*

A diagram of the *pombe* mitotic commitment pathway. The Cdc2-Cdc13 complex can trigger mitosis. This complex is inhibited by the kinase Wee1 and activated by the phosphatase Cdc25. Activated Cdc2-Cdc13 complex has feedback loops that hyperactivate Cdc25 and inhibit Wee1.

cell get further away from the center of the cell, the Pom1 concentration at the center of the cell decreases, leading to a decrease in Wee1 activity. While this mechanism is important in the morphology of the cell, recent results show that cells deleted for *pom1* still maintain size homeostasis suggesting that Pom1 does not have an essential role in cell size control in *S. pombe* (Wood & Nurse, 2013).

Wee1 and Cdc25 have been implicated in cell size control of fission yeast. Mutants lacking Wee1 activity are half the size of wild type cells while mutants lacking Cdc25 activity continue to grow and are unable to enter mitosis (Nurse, 1975; Nurse & Thuriaux, 1977). Adding extra copies of Wee1 produces a dose-dependent increase in cell size (Russell & Nurse, 1987). Overexpression of Cdc25 produces small cells similar to Wee1 mutants (Russell & Nurse, 1986). How cell size feeds into and regulates this pathway is still not understood.

Size Control in Multicellular Organisms

Size control is more complicated in organisms with multiple cell types. Different cell types have characteristic cell sizes which may be necessary

for proper cell function (Smith, 1971; Giordano et al., 1993; Pende et al., 2000; Ruvinsky et al., 2005; Miettinen et al., 2014). The state of differentiation and environment within the organism affects cell size control. The complicated regulation of cell differentiation and development mean there are more factors that dictate the terminal size of the cell. Growth factors, mitogens, and cytokines can greatly affect the size of the cell. Thus, dissecting the sizing pathway in multicellular organisms presents added challenges when compared to yeast and other single cell organisms.

Cell size control is evident in fertilized eggs, before cells have had a chance to differentiate. Embryos go through a number of synchronous divisions after fertilization until the cells drop below a critical size. During these synchronous divisions the cell cycle is short, with no G1 or G2 phases. When the cells reach a critical size the cells go through the mid-blastula transition where the cell cycle lengthens and zygotic transcription begins. Recently, two models have proposed how this transition is regulated in a size dependent manner in *Xenopus laevis*. One model suggests that four DNA replication factors are limiting for replication initiation as the nuclear to cytoplasmic ratio increases as the cells get

smaller (Collart et al., 2013). Another model suggests that titration of excess histones against the increasing amount of DNA in the embryo determines when the embryo goes through the mid-blastula transition (Amodeo, Jukam, Straight, & Skotheim, 2015). A third model suggests that nuclear volume has an input into mid-blastula transition such that the ratio of nuclear volume to cytoplasmic volume has a input on the regulation of the mid-blastula transition (Jevtić? & Levy, 2015). Though each focuses on different determining factors for the mid-blastula transition, these three models invoke the nuclear to cytoplasmic ratio as the basis for the mid-blastula transition. The nuclear to cytoplasmic ratio sets a basis from which differentiating cells modify their target cell size.

Early observations of mouse fibroblasts showed that cells entered S phase with less variance in cell size compared to cells that just divided (Killander & Zetterberg, 1965). Similar to *cerevisiae*, small fibroblast cells spent longer in G1 compared to larger cells to reach a critical size to enter S phase. Similarly, work with erythroblasts showed that altering the growth factors, to change the desired size of the cell, affected the length of the following cell cycle as cells adjusted to the new size threshold (Dolznig et al., 2004).

Interestingly, results from mouse lymphoblasts and pro-B-lymphoid cells showed that growth rate at the start of G1 and length of time in G1 were inversely proportional. Additionally, cells exiting G1 had similar growth rates (Son et al., 2012). In general, small cells have a slower growth rate than larger cells. Since a small cell would have a slower growth rate, it would spend a longer time in G1, until it had grown sufficiently that its growth rate increased to a point to surpassed the growth rate threshold. These results suggest that it is a growth rate threshold and not a size rate threshold that dictates exit from G1. To complicate matters, it was also noted that with cells of similar size, the older cell was more likely to divide. The fact that the age of the cell has an affect on cell cycle progression suggests that there is a “timer” in addition to a sizer (Son et al., 2012; Kafri et al., 2013).

Some evidence hints at metazoan cells having a similar sizer mechanism as *S. cerevisiae*. Cyclin E has many similar properties to Cln3. Cyclin E was originally discovered by complementation, rescuing a Cln3 deletion in *S. cerevisiae* (Lew, Dulić?, & Reed, 1991). Over-expression of Cyclin E results in small cells (Neufeld et al., 1998). However, more work needs to

be done to determine if the sizing mechanism is conserved between *S. cerevisiae* and metazoan cells.

Challenges and Future Directions

Despite decades of research, there is no general understanding of the mechanism of how cells regulate size. This lack of mechanistic understanding is due to a number of challenges inherent in cell size research. The main challenge facing size research is accurately measuring small changes in cell size and protein concentration. Cell size changes only two-fold during a typical cell cycle. Using genetic blocks or inhibitors of the cell cycle, the change in size can be increased, but only to a certain degree before the cell exhibits adverse effects of the block (Tzur et al., 2009; Zhurinsky et al., 2010). Due to this caveat, mechanisms regulating cell size are expected to change two-fold to mirror the change in cell size. Techniques that offer more sensitivity to measure cell size or protein change need to be developed in order to overcome this barrier.

Cell size is intimately linked to other cell functions. Attempts to do genome-wide screens for cell size mutants reveals many genes in

pathways that affect cell nutrition or morphology (Jorgensen, Nishikawa, Breitkreutz, & Tyers, 2002). Therefore, it is challenging to discover genes directly involved in the size regulation mechanism. Testing candidate genes has been the most successful strategy thus far for investigating size control. Perhaps in the future new techniques will overcome this caveat of genome wide cell size screens.

The mechanisms proposed for cell size control in various organisms have many similarities. There are direct correlations between metazoan cells and *S. cerevisiae* cells, and the fact that complementation of metazoan genes can rescue size mutations in *S. cerevisiae*, suggest that these pathways are well conserved. Furthermore, the implication of the mitotic pathway in *S. pombe* as the G2/M sizer suggests that cell size regulation is a well conserved mechanism. Any progress in cell size control, regardless of the organism, will provide insight into the mechanisms that control cell size.

Chapter 2: Protein Accumulation of Cdc25 and Cdc13 Redundantly Regulate Cell Size

Introduction

How cells coordinate growth and division to regulate cell size is unknown. Despite decades of research, no direct mechanism has been discovered. Previous results have shown that there are size checkpoints which prevent the cell from progressing to the next phase of the cell cycle without obtaining a minimum size. These results suggest there is a sizer that regulates the cell cycle machinery in a size dependent manner. This study tries to understand the mechanism of the sizer and how it affects the cell cycle.

Yeast have a relatively simple cell cycle, with only one CDK, the cyclin dependent protein kinase that drives cell cycle transitions. Many checkpoints act on CDK activity to arrest the cell from further progression through the cell cycle and the basic cell cycle functions are well

understood in yeast. Therefore, yeast make a great model organism to study how cell size affects progression through the cell cycle.

The fission yeast *Schizosaccharomyces pombe* is an attractive model organism to study cell size based on its genetic tractability and its bipolar growth, which means length measurement correlates with the volume of the cell. Previous work has characterized two size checkpoints in the *S. pombe* cell cycle. The size checkpoint at G1/S phase transition is usually not invoked in exponentially growing cells because freshly divided cells fulfill the size requirements to bypass the checkpoint. The G1/S phase size checkpoint is invoked when the cell is too small after division to pass the G1/S phase size checkpoint, such as in a *wee1Δ* mutant (Nurse & Thuriaux, 1977). The size checkpoint at the G2/M boundary determines the size of exponentially growing cells (Nurse, 1975; Fantes, 1977; Fantes & Nurse, 1978).

In fission yeast, mitosis is triggered by the CDK Cdc2 in complex with the main B-type cyclin Cdc13. This complex can be inhibited by the kinase Wee1, which phosphorylates Tyrosine 15 on Cdc2 (Featherstone & Russell, 1991; Aligue et al., 1997; Kim & Ferrell, 2007). This inhibitory

phosphorylation can be removed by the phosphatase Cdc25 (Russell & Nurse, 1986; Millar et al., 1991). The Cdc2-Cdc13 complex has feedback loops which inhibit Wee1 and activate Cdc25 by hyperphosphorylation (Pomerening et al., 2003; Pomerening et al., 2005; Lu et al., 2012). These feedback loops provide the switch-like behavior necessary for a succinct transition from G2 into mitosis (Figure 2.1).

Previous results have shown that Wee1 is a dose-dependent inhibitor of mitosis. Each additional copy of *wee1* produces a linear increase in cell size (Russell & Nurse, 1987). Deletion of *wee1* produces small cells that divide at half the size of wild type cells (Nurse, 1975). Conversely, overexpression of the phosphatase Cdc25 results in small cells, similar to a wee1 deletion (Russell & Nurse, 1986). Deletion of *cdc25* results in elongated cells that do not divide and are inviable. Many pathways, such as DNA damage, morphology, and nutritional pathways, act on these proteins to regulate when the cell enters mitosis (Belenguer, Pelloquin, Oustrin, & Ducommun, 1997; Kanoh & Russell, 1998; Rhind & Russell, 2001; Rhind & Russell, 2012). Thus, the interplay between Cdc25 and Wee1 represent the last point at which the cell can regulate the entry into mitosis.

Figure 2.1 *Schizosaccharomyces pombe* Mitotic Commitment Pathway

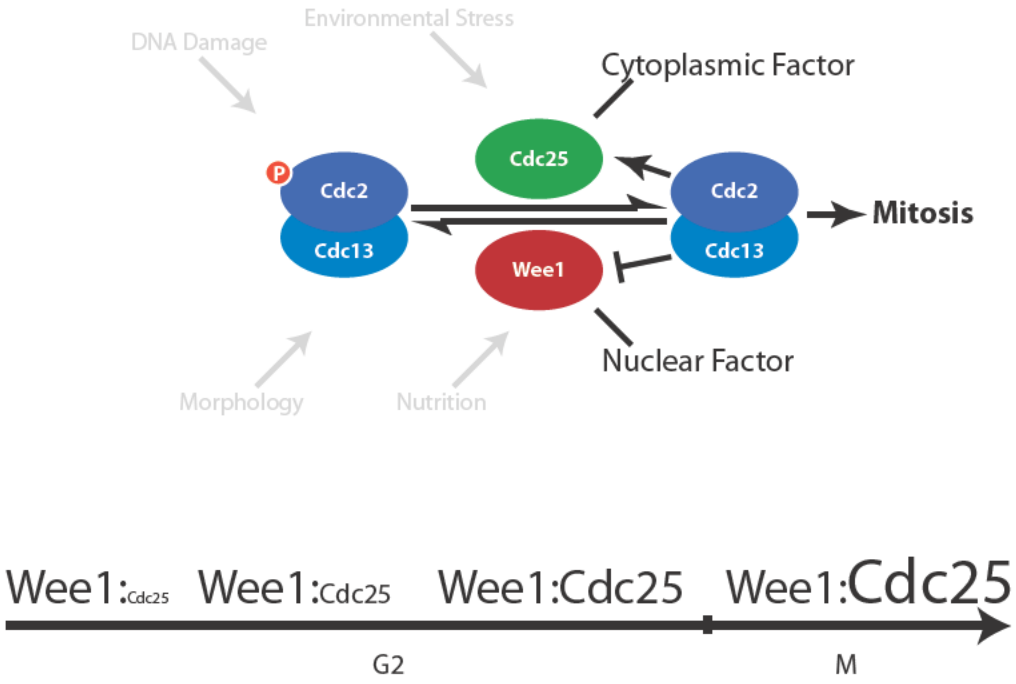


Figure 2.1 *Schizosaccharomyces pombe* Mitotic Commitment Pathway

A diagram of the *S. pombe* mitotic commitment pathway. The Cdc2-Cdc13 complex can trigger mitosis. This complex is inhibited by the kinase Wee1 and activated by the phosphatase Cdc25. Many pathways feed into the mitotic pathway, such as nutrition and morphology pathway, to modulate entry into mitosis. The lower part of the figure shows a graphical representation of the hypothesis that Cdc25 concentration increases as the cell size increases. When Cdc25 overcomes the inhibition by Wee1, mitosis is triggered.

The characteristics of Cdc25 and Wee1 make these two proteins potential surrogates for the nuclear to cytoplasmic ratio. In this hypothesis, Wee1 maintains a constant concentration in the cell and represents the nuclear factor due to its ability to inhibit mitosis while Cdc25 concentration increases with cell size and represents the cytoplasmic factor due to its ability to induce mitosis. In other words, this model predicts that when the cell is small the amount of Cdc25 is low and unable to overcome the inhibition by Wee1. As the cell grows the amount of Cdc25 grows to a critical level where it can overcome the inhibition by Wee1.

To test this hypothesis, I have measured the levels of Cdc25 and Wee1 throughout the cell cycle. My results show that Cdc25 levels are produced in a size-dependent fashion, which requires unusual kinetics, while Wee1 levels are constant during G2. Interestingly, cells still show size control when Cdc25 production is compromised, suggesting there is a redundant size control mechanism. Cdc13 has many similar expression characteristics to Cdc25 and its role in activating Cdc2 make it a good candidate for a redundant sizer. I provide evidence that Cdc13 also has properties of a sizer.

Results

Accurately measuring protein levels by luciferase assay

In order to measure Cdc25 and Wee1 protein expression throughout the cell cycle, I developed a luciferase-based assay. This assay has distinct advantages compared to other techniques used to measure protein levels. First, since the luciferase assay is based on an exogenous chemical reaction to excite the luciferase molecule, there is very little background. While fluorescent molecules, such as Green Fluorescent Protein (GFP), are much brighter compared to the luminescent reactions of luciferase, fluorescent molecules require a light source to excite the molecules to produce a photon of light which causes widespread background fluorescence. This advantage means the relative signal for luminescent reactions is much higher compared to fluorescent reactions and is a big advantage for detecting less than twofold changes in protein levels. This sensitivity is necessary for studying a problem like cell size where cell size doubles during the cell cycle; a protein involved in measuring cell size is predicted to only double in concentration. Additionally, many cell cycle related proteins, Wee1 and Cdc25 in particular, are expressed at such low amounts that they can be difficult to detect quantitatively. Luminescent

based assays provide the sensitivity needed to detect and quantitate low expressed proteins. In particular, luminescence is more sensitive and accurate than Western blots for quantitating protein levels.

I took advantage of the orthogonal chemistry of beetle luciferase and *Renilla* luciferase to assay these enzymes independently in the same samples. For this project, beetle luciferase was fused to the C-terminus of Cdc25 and *Renilla* luciferase was fused to the C-terminus of Wee1 and Cdc13. Mutants of *wee1*, *cdc13*, and *cdc25* have a significant effect on cell size. The luciferase tags do not affect cell size, and therefore do not significantly compromise function. This assay allowed direct measurement of the relative Cdc25 to Wee1 ratio in cells. To measure Cdc13, Cdc25, or Wee1 independently, Ade4, an internal control, was tagged with the complementary luciferase. Ade4 was selected as an internal control to normalize Cdc25 or Wee1 expression because it is expressed at a constant rate as assayed by transcript expression (Rustici et al., 2004), it is not a common auxotrophic marker, it is non-essential, and it is cytoplasmic to easily detect in cell lysates. Originally total protein was used to normalize Cdc25 and Wee1 but the assays for total protein proved

to be too noisy to get an accurate measurement of Cdc13, Cdc25 and Wee1, which led to using Ade4 as an internal control.

Cdc25 concentration increases during G2

To determine the relative concentrations of Cdc25 and Wee1 across the cell-cycle, luciferase-tagged cells were synchronized in early G2 by centrifugal elutriation and followed through two cell cycles. Centrifugal elutriation was used to synchronize cells because it is a less perturbative method than cell cycle arrest by a small molecule or temperature sensitive allele of an essential cell cycle protein. Cells continue to grow during cell cycle arrests and therefore would produce non-physiological cell sizes. Centrifugal elutriation, like other size selection methods of synchronizing cells, isolates a subpopulation of unperturbed cells with a similar size from the cell culture. In the case of *S. pombe*, the smallest cells in the culture are in early G2 due to septation from the previous mitosis occurring in mid-S phase (Walker, 1999).

Both Cdc25 and Wee1 are expressed during G2, which has previously been observed (Moreno, Nurse, & Russell, 1990; Ducommun, Draetta, Young, & Beach, 1990; Aligue et al., 1997). G2 accounts for about

two-thirds of the *S. pombe* cell cycle and cell size almost doubles during this phase. Therefore, it is predicted that a protein produced in a size-dependent manner will double during this period, and a protein produced in a nuclear-dependent manner will not change during G2, since nuclear content does not change during this period. This dynamic is exactly what we see with levels of Cdc25 and Wee1: Cdc25 levels double, while Wee1 levels remain relatively constant (Figure 2.2).

To directly compare Cdc25 and Wee1, I monitored the Cdc25 to Wee1 ratio. The ratio of Cdc25 to Wee1 peaks in late G2/early mitosis, when the cells have grown large enough to enter mitosis. The ratio of Cdc25 to Wee1 increases approximately twofold during G2, as predicted by the model where Cdc25 production is size-dependent. The proteins are then actively degraded in an APC-dependent manner to reset the ratio for the next cell cycle (Figure 2.3) (Wolfe & Gould, 2004). The second ratio during the second cycle does not increase two fold due to the slight loss in synchrony of the culture. The fact that the peak of the Cdc25 to Wee1 ratio correlates with entry into mitosis suggests that the ratio of Cdc25 to Wee1 is involved in mitotic control and may influence the timing of mitosis.

Figure 2.2 Cdc25 and Wee1 Dynamics During G2

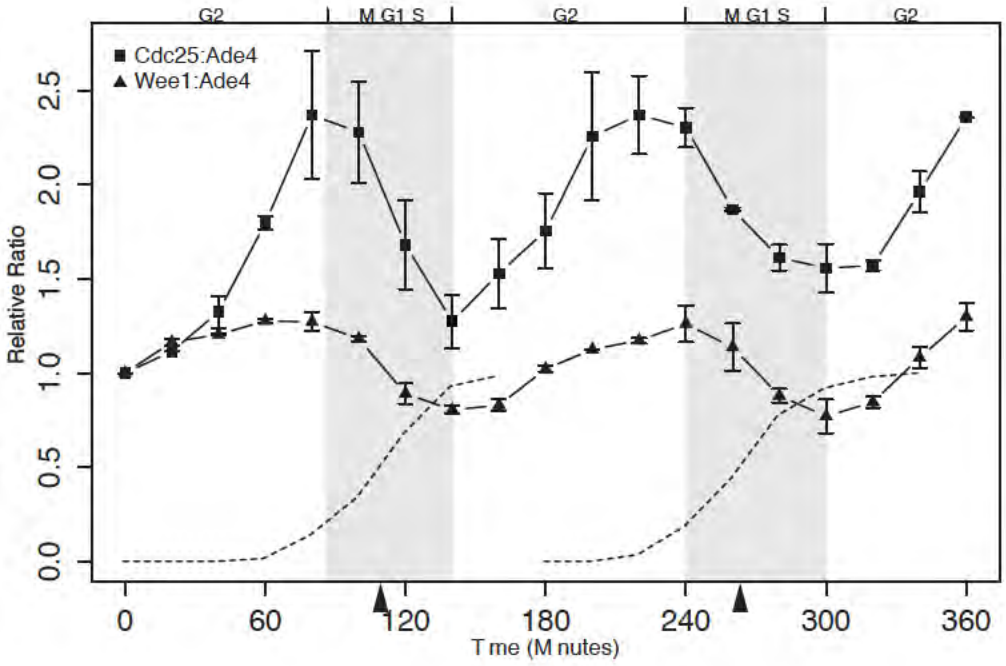


Figure 2.2 Cdc25 and Wee1 Accumulate During G2

Cdc25 and Wee1 protein accumulation were monitored during two cell cycles. Cells were synchronized by centrifugal elutriation. Protein concentration was measured by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the zero time point and Ade4, an internal luciferase-tagged control. The dotted lines show the average mitotic index as a fraction of the culture that has completed mitosis during each cell cycle. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated as the time between 0.2 and 0.8 of the mitotic index. The arrowheads represent the time at which half of the culture had gone through mitosis. Error bars indicate the Standard Error of the Mean (SEM) for 2 repeats of each strain.

