Investigation of Temperature Sensitive Mutant Stains RCY1110, RCY1120, RCY1123, RCY1124, RCY1125, and RCY1126 in Kluyveronmyces lactis for cdc Mutations and Potential for Cloning by Complementation

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Investigation of Temperature Sensitive Mutant Strains RCY1110, RCY1120, RCY1123, RCY1124, RCY1125, and RCY1126 in Kluyveromyces lactis for cdc Mutations and Potential for Cloning by Complementation

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Abstract

Gaining a better understanding of genes, that when mutated, cause the cell-division cycle to malfunction will allow for better and more specialized cancer treatments. This project screened strains with potential mutations in cell-division cycle (*cdc*) genes in the budding yeast *Kluyveromyces lactis*. The goal was to confirm a Cdc phenotype in several temperature sensitive (*ts*) mutants in the manner introduced by Hartwell and colleagues in the 1970's. Although initially identified as a *cdc* mutant, RCY1125 was found not to be a *cdc* mutant upon rescreening. Screening for a *cdc* mutation in RCY1126 was inconclusive, as there were insufficient cells to be scored; however it showed interesting enlarged cell and elongated cell phenotypes. Four haploid mutant strains, RCY1110, RCY1120, RCY1123, and RCY1124 did show a Cdc phenotype so further analysis was performed to confirm that the *cdc* mutation was in a single gene and recessive. Having a recessive mutation in a single gene is necessary to allow for cloning by complementation in later stages of the project. Each haploid was mated with LSY25, a wild type strain, yielding diploid strains that were used for recessiveness testing and tetrad dissection. Through recessiveness testing we were able to confirm recessive mutations in RCY1110 and RCY1124. Analysis on RCY1120 and RCY1123 was discontinued during recessiveness testing due to the haploids no longer showing a temperature sensitive phenotype, possibly due to osmotic effects. Tetrad dissections so far have shown a 2:2 segregation of the temperature sensitive phenotype for RCY1110 and RCY1124, indicating a mutation in a single gene; however more tetrad analysis is still needed on both RCY1110 and RCY1124. Preliminary tetrad analysis indicates linkage of the *ts* locus in RCY1110 to *ura3* and the *ts* locus of RCY1124 to *ade1*. Currently, RCY1110 and RCY1124 appear to be good candidates for cloning by
complementation and we hope they will fall into complementation groups not yet found by this lab.

Introduction

In order for organisms to grow and develop they must maintain the ability to duplicate their cells. The finely tuned process of cell duplication is known as the cell-division cycle. In budding yeast, cell duplication occurs in which a bud emerges from the mother cell. The bud becomes larger throughout the cycle until it is a perfect replica of the parent cell, then the parent and the bud separate. The size of the bud is indicative of the extent of the cell's progression through the cell cycle (Figure 1) (Hartwell et al., 1970).

![Diagram of the cell cycle in budding yeast](Index_of/research/budding_yeast_model/gif_files)

**Figure 1: The Cell Cycle in Budding Yeast:** As the cell progresses through the cycle, bud size increases predictably.

The cell cycle is composed of four distinct phases G1, S, G2, and M. G1 is a time for cell growth, as well as when the cell closely monitors its environment to determine if the conditions are right to allow it to commit to carrying out mitosis (Alberts et al., 2007). If the cell receives signals that the internal and external conditions are acceptable, it will pass the Start point and commit to S (DNA synthesis) phase. In S phase the cell replicates the
entirety of its DNA and continues to grow. Following S phase is G2 where the cell has another opportunity to grow, store up resources, and monitor its internal and external environment. Again, if conditions are right it will progress to M (mitosis) phase where segregation of one set of the duplicated chromosomes into the daughter cell is followed by cytokinesis, when the two cells are split apart from each other.

The cell division cycle is a very involved process that the cell must closely regulate to ensure that each step is carried out with accuracy (Alberts et al., 2007). The cell manages control through the use of positive regulators, “transition regulators,” that push the cell cycle forward to the next step and negative regulators, ”checkpoint regulators,” which stop the progress of the cycle when they detect problems. There are at least three places in the cell cycle where these regulatory mechanisms act, known as cell cycle checkpoints. The three major checkpoints include Start, the G2/M checkpoint and the Metaphase/Anaphase transition. Transition regulators push the cell past the checkpoint to the next stage, and checkpoint regulators cause the cell cycle to arrest if they detect a problem.

Positive transition regulators act on other factors in the cell to push the cell forward through the cell cycle (Alberts et al., 2007). The main players in the transition regulation of the cell cycle are proteins called cyclin-dependent kinases (Cdks). These proteins regulate progress through the cell cycle through phosphorylation of different cell cycle factors. For example, at the G2/M checkpoint the activated Cdks phosphorylate proteins that are involved in chromosome condensation, nuclear envelope breakdown and spindle assembly. Cells may have one to several different Cdks, but often have many types of cyclins that activate the Cdks at different times in the cell cycle to determine what targets the cyclin-
Cdk complex will phosphorylate. Cdns can also be inactivated by proteins called cyclin-dependent kinase inhibitors (CKIs) and through phosphorylation of the inhibitory site on the Cdk itself. Finally, Cdns also have the ability to phosphorylate inhibitors on other inactive Cdns or cyclins on other active Cdns. For example in the G1 to S transition, phosphorylation of an inhibitor, Sic1, allows a ubiquitin ligase to tag the inhibitor to be degraded by a proteosome and therefore activate the previously inactive Cdk.

Phosphorylating the cyclin on an active Cdk allows for a ubiquitin ligase to tag the cyclin and when it is degraded by a proteosome the Cdk becomes inactive. The ability to inactivate Cdns allows for terminating functions of one stage of the cell cycle so that a different cyclin can reactivate the Cdk for functions of the next stage of the cycle.

It is important to understand the mechanisms that control in the cell cycle because when they fail in humans, the result is often cancer (Pray, 2008). Cancer is caused when a mutation occurs in a gene that regulates the cell cycle and the cells begin to proliferate uncontrollably. This mutation could occur in a gene encoding a transition regulator, a checkpoint gene, or a DNA repair gene. Transition regulator gene products are responsible for stimulating cell division based on the signals they receive and can cause overstimulation if mutated. Checkpoint factors normally stop the cell cycle when they receive signals indicating that the conditions are not optimal and can cause lack of inhibition if mutated. DNA repair gene products repair damage detected in the DNA at checkpoints and if coupled with mutated checkpoints may increase the number of defective cells. Activating mutations in positive transition regulators and loss of function mutations in checkpoint genes can cause the cell cycle to proceed under conditions when normal gene products would cause proliferation to cease.
An example of a transition regulator involved in entry into S phase in humans is cyclinD1. Overexpression of cyclinD1 can be a cause of cancer. The activated Cdk4-cyclinD1 complex is responsible for phosphorylating the Rb protein, which in turn activates transcription factors for S phase genes (Muschgrove et al., 2011). Overexpression or amplification on the CCDN1 gene, which codes for the cyclinD1 protein, can lead to more cells entering the cell cycle than normally would. This increased cell proliferation due to cyclinD1 overabundance has been observed in many kinds of cancers including head and neck squamous cell carcinoma, non-small-cell lung cancer, melanoma, colorectal, pancreatic, and breast cancers (Musgrove et al., 2011). Though this transition is regulated differently in yeast, the presence of Cdk4 and cyclins in all eukaryotic cells makes yeast an initial starting point for discovering factors involved in the cell cycle. A better understanding of how different kinds of mutations lead to cancer will allow for more specialized treatment options with drugs that can be tailored to target specific types of mutations (Pray, 2008).