Figure 2.3 The Ratio of Cdc25 to Wee1 Cycles

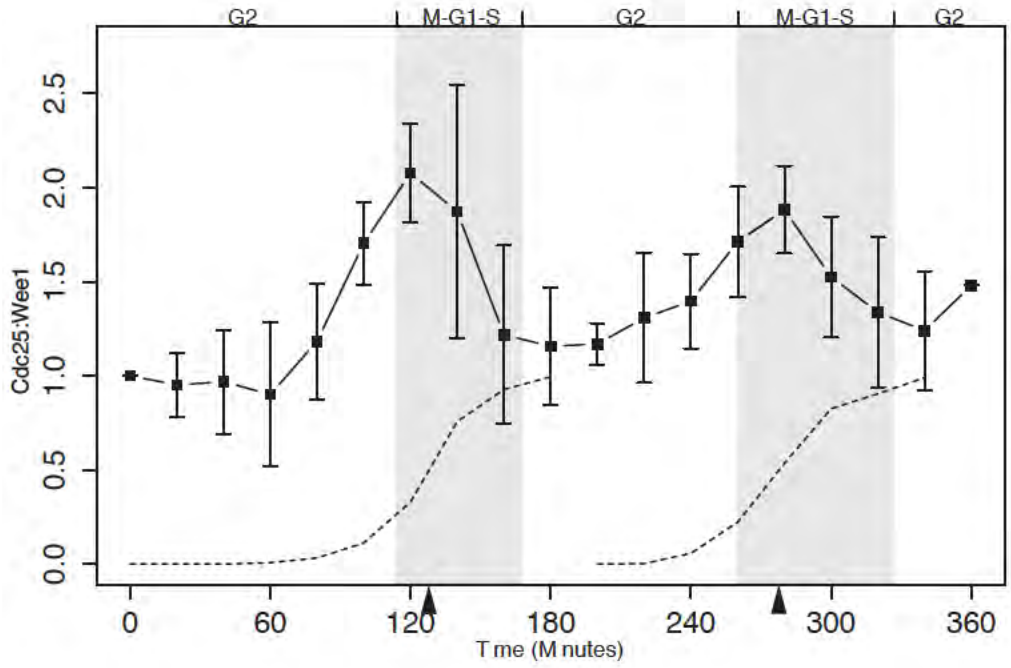


Figure 2.3 The Ratio of Cdc25 to Wee1 Cycles

The ratio of Cdc25 to Wee1 was monitored over two cell cycles. Cells were synchronized by centrifugal elutriation. Protein concentration was measured by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the zero time point. The dotted lines show the mitotic index as a fraction of the culture that has completed mitosis during each cell cycle. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated as the time between 0.2 and 0.8 of the mitotic index. The arrowheads represent the time at which half of the culture had gone through mitosis. Error bars indicate the SEM for 4 repeats of the time course.

To further investigate the dynamics of Cdc25 and Wee1 during the G2 phase of the cell cycle, the cells were synchronized in early G2 by centrifugal elutriation and then prevented from entering mitosis, using a temperature sensitive allele of *cdc2*, grown at the non-permissive temperature. The cells continue to grow during the block and increased in size 3.5 fold during the time course. Interestingly, the ratio of Cdc25 to Wee1 increased proportionally during this time course, providing strong evidence that the ratio of Cdc25 to Wee1 is proportional to cell size over a wide range of sizes (Figure 2.4).

The data in Figure 2.2 suggest that the change in the Cdc25:Wee1 ratio is driven by an increase in Cdc25 levels. To confirm that Cdc25 levels continue to rise in the cdc2 arrest, I assayed Cdc25 and Wee1 independently, relative to Ade4. Cdc25 levels increase over time while Wee1 levels do not change, showing that Cdc25 is driving the increase in the Cdc25 to Wee1 ratio over a wide range of cell sizes (Figure 2.5). It is easy to explain how a protein could exhibit dynamics like Wee1, where the protein reaches an equilibrium concentration relatively quickly after start of expression and remains at a constant concentration as the cell grows. However, it is difficult to explain how a protein could exhibit dynamics like

Figure 2.4 Increase of Cdc25:Wee1 Ratio During a Prolonged G2 Arrest

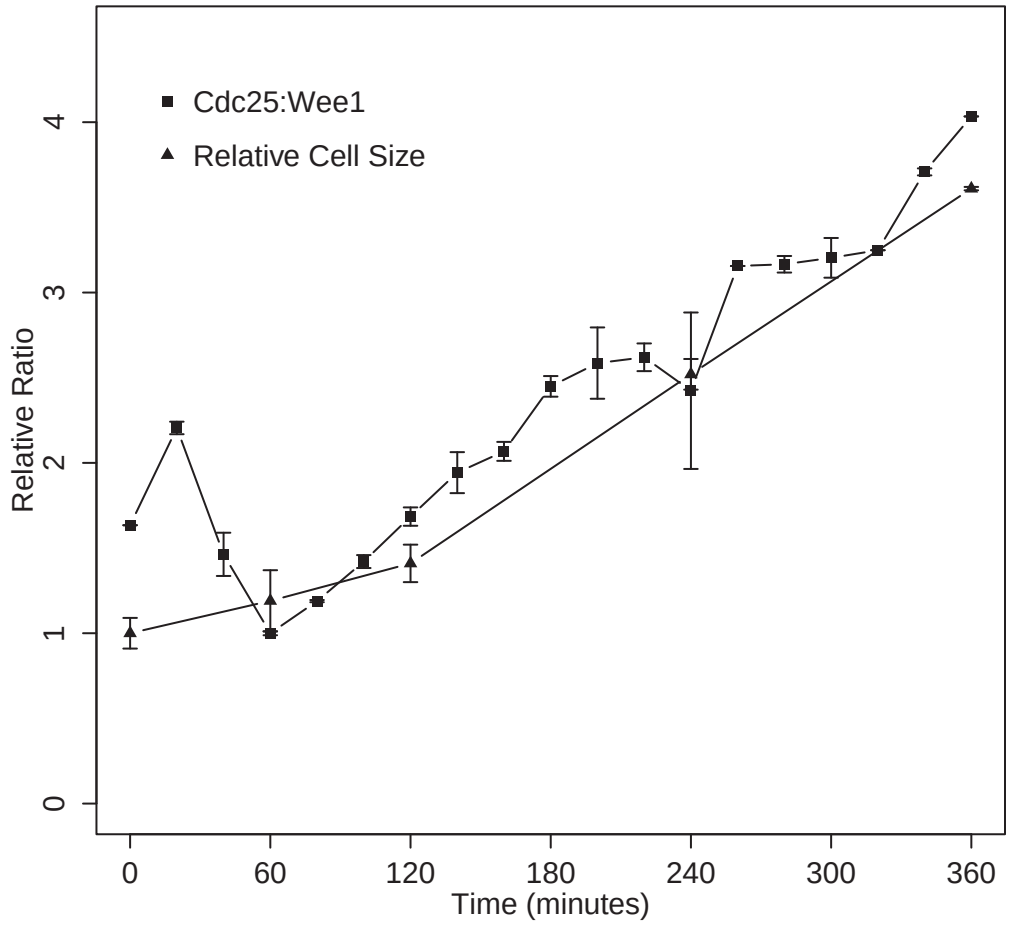


Figure 2.4 Increase of Cdc25:Wee1 Ratio During a Prolonged G2 Arrest

The ratio of Cdc25 to Wee1 and cell size were monitored during a Cdc2 arrest. Cells were synchronized by centrifugal elutriation and arrested in G2 using a temperature sensitive allele of Cdc2, *cdc2-L7*, grown at 35℃. The ratio of Cdc25 to Wee1 was measured using the luciferase assay. The length of the cells were measured by microscopy, using ImageJ (NIH) to analyze images. Both the Cdc25 to Wee1 ratio and cell size were normalized to the lowest expression. Error bars indicate the SEM for 3 repeats of the time course.

Figure 2.5 Dynamics of Cdc25 and Wee1 During a Prolonged G2 Arrest

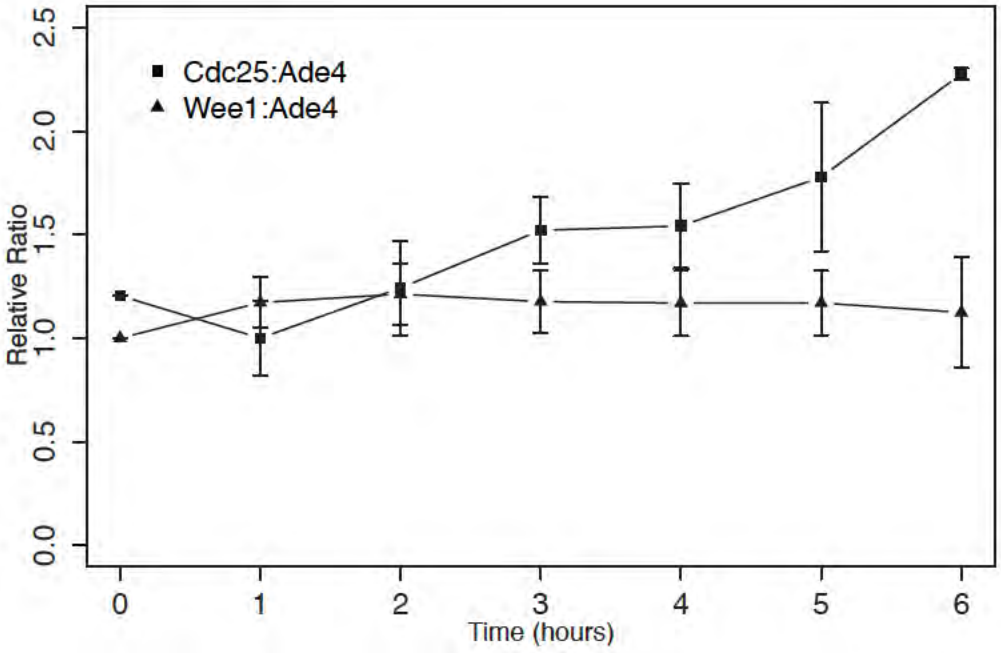


Figure 2.5 Dynamics of Cdc25 and Wee1 During a Prolonged G2 Arrest

The relative concentration of Cdc25 and Wee1 were monitored during a Cdc2 arrest. Cells were synchronized by centrifugal elutriation and arrested in G2 using an analog sensitive allele of Cdc2, cdc2-as, with the analogue added immediately after elutriation (Dischinger, Krapp, Xie, Paulson, & Simanis, 2008). The concentration of Cdc25 and Wee1 was measured using luciferase assay and was normalized to the lowest expression and Ade4, an internal luciferase-tagged control. Error bars indicate the SEM for 3 repeats for each strain.

Cdc25, where the concentration increases as the cell size increases. The observation that Cdc25 concentration increases over a wide range of cell size suggests that Cdc25 concentration is regulated by cell size.

Cdc25 is an unstable protein and recovery correlates with entry into mitosis

A protein with the dynamics of Cdc25 was predicted over 40 years ago in experiments investigating the effects of cell-cycle perturbation on cell size (Herring, 1972; Sudbery & Grant, 1975). In these experiments, short pulses of cycloheximide, which inhibits translation of proteins, at different points in the cell cycle resulted in different lengths of delays in mitotic entry. Pulses of cycloheximide early in the cell cycle produce a delay proportional to the length of the pulse of cycloheximide. However, as the pulse of cycloheximide gets closer to mitosis the length of the delay gets progressively longer resulting in an excess delay. At mitosis, the cells reset and the excess delay disappears.

From these experiments it was concluded that there is an unstable inducer of mitosis that is responsible for the excess delay (Wheals & Silverman,

1982; Tyson, 1983). In this model, mitosis would be initiated when the mitotic inducer reaches a sufficient concentration to overcome an inhibitor of mitosis (Figure 2.6 i). This initiation of mitosis would be size-dependent based on the threshold set by the mitotic inhibitor. It is predicted that the unstable inducer of mitosis would have a relatively short half-life and maintain a size-dependent concentration. Conversely, the inhibitor of mitosis would be relatively stable and have a constant concentration. During the pulse of cycloheximide, the inducer concentration would decrease more than the inhibitor. If the cycloheximide pulse was done early enough in the cell cycle, the inducer would have sufficient time to return to a size-dependent concentration with a delay in mitosis would only be as long as the delay in growth caused by the pulse of cycloheximide (Figure 2.6 ii). However, if this pulse is moved closer to mitosis, the inducer does not have sufficient time to return to a size dependent concentration and results in an excess delay. The length of the excess delay depends on the recovery dynamics of the inducer and how close to mitosis the cells are when they are pulsed with cycloheximide (Figure 2.6 iii and iv).

Figure 2.6 Diagram of Excess Delay Model

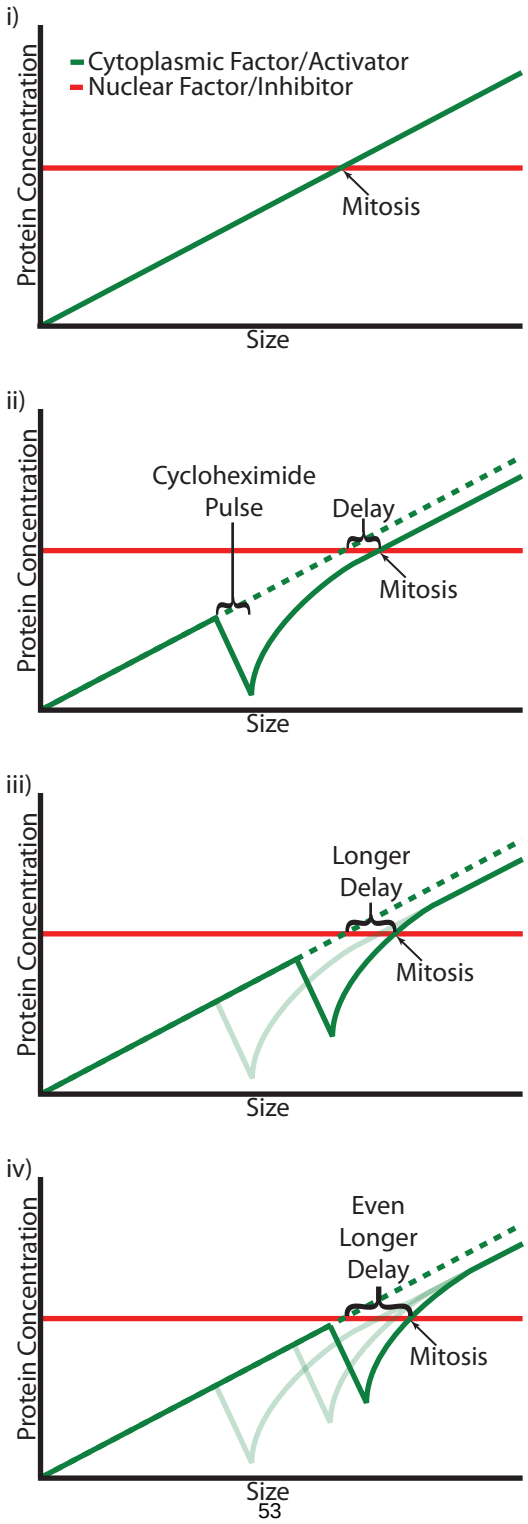


Figure 2.6 Diagram of Excess Delay Model

In the excess delay model there is a stable, nuclear dependent inhibitor of mitosis that sets a threshold which the unstable, size dependent inducer of mitosis has to overcome to trigger mitosis (i). If the cell is pulsed with cycloheximide well before mitosis, the inducer decreases in concentration due to its instability. After the pulse the inducer quickly returns to a size dependent equilibrium which would be offset by the length of the cycloheximide pulse compared to the untreated cell. This would cause a delay in mitosis (ii). If the pulse of cycloheximide is moved closer to mitosis to the point where the inducer does not have time to return to a size dependent equilibrium, then the delay of mitosis would be longer (iii). If the pulse of cycloheximide is moved even closer to mitosis, the delay mitosis would be even longer (iv).

The difference in delay caused by the inducer not having time to return to equilibrium is termed the excess delay.

To determine if Cdc25 is unstable I measured the half-life of Cdc25 and Wee1. In asynchronous cells, Cdc25 has a half-life of 15 minutes and Wee1 has a half-life of 88 minutes (Figure 2.7). Previous studies have shown that dephosphorylated Cdc25 is relatively stable (Wolfe & Gould, 2004). The relatively short half-life I measured in asynchronous cells seems to contradict the results that dephosphorylated Cdc25 is stable. Looking at my half-life data, about 20% of Cdc25 protein is potentially more stable than the rest of Cdc25 and therefore does not fit on the estimated half-life curve (Figure 2.7). However the other 80% of Cdc25 protein is relatively unstable. To accurately determine the difference in these two populations, the experiments should be repeated with populations of cells isolated from different points in the cell cycle.

My data that measures the peak of the ratio of Cdc25 to Wee1 supports the unstable inducer of mitosis model. There is a correlation between the delay in the peak of the Cdc25 to Wee1 ratio and the delay in mitosis (Figure 2.8). Therefore, Cdc25 and Wee1 fit the prediction of inducer and

Figure 2.7 Half Life Measurement of Proteins

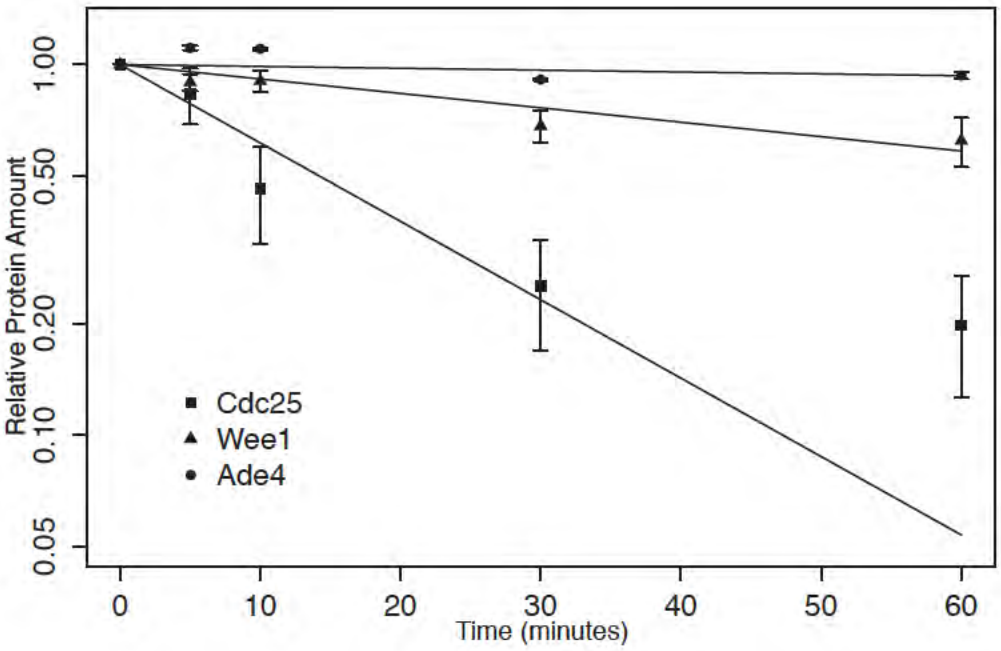


Figure 2.7 Half-life Measurement of Proteins

The half-life of Cdc25, Wee1, and Ade4 were measured using the luciferase assay. Asynchronous cultures in log phase were treated with 100 µg/mL of cycloheximide. Protein amount was normalized to the zero time point. The half-life for Cdc25 = 15 minutes, Wee1 = 88 minutes, Ade4 = 204 minutes. The last time point for Cdc25 was not included in the calculations for a better fit. Error bars indicate the SEM for 3 repeats of the time course.