The goal of this project is to study cells in which the cell cycle is malfunctioning and to work backwards to identify which component of the cell cycle is not functioning properly. Cells mutated in a gene involved in the cell cycle that arrest their cell cycle as a result of the mutation are called cell-division cycle (cdc) mutants. Observing the phenotypes of mutated cell-division cycle genes can give us a clue as to the function of the gene in the cell. We have chosen to study the cell division cycle in yeast, as it is an economic and easily genetically manipulated organism that shares many homologous genes with humans (Botstein et al., 1997). An understanding of the cell cycle in yeast is valuable to an understanding the cell cycle in humans because genes found in yeast likely have the same
or similar function in humans. As of 1997, at least 31% of genes in the yeast *Saccharomyces cerevisiae* had been found to have a mammalian homolog and at least 71 human genes were able to complement mutant yeast genes, though this is likely an underestimate (Botstein *et al.*, 1997). Some important genes that have already been identified as homologous between humans and yeast include *MSH2* and *MLH1*, which cause hereditary nonpolyposis colon cancer, *IRA2* which is responsible for neurofibromatosis type I, and *SGS1* which causes Werner's Syndrome (Botstein *et al.*, 1997). The 60% of the yeast genome whose function is yet to be determined illustrates the pertinence of such studies as the *cdc* mutant search (Botstein *et al.*, 1997).

The first project to undertake a *cdc* mutant search was led by Leland Hartwell *et al.* in the 1970's (Hartwell *et al.*, 1970; Hartwell *et al.* 1973). His work eventually garnered him the Nobel Prize in 2001. Hartwell *et al.* (1970) introduced the method of a temperature sensitive (ts) mutant search in the yeast species *Saccharomyces cerevisiae*, and were able to identify three *cdc* mutant genes. Hartwell's search was then expanded to yield 148 *cdc* mutants (Hartwell *et al.*, 1973). A temperature sensitive mutant, a type of conditional mutant, has gene products that function at the normal growth temperature. However, when they are exposed to higher or lower temperatures, one or multiple proteins denature and a lack of growth is observed due to loss of essential protein function (Tan *et al.*, 2009). The use of a conditional mutant search allowed Hartwell *et al.* to identify genes that, when mutated, would cause cell death, but only under certain conditions, allowing for isolation of the mutant and study of its defect. This was accomplished by growing one culture of *S. cerevisiae* under permissive growth conditions (low temperature), and another identical culture at restrictive growth conditions (higher temperature) that would kill any
temperature sensitive (ts) mutants whose mutant protein products could not function at the higher temperature (Hartwell et al., 1970). Hartwell could then identify which were the temperature sensitive mutants in the permissive culture by their absence in the restrictive culture.

Hartwell et al. (1970) could determine if the temperature sensitive mutants were cell-division cycle mutants by examining a liquid culture for uniform bud size upon cell cycle arrest once shifted to the restrictive temperature. Comparatively, cultures of non-cdc mutants display a variety of bud sizes because they arrest at varying stages of the cell cycle. The Cdc phenotype occurs because a factor that normally pushes the cell cycle forward fails to act at a point in the cell cycle uniform throughout all cells. Subsequently, because bud size is indicative of progression through the cell cycle, all cells will stop at the same point in the cell cycle, displaying a uniform bud size. Therefore, bud size can be interpreted as an indication of where in the cycle the cell is arrested.

Often, bud morphologies are observed that correspond to cell cycle checkpoints. Hartwell et al. (1970) distinguish between the point in the cycle where the function of the defective gene is normally performed, "the execution point," and the point in the cycle at which mutated cells will uniformly arrest, the "termination point." Instead of being directly responsible for cell cycle control, a gene product may act in cell cycle events between checkpoints. However, the failure of that process may not disrupt the cycle drastically enough to cause immediate arrest, but the disruption will be sensed when the cell reaches the next checkpoint, causing arrest at the checkpoint (Pringle et al., 1997). Therefore, it is necessary for this type of mutant search that the cells have intact checkpoints, otherwise there will be a lesser chance of finding mutants with a uniform arrest phenotype.
In addition to Hartwell's work, other notable work was performed on \textit{cdc} mutants by Lee and Nurse in the 1980's when they discovered a human homologue of the yeast \textit{Schizosaccharomyces pombe} \textit{cdc2} gene (Lee and Nurse, 1987). Through the use of a human cDNA library, they identified a human gene that could rescue a \textit{cdc2} mutant of \textit{S. pombe}, a fission yeast. The functionality of the human gene within the cell was similar enough to the \textit{S. pombe} gene to make up for the loss of function due to the mutation. This indicates that the function of cell cycle control exerted by \textit{cdc2}, a Cdk coding gene, is most likely conserved between \textit{S. pombe} and humans (Lee and Nurse, 1987). This study reinforces the idea that generating \textit{cdc} mutants in yeast gives us a tool to isolate and study genes from other species, especially humans due to the similarities between the yeast and human genomes.

For our \textit{cdc} mutant search, we used \textit{Klyveromyces lactis}, which, like \textit{S. cerevisiae}, is a budding yeast. There are several advantages to using \textit{K. lactis} because the majority of the \textit{cdc} mutant searches have been performed in \textit{S. cerevisiae}. Due to the similarity between the two species, we can use similar techniques and easily express genes from one yeast in the other to allow for comparison. The ways in which \textit{K. lactis} differs from \textit{S. cerevisiae} can also be advantageous for the \textit{cdc} mutant search.

As illustrated by Wolfe and Shields (1997), 13\% of the protein products in \textit{S. cerevisiae} come from genes that were at one time duplicated in the genome. In a comparative genome study, Dujon \textit{et al.} (2004) identified 56 blocks of duplicated genes in \textit{S. cerevisiae} and only 5 duplicated blocks (501 sets of paralogs) in \textit{K lactis}. Fitch \textit{et al.} (1992) showed an instance of this gene redundancy in \textit{S. cerevisiae} in the closely related \textit{CLB1} and \textit{CLB2} genes as well as the \textit{CLB3} and \textit{CLB4} genes. The \textit{CLB} genes encode B-type
cyclins. *CLB1* and *CLB2* are closely related and function around the time of nuclear division, whereas *CLB3* and *CLB4* are also closely related to each other and function from S phase through anaphase. The *CLB* genes are needed to carry out mitosis and are likely involved in spindle formation. As a result of duplicated sections of the genome, a mutated gene may have a "back up" copy that can take over its function and make detection by phenotype impossible because no mutant phenotype will be observed. All combinations of single and double mutations of the *CLB* genes in *S. cerevisiae* were viable indicating that they have a great capacity to compensate for loss of other *CLB* genes (Fitch *et al.*, 1992). The duplications in the *S. cerevisiae* genome occurred after the split from *K. lactis*, meaning that *K. lactis* is unlikely to have duplications in the same genes as *S. cerevisiae*, allowing these mutants to be more easily detected by their mutant phenotype.

Another way that *K. lactis* will be beneficial to this study is that we will be working with a different screen bias, hopefully revealing new genes. The difference in screen bias may be attributed to structural or functional differences between the genes in the two species, as discussed below. In previous *ts* screens performed by Kaback *et al.* (1984) and Hartwell *et al.* (1973) a bias has been observed in the ability to obtain *ts* and *cdc* mutants. Hartwell *et al.* (1973) observed an average of 4.6 genes per complementation group, illustrating that their screen was saturated, meaning that finding mutations in new genes was highly unlikely. The mutations they observed did not occur in each gene equally or randomly, but were far more likely in some genes than others, indicating that their screening method was not allowing for identification of all cell cycle genes (Hartwell *et al.*, 1973). Additionally, Kaback *et al.* (1973) performed a temperature sensitive mutant search in a strain of *S. cerevisiae* that was monosomic for chromosome I and disomic for other
chromosomes. Though Kaback et al. (1973) expected to see a plethora of mutations in the 60-100 genes that are located on chromosome I, they were only able to find temperature sensitive lethal mutations in three genes on chromosome I, CDC15, CDC24, and CDC19, all of which have been previously identified, an illustration of the bias of cdc screens.