Figure 2.8 Two Examples of Excess Delay Experiment

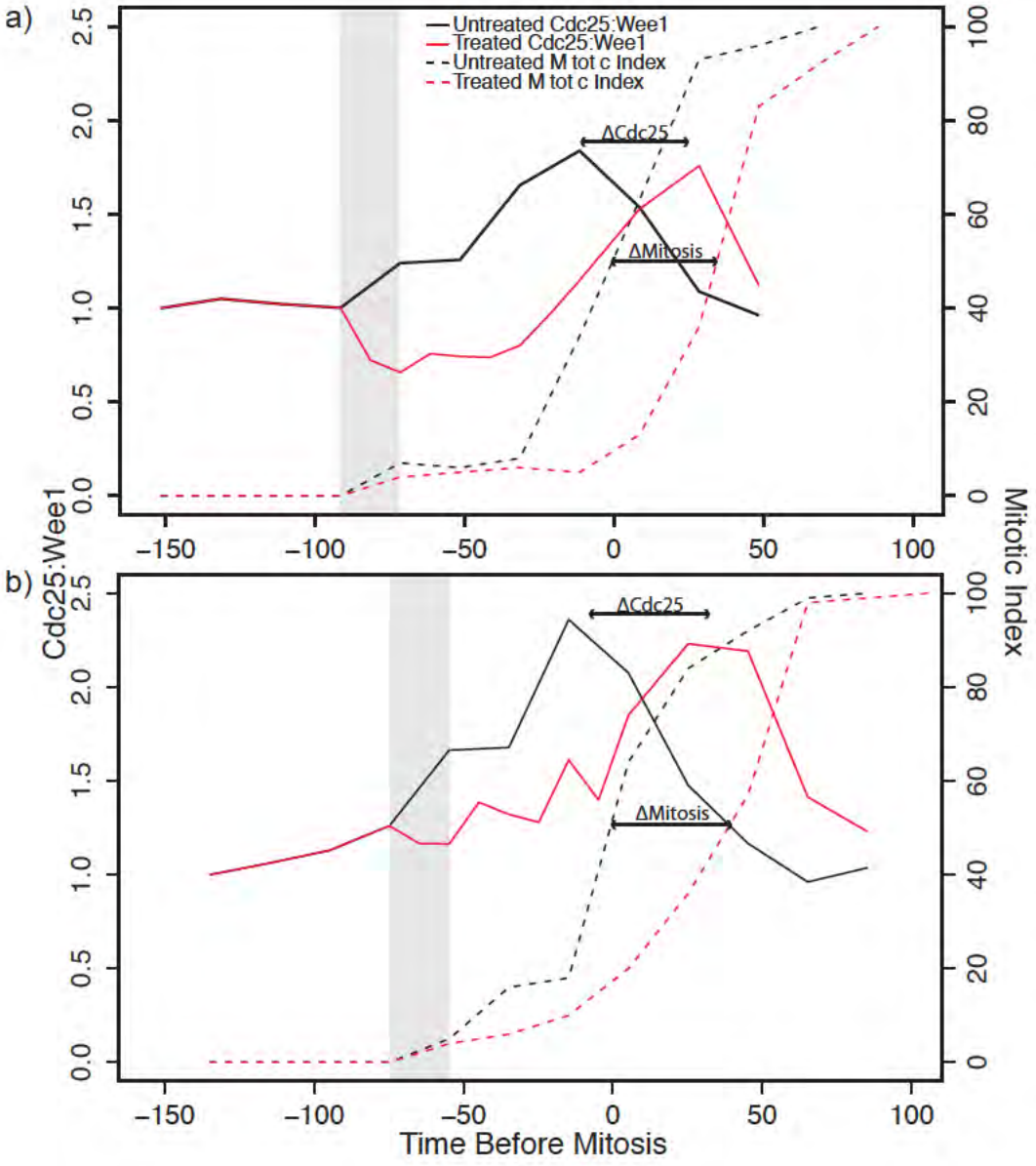


Figure 2.8 Two Examples of Excess Delay Experiment

Two examples of time courses with cycloheximide pulses. Cells were synchronized in early G2 by centrifugal elutriation and followed through one cell division. In the top time course the cells were pulsed for 20 minutes with 100 μg/mL cycloheximide 92 minutes before mitosis. This resulted in a 37 minute delay in the peak of Cdc25:Wee1 and a 34 minute delay in mitosis. In the bottom time course the pulse of cycloheximide was 75 minutes before mitosis. This resulted in a 43 minute delay in the peak of Cdc25:Wee1 and a 38 minute delay in mitosis. Protein concentration was measured by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the first time point. The grey band indicates the time of the cycloheximide pulse.

inhibitor of mitosis, respectively, in the unstable inducer model. That is, a short 20 minute pulse of cycloheximide affects the concentration of Cdc25 more than the concentration of Wee1. A pulse of cycloheximide 92 minutes before mitosis results in a 34 minute delay in mitosis and a 37 minute delay in the Cdc25 to Wee1 ratio (Figure 2.8a). A pulse of cycloheximide 75 minutes before mitosis results in a 38 minute delay in mitosis and a 43 minute delay in the Cdc25 to Wee1 ratio (Figure 2.8b). There is an increase in the delay as the cells progress through the cell cycle with the longest delay of about 45-50 minutes happening right before the cells commit to mitosis. Thus the maximum excess delay observed is 25-30 minutes (Figure 2.9). This maximum excess delay is slightly shorter than the 40 minute maximum excess delay seen in the original experiments (Herring, 1972). This difference is most likely due to technical differences in the experiments due to differences in strains and synchronization techniques. However, the general trend is recapitulated in these recent experiments. The excess delay disappears once the cells have committed to mitosis (see last data point in Figure 2.9). Therefore, the closer to mitosis the cells are pulsed with cycloheximide the longer the delay in the peak of the Cdc25 to Wee1 ratio and mitosis.

Figure 2.9 Excess Delay Increases During Cell Cycle

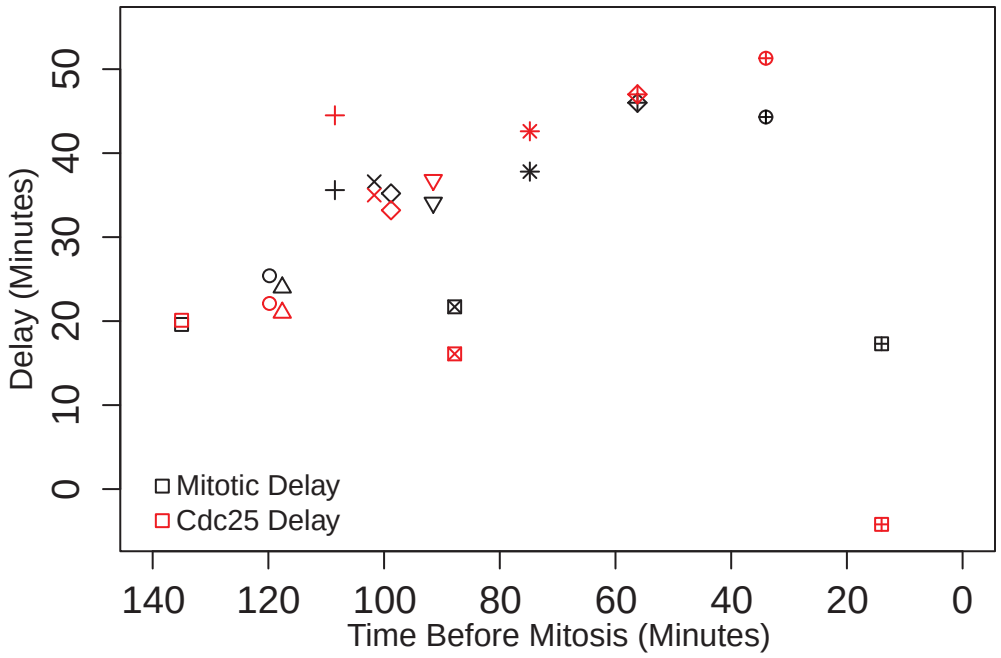


Figure 2.9 Excess Delay Increases During Cell Cycle

The excess delay of both the peak of Cdc25 accumulation and mitosis increases for cycloheximide pulses up to about 35 minutes before septation. For each cycloheximide pulse experiment, the delay of mitosis and the peak of Cdc25:Wee1 of the treated culture compared to the untreated culture was plotted versus the time before mitosis the pulse of cycloheximide was administered. Each symbol represents the two data points from an independent time course experiment.

Although these experiments only quantified the Cdc25 to Wee1 ratio, there could be other factors that have an affect on the delay in mitosis in response to pulses of cycloheximide. However, the correlation between the delay of the peak of the Cdc25 to Wee1 ratio and the delay mitosis in response to a pulse of cycloheximide provides evidence that Cdc25 has the dynamics of an unstable inducer of mitosis predicted by the original cycloheximide experiments. These dynamics show that Cdc25 is at a size dependent equilibrium. Since Cdc25 has the characteristics of an unstable inducer of mitosis, it argues against a timer model for cell size control. If Cdc25 accumulation was time dependent and not size dependent, I would expect the protein to be stable and therefore the recovery in response to a pulse of cycloheximide would produce a more constant excess delay in mitosis. Testing other proteins to see if Cdc25 dynamics are unusual could strengthen this result. This observation paired with the previous observation that the concentration of Cdc25 increases as the cell size increases provides mounting evidence that Cdc25 has the characteristics of sizer.

Size-dependent accumulation of Cdc25 protein is regulated by its promoter

To determine if the size-dependent accumulation of Cdc25 is regulated at the transcript level or by a post-transcriptional mechanism, I examined the accumulation of the *cdc25* transcript. I synchronized cells in early G2 by elutriation, as before, and monitored the levels of several cell-cycle transcripts by fluorescent hybridization single-transcript counting in whole-cell extracts using the Nanostring nCounter approach (Figure 2.10). This approach uses fluorescently labeled probes to capture and count individual transcripts. The method relies on base pair hybridization to capture the transcripts and, therefore, has no enzymatic bias and is highly quantitative (Geiss et al., 2008). *cdc25* transcript levels increase about twofold during G2. It is striking how the increase in Cdc25 protein concentration mirrors the increase in *cdc25* transcript (compare Cdc25 protein in Figure 2.2 to *cdc25* transcript in Figure 2.10). The correlation between Cdc25 protein and *cdc25* transcript is consistent with the short half-life of Cdc25 that can quickly adapt to the changing levels of *cdc25* transcript (Mehra, Lee, & Hatzimanikatis, 2003). *cdc13* transcripts also

Figure 2.10 Transcript Accumulation Throughout the Cell Cycle

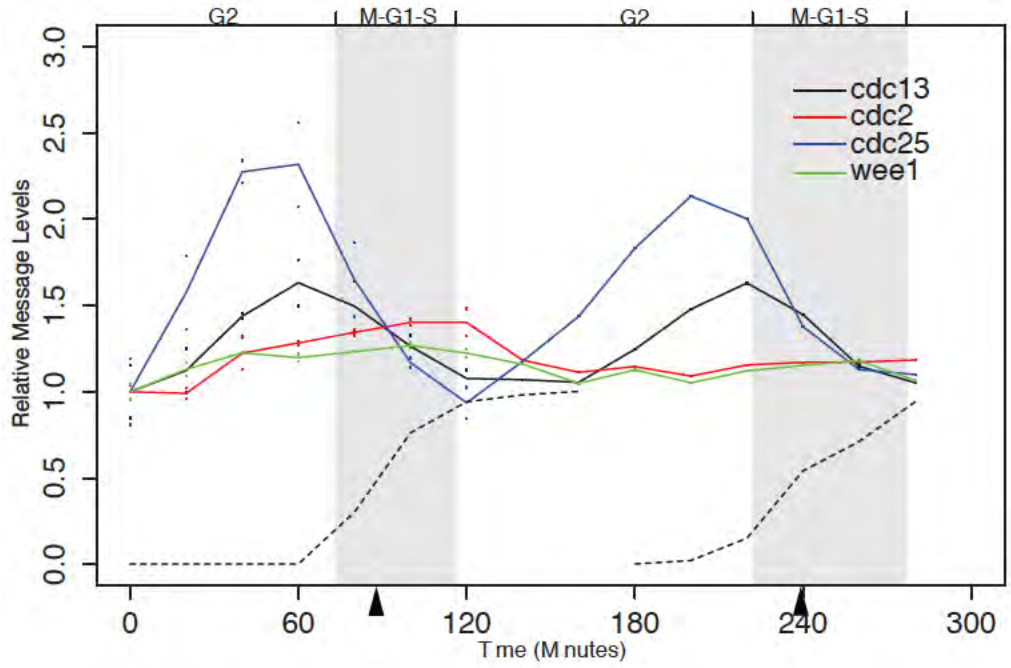


Figure 2.10 Transcript Accumulation Throughout the Cell Cycle

Transcript accumulation of *cdc25*, *wee1*, *cdc13* and *cdc2* were measured throughout the cell cycle. Cells were synchronized by centrifugal elutriation and followed through two cell cycles. Transcripts were measured by counting single molecules using hybridized probes developed by nanoString (Seattle, WA). The graph represents the average of two time courses. One time course was one cell cycle in length while the second cell cycle was two cell cycles in length. The dots represent the individual measurements of the two time courses. The dotted lines show the mitotic index as a fraction of the culture that has completed mitosis during each cell cycle. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated from the mitotic index. The arrowheads represent the 0.5 mitotic index.

increase during G2, consistent with its role as the G2 cyclin. In contrast, transcript levels for *wee1* and *cdc2* are relatively constant.

All the transcripts for the proteins involved in the mitotic commitment pathway have relatively similar half-lives between 5 and 25 minutes (Figure 2.11). The similarity in the half-life of these transcripts suggests that size-dependent expression does not involve some general mechanism based on differences in transcript longevity. One model that was put forward predicted that a long lived transcript could accumulate in a size dependent manner since, in general, transcription increases as cells increase in size. However, that is not observed and therefore the increase in *cdc25* transcript must be regulated by expression and not by general accumulation due to a long half-life.

Previous work showed that the 5' untranslated region (5' UTR) of cdc25 and cdc13 are unusual for fission yeast (Daga & Jimenez, 1999). The *cdc25* 5' UTR is relatively long with many small open reading frames, small hairpins, and a spliced intron. The essential translation initiation

Figure 2.11 Half-life Measurement of Transcripts

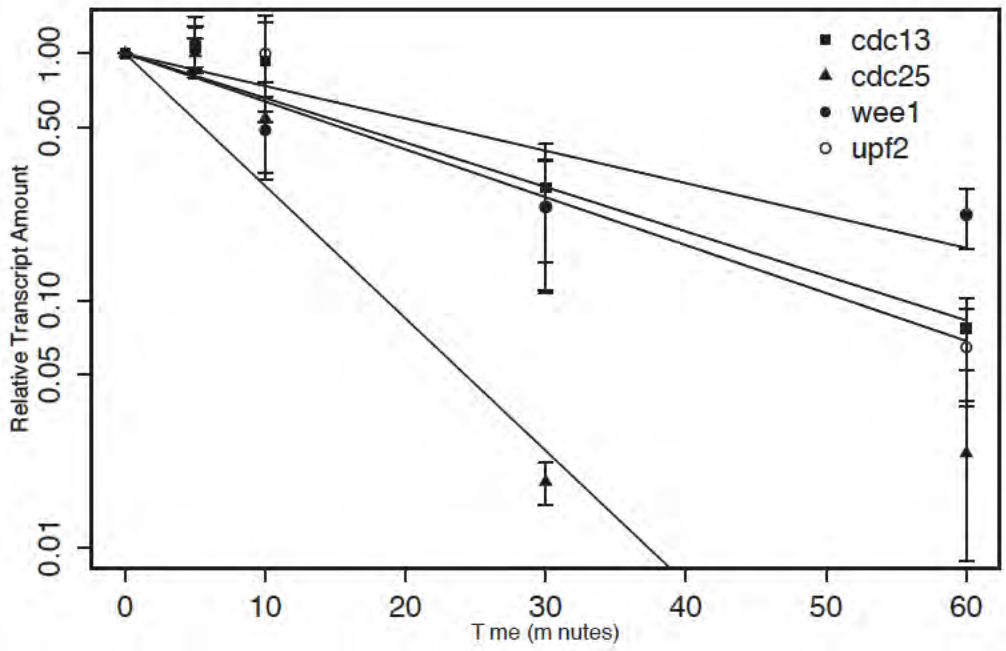


Figure 2.11 Half-life Measurements of Transcripts

The half-life of *cdc25*, *cdc13*, and *wee1* were measured as well as *upf2* and *srp7* as controls for short and long transcript half-life respectively. Asynchronous cultures were treated with 15 µg/mL Thiolutin to inhibit transcription. Transcripts were normalized to the zero time point and *srp7* as an internal control. The half-life for *cdc25* = 5 minutes, *wee1* = 26 minutes, *cdc13* = 15 minutes, and *upf2* = 14 minutes. The last time point for Cdc25 was not included in the calculations for a better fit. Error bars indicate the SEM for 3 repeats of the time course.

factor 4a *tif1* is required for translation initiation through its RNA helicase activity. It was shown that translation of *cdc25* was sensitive to the activity of *tif1* (Daga & Jimenez, 1999). Deletion of a majority of the 5' UTR alleviated the sensitivity to *tif1* activity. To see if the 5' UTR, and thus translation, has any effect on Cdc25 size-dependent accumulation I used the luciferase assay to monitor the accumulation of Cdc25 during the cell cycle in a strain with the *cdc25* 5' UTR deleted. The results show that Cdc25 accumulation in the absence of the 5' UTR is similar to wild-type Cdc25 (Figure 2.12 compared to Figure 2.2). Therefore, the 5' UTR is not required for the size dependent accumulation of Cdc25.

To test whether transcriptional regulation is necessary for the size-dependent accumulation of Cdc25 protein, I made chimeric alleles in which the *cdc25* promoter and 5'UTR were replaced by the corresponding regions of the *nmt1* gene (Figure 2.13). The *nmt1* (no message in thiamine) promoter can be repressed by the addition of thiamine to the media. The repressed expression is 10 – 1000 fold lower than the induced expression depending on the target gene. There are two additional hypomorphs of this promoter that allow for lower expression control: while

Figure 2.12 Accumulation of Cdc25 Protein in a Strain With a Deletion of the *cdc25* 5'UTR

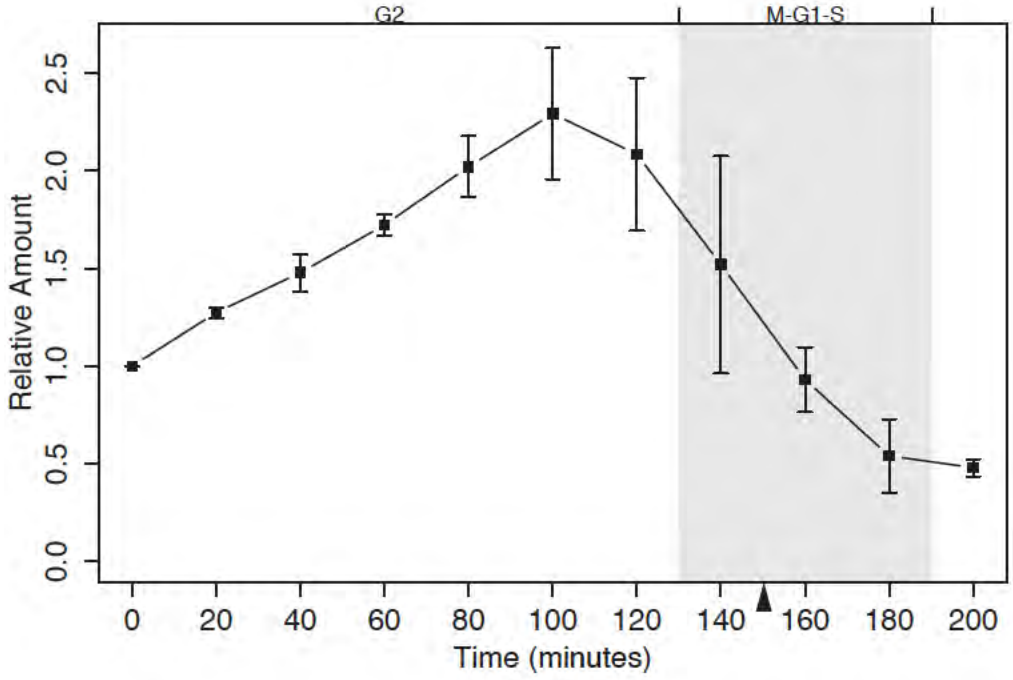


Figure 2.12 Accumulation of Cdc25 Protein in a Strain With a Deletion of the *cdc25* 5'UTR

Accumulation of Cdc25 protein during the cell cycle in a strain with a deleted *cdc25* 5'UTR. Cells were synchronized by centrifugal elutriation and followed through two cell cycles. Protein concentration was measures by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the zero time point and Ade4, an internal luciferase-tagged control. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated from the mitotic index. Error bars indicate the SEM for 2 repeats of the time course.