To acquire a ts mutant it is necessary that the mutant has a protein product which functions normally at the permissive temperature, but will denature at a higher temperature and fail to perform its cellular function. The higher temperature is only about 10°C higher than the preferred temperature of growth, so it is difficult to find proteins that will denature with such a small change, thus making it difficult to find all potential cdc genes (Kaback et al., 1984). The protein structure of similar genes may be slightly different in S. cerevisiae and K. lactis making one gene easier to mutate into a temperature sensitive mutant than the other, allowing us to detect it in our screen (Kaback et al., 1984). The difficulty in producing temperature sensitive mutants should persist in both K. lactis and S. cerevisiae. However, because of the slightly different structures of their homologous proteins, we are hoping that K. lactis has different proteins that are prone to denaturation at the higher temperature than S. cerevisiae. This does not mean that we will be able to find all the genes there are to find, but we should find a different set of mutants than those found in S. cerevisiae. So, though no screen is unbiased, hopefully K. lactis will offer a different screening bias than S. cerevisiae.

The difference in physiology between S. cerevisiae and K. lactis may lead to phenotypic differences for mutations in the same gene, one of which is detectable and the other not. For example, CPF1 is a gene involved in chromosome segregation and transcription activation that is found in both S. cerevisiae and K. lactis. Disrupting this gene
in *S. cerevisiae* results in slow growth, but disruption of this gene in *K. lactis* is lethal (Mulder *et al*., 1994). Another important difference between *S. cerevisiae* and *K. lactis* is that *S. cerevisiae* prefers to live as a diploid, while *K. lactis* prefers to live as a haploid (Murray and Hunt, 1993; Herman and Roman, 1966). This difference will put different selective pressures on each species. For example, *K. lactis* may be more eager to enter S phase and replicate its DNA because this means it will now have a backup copy of each gene that can be used to fix mistakes. Conversely, *S. cerevisiae* may be more hesitant to enter S phase because it already has a second copy of each gene and it is more important for it to prioritize nutrient requirements so that it can successfully complete mitosis (Murray and Hunt, 1993). Therefore, each species might have slight differences in their mechanisms of regulation and control of the cell cycle that can be observed in phenotypic differences.

Though this project is based on the methods outlined by Hartwell *et al*., a change has been made between this experiment and Hartwell's regarding the method of mutagenesis. Hartwell generated mutants using N'-methyl-N'-nitro-N-nitrosoguanidine (NG) and ethylmethane sulfonate (EMS) (Hartwell *et al*., 1970; Hartwell *et al*., 1973). The mutants being analyzed in this project were generated using ultraviolet light. A study in *E. coli* indicated that NG is highly specific for G•C → A•T transition (Coulondre and Miller, 1977). EMS is also highly specific for the G•C → A•T transition. UV light does not show the mutational specificity that NG or EMS show and creates less specific base substitutions. Similar results were also reported by Prakash and Sherman (1973) who tested mutagenic specificity in yeast. They also found that EMS generated G•C → A•T transitions while UV caused many different mutations. Todd and Glickman (1982) have illustrated that UV light
also heavily targets areas of the DNA with secondary structure in *E. coli* (Todd and Glickman, 1882). With this change in mutagen we are hoping to generate different kinds of mutants than have previously been created by mutating the DNA in a different location. A different mutation in the DNA will hopefully cause a different mutation in the protein, possibly causing it to be temperature sensitive.

This lab's work aims to identify new factors in the cell cycle through searching for and classifying temperature sensitive cell cycle mutants in *K. lactis*. This will allow for discovery of new mutants, but will also help to better understand mutations that have previously been found in *S. cerevisiae* and *S. pombe*. The value of studying the function of the same gene in two different species has already been illustrated by Sorger *et al.* (1992). Previous research by Gould and Nurse (1989) showed that in *S. pombe* mutations in *cdc2* that do not allow for inhibitory phosphorylation of its gene product, a Cdk, cause deregulation of cell cycle progression. Sorger *et al.* (1992) investigated if this same effect was seen in the gene product of the *cdc2* homologue, *CDC28*, in *S. cerevisiae*. First, in *S. pombe* this lack of phosphorylation of *cdc2* causes too many cells to proceed into mitosis (Gould and Nurse, 1989), whereas in *S. cerevisiae*, failure to phosphorylate the *CDC28* gene product, also a Cdk, does not cause premature entry into mitosis (Sorger *et al.*, 1992). Secondly, the mutation in *S. pombe* does not allow for arrest at the end of G2 in the presence of damaged or unreplicated DNA (Enoch and Nurse, 1990). In *S. cerevisiae*, the cells do arrest at the end of G2 in the presence of damaged or unreplicated DNA (Sorger *et al.*, 1992). If this same phenomenon of phenotypic differences is observed in *K. lactis* and *S. cerevisiae*, it will yield a more complete understanding of cellular functions of homologous proteins.
We are optimistic about the number of \textit{cdc} mutants there are still to find in \textit{K. lactis} because Hartwell \textit{et al.} was able to identify 148 \textit{cdc} mutants in \textit{S. cerevisiae} that fell into 32 complementation groups (Hartwell \textit{et al.}, 1973). Hartwell \textit{et al.} (1973) saw an average of 4.6 mutants per complementation group, indicating that his screen was saturated after screening 1,500 temperature sensitive mutants. So far our lab has identified 10 \textit{cdc} mutants that fall into 8 complementation groups. This project has only generated an average of 1.25 mutants per complementation group, so we are not yet repeatedly generating the same results. Mutations have been confirmed in \textit{cdc14} (a phosphatase involved in mitotic exit) and \textit{cdc15} (a MEN protein involved in mitotic exit), and are extremely probable in \textit{tub2} (tubulin) and \textit{cdc16} (subunit of APC). The \textit{tub2} and \textit{cdc16} mutants are still probable because DNA containing \textit{TUB2} and \textit{CDC16} complement the mutants, but there are also other genes in the pieces of complementing DNA. All of the mutants are large budded at arrest, indicating that there are more still to find based on the range of arrest morphologies seen in the Hartwell \textit{et al.} study (1973) (Figure 2). The phenotypes our lab has seen so far include large budded with one nucleus (\textit{tub2, cdc16}) and large budded with two nuclei (\textit{cdc14, cdc15}). Hartwell \textit{et al.} (1973) saw many more phenotypes including unbudded with one nucleus, tiny budded, unbudded with two nuclei, large budded with one nucleus, and large budded with two nuclei. Some cells had abnormally elongated buds, and some cells displayed rebudding (Figure 2). It is reasonable to expect that we will see a similar variety of phenotypes in \textit{K. lactis}.

We can expect our screen to find cytokinesis mutants (chains of cells), mitotic exit mutants (large budded, two nuclei), Start mutants deficient for G1-Cdk activation or that give false signals indicating nutrient scarcity or a mating state (unbudded), DNA
replication, anaphase promoting complex, and spindle mutants (all large budded) (L. Silveira, personal communication). Also, we should be able to pinpoint mutants that arrest before Start as unbudded cells, and cells that arrest at the G2 to M transition, Metaphase to anaphase transition, and mitotic exit as large budded cells. The lack of phenotypic variety observed so far indicates that this screen still has potential to find new mutants.

![Figure 2. Cdc+ phenotypes observed by Hartwell et al. (1973)](image)

Our project will add to the overall understanding of the cell cycle through investigation of six previously identified cdc mutants that are in need of more analysis, as well as looking for a Cdc+ phenotype in previously identified ts mutants. Some of the mutants we investigated are pictured in Figure 3. Through a second round of cdc screening we hoped to confirm the phenotypes of RCY1110 (unbudded), RCY1123 (small budded), RCY1124 (large budded), RCY1120 (tiny buds), RCY1126 (unbudded but swollen) and RCY1125 (large budded) (Figure 3) (Heitz, 2012; Callaghan, 2012).
As stated above, the arrest morphologies of the strains we re-screened included unbudded, tiny budded, small budded, and large budded. Of these phenotypes, unbudded, tiny budded, and large budded were detected by the Hartwell et al. screen (Hartwell et al., 1970). The tiny budded phenotype only appeared once, and it indicated a cdc1 mutation. Our novel phenotypes could be indicators of true novel arrest morphologies or indicators that we do not actually have cdc mutants. It is important to redo the screen to confirm that these strains are cdc mutants and are not mutated in some other cellular function. A second screening will ensure that errors were not made in the first round of cdc screening by Heitz (2012) and Callaghan (2012).