Figure 2.13 Diagram of Different *cdc25* Constructs

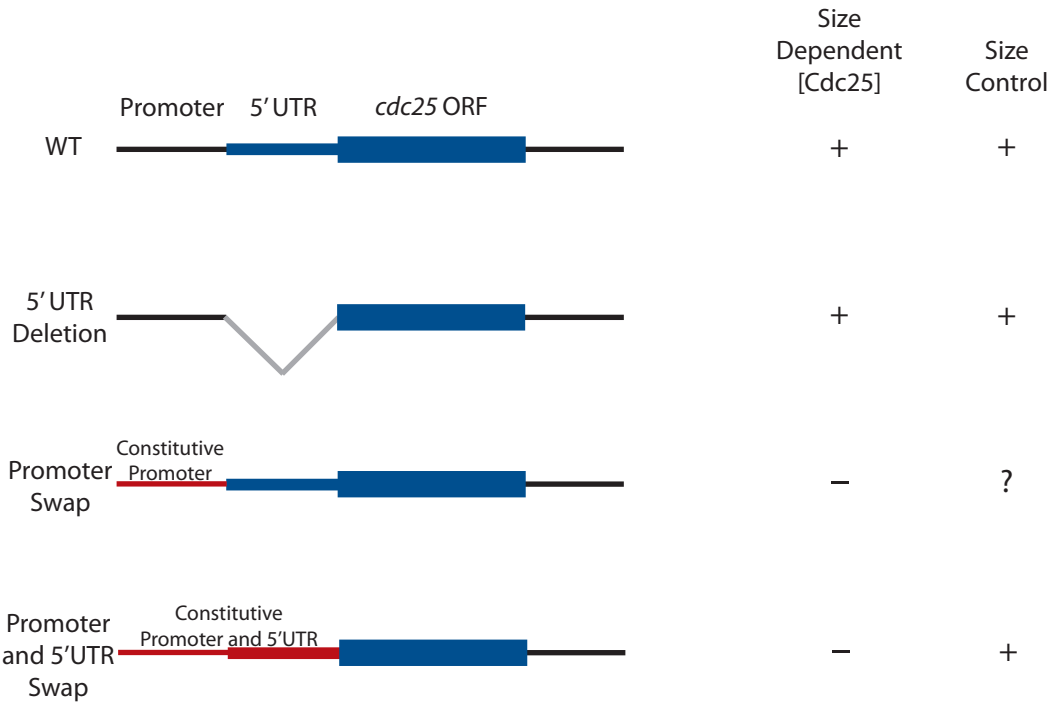


Figure 2.13 Diagram of Different *cdc25* Constructs

Graphic table of constructs made to identify the cause of size dependent expression of cdc25. Each construct was assessed both for size dependent accumulation of Cdc25 by luciferase assay. The ability of the cells to regulate their cell size was assayed by measuring the distribution of cell size at septation and monitoring the how the cells return to normal size after an HU arrest.

nmt1 is used for high expression, *nmt41* can be used for medium expression, and *nmt81* is used for low expression (Maundrell, 1990; Basi, Schmid, & Maundrell, 1993). I tested for function in size control by strain viability, variation of size at septation. I also tested for size control by monitoring recovery from an hydroxyurea block. I blocked the cells from progressing through the cell cycle so they grew to a longer than normal size and observed if they could return to their pre-hydroxyurea size (Figure 2.14). Replacement of the *cdc25* promoter with the thiamine-regulated *nmt41* promoter results in cell cycle independent expression of Cdc25 (Figure 2.15). Interestingly, repressed *nmt41:cdc25* is expressed at one-tenth the wild-type expression level but the cells are still viable.

The fact that the *nmt41:cdc25* strain is still alive was surprising as the model predicts that the strain would be inviable with a low, constitutive expression of Cdc25. The expected outcome, based on the model, would be that Cdc25 would not be able to overcome the inhibition by Wee1 due to the lack of increase of Cdc25 concentration. The cells would think they are not large enough to divide, and thus would continue to grow without

Figure 2.14 Evidence for Size Control

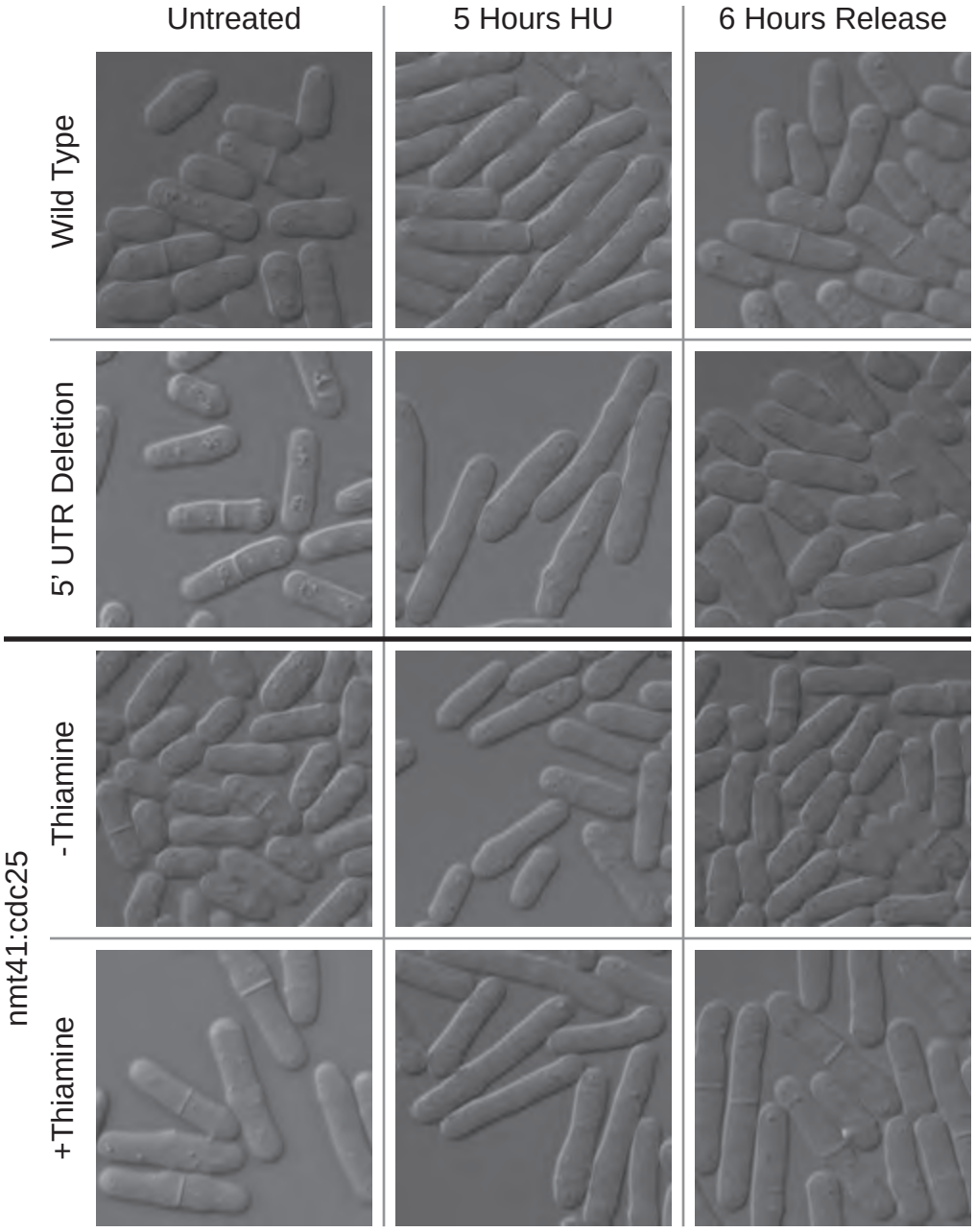


Figure 2.14 Evidence for Size Control

Asynchronous cultures were arrested with Hydroxyurea (HU) for five hours to allow cells to grow to an abnormal length. Cells were then released from the HU block and allowed to go through unhindered cell cycles for six hours to see if they return to a normal size. The 5' UTR deletion is a strain with the 5' UTR of *cdc25* deleted. For *cdc25* expressed under the *nmt41* promoter, cells were grown in minimal media in the absence or presence of 15μM thiamine.

Figure 2.15 Protein Accumulation of *nmt41:cdc25* Construct

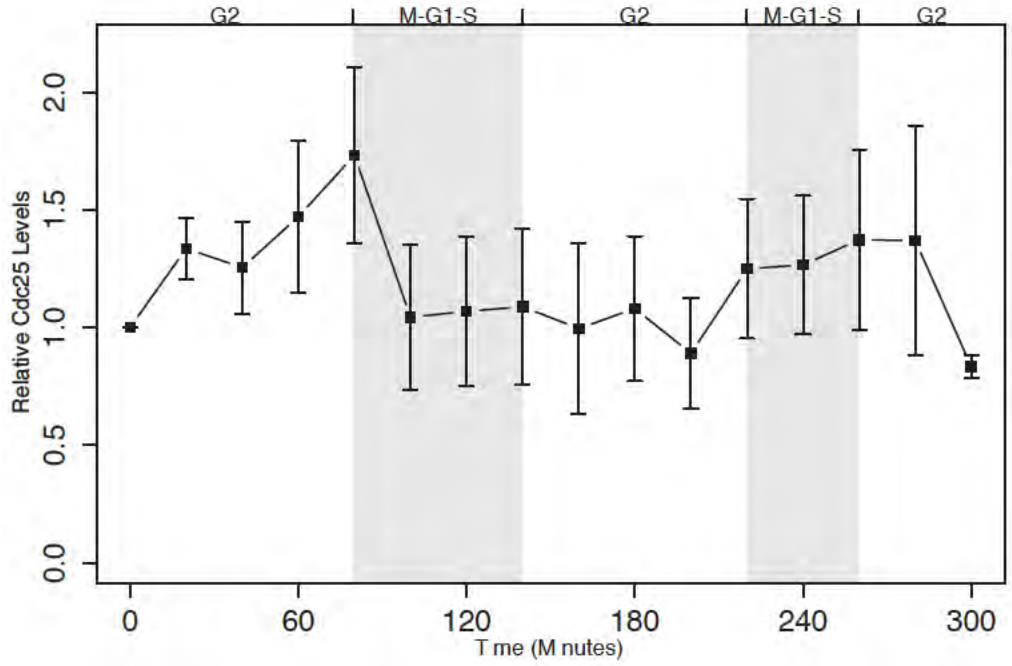


Figure 2.15 Protein Accumulation of *nmt41:cdc25* Construct

Cdc25 protein expressed from a constitutively active *nmt41* promoter during two cell cycles. Cells were synchronized by centrifugal elutriation and followed through two cell cycles. Protein concentration was measured by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the zero time point and Ade4, an internal luciferase-tagged control. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated from the mitotic index. Error bars indicate the SEM for 5 repeats of the time course.

dividing. Since the cells are still viable, it suggests there is another sizer that can regulate cell size. Either Cdc25 is not involved in size control or there are two sizers, one being Cdc25, that can redundantly regulate cell size.

To test if the *cdc25* promoter is sufficient for size dependent expression, I created a construct with the *cdc25* promoter and 5' UTR driving the expression of beetle luciferase. To destabilize beetle luciferase to ensure its expression dynamics are similar to *cdc25*, I added an N-end degron to beetle luciferase (Houser et al., 2012). The *cdc25* promoter:luciferase construct cycles in a similar manner to wild-type *cdc25,* though the amplitude is higher and the accumulation is not as constant as compared to Cdc25 (Figure 2.16). The higher amplitude and fluctuation in the accumulation of luciferase during G2 could be due to differences in degradation pathways used to degrade Cdc25 versus the luciferase construct. The construct data shows the promoter is sufficient to drive cell cycle dependent expression. This data suggests that *cdc25* is regulated via transcription although the molecular mechanism is still unknown.

Figure 2.16 Accumulation of Luciferase Driven by *cdc25* Promoter Throughout the Cell Cycle

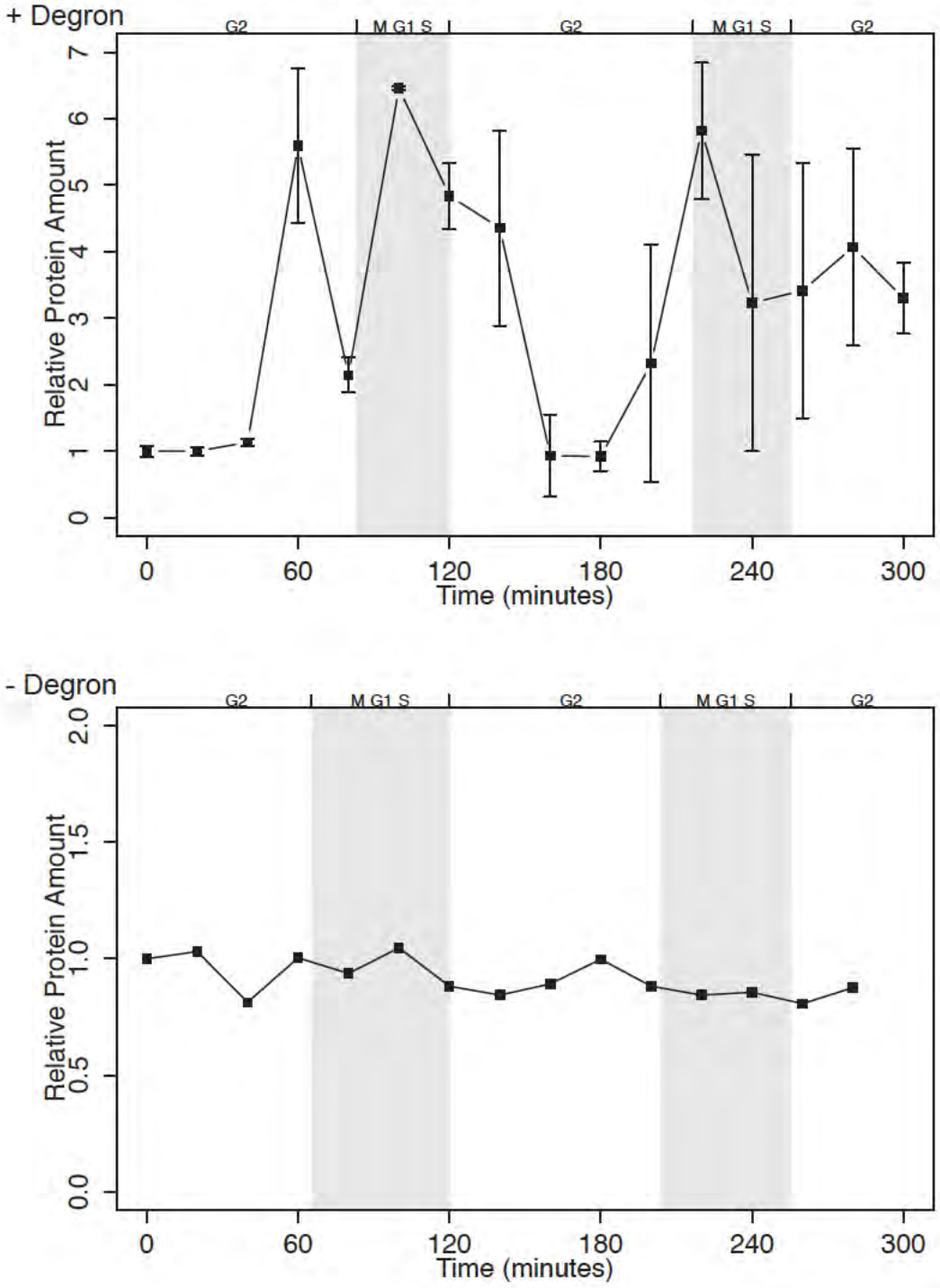


Figure 2.16 Accumulation of Luciferase Driven by *cdc25* Promoter Throughout the Cell Cycle

In the top panel, luciferase accumulation driven by the *cdc25* promoter and an N-end rule degron was monitored throughout the cell cycle. Cells were synchronized by centrifugal elutriation and followed through two cell cycles. Protein concentration was measured by luciferase assay using protein fusions to endogenous loci. Protein concentration was normalized to the zero time point and Ade4, an internal luciferase-tagged control. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated from the mitotic index. Error bars indicate the SEM for 2 repeats of the time course. The bottom panel shows luciferase expression driven by the cdc25 promoter in the absence of the N-end rule degron.

Cdc13 concentration increases during G2

One obvious candidate for a second sizer is the only essential B-type cyclin Cdc13. Ultimately mitosis is triggered by high Cdc2 activity. Two factors determine Cdc2 activity, one being tyrosine phosphorylation status of Cdc2 and the other being binding of Cdc13. In the event that Cdc25 expression is compromised, Cdc13 binding to Cdc2 could potentially increase Cdc2 activity in a size dependent manner. Therefore, the model predicts that Cdc13 would have similar dynamics to Cdc25.

Cdc13 protein cycles similar to Cdc25 (Figure 2.17) as well as the transcript (Figure 2.10). Again, similar to Cdc25, it is striking how similarly protein expression of Cdc13 mirrors transcript accumulation. Replacing the *cdc13* promoter with the *nmt41* promoter results in a cell that is only viable when the *nmt41* promoter is induced. When the *nmt41* promoter is repressed, the cells have an elongated, cdc phenotype due to the low expression of *cdc13* (Figure 2.18).

Figure 2.17 Accumulation of Cdc13 Protein During the Cell Cycle

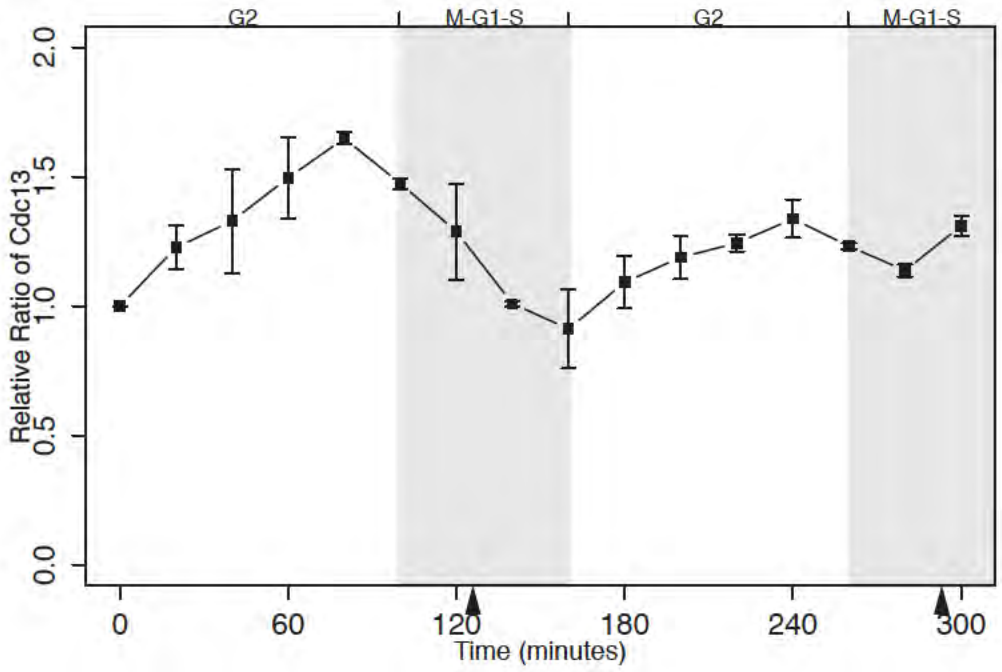


Figure 2.17 Accumulation of Cdc13 Protein During the Cell Cycle

Cdc13 protein accumulation was monitored during two cell cycles. Cells were synchronized by centrifugal elutriation. Protein concentration was measured by luciferase assay using protein fusions to the C-terminus of the endogenous loci. Protein concentration was normalized to the zero time point and Ade4, an internal luciferase-tagged control. The shaded areas represent the Mitosis-G1-S phases of the cell cycle estimated from the mitotic index. The arrowheads represent the 0.5 mitotic index. Error bars indicate the SEM for 3 repeats of the time course.

Figure 2.18 Synthetic Interaction Between *cdc25* and *cdc13*

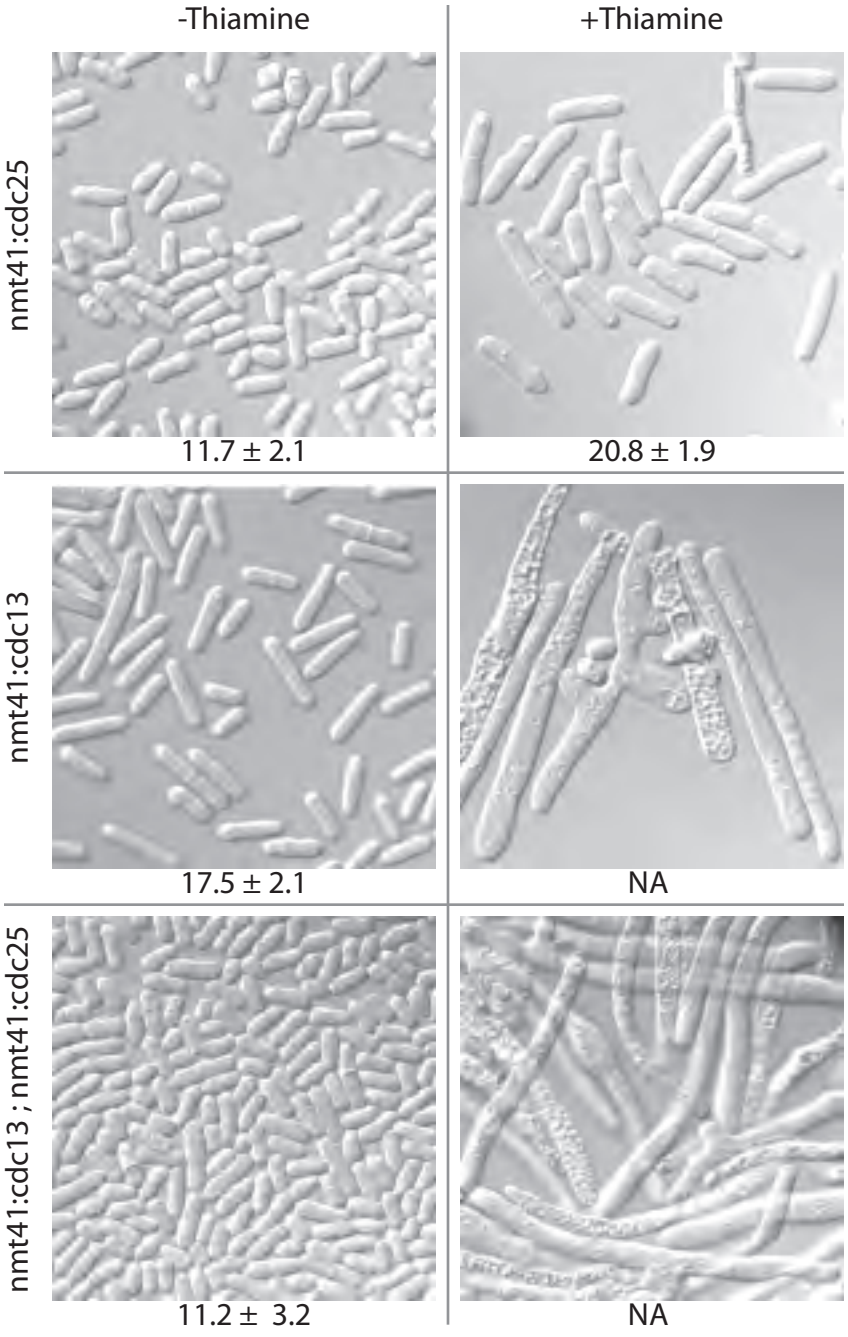


Figure 2.18 Synthetic Interaction Between *cdc25* and *cdc13*

Asynchronous cultures were grown in minimal media in the absence or presence of 15μM thiamine overnight to turn the promoter on or off, respectively. The average size at septation is listed below each strain type and thiamine treatment. For each strain in each condition, 50 cells were measured to get the mean and standard deviation of the population of cells.

To determine if there is a size-monitoring genetic interaction between *cdc25* and *cdc13*, I made a double mutant with *nmt41:cdc25* and *nmt41:cdc13*. This double mutant is alive when the *nmt41* promoter is induced but dead when the *nmt41* promoter is repressed (Figure 2.18). When the cells are grown under inducing conditions, the cell lengths of the double mutant is the same as the *nmt41:cdc25* single mutant. Conversely, when the cells are grown under repressive conditions, the cell lengths of the double mutant are the same as the *nmt41:cdc13* single mutant. Unfortunately, these results are difficult to interpret how Cdc25 and Cdc13 regulate cell size in relation to each other. Ultimately, the critical experiment is to express both Cdc25 and Cdc13 in a size independent manner where I can modulate the expression such that Cdc25 and/or Cdc13 is expressed at the lowest possible amount to see if there is any genetic interaction. However, given the tools available, I was unable to do this experiment.

Discussion

In this study I present evidence that the protein Cdc25 increases in a size dependent manner. This size dependent increase is regulated by

transcription of the *cdc25* gene. When the cells are small there is relatively little Cdc25 in the cell. As the cells grow in size, the amount of Cdc25 increases. I propose that when the cells reach a critical size, the Cdc25 concentration increases to the point where Cdc25 activity can overcome the activity of Wee1 and activate Cdc2 to trigger mitosis. Cdc2 can then hyperactivate Cdc25 and inhibit Wee1 through feedback loops to succinctly switch cells into mitosis.

The model where Cdc25 accumulation measures cell size predicts that size independent expression should be lethal because Cdc25 would not be able to overcome the inhibition of Wee1 and therefore the cells would continue to grow and not divide. However, that is not the case. One possibility is that Cdc25 is not involved in size control and the size dependent accumulation is a coincidence. Another possibility is that size control is redundant and another sizer exists. In a situation where size dependent Cdc25 accumulation is compromised, one potential way to trigger mitosis is to create enough Cdc2 to trigger the feedback loops which would in turn activate Cdc2 and trigger mitosis. In the case of compromised Cdc25 production, Cdc2 would be inhibited by Wee1 phosphorylation. Accumulation of Cdc13 as the cell cycle progresses

would create more active Cdc2 complex and could trigger the feedback loops, which could hyper-activate the low level of Cdc25. My results suggest that this may be the case, although more research on Cdc13 expression and its interactions with Cdc25 is needed. One assumption that is testable by kinase assays is that phosphorylated Cdc2 (inhibited) has some basal level of activity that, if enough Cdc2 complex was formed, could trigger the feedback loops. The hyper-activated Cdc25 could then dephosphorylate Cdc2 and thus trigger mitosis.

Replacing the promoter of *cdc25* abolished the size-dependent increase in protein concentration suggesting that *cdc25* is transcriptionally regulated. *cdc25* and *cdc13* have been shown to be cell cycle regulated (Creanor & Mitchison, 1996; Oliva et al., 2005; Rustici et al., 2004; Peng et al., 2005) which is in agreement with my transcript and protein data. *cdc25* and *cdc13* do not cluster with defined transcript groups, such as *fkh2*, and peak around the same time in late G2/M. *cdc13* expression is regulated by the MAPK pathway in response to cellular stress (Bandyopadhyay, Dey, Suresh, & Sundaram, 2014). However, the mechanism that controls expression of *cdc25* and *cdc13*, under normal growth conditions, is still unknown. There could be a size-dependent transcription factor that

regulates *cdc25* and *cdc13* in a size-dependent manner. Transcription has been shown to increase with the increase in cell size to accommodate the demands of the bigger cell (Zhurinsky et al., 2010; Wu, Rolfe, Gifford, & Fink, 2010). As a more general mechanism, regulation of *cdc25* and *cdc13* expression could be tied to the general increase in transcription, but this requires the *cdc25* and *cdc13* transcripts to have characteristics necessary to reflect the increase in transcription. Since *cdc25* and *cdc13* transcripts have relatively short half-lives, it suggests a model where transcription initiation events could be the mechanism that monitors the increase in transcription and ultimately the increase in cell size.

Therefore, I propose a two trigger model where both Cdc25 and Cdc13 are sizers. Cdc25 will trigger mitosis in a normal cell under normal growth conditions in a size dependent manner. However, if Cdc25 expression is compromised, Cdc13 can accumulate to a point where there is enough Cdc2-Cdc13 complex to overcome the inhibition by Wee1 and trigger mitosis. The model requires that there is some minimal amount of Cdc25 that is necessary to trigger the feedback loop to activate Cdc2 and a minimal amount of Cdc13 in order to form some Cdc2-Cdc13 complex.

The two trigger model does not invoke any localization of the factors involved in sizing the cell but instead relies on total cellular concentration. However, the factors involved in triggering mitosis have distinct patterns of localization suggesting that there is a spatial regulation of the mitotic commitment pathway (Audit et al., 1996; Lopez-Girona, Furnari, Mondesert, & Russell, 1999; Moseley et al., 2009; Masuda, Fong, Ohtsuki, Haraguchi, & Hiraoka, 2011; Deng & Moseley, 2013). To test if localization plays a part in triggering mitosis in a size dependent manner, factors, such as Cdc25 and Wee1, can be mislocalized to see if the model is dependent on localization.

This study provides evidence for size dependent increase in protein concentration as a model for how cells control cell size. These results suggest that size control is an intrinsic property of the basic cell cycle machinery. The basic mitotic entry pathway is well conserved and provides an intriguing possibility that the size sensing aspect that I see in *S. pombe* could be conserved.

Chapter 3: Mitotic Activity of the B-type Cyclin Cig2

Introduction

Cyclin dependent kinases (CDK) and cyclin, its obligate binding partner, regulate progression through the cell cycle. The exact role of cyclins in regulating CDK activity is still debated. Cyclins could be functional regulators of CDK activity, helping to target CDK to substrates. Conversely, cyclins could modulate the activity of CDK to regulate progression through the cell cycle. Although there is likely truth to both views of cyclin function, mounting evidence suggests that, in general, cyclins quantitatively regulate the activity of CDK to regulate the cell cycle and that there is significant overlap of substrate specificity between B-type cyclins.

S. cerevisiae has nine cyclins, three G1 cyclins termed Cln type cyclins and six B-type cyclins that are active during S-G2-M phases. At least one Cln type cyclin (Richardson, Wittenberg, Cross, & Reed, 1989; Cross,

1990) or over expression of the B-type cyclin Clb5 (Epstein & Cross, 1992; Schwob & Nasmyth, 1993) is necessary for proper G1 progression. Overexpression of Clb1 or expression of Clb2 from a Clb5 promoter in the absence of the inhibitor Swe1 can compensate for deletion of all other Clb genes (Fitch et al., 1992; Richardson, Lew, Henze, Sugimoto, & Reed, 1992; Haase & Reed, 1999; Hu & Aparicio, 2005). These results suggest that, to a certain degree, there is an overlap of activity among Cln and Clb cyclins. However, there are examples of cyclin specific targets which suggest a more complicated regulation of CDK activity, where CDK activity and substrate specificity both play a role in cell cycle regulation (Oehlen & Cross, 1998; Wu, Leeuw, Leberer, Thomas, & Whiteway, 1998; Loog & Morgan, 2005; Strickfaden et al., 2007; Koivomagi et al., 2011; Bhaduri & Pryciak, 2011).

S. pombe has four cyclins. Three cyclins are involved in the G1 to S phase transition: Cig1, Cig2, and Puc1. Cig1 and Cig2 are B-type cyclins while Puc1 is a Cln type cyclin. Cig1, Cig2, and Puc1 are nonessential and deletion of any combination of the three cyclins has very little effect on the morphology or progression of cells through the cell cycle (Bueno, Richardson, Reed, & Russell, 1991; Bueno & Russell, 1993; Forsburg &

Nurse, 1991; Connolly & Beach, 1994). The fourth cyclin, Cdc13, is a B-type cyclin involved in entry and progression through mitosis. Deletion of *cdc13* results in cells arresting in late G2 before entry into mitosis and going through rounds rereplication (Nurse, Thuriaux, & Nasmyth, 1976; Booher & Beach, 1987; Booher & Beach, 1988; Booher, Alfa, Hyams, & Beach, 1989; Moreno, Hayles, & Nurse, 1989; Hayles, Fisher, Woollard, & Nurse, 1994). A double mutation of *cig2* and *cdc13* causes the cells to arrest in G1 without rereplication, suggesting that Cig2 or Cdc13 is necessary for S phase progression (Mondesert, McGowan, & Russell, 1996). Interestingly, deleting Cig1, Cig2, and Puc1 is not detrimental to the cells. The cells are viable with the only phenotype being a delayed entry into S phase (Fisher & Nurse, 1996; Martin-Castellanos, Blanco, de, J M, & Moreno, 2000). This means that Cdc13 can drive the entire cell cycle. Like *S. cerevisiae*, there is overlap in the function of the different cyclins in *S. pombe*.

From this data, a model was proposed where CDK activity regulates the G1/S and G2/M transition (Fisher & Nurse, 1996; Mondesert et al., 1996). In this quantitative model the level of activity of the CDK-Cyclin complex determines the phase of the cell cycle. Cells that have no cyclin, and

therefore no CDK activity, are in G1. In cells where cyclin bound CDK is inhibited by phosphorylation, a small amount of CDK activity transitions the cell from G1 to S phase. In cells where cyclin bound CDK is not inhibited by phosphorylation, a large amount of activity transitions the cell from G2 to mitosis. Recent results gave striking evidence that supports this model. Cdc2, the main CDK in *S. pombe*, was fused to Cdc13 and driven by the *cdc13* promoter in order to simplify the cell cycle machinery. This CDK fusion protein, in a background where the other cyclins were deleted, was able to drive the cell cycle (Coudreuse & Nurse, 2010). Manipulating the activity of CDK fusion protein artificially changed the phase of the cell cycle the cell was in, thus providing strong evidence for the CDK activity model.

To further test the model, two inhibitory kinases, *wee1* and *mik1*, that phosphorylate Cdc2 on tyrosine 15 were deleted in the strain expressing the Cdc2-Cdc13 fusion protein to make a situation where there was no inhibitory CDK phosphorylation. Wee1 is active during S phase and G2, while Mik1 is active during S phase. In wild type cells, *wee1* and *mik1* deletions are synthetically lethal due to uncontrolled entry into mitosis (Lundgren et al., 1991). These cells have high CDK activity late in S phase

due to the lack of inhibitory phosphorylation and thus have no G2 growth phase and go through mitosis when the cells are too small. It was surprising that the CDK fusion protein can rescue this synthetic lethality and suggests the CDK fusion protein may have altered activity compared to the wild-type proteins. Additionally, the cells were wild type length from which the authors concluded that the accumulation of Cdc13 regulated cell size (Coudreuse & Nurse, 2010). However, there were no cyclins in the background for the CDK fusion protein and therefore it is possible that the lack of one of the cyclins could be the reason for the suppression of the *wee1Δ mik1Δ* synthetic lethality. This would mean that mitosis could be triggered by a different cyclin other than Cdc13 binding to and activating Cdc2. If true, these results could provide evidence for a quantitative model of cell cycle progression where the activity of Cdc2 determines the phase of the cell cycle. The best candidate for possible rescue of the synthetic lethality is the S phase B-type cyclin *cig2*. The results show that the activity of Cdc2, regulated by Cig2 or Cdc13 cyclin binding in the absence of Cdc2 phosphorylation, can regulate entry into mitosis. Building on the results from the previous chapter, the accumulation of Cdc13 activity, presumably by the size dependent increase in Cdc13 protein, could

regulate entry into mitosis in the event that Cdc25 expression is compromised.

Results

To test whether the lack of Cig2 could be responsible for the viability of the *cdc13-cdc2 wee1-50 mik1*∆ strain, I tested if Cig2 drives lethality in *wee1-50 mik1*∆ cells. I made a *wee1-50 mik1Δ cig2Δ* triple mutant and compared it to a *wee1-50 mik1Δ* double mutant. The *wee1-50* allele is a temperature sensitive allele that is functional at 25℃, has almost no activity at 30℃, and has no activity at 35℃ (Rhind & Russell, 2001). The *wee1-50 mik1*∆ double mutant is alive at 25℃ and dead at 30℃ and 35℃. The triple mutant is alive at 25℃, alive at 30℃, and dead at 35℃ (Figure 3.1). The size of the *wee1-50 mik1Δ cig2Δ* triple mutant is similar to a *wee1-50* single mutant at 30℃. (Figure 3.2)

Since the triple mutant cells are alive at 30℃ but not 35℃, it suggests that *cig2Δ* can partially rescue the synthetic lethality of *wee1-50 mik1Δ*. The fact that a deletion of *cig2* can rescue the synthetic lethality of *wee1-50*

Figure 3.1 Genetic Interactions of *cig2* and *cdc13*

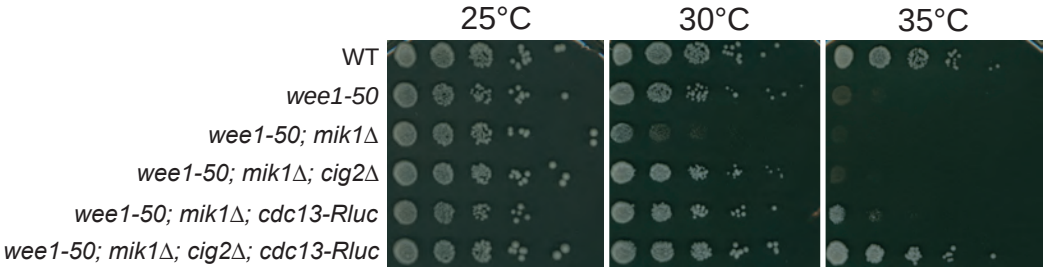


Figure 3.1 Genetic Interactions of *cig2* and *cdc13*

A 3-fold serial dilutions of *cig2* and *cdc13* mutant strains were plated on YES and grown at indicated temperatures to assay for strain viability. *cig2Δ* and *cdc13-Rluc* partially rescue *wee1-50 mik1Δ* at 30℃ but not 35℃. The combination of *cig2Δ* and *cdc13-Rluc* rescue the synthetic lethality at 35℃.

Figure 3.2 Phenotypes of Cyclin Mutants

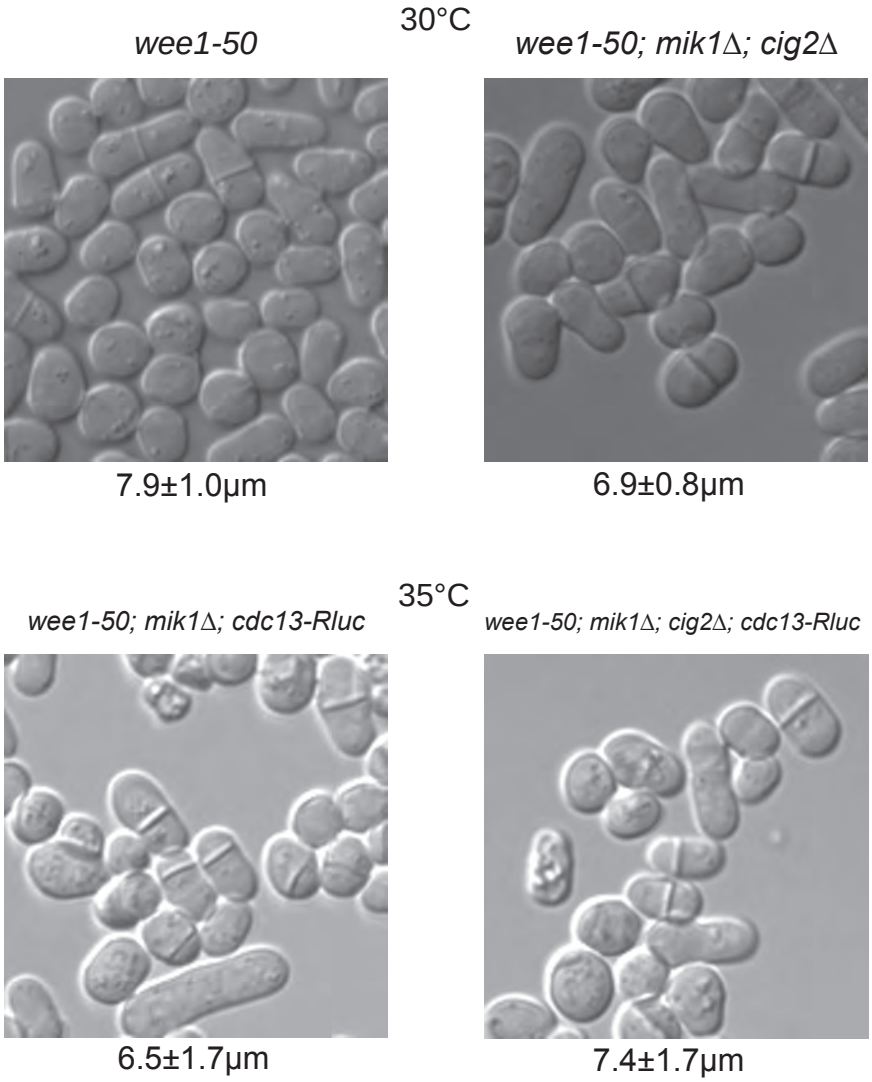


Figure 3.2 Phenotypes of Cyclin Mutants

Cyclin mutants grown at indicated temperature. Average length ± standard deviation of strain indicated below picture. All strains show a wee phenotype, although some are stronger than others. For each strain in each condition, 50 cells were measured to get the mean and standard deviation of the population of cells.

mik1Δ suggests that Cig2 can activate Cdc2 to trigger an inappropriate mitosis when the cells are too small. The difference in phenotype between the two temperatures is likely due to the small amount of Wee1 activity at 30℃. At 35℃ the activity of Cdc2 would be unchecked by phosphorylation and the cells would enter mitosis early. However, at 30℃, the little bit of Wee1 activity would prevent *wee1-50 mik1*Δ *cig2*Δ, but not *wee1-50 mik1*Δ cells from going through mitosis while they are too small.