Figure 3: Potential cdc mutants. Visualization of cdc mutants under a phase microscope after seven hours at the restrictive temperature. A) RCY1123- small budded at arrest, 1000X. B) RCY1124- large budded at arrest, 1000X. C) RCY1120- tiny budded at arrest, 400X. D) RCY1125- large budded at arrest, 1000X. (Callaghan, 2012)
Of particular interest to us are the unbudded, small budded, and tiny budded phenotypes, as they are different from the large budded phenotype already identified by this lab. The unbudded phenotype would indicate that we may have a mutant arresting at the Start checkpoint. The small budded phenotype was not found in Hartwell’s screen, presumably because there is no known checkpoint in *S. cerevisiae* that acts at a time in the cell cycle in which the cell has a small bud. A small budded mutant in *K. lactis* may be an indication that *K. lactis* utilizes a slightly different mechanism of cell cycle control, or that the mutants were improperly screened the first time.

Hartwell was the first to describe the tiny budded phenotype indicative of a mutation in *cdc1* in *Saccharomyces cerevisiae* in 1970 (Hartwell *et al.*, 1970). Though the mode of action of *cdc1* has not yet been determined, it is involved in cell cycle progression and organelle distribution (Saccharomyces Genome Database). More specifically, it is a transmembrane protein found in the endoplasmic reticulum, possibly a lipid phosphatase, that is responsible for Golgi inheritance through actin transport (Losev *et al.*, 2008). When *cdc1* is mutated, defects in actin polarization and Golgi inheritance are observed. It appears that the defect in normal calcium signaling in *cdc1* mutants is responsible for the mutant phenotypes (Losev *et al.*, 2008). Paidhungat and Garrett (1998) investigated a discrepancy that the phenotype of a *cdc1* mutation was sometimes reported as tiny budded and other times as small budded. What they discovered was that at 30° C the cells accumulated with a small bud, but at 36° C there were more cells without a bud (Paidhungat and Garrett, 1998). It appears as though the phenotype seen as a result of a mutation in *cdc1* differs based on the temperature it is arrested at, at least in *Saccharomyces cerevisiae*. Rossanese *et al.* (2001) propose that the range of phenotypes observed indicates that *cdc1* is involved
in multiple stages of the cell cycle, and that the general growth defects caused by the depolarized actin cytoskeleton could be the reason why.

Another interesting phenotype observed by Heitz (2012) and Callaghan (2012) was that of RCY1126, dubbed “Frankenyeast.” It was described as having mostly unbudded but very swollen cells (Heitz, 2012). Though the numbers were never quantified, they believed that upon rescreening, this mutant would prove to be cdc, prompting us to screen it again. Similar phenotypes have been described as “monster” cells by Smith and Blackburn (1999), or uge mutants by Zhang et al. (2002). “Monster” cells exhibit defects in telomere capping, and uge mutants show problems with genes involved in transcription regulation, signal transduction, and cell cycle regulation (Smith and Blackburn, 1999; Zhang et al., 2002).

The goal of this project was to perform further analysis on cdc mutants identified by Heitz (2012) and Callaghan (2012) which included RCY1116, RCY1120, RCY1124, RCY1123, RCY1125, and RCY1126. We determined if the mutation in each strain was due to a recessive mutation in a single gene so that the gene could be identified later through cloning by complementation. Cloning by complementation includes transforming each mutant with a K. lactis genomic library and finding the gene that complements the cdc mutation. Therefore if the cdc mutations are dominant they cannot be rescued by a gene from the library. Similarly, if the Cdc- phenotype is due to mutations in multiple genes, the mutant will not be rescued by a single gene from the DNA library. Any strains that were not confirmed to have a recessive mutation were excluded from further analysis because cloning by complementation would not be feasible for those strains.

In addition to analyzing previously identified cdc mutants, we performed preliminary cdc screens on temperature sensitive strains identified by Heitz (2012) and
Callaghan (2012) that had not yet been screened for Cdc phenotype. This allowed for identification of additional potential cell-division cycle mutants from the large pool of temperature sensitive mutants generated by this lab thus far.