Cdc13 is most likely activating Cdc2 in the *wee1-50 mik1Δ cig2Δ* triple mutant at 35℃ to cause the lethality. To test the genetic interaction of the *wee1-50 mik1Δ cig2Δ* triple mutant with *cdc13*, I used a *cdc13* allele tagged with *Renilla* luciferase as a hypomorphic allele due to its slow growth. A *wee1-50 mik1Δ cdc13-Rluc* triple mutant was alive at 30℃ but dead at 35℃. However, a quadruple mutant of *wee1-50 mik1Δ cig2Δ cdc13-Rluc* was alive at both 30℃ and 35℃ (Figure 3.1) with a wee phenotype (Figure 3.2). These results reinforce the conclusion that S-phase expression of Cig2 contributes to the lethality due to premature mitotic entry due to the lack of tyrosine phosphorylation.

The *cdc13* hypomorphic allele has the same genetic interaction with *wee1* and *mik1* as *cig2*. Although *cig2Δ* or *cdc13-Rluc* partially rescue the synthetic lethality of *wee1-50 mik1Δ*, the combination of *cig2Δ* and *cdc13-Rluc* do completely rescue the synthetic lethality of *wee1-50 mik1Δ*. These results highlight the fine control of CDK activity to control the progression through the cell cycle. The results also show that, like *S. cerevisiae*, there is significant overlap in cyclin activity and that Cig2 may have the ability to substitute for Cdc13 since the phenotype at 30℃ is the same for the *cig2Δ* triple mutant and the *cdc13-Rluc* triple mutant.

To test if Cig2 activity is capable of driving a viable mitosis in the absence Cdc13, I made a *wee1-50 mik1Δ cdc13-117* strain and a *wee1-50 mik1Δ cdc13-117 cig2Δ* strain. I inactivated *wee1* and *cdc13* by shifting an asynchronous culture to the nonpermissive temperature at 35℃. In the *wee1-50 mik1Δ cdc13-117 cig2Δ* quadruple mutant I saw the same elongated cell arrested in mitosis phenotype as in a *cdc13-117* single mutant strain, demonstrating that the lack of both Cdc13 and Cig2 prevent the cells from entering mitosis when there is no tyrosine phosphorylation of Cdc2. In the *wee1-50 mik1Δ cdc13-117* triple mutant, where there was still active Cig2, I saw a multiseptated phenotype where each cell had

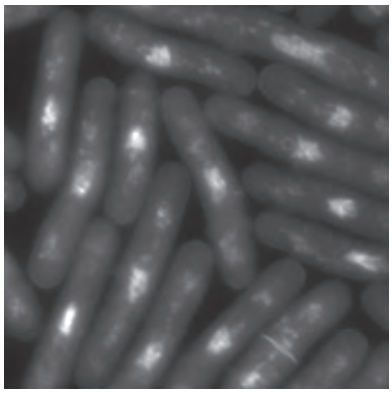
multiple septum and often the nucleus had not segregated properly. In some cases the nucleus was intact at one end of the cell, while in other cases there were bits of nuclear material in each septated compartment (Figure 3.3). Therefore, Cig2 can trigger mitosis, although there are many problems with chromosome segregation and septation regulation.

Conclusions

Previously it was thought that Cdc13 was the only cyclin that could activate Cdc2 to trigger mitosis in *S. pombe*. To counteract the activity of Cdc2, tyrosine phosphorylation of Cdc2 by *wee1* and *mik1* regulates entry into mitosis. A *wee1-50 mik1Δ* double mutant is lethal due to the unregulated activity of Cdc2. However, a fusion between Cdc13 and Cdc2 was shown to not require tyrosine phosphorylation to regulate entry into mitosis (Coudreuse & Nurse, 2010). However, I have shown that Cig2, the other B-type cyclin in *S. pombe*, can partially rescue the synthetic lethality of *wee1-50 mik1Δ* double mutant. This partial ability to rescue the *wee1-50 mik1Δ* synthetic lethality may be the reason the Cdc13-Cdc2 fusion does not require tyrosine phosphorylation. I have also shown that

Figure 3.3 Multiple Septum Phenotype of Cig2 Driven Mitosis

wee1-50; mik1Δ; cdc13-117; cig2Δ



wee1-50; mik1Δ; cdc13-117

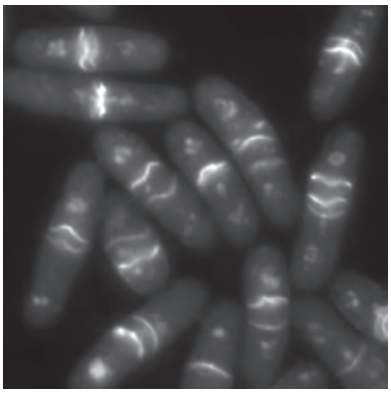


Figure 3.3 Multiple Septum Phenotype of Cig2 Driven Mitosis

In the absence of CDK phosphorylation and Cdc13, Cig2 triggers an aberrant mitosis with the formation of multiple septa. Cells were grown at 35℃ for 4 hours and stained with DAPI and Calcofluor to visualize DNA and septums.

Cig2 and Cdc13 overlap in their ability to trigger mitosis. The activity of Cig2 is enough to trigger an aberrant mitosis with problems in chromosome segregation and septation regulation.

These results highlight how sensitive the cell cycle is to CDK activity. I hypothesize that deleting *cig2* delays the increase in CDK activity enough to partially rescue the *wee1-50 mik1Δ* synthetic lethality. In order to fully rescue the *wee1-50 mik1Δ* synthetic lethality, *cdc13* activity also has to be impaired. In the case of the *wee1-50 mik1Δ cig2Δ* triple mutant at 30℃ and the *wee1-50 mik1Δ cig2Δ cdc13-Rluc* quadruple mutant at 35℃ the cells are wee and therefore the activity of CDK is barely inhibited to allow the cells to grow enough to go through a successful mitosis (Figure 3.4).

The regulation and activity of Cig2 can regulate CDK activity to prevent an early mitosis. Cig2 is normally expressed in S phase during the time that both *wee1* and *mik1* are expressed. Thus, both kinases can regulate the activity of Cdc2 when it is bound to Cig2. Additionally, the activity of Cig2 may be lower than Cdc13. It has already been shown in *S. cerevisiae* that even though cyclin activity may overlap, one cyclin may be better for phosphorylating a target (Loog & Morgan, 2005).

Figure 3.4 Model for CDK Activity in Response to Cyclin Binding

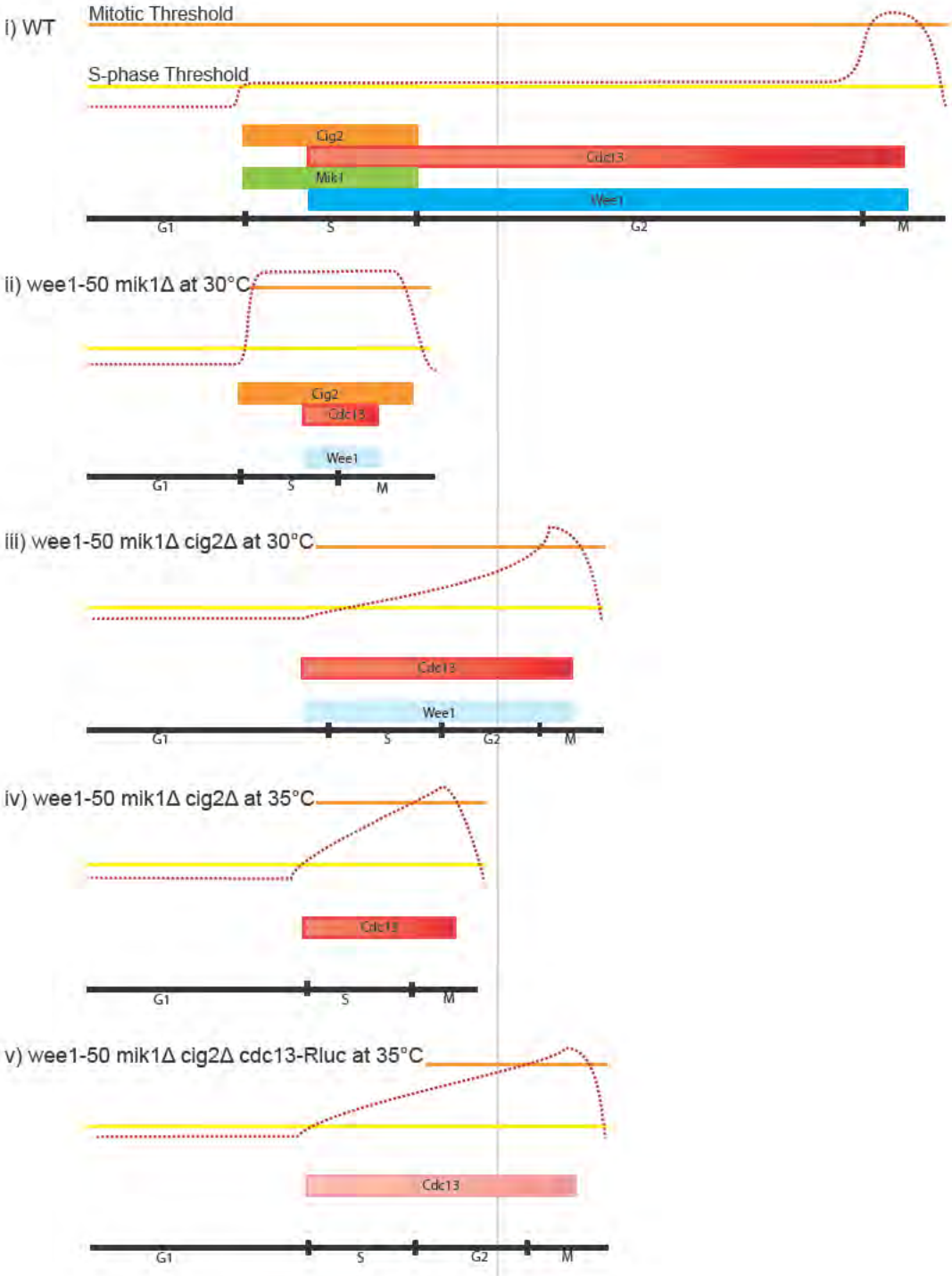


Figure 3.4 Model for CDK Activity in Response to Cyclin Binding

A diagram illustrating the effect cyclin mutants on CDK activity. The bars in the lower part of each panel represent time of expression of indicated proteins. The red dotted line represents CDK activity. The vertical grey line represents the minimal length of cell cycle necessary for the cell to be viable. i) Wild type cell cycle. Cig2 causes a small increase in CDK activity to trigger S phase. This activity is kept in check by Wee1 and Mik1. The increase of Cdc13 causes CDK activity to rise to level necessary to trigger mitosis. ii) A *wee1-50 mik1Δ* cell grown at 30℃ will have high CDK activity too early and will go through a premature mitosis. iii) A *wee1-50 mik1Δ cig2Δ* cell grown at 30℃ will have a small amount of Wee1 activity which will keep CDK activity in check just long enough to keep the cells viable. iv) A *wee1-50 mik1Δ cig2Δ* cell grown at 35℃ will have a high CDK activity due to Cdc13 and will have a premature mitosis. v) A *wee1-50 mik1Δ cig2Δ cdc13-Rluc* cell grown at 35℃ will have slowly rising CDK activity due to the hypomorphic allele of Cdc13 that will delay mitosis just long enough to keep the cells viable.

Further experiments need to be done to test the activity of Cig2. Ectopically expressing *cig2* in G2 and M phases may determine if Cig2 can fully substitute for Cdc13. Two possible ways to express *cig2* ectopically are to swap promoters with *cdc13* and/or swap the destruction box with *cdc13*. I made the *cig2-cdc13* promoter swap construct but was unable to transform it into cells. Secondly, swapping the *cig2* destruction box for the *cdc13* destruction box did not affect the expression of *cig2* (Coudreuse, personal communication). It is possible that these two strategies need to be combined to ectopically express *cig2*.

Furthermore, testing the kinase activity of Cdc2 bound to Cig2 or Cdc13 will determine the relative activity of these two cyclins. Isolated complexes can be assessed for their ability to phosphorylate different cell cycle targets to see if there is differential kinase activity. The kinase assay can be used to assess if the overlap in Cig2 and Cdc13 activity is due to a qualitative overlap in targets or simply a quantitative ability to activate Cdc2.

On one hand, the ability of Cig2 to trigger mitosis supports a quantitative model for cell cycle progression in *S. pombe*, as was previously proposed

(Coudreuse & Nurse, 2010). The binding of Cig2 or Cdc13 to Cdc2 provides enough CDK activity to trigger mitosis in the absence of tyrosine phosphorylation. Also, since *cig2Δ cdc13-Rluc* rescues the synthetic lethality of *wee1-50 mik1Δ*, it suggests that the reason the Cdc13-Cdc2 fusion does not require tyrosine phosphorylation is that the fusion is hypomorphic, and thus the activity of CDK takes longer to rise to a level necessary for entry into mitosis when compared to a wild type cell.

On the other hand, the mitosis triggered by Cig2 is defective. This could be due to either the Cig2-Cdc2 complex having insufficient activity of to drive CDK or the Cig2-Cdc2 complex cannot target key effectors of mitosis. On the former point, the Cig2-Cdc2 complex may have enough activity to trigger entry into mitosis but not enough activity to complete mitosis. On the later point, there could be certain targets that the Cig2-Cdc2 complex is unable to phosphorylate. The proposed experiments above will hopefully sort these two points out.

Chapter 4: Final Summary and Discussion

Cell size regulation, or coordination between growth and division, is essential for proper cell function. Without regulation of cell size, the small fluctuations in these biological processes would lead to a large distribution of cell size in a given population of cells. Despite repeated efforts over the past forty years to define this mechanism of size regulation, we still do not understand how cells measure size.

My initial goal for this thesis project was to determine how *Schizosaccharomyces pombe* regulates cell size. Early research on *S. pombe* cell cycle showed clear size control checkpoints at two points in the cell cycle, the G1/S transition and the G2/M transition (Nurse & Thuriaux, 1977; Nurse, 1975; Fantes & Nurse, 1977; Fantes & Nurse, 1978). Cdc25 and Wee1 were long implicated in G2 cell size regulation in *S. pombe*, however there was no mechanism to describe how cell cycle regulation works (Novak & Tyson, 1993; Novak & Tyson, 1995; Sveiczer,

Csikasz-Nagy, Gyorffy, Tyson, & Novak, 2000; Sveiczer, Tyson, & Novak, 2004).

In chapter two, I show that Cdc25 accumulates in a size dependent manner in G2 while Wee1 levels stay relatively constant. This accumulation of Cdc25 occurs over a large range of cell sizes. Additionally, experiments with short pulses of cycloheximide have shown that Cdc25 is inherently an unstable protein that quickly returns to a size dependent equilibrium in the cell, suggesting that Cdc25 concentration is dependent on size and not time. Furthermore, transcript levels mirror protein concentration suggesting that transcriptional and post transcriptional regulation play a part in regulating the size dependent Cdc25 protein concentration.

However, cells are still viable when Cdc25 is constitutively expressed suggesting that there is another sizer in the case that Cdc25 expression is compromised. Cdc13 is a likely candidate as a second sizer due to the similar characteristics to Cdc25 and its ability to activate Cdc2. Cdc13 protein and transcript accumulates during the cell cycle in a similar manner to Cdc25.

There are two possible ways to interpret these results. One possibility is that Cdc25 is not a sizer and it is simply a coincidence that Cdc25 accumulates with the increase in cell size. If this is true, it is intriguing that Cdc25 does increase in a size dependent manner and thus is still worth studying as it could be related to size control in the cell. Figuring out the mechanism of size dependent expression of Cdc25 could lead to the sizing mechanism of the cell. One plausible mechanism is a size dependent transcription factor that regulates a number of size related genes. This could explain why Cdc25 and Cdc13 have such similar expression profiles.

The second possibility is a two sizer model where Cdc25 is the main sizer with Cdc13 as a backup sizer in the event of Cdc25 expression being compromised. Ultimately, high CDK activity drives entry into mitosis. In a normal functioning cell, the balance of Cdc25 and Wee1 regulate CDK activity in a switch-like fashion (Pomerening et al., 2003; Pomerening et al., 2005; Lu et al., 2012). Wee1 provides the threshold that Cdc25 has to overcome in a size dependent manner to trigger mitosis. In the event that Cdc25 expression is compromised, the accumulation of Cdc13 could form

enough Cdc13-Cdc2 complex to trigger the feedback loops to produce high CDK activity.

There are a couple of things to consider about this model. There is no obvious factor to set a threshold for Cdc13 to overcome in the case where Cdc25 expression is compromised. One possibility that could set the threshold for Cdc13 is Wee1. The assumption would be that phosphorylated Cdc2 would have some activity and the increasing amount of Cdc13 would create enough Cdc2-Cdc13 complex to trigger mitosis. One way to test if Wee1 sets the threshold for Cdc13 in a similar manner to Cdc25 is to see if the size of the cell increases with the dose of Wee1 in a strain where Cdc25 expression is compromised (Russell & Nurse, 1987). Additionally, many pathways that affect cell size, such as nutrition and morphology, signal through Cdc25 and/or Wee1, and therefore Cdc13 cannot adjust to changes in the cellular environment. Developmentally, Cdc13 could be an ancestral sizer and Cdc25 and Wee1 evolved to better respond to perturbations from the cellular environment.

Another consideration is investigating how relatively low expressed genes can provide a robust sizing mechanism. Cdc25 and Wee1 are expressed

at relatively low levels in the cell with only a few transcripts per cell (Marguerat et al., 2012). The low number of transcripts per cell suggests that any stochastic variance in expression could have a large impact on cell size. However, that is not what is observed and thus the cell must have some way to ensure a robust sizer with low expressed components. My data shows clear trends in expression of Cdc25 and Wee1 (Figure 2.10) providing evidence for transcriptional regulation is tightly controlled to ensure proper expression.

In chapter three, I show that deletion of *cig2* or a hypomorphic allele of *cdc13* can partially rescue the synthetic lethality of *wee1-50 mik1Δ*. Together, cig2*Δ* and the hypomorphic allele of *cdc13* can fully rescue the synthetic lethality of *wee1-50 mik1Δ*. The ability of *cig2* to partially rescue the synthetic lethality of *wee1-50 mik1Δ* suggests that the *wee1-50 mik1Δ* dies due to premature mitosis caused by Cig2-Cdc2 complex and therefore *cig2* can trigger mitosis. I show that *cig2* can trigger mitosis, although the fidelity of the mitosis is low. These results support the quantitative model of cell cycle progression where CDK activity determines the phase of the cell cycle. The overlap in *cig2* and *cdc13* activity suggests that general cyclin binding to Cdc2 determines CDK

activity and progression through the cell cycle. However, further experiments to assay the activity of Cdc2 when in complex with Cdc13 or Cig2 are needed to completely rule out a qualitative model of cell cycle progression.

The results I present in this dissertation provide the groundwork for understanding how cells regulate size and how this size regulation affects cell cycle control in *S. pombe*. The results show how the intrinsic cell cycle machinery can act as a sizer for the G2/M transition in *S. pombe*.

With recent results from *S. cerevisiae* (see chapter 2 introduction), the protein concentration model is proving to be a general model for how cells regulate cell size. Even though both models from *S. cerevisiae* and *S. pombe* rely on protein concentration as an effector for the sizer, there is a fundamental difference that separates these two models. In *S. cerevisiae*, inhibitor dilution regulates the transition from G1 to S phase. In *S. pombe* activator accumulation regulates the transition from G2 to M phase. Thus two different concentration models may regulate different phases of the cell cycle in eukaryotes.

Future work should focus on deciphering a mechanism to confirm whether or not the protein concentration model is the general model for size control. Understanding the mechanism of how protein concentration is regulated in a size dependent manner will give much needed insight into how cells control size. Elucidating the mechanism for size control will capitalize on decades of research and deepen our understanding of basic cell biology.

Interestingly, this mitotic commitment pathway is well conserved suggesting a general solution for size control in eukaryotes at the G2/M transition. Further experiments can establish if the same dynamics seen in *S. pombe* are conserved in multicellular organisms. If the Cdc25 dynamics are conserved, it provides a possible mechanism to how cells can adjust size. In *S. pombe*, there are many pathways that sense the cellular environment and adjust the expression of Cdc25 to adjust the size of the cell. These signaling networks could be conserved in multicellular organisms and used to adjust the size of different cell types. Obviously many questions need to be addressed in order to have a good understanding of cell size control, but the observations I have made provide a starting point to understand the mechanism of cell size control.

Methods

Cell Culture

Strains were created and cultured using standard techniques (Forsburg & Rhind, 2006). Briefly, cells were grown in yeast extract plus supplements (YES) at 30°C. For strains with nmt expression, cells were either grown in Edinburgh minimal media for induced expression or EMM plus 15μM thiamine for repressed expression. Strains with temperature-sensitive alleles were grown at 25°C for permissive growth and switched to 35°C for non-permissive growth unless otherwise noted. To inhibit the analogue sensitive allele of cdc2, 1μM of 1NM-PP1 was added to the culture to block the cells in G2.

Synchronization and Time Course

Cells were synchronized by centrifugal elutriation. Cells were grown overnight to a concentration of 1 – 1.5 OD per mL. For the small chamber, ~400 OD of cells were loaded into the elutriator at 4100 rpm at 40 mL per minute flow. For the larger chamber, ~4000 OD of cells were loaded into the elutriator at 4100 rpm at 100 mL per minute flow. Cells were harvested

from the elutriator into fresh media at 0.10 – 0.20 OD per mL by either slowing down the rotor speed or increasing the flow of media.

For time points, time points were taken every 20 minutes to measure septation and take samples for protein measurement. Septation was monitored by counting unseptated, septated, and undivided pairs on a hemocytometer. Mitotic index was calculated by calculating the ratio of septated + undivided pairs divided by total count for that time point. For the luciferase assay, samples were first spun down to remove media. The samples were then collected in a screw top eppendorf, excess media was removed, and samples were put in liquid nitrogen for later processing.

Luciferase Assay

To process cell pellets for the luciferase assay I followed a modified procedure based on the Dual-Luciferase Reporter Assay (Promega, Madison WI). 5 – 10 OD cell pellets were lysed into 200µL 1X Passive Lysis Buffer by bead beating to a point where ~80% of the cells were lysed and kept on ice. Lysates were spun down at 16,000g at 4°C to remove cellular debris. 10µL of cleared lysate was loaded per well in a 96-well plate and each sample was read in triplicate at room temperature. For

each well, 50μL of Luciferase Assay Substrate and Stop and Glow Buffer are added sequentially to assay for beetle followed by Renilla luciferase. After the addition of each Substrate, the samples rest for 2 seconds followed by a 10 second measurement for luminescence.

Transcript Quantitation

For Nano String (Seattle, WA) quantitation, $1x10^7$ cells were fixed with 70% methanol and stored at -80°C in 1mL of RNALater (Ambion). For processing, cells were spun down to remove the RNALater. Cells were then resuspended and bead beat in 600μL RLT buffer (Qiagen) with 1% β-Mercaptoethanol. 200μL of lysate was spun down at 16,000g and 3μL of supernatant was processed according to Nano String protocol.

Transcript Half Life and RT-qPCR

For calculation of transcript half-life, yFS105 cells from a log phase culture grown in YES at 30°C were treated with 15 μg/mL Thiolutin to inhibit polymerase II and 10 OD samples were taken at 0, 5, 10, 30, and 60 minutes. Half life curves were fit to an exponential curve using Igor Pro (WaveMetrics) to estimate tau and half life. Samples were collected in screw top eppendorf tube and stored in liquid nitrogen. Total RNA was

isolated from pellets using the Direct-zol kit (Zymo Research, Irvine, CA) First strand synthesis was performed using random hexamers and SuperScript III first strand synthesis kit (Invitrogen). qPCR was performed using Kapa SYBR Fast qPCR kit (Wilmington, MA). Transcripts were normalized to 0 time point and *srp7* as an internal control for a stable transcript. Primers for each target are as follows: *cdc25* - DK38, DK39; *wee1* - DK36, DK37; *cdc13* - DK368, DK369; *upf2* - DK374, DK375; *srp7* - DK372, DK373.

Excess Delay Assay and Protein Half Life

To measure half life of proteins, strains with luciferase tagged protein of interest was grown to log phase in YES at 30°C. 100 µg/mL of the translation inhibitor cycloheximide was added to the culture and samples of 10 OD were taken at 0, 5, 10, 30, and 60 minutes. Half life curves were fit to an exponential curve using Igor Pro (WaveMetrics) to estimate tau and half life. Samples were collected in screw top eppendorf tube and stored in liquid nitrogen. Samples were processed by luciferase assay.

To assay for excess delay a time course, as described above, was modified by splitting the synchronized culture into two subcultures. One

subculture was treated with a 20 minute pulse of 100 µg/mL of cycloheximide. Cycloheximide was removed by filtration and cells were put into new, warmed media. The time of the pulse of the cycloheximide was chosen prior to starting time course in order to calculate how much culture was needed for the experiment.

Hydroxyurea Arrest and Release to Test for Size Control

To assay for size control cells were were grown to log phase in YES or EMM if a gene was expressed using an nmt promoter. Cells were treated with 3 mM Hydroxyurea to block cells in early S phase for 5 hours to allow cells to elongate past normal size. The Hydroxyurea was then washed out by pelleting the cells and resuspending them in fresh media.

Microscopy

Cells were imaged by DIC microscopy using an Axioscop 2plus microscope fitted with a 100x oil immersion lens and Spot RT Monochrome digital camera with Spot software. A micrometer was used to set a scale for images. ImageJ (NIH) was used to analyze images and measure cells.

For DAPI and Calcofluor staining cells were fixed with 70% methanol at -20°C. Cells were then washed in PBS and stain with a DAPI and Calcofluor solution that contained 1 µg/mL DAPI, 1 mg/mL Calcofluor, 1 mg/mL p-phenylenediamine in 20% PBS / 80% glycerol solution.

Appendix

Screen for G1 Cell Size Control Factors

The mechanisms that regulate cell size in eukaryotes is still unknown. Two size checkpoints have been identified, one at the G1/S boundary and one at the G2/M boundary. Many eukaryotes use the G1/S boundary as the main size checkpoint during the cell cycle.

Previous work has characterized the two size checkpoints in the *S. pombe* cell cycle. The size checkpoint at the G2/M boundary is the main size checkpoint in exponentially growing cells. The CDK inhibitory kinase Wee1 has been implicated in the G2/M size checkpoint by inhibiting CDK in a size dependent manner. Deletion of *wee1* removes the inhibition of CDK, ablating the G2/M size checkpoint. Cells with a *wee1* deletion have no way to regulate CDK activity during G2 phase and therefore the cells enter mitosis shortly after S phase with little to no G2 phase. Since these cells have no G2 phase, they do not have sufficient time to grow to a normal size and enter mitosis at a small size.

The size checkpoint at G1/S boundary is usually not invoked in exponentially growing cells because freshly divided cells fulfill the size requirements to bypass the checkpoint. The G1/S size checkpoint is invoked when the cell is too small after division to pass the G1/S phase size checkpoint, such as in a *wee1Δ* mutant (Nurse & Thuriaux, 1977). Thus, in *S. pombe*, the G1/S size checkpoint is nonessential and can be manipulated by genetic techniques, providing a method to assay for genes that are essential for the G1/S size checkpoint.

With current genetic tools it is possible to screen for genes that are involved in the G1/S size checkpoint. Using *wee1-50*, a temperature sensitive allele of *wee1*, I can conditionally inactivate the G2/M size checkpoint. These cells will rely on the G1/S size checkpoint to regulate cell size in the absence of the G2/M size checkpoint. Therefore a gene involved in the G1/S size checkpoint will be synthetically lethal with the *wee1-50* allele at the nonpermissive temperature since the cell will have no way to regulate cell size.

Two methods have been developed to systematically assay double mutants for genetic interactions. These methods rely on the use of the *S.*

pombe deletion collection (Kim et al., 2010), where every nonessential gene has been deleted, allowing for the systematic generation of double mutants of *wee1-50* with a specific nonessential gene. In order to systematically generate double mutants there are two points that need to be addressed: i) select for the double mutants to eliminate single mutants and wild-type cells; and ii) eliminate unmated vegetative cells and diploid cells to select for spores.

The first point is solved in the same way in the two methods and is easy to solve by simply tagging the two gene deletions with different selective markers. In the case of the *S. pombe*, the deletion collection is marked with a G418 resistant marker. To compliment this, I tagged the *wee1-50* allele with a Nourseothricin (NAT) resistance marker. Therefore, double mutants can be selected by plating on a plate that contains both G418 and NAT.

Where the two methods differ is the way they eliminate the vegetative and diploid cells. In one strategy, termed the *Sp*SGA for S. pombe Synthetic Genetic Array, vegetative and diploid cells are eliminated by treating the mating plaque, which contains a mixture of spores and unmated cells, at

42°C which selectively kills the vegetative and diploid cells but not the spores (Dixon et al., 2008). In the other strategy, termed PEM-2 for *pombe* epistasis mapper, a recessive cycloheximide resistant marker is tied to one mating type (Roguev, Wiren, Weissman, & Krogan, 2007). Selection on cycloheximide will isolate only cells with one mating type, killing the cells with the other mating type and diploid cells.

To test these two strategies I constructed the appropriate strains containing the *wee1-50* allele tagged with NAT and the appropriate background for the PEM-2 strategy (yDK101 for the *SpSGA* strategy and yDK104 for the PEM-2 strategy). I then selected a few strains to do a test run of the screen to see which strategy worked better. I selected *his3Δ* as a control that would not have a genetic interaction with *wee1-50* and *rum1Δ* and *rad3Δ*, which have known synthetic lethal interactions with *wee1-50*.

The test runs showed that there is a technical problem with the screen. In both strategies, there were colonies that grew in the *rum1Δ* and *rad3Δ* crosses. The number of colonies growing in the *rum1Δ* and *rad3Δ* crosses were fewer than in the *his3Δ*, suggesting that the selection is working to

some degree. When I examined the the colonies that did grow in the *rum1Δ* and *rad3Δ* crosses, all the colonies were diploid. Interestingly, when I compared the number of colonies in the *rum1Δ* and *rad3Δ* crosses in the *Sp*SGA and PEM-2 strategy, the PEM-2 strategy had a quarter of the number of colonise as the *Sp*SGA. From these results, I hypothesize that the “escaper” colonies arise from diploid spores that result from an error in meiosis. At some frequency, all matings will have diploid spores that result from an error in meiosis. The fact that the PEM-2 strategy has a quarter of the escaper colonise as the *Sp*SGA strategy makes sense because the PEM-2 will select for diploids that are homozygous for a h+ mating type. The *Sp*SGA will have diploids that have a Mendelian distribution of mating types.

To overcome this technical problem there are two approaches one could take. One approach is to add another selection against diploids in the PEM-2 strategy. The chance of two selection markers segregating together will decrease the number of escapers by a factor of 4. This rate of escapers could be low enough to run the assay. However, there will still be escapers which will cause a background that may make it hard to interpret results. A second approach would be to limit the number diploids

that are formed. A *wee1* deletion is known to have a high rate of diploidization. One potential way to lower the rate of diploidization in a *wee1* mutant is to delete *cig2* to possibly prevent the cells from entering mitosis early (see chapter 3). This could give the cells more time to finish S phase and allow them to properly segregate the chromosomes. The first step would be to test if *cig2Δ* does lower the rate of diploid formation in a *wee1-50* cell at the nonpermissive temperature.

pFS	pDK	Short Description	Construction Details
345	pDK1	pFA6a with luc+ tagging cassette and Kan	Bluc+ from promega pGL3 was cloned into pFS230 in place of GFP using DK20 and DK21
346	pDK2	pFA6a with Rluc+ tagging cassette and Nat	Rluc+ from promega pRL-TK was cloned into pFS275 in place of GFP using DK22 and DK23
386	pDK6	pFA6a with IAA17 (Auxin degron) with NatR Marker	IAA17 Auxin Degron cloned into pFS275 in place of GFP using PacI and BglII restriction sites using DK75 and DK76
428	pDK18	nmt41:cdc25:Bluc	Bluc (DK140, DK141, pDK345) inserted at NotI and PspOMI site of pFS380. Then cdc25(DK142,DK143) inserted at NotI and XhoI.
429	pDK31	nmt41 promoter:cdc25(including 5'UTR):Bluc	Bluc (DK140, DK141, pDK345) inserted at NotI and PspOMI site of pFS380. Then cdc25+5'UTR(DK148,DK143) inserted at NotI and XhoI.
430	pDK22	cdc25 cloned into Strata vector	cdc25(DK142,DK143) strata cloned
431	pDK30	cdc25 with 5' UTR cloned into strata vector	Cdc25 + 5'UTR(DK148,DK143) strata cloned
432	pDK97	nmt41:cdc25:Bluc Flip in Plasmid	Constructed in three steps: 1. Stepwise addition of cdc25 fragments by traditional cloning: cdc25 promoter (DK186,187, EcoRI-KpnI into pUC19 (pDK69)) cdc25 5'UTR (DK188.189, PmeI-KpnI (pDK70)) cdc25 ORF (DK190,191, NcoI-KpnI (pDK71)). 2. Gibson assembly of the rest of cdc25 construct: luc (DK210, 211) cdc25 3'UTR (DK212, 213) and cdc25 down stream (DK214, 215) (pDK72). 3. Cloned nmt promoter and 5'UTR into EcoRI-NcoI site (pDK75). Added 300 bases upsteam of construct for flip-in (DK318,319 EcoRI-PstI)
433	pDK98	nmt41:cdc13 Flip in Plasmid	Constructed in two steps: 1. Gibson assembly: promoter (DK223, 224), cdc13 5'UTR (DK225, 226), cdc13 ORF (DK227, 228), cdc13 3'UTR (DK229, 230), downstream (DK231, 232) (pDK80). 2. nmt promoter inserted (DK284, 285, pDK75) and 300 bases upstream of construct for flip-in (DK316, 317).
434	pDK100	nmt1:cdc25:Bluc Flip in Plasmid	Constructed in three steps: 1. Stepwise addition of cdc25 fragments by traditional cloning: cdc25 promoter (DK186,187, EcoRI-KpnI into pUC19 (pDK69)) cdc25 5'UTR (DK188.189, PmeI-KpnI (pDK70)) cdc25 ORF (DK190,191, NcoI-KpnI (pDK71)). 2. Gibson assembly of the rest of cdc25 construct: luc (DK210, 211) cdc25 3'UTR (DK212, 213) and cdc25 down stream (DK214, 215) (pDK72). 3. Cloned nmt promoter and 5'UTR into EcoRI-NcoI site (pDK73). Added 300 bases upsteam of construct for flip-in (DK318,319 EcoRI-PstI)

435	pDK110	Cdc25 5'UTR:UBI-YdK-Bluc:cdc25 3'UTR	Constructed in two steps: 1 luc cloned in place of cdc25 ORF (DK193, 206, pDK1) intos pDK97 at AgeI-SalI. 2. UBI tag added to N-term of luc (DK362, 363, pNC1124 http://www.ncb .n m.n h.gov/pubmed/23172645).
436	pDK109	nmt1:cdc13 Flip in Plasmid	Constructed in two steps: 1. Gibson assembly: promoter (DK223, 224), cdc13 5'UTR (DK225, 226), cdc13 ORF (DK227, 228), cdc13 3'UTR (DK229, 230), downstream (DK231, 232) (pDK80). 2. nmt promoter inserted (DK284, 285, pDK73) and 300 bases upstream of construct for flip-in (DK316, 317).

yFS	Genotype	Construction Details
735	h- leu1-32 ura4-? wee1-50 (natMX)	yFS132 transformed with cassette(NR172, NR173, pFS272)
810	h- leu1-32 ura4-D18 ade4-Bluc(KanMX) wee1-Rluc(NatMX)	yFS866 crossed with yFS871
865	h- leu1-32 ura4-D18 wee1-Rluc(NatMX)	yFS105 transformed with cassette(NR172, NR173, pFS346)
866	h+ leu1-32 ura4-D18 wee1-Rluc(NatMX)	yFS104 transformed with cassette(NR172, NR173, pFS346)
867	h+ leu1-32 ura4-D18 cdc25-Bluc(KanMX)	yFS104 transformed with cassette(DK6, DK7, pFS345)
868	h- leu1-32 ura4-D18 cdc25-Bluc(KanMX)	yFS105 transformed with cassette(DK6, DK7, pFS345)
869	h+ leu1-32 ura4-D18 wee1-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS865 crossed with yFS867
870	h- leu1-32 ura4-D18 wee1-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS865 crossed with yFS867
871	h- leu1-32 ura4-D18 ade4-Rluc(NatMX)	yFS105 transformed with cassette(DK26, DK27, pFS346)
872	h- leu1-32 ura4-D18 ade4-Bluc(KanMX)	yFS105 transformed with cassette(DK26, DK27, pFS345)
873	h+ leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS867 crossed with yFS871
874	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS867 crossed with yFS871
875	h+ leu1-32 ura4-D18 ade4-Bluc(KanMX) wee1-Rluc(NatMX)	yFS866 crossed with yFS872
876	h+/h- leu1-32 / leu1-32 ura4-D18 /ura4-D18 ade6-210/ade6-216 wee1-Rluc(NatMX)/wee1-Rluc(NatMX) cdc25-Bluc(KanMX)/cdc25-Bluc(KanMX)	yDK145 crossed with yDK148
877	h- leu1-32 ura4-D18 cdc2-Bluc(KanMX)	yFS105 transformed with cassette(DK68, DK69, pFS345)
878	h- leu1-32 ura4-D18 cdc2-Rluc(NatMX)	yFS105 transformed with cassette(DK68, DK69, pFS346)
879	h+ leu1-32 ura4-D18 ade4-Rluc(NatMX)	yFS871 crossed with yFS104

880	h+ leu1-32 ura4-D18 ade4-Bluc(KanMX)	yFS872 crossed with yFS104
881	h+ leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc2-Bluc(KanMX)	yFS877 crossed with yFS879
882	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc2-Bluc(KanMX)	yFS877 crossed with yFS879
883	h+ leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc2-Rluc(NatMX)	yFS878 crossed with yFS880
884	h- leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc2-Rluc(NatMX)	yFS878 crossed with yFS880
885	h+ leu1-32 ura4-D18 ade6-? his7-? ade4-Rluc(NatMX) cdc25-d1-Bluc(KanMX)	yFS739 transformed with cassette(DK6, DK7, pFS345) and crossed with yFS871
886	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) nmt1::pFS429(cdc25(+ 5'UTR) ura4)	pFS429 transformed into nmt locus of yFS879
887	h- leu1-32 ura4-D18 nmt41(KanMX):cdc25	yFS105 transformed with cassette(DK60, DK61 pFS160)
888	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc25::pFS435(cdc25 promoter and 5'UTR:Bluc ura)	pFS435 transformed into cdc25 locus using EcoRI of yFS879
889	h- leu1-32 ura4-D18 cdc13-Rluc(NatMX)	yFS105 transformed with cassette(DK64, DK65, pFS346)
890	h+ leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc13-Rluc(NatMX)	yFS890 crossed with yFS880
891	h- leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc13-Rluc(NatMX)	yFS890 crossed with yFS880
892	h+ leu1-32 ura4-D18 cdc2-L7 wee1-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS123 crossed with yFS869
893	h- leu1-32 ura4-D18 cdc2-L7 wee1-Rluc(NatMX) cdc25-Bluc(KanMX)	yFS123 crossed with yFS869
894	h+ leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc2-as cdc25-Bluc(KanMX)	yFS757 crossed with yFS874
895	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) cdc2-as cdc25-Bluc(KanMX)	yFS757 crossed with yFS874
896	h+ leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc2-as wee1-Rluc(NatMX)	yFS757 crossed with yFS875
897	h- leu1-32 ura4-D18 ade4-Bluc(KanMX) cdc2-as wee1-Rluc(NatMX)	yFS757 crossed with yFS875

898	h- leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cig2::LEU2	yFS140 crossed with yFS155
899	h+ leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cig2::LEU2	yFS140 crossed with yFS155
900	h+ leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cdc13-117	yFS140 crossed with yFS153
901	h+ leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cdc13-117	yFS140 crossed with yFS153
902	h+ leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cig2::LEU2 cdc13-117	yFS899 crossed with yFS153
903	h- leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cig2::LEU2 cdc13-117	yFS899 crossed with yFS153
904	? leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cdc13-Rluc(NatMX)	yFS140 crossed with yFS889
905	? leu1-32 ura4-D18 ade6-? his7-? wee1-50 mik1::ura4 cig2::LEU2 cdc13-Rluc(NatMX)	yFS899 crossed with yFS889
906	h+ leu1-32 ura4-D18 nmt41:cdc13	yFS104 transformed with pFS436 at cdc13 locus and WT locuse subsequently fliped out.
907	h? leu1-32 ura4-D18 nmt41:cdc13 nmt41(KanMX):cdc25	yFS906 crossed with yFS887
908	h- leu1-32 ura4-D18 ade4-Rluc(NatMX) nmt1:cdc25:luc:cdc25cdc25::pFS434(nmt1:cdc25:lucura4+)	pFS434 transformed into cdc25 locus using EcoRI of yFS879

Name	Sequence
DK1	GGAAGATTGGCAGGTGAATG
DK2	GTACAGCGTAAAATAGCGAGAG
DK3	TGGTTGAGGAAGTGGAACGT
DK4	CGTGCACCGTGTTACTCAGTAT
DK5	AGAATTGGATAGCAGCACCC
DK6	TTGGCCAAAGTGTGTTAGCTTCCCCAGACGTTAATGATTCTCCTACTGCCATGCATTCCCTCTCTACACTTAGAAGATTTCGGATCCCCGGGTTAATTAA
DK7	AGAAAAAACTTAGGTTTAGAAAGTTGAATATATAAGAGTATACTTCAGGCTAGGTAAAGTATTGAGTCAGCCTAAAATCAGAATTCGAGCTCGTTTAAAC
DK8	AAACCTCTTACTCATCGGGTTG
DK9	GAGCAATTAGAATGGACTTCGG
DK10	AGTGAGCGCAACGCAATTAATG
DK11	ATTCAGGCTGCGCAACTGTT
DK12	TTTGGAATGGCCAGTGTTTGGCCT
DK13	TGATGAGGTTTGCTGGGT
DK14	GGCAAACGGAATGTATTGCGTC
DK15	CACCAAAATCACTGTTGGGACG
DK16	GTCCCAACGCTCCATGAATCTA
DK17	AAACCCAAGCTTGCGTGCAGTA
DK18	GCATTAATTAACACTTCGAAAGTTTATGATCCAG
DK19	GCAGGCGCGCCCTATTGTTCATTTTTGAGAACTCGC
DK20	GCATTAATTAACGAAGACGCCAAAAACATAAAGA
DK21	GCAGGCGCGCCCTACACGGCGATCTTTCCGCCCTTC
DK22	GTGGATTTAATTAACTCTGGTTTTGCTAATGAACTTGGTCCTCGTCTTATGGGTAAATAGGGCGCGCCTGAGGG
DK23	CCCTCAGGCGCGCCCTATTTACCCATAAGACGAGGACCAAGTTCATTAGCAAAACCAGAGTTAATTAAATCCAC
DK24	ATCTTTTGCAGTCGTGTCGTCC
DK25	CATCTGTTCCGTTTGCATTCC
DK26	TTGCAGAAGATGAGGAACGTGAAGCTCCCGAAGACATTTCTCTCCATAACACACATTCAGATGTTACTTTTGATTTTGTTCGGATCCCCGGGTTAATTAA
DK27	GATTAGAGCATCAATCTAGACAAAGTAAATGGAGGATTGGTTATTATAATAAAGCACTAAGCATTGAATAAATTGGGGAAGAATTCGAGCTCGTTTAAAC
DK28	GGAAAAACTGAGGATGAGGTTG
DK29	ATGGGGCGATTGTACATTCTAT
DK30	GCATTCCAATTCAATTTAATTAAATCAAAAATTTCATATCTATTTTTTTGTTAAATTGCCACATTTTCCATACAGAAAACGAATTCGAGCTCGTTTAAAC
DK31	GTAGCACGATTTAGATTCATGGAGCGTTGGGACCGCCGTAAGCCATAAGATCTATGACTGCTGGTATTAGAAGAAGAGCTCATGATTTAACAAAGCGACTATA
DK32	ATTACGAGCATTGGCTCAATTT
DK33	ATCATACAGGCTCGATGGAGTT
DK34	TGTCGTGTTTTCTTACCGTATTGTCCTACCAAGAACCTCTTTTTTGCTTGGATCGAAATTAAAGGTTTAAAAGCAAAGTTATGAGCTCTTCTTCTAATACCA
DK35	AAAACGCCTAGGAAAACAAACGCAAACAAGGCATCGACTTTTTCAATAACCAACCAAAAAAATTTTACATTAGTCTTTTTTTAAACATTCACCTGCCAATCT
DK36	GATGAGGTTTGCTGGGTTGA
DK37	CATTCACCTGCCAATCTTCC
DK38	ATGACCTGCACCAAGGCTAT
DK39	TCATTAACGTCTGGGGAAGC
DK40	GCTGAGCTGACCGAATTTGA
DK41	AATGTCTTCGGGAGCTTCAC
DK42	AACCCGGCCTTCCGACGTGATTTTCGCATTCTCTCCTTATTTATGAAAAATAGTTTCCCCTCCCTTTTAATCAGCTGTTACGGATCCCCGGGTTAATTAA
DK43	AAAACAAAGAAACGGAACCACAAAATTTTCAGCAAATTGAACGTCTAAAGCCTATAAAGAAAGAAGCAGATAAGGACGCTGAATTCGAGCTCGTTTAAAC
DK44	CACTACACTACACTACGCTG
DK45	AGGTTAGCAAACACGGAAGG
DK46	ATGTCAGGACGCGTGCAAAA
DK47	CGTCGAAGCCCCTAATTTGT
DK48	ATCCTCGTAAGGTTGCTTGG
DK49	GCTAACCCGTTTCATGGCAT
DK50	GAGTTAATTAACGTAAAGCGTGAGAAAAATGTC
DK51	GAGAGATCTCTAACCGCCGGCCTTCACCA
DK52	CCCAGATACAGGCACAACCT
DK53	CTGGGCAACAGGAGAAAGAG
DK54	GGAATGTATTGCGTCCTGCT
DK55	CAGGTGAAGCTGTCGAACAA
DK56	GTCGTAAATCGGTTCGTCGT
DK57	GTCGTAAATCGGTTCGTCGT
DK58	TCAATTTACCGAACCCGAAG
DK59	TTTGCATGGCTGAAGATGAG
DK60	TAGTTCTTTTTGCAGTGTACTTGGTTTAAATTAAATTTACCATTTTGTCTGCTTTTAATAATAGTTAAACCTCAACTAAAGAATTCGAGCTCGTTTAAAC
DK61	TCACGGGCAGCAGGACGCAATACATTCCGTTTGCCAGATAGAGTGTTGGTAAAGGAAAGTGAAGAAAGCGGAGAATCCATGATTTAACAAAGCGACTATA
DK62	CTCAATCTTTTGCAGTCGTGTC
DK63	CGGGAGAGGTGAAACAATAGTC
DK64	CTCTTGGCGATGACGCTGATGAAGATTATACTTTTCACAAGCAAAAACGTATACAACATGACATGAAAGATGAAGAATGGCGGATCCCCGGGTTAATTAA
DK65	CACATATAAAGAGCGCTTGAACAAGTTGGAATATTCACAATTGAAAGAGGTTGAGATAGTGATATGCACAATACACTAAAGAATTCGAGCTCGTTTAAAC
DK66	CTTTTTGTTCGTGACTGGATCA
DK67	AGCAGCATCATTAGCAGAAACA
DK68	AGGCTATGCTTGTTTATGACCCTGCCCATCGCATTAGTGCAAAACGAGCTTTGCAACAAAATTATCTTCGTGATTTTCATCGGATCCCCGGGTTAATTAA
DK69	AACTGATATCAAGAAACACAGCAAAGTACAGATAAAGTCAAGGATAGCGTTTTTAAAGGTTTAATAATAAGAGACGAAAAGAATTCGAGCTCGTTTAAAC
DK70	CGTTGGATGTATTTTTGCTGAA
DK71	TATGTTTTGAACAAACGCCAAG
DK72	CCTTAATTAACCAGAAGCGAATAGCCTTGTC
DK73	ATAGATCTTGAATGATCGTTCCACTTTT
DK74	CGTTAATTAACACGTACTTCCCGGAGGAGGT
DK75	GGTTAATTAACATGATGGGCAGTGTCGAGCT
DK76	CCAGATCTTCAAGCTCTGCTCTTGCACT
DK77	AAATGCGTTTACCTGCGTATTCATCACCACAAAAATCACGATCTAATACAAAAGATGAAAACAGGCACAATTTATTACGACGGATCCCCGGGTTAATTAA
DK78	ATGAATAAGGCAGAAGAGTATTTCGTGATTGGGCATTTATATAAACGGTATCAAACACAATTACAAAATGCGAAAAAAAGGAATTCGAGCTCGTTTAAAC
DK79	TTCAGGCCTGGAACTGATTTAT
DK80	ATGCCGTAAAAGGTTGCTAAAA
DK81	TCTGGAAGCTGTTTGATTCTAAATCAAAACACTCCGCTTCCACTATGAGTTCTCCATTTGACCCTACAATGAAAATACGGCGGATCCCCGGGTTAATTAA
DK82	AAAGCGACAAAGGTGTAATATTTTAGTAATAAGATGACAAATATAATGTACAAAAGACTTCAAAGAGTGAATGTGTTGCAGAATTCGAGCTCGTTTAAAC
DK83	TAGTGTTTCACGTGTTGGAACC
DK84	CAACCAAAAGGACATATGCTGA
DK85	AAGGTCTTACCGGAAAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGATCGCCGTGCGGATCCCCGGGTTAATTAA
DK86	ATTTTTCGCAAGAAGATGCACCTGATGAAATGGGAAAATATATCAAATCGTTCGTTGAGCGAGTTCTCAAAAATGAACAACGGATCCCCGGGTTAATTAA
DK87	AGATTACGCTGCTCAGTGCT
DK88	GGGGGTGCGTTGCCTGATGATCGAGAACGTGAGACGTTAGGACGAAGGTAGGAATCAAAAGAAGAACTCGATAATGACGAACGAGTAGCTTCGCTTTTGGTGGTG
DK89	CACCACCAAAAGCGAAGCTACTCGTTCGTCATTATCGAGTTCTTCTTTTGATTCCTACCTTCGTCCTAACGTCTCACGTTCTCGATCATCAGGCAACGCACCCCC

Name	Sequence
DK90	GTCGGTTTGTGTATTGGAAGGAGTCGAGGGTGATGGAGGCGAATCTGTGGAGGAAGAATGTGGACGAACAAAGGTAGAGGGTTTTGAAGGAGTACTAGGAGGGAGAGGTG
DK91	ACAACACTCTCACTAGCAAGCATGTAATCCTTTCCTTCGGGGGTATCACGTTCACCGATCACTAAAGGACGAATGCCATTGGGATCACGGAATCCCAAAACACCCAAGCC
DK92	ACGAATAGCTGTGCTAGGAACTCCCTCAGATTCATCTTCAAGGCGAATTTTTTTTCATGGCCACAATACGCCCTGACAATTTATGTCTTGCTTTATAAACAACGCCATAGG
DK93	ACAAAATCCTCTAGAAGCGGCCATGCGATGACCTGACAAGCAAGCTGAGCGGCCCGTGCAAGGAGCGCTGAAATTTTGAAACAAGCCGAGGATTCTAAGGTTCCCGATTT
DK94	ATCTTAATTAACATGGTGAGCAAGGGCGAGGA
DK95	ATCGGCGCGCCTTACTTGTACAGCTCGTCCA
DK96	CGAAAACCGAACTTTCAAGC
DK97	GGCCGCTTCTAGAGGATTTT
DK98	GCTTGGCTTCAAGAAGGTTG
DK99	CAGAGTTGTTTCCAGCGACA
DK100	GGTGAGCTCCATTCTTGCCGAGCATCTTAAG
DK101	CCAGGTACCGGGGAAGCATGGTCGTTATTCAT
DK102	TCCGGATCCCGTGATTTTCGCATTCTCTCC
DK103	CCTGAATTCTCCCGTGCTGTTGTCAAAG
DK104	ATTCAGCTCGACACTGCCCAT
DK105	GAGTTTTGTTATATTTTGTATGTTGAAGTGTAATAATAGCGGTATCTTTGTTGAAGATATCCGAGTTTAACAAGACAACTGAATTCGAGCTCGTTTAAAC
DK106	TATGCTTCGTCGGCATCTCT
DK107	AACGCAAACAAGGCATCGAC
DK108	CAACAGTGTTGAGTTTCCCG
DK109	ACAGCGACAACTCGGTCATA
DK110	TATGCGGCATCAGAGCAGAT
DK111	TAGTTCTTTTTGCAGTGTACTTGGTTTAAATTAAATTTACCATTTTGTCTGCTTTTAATAATAGTTAAACCTCAACTAAACGGATCCCCGGGTTAATTAA
DK112	GCATTCCAATTCAATTTAATTAAATCAAAAATTTCATATCTATTTTTTTGTTAAATTGCCACATTTTCCATACAGAAAACCGGATCCCCGGGTTAATTAA
DK113	TTACAACTTTGGTATTCTTAAGAATAATATTTTCAATGTAATACACTTGATCTAAAGGAGAAGAGCTTGAAGCGCTGTAGTGATGATATGCCAGGATTCCTC
DK114	TTTTTTCCTCTCGGAGGCAGAGCTGAGATTTCTCTCTACCCTCAACTATCTACAGCGCTTCAAGCTCTTCTCCTTTAGATGAATTCGAGCTCGTTTAAAC
DK115	GAGTGTTGGTAAAGGAAAGTGAAGAAAGCGGAGAATCCATTTTAGTTGAGGTTTAACTATTATTAAAAGCAGACAAAATGAGAAGTGGCGCGTAAGATCG
DK116	TTGCGTTACCCCGACAACCCCATAGTGTTTACGCCCTCTACGATACTCCTTTCAGTCGACTCCACTACCACGAGCTGTCCGAATTCGAGCTCGTTTAAAC
DK117	TTGCCAATAGGTGCTGGCGAGTTAAACGACGGGTAGTCATGAGGAAAAGAAGAAACGAATTCTGTCGGGGATTGAAAGAGAGAAGTGGCGCGTAAGATCG
DK118	TTATCTTCTCCGTCCCGTAC
DK119	TAGGTGCTGGCGAGTTAAAC
DK120	ATTGTGGTTCCATATCTCCG
DK121	ATTAGATCTACTAAAGTGGGTGTACTGGC
DK122	CGGTTAATTAAGAGGAAAAGAAGAAACGAATTC
DK123	CAACTATTAGTGTATCAGTGTCTTTAATTCGTCGTCTATATATGCCACATTGTTCTCTCCCTCATTTTTTCTCTCATATTGAATTCGAGCTCGTTTAAAC
DK124	AACAAGTGTGTTTTCATCTTGATAACTATGCTTATTGATTTTAGAACCAACAGGCTTTGAAATTGAATAGAGAGCCATGAGGAAAAGAAGAAACGAATTC
DK125	TTCTTTTTGCAGTGTACTTGGTTTAAATTAAATTTACCATTTTGTCTGCTTTTAATAATAGTTAAACCTCAACTAAAATGCGGATCCCCGGGTTAATTAA
DK126	GGGATTTTTCATCCCCTCAG
DK127	TATGGAGCTGGTCGTAATCC
DK128	CCTCCAAGGCAGAAGCAACA
DK129	GGAGGCTGAAGCTATCGAAG
DK130	AAAAACGCGAACGCGTCATG
DK131	GGACGATTTCTTTCCCTTTC
DK132	ACATGTTTCCCAACGGGCAG
DK133	TCTGTTACCTGAACCAATTAATATTTTAATTTCCCTTCCTCTCTTTCAATCCCCGACAGAATTCGTTTCTTCTTTTCCTCGAATTCGAGCTCGTTTAAAC
DK134	ATATGGTTTGAAGGATGATTTTCGTCATTGTTGCCCAAGGTATTTGCCAATAGGTGCTGGCGAGTTAAACGACGGGTAGTCATGATTTAACAAAGCGACTATA
DK135	GTCTCAAAAGCTCTTGCCAAAT
DK136	ATTAAAAACAGAAGGTTGGCGA
DK137	TAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACC
DK138	ACGCTAACATTTGATTAAAATAGAACAACTACAATATAAAAAAACTATACAAATGACAAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGATTA
DK139	CCACCGCGGTGGCGGCCGCATGGAAGACGCCAAAAACATAAAGA
DK140	CCACCGCGGTGGCGGCCGCGAAGACGCCAAAAACATAAAGAAAGGC
DK141	CCGATCGATTGGGCCCTCCACGGCGATCTTTCCGCCCTTC
DK142	GGCTACTTGACTCGAGAATGGATTCTCCGCTTTCTTCAC
DK143	CCGATCGATTGCGGCCGCCAAATCTTCTAAGTGTAGAGAGGGA
DK144	AGGAAGAGGAATCCTGGC
DK145	CCGCATAGTCAAGAACATCG
DK146	GGCTACTTGACTCGAGATGGATCTTGTGTGCGTTACGCCA
DK147	CCGATCGATTGCGGCCGCCTTGGCTTTTGAAAAGCCCTAG
DK148	GGCTACTTGACTCGAGACAACAATAGTTGTCATGGCTGAGG
DK149	TCATGGTTGTACCCATGAGC
DK150	TCCTCCTACCAAGATTCCTC
DK151	GTTGTGTGGAATTGTGAGCGG
DK152	TCTGATCCTCTAGAGTCGACC
DK153	GACCATATCTCAAATTCAAAAATTTGACTTCTATTTTACATTATTAAATCGTTCCATATAATTTTTTTAATTATTAACTCGAATTCGAGCTCGTTTAAAC
DK154	ATGTCGTTTAAGGGTAAAAACTCGTCAAGATGCTGGTTAAGGACAGCTCGTGGTAGTGGAGTCGACTGAAAGGAGTATCGGATGATATGCCAGGATTCCTC
DK155	AACAAGTGTGTTTTCATCTTGATAACTATGCTTATTGATTTTAGAACCAACAGGCTTTGAAATTGAATAGAGAGCCATCGGATCCCCGGGTTAATTAA
DK156	CTGCAGACTAAAGTGGGTGTACTGGC
DK157	CTCGAGGAGGAAAAGAAGAAACGAATTC
DK158	AGATCTCATGACTACCCGTCGTTTAAC
DK159	GCGGCCGCCCCATTCTTCATCTTTCATGTC
DK160	CTCGAGATGGCTCTCTATTCAATTTC
DK161	GCGGCCGCCGTGACCATCATTTGTTAAAG
DK162	AGATCTCGATACTCCTTTCAGTCGAC
DK163	CCTAGGTACTGAGAGTGCACCATACG
DK164	CTGCAGCGGTATTTCACACCGCATCG
DK165	GGATTTGTAGCTAAGCTTGC
DK166	GCAAATGGTAAGGAGTGGCC
DK167	TCTGTTACCTGAACCAATTAATATTTTAATTTCCCTTCCTCTCTTTCAATCCCCGACAGAATTCGTTTCTTCTTTTCCTCCGGATCCCCGGGTTAATTAA
DK168	GAGTTTTGTTATATTTTGTATGTTGAAGTGTAATAATAGCGGTATCTTTGTTGAAGATATCCGAGTTTAACAAGACAACTGCGGATCCCCGGGTTAATTAA
DK169	TATTATTTGATGTTTGTATTTGTTTCCCAGCTTGCCAACGTTGCGTTACCCCGACAACCCCATAGTGTTTACGCCCTCTACGGATCCCCGGGTTAATTAA
DK170	CCTGCAGGCTTCTGATATTCTTGCCGAGC
DK171	GCGGCCGCTTTAGTTGAGGTTTAACTATTAT
DK172	AACTCGATGACTTGTGCAGC
DK173	CTCAAGCTCGACTTGTTGCA
DK174	GTAGAAACGTAGACGGGTGC
DK175	CATAGACTCGGGTTGGGTAG
DK176	TATGTGGCGCGGTATTATCC
DK177	CTGTCATGCCATCCGTAAGA
DK178	TTATGTCGAGCCTGCCAACC

Name	Sequence
DK179	ATTACGCTGCTTAGTCGACC
DK180	ATAGTCGAAGGCGATACAGC
DK181	ATCATCTGATGGAGGACTCG
DK182	TACACAGCGACAACTCGGTC
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DK372	GTGCATGTTCGGTGGTCTCG
DK373	AAGACCCGGTAGTGATGTGC
DK374	ATCCGCCAAAGCGTGGTATC
DK375	AAGCGCACTAAGCAGACGAG

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