Materials and Methods

Media
Liquid YPD, YPD plates, and acetate sporulation media were prepared as in Ausubel et al. (1993). SD plus supplements plates were prepared with supplement concentrations as in Guthrie and Fink (1991). Malt extract plates were prepared as in Wolf et al. (2003).

Strains
Table 1. Control and cdc Analysis Strains Used in this Project.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG1888</td>
<td>MAT a ura3</td>
<td>Zonneveld and Van der Zanden (1995)</td>
<td>Wild type control</td>
</tr>
<tr>
<td>RCY303</td>
<td>MAT a ura3 ts cdc</td>
<td>EMS mutant of GG1888 by Silveira, Wallace (2003), and Oldroyd (2002)</td>
<td>Positive control for cdc screens</td>
</tr>
<tr>
<td>LSY25</td>
<td>MAT a his7 ade1</td>
<td>B. Zonneveld</td>
<td>Wildtype control, backcross</td>
</tr>
<tr>
<td>RCY1110</td>
<td>MAT a ura3 ts</td>
<td>UV mutant of GG1888 by Dror (2010)</td>
<td>cdc analysis and screening, backcross, tetrad analysis</td>
</tr>
<tr>
<td>RCY1119</td>
<td>E. coli</td>
<td></td>
<td>Accidental inclusion. Removed from project.</td>
</tr>
<tr>
<td>RCY1120*</td>
<td>MAT a ura3 ts</td>
<td>UV mutant of GG1888 by Heitz (2012) and Callaghan (2012)</td>
<td>cdc screening and analysis, backcross</td>
</tr>
<tr>
<td>RCY1122</td>
<td>MAT a ura3 ts</td>
<td>UV mutant of GG1888 by Heitz (2012) and Callaghan (2012)</td>
<td>cdc screening and analysis</td>
</tr>
<tr>
<td>RCY1123*</td>
<td>MAT a ura3 ts</td>
<td>UV mutant of GG1888 by Heitz (2012) and Callaghan (2012)</td>
<td>cdc screening and analysis, backcross</td>
</tr>
</tbody>
</table>
RCY1124 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening and analysis, backcross, tetrad analysis

RCY1125 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening and analysis

RCY1126 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1129 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1130 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1131 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1132 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1133 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1134 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

RCY1135 | \textit{MAT}^a \textit{ura}3 \textit{ts} | UV mutant of GG1888 by Heitz (2012) and Callaghan (2012) | \textit{cdc} screening

* Strains failed to show a temperature sensitive phenotype on solid YPD.

Cell Division Cycle Mutant Screening to Confirm Previous Study Results and Preliminary Screening of Novel Temperature Sensitive Mutants

Screen 1: The Strains RCY1119, RCY1120, RCY1124, RCY1125, GG1888, and RCY303 were retrieved from a -70°C freezer and streaked on YPD plates to grow at room temperature for 4-5 days. RCY1119, RCY1120, RCY1124, and RCY1125 were previously identified to be \textit{cdc} mutants by Heitz (2012) and Callaghan (2012). GG1888, a wildtype strain, served as the negative control. RCY303, a known large budded \textit{cdc} mutant, served as
the positive control (Oldroyd, 2002). Two test tubes per strain containing 4 mL of liquid YPD were inoculated with a ~0.2 cm² size and a ~0.4 cm² size amount of that strain. The tubes were placed on a rotating culture wheel at room temperature for about 24 hours.

A 1:15 dilution to a final volume of 1 mL was performed for the cloudiest liquid culture. The OD₆₀₀ was determined on a Jasco V-530 UV/VIS Spectrometer (Easton, MD). An OD₆₀₀ range of about 1-3 indicates that the cells are in log phase growth (actively dividing). If the cells are above an OD₆₀₀ of 3, they may be entering or in stationary phase, which would cause the whole culture to be unbudded cells, appearing to be a cdc mutant when it is not. The cloudiest culture was in log phase growth, allowing us to assume that tubes less cloudy were also still in log phase growth, so one tube for each strain was shifted to the restrictive temperature. The culture of GG1888 was diluted 50 fold in YPD before being shifted to allow for its continued growth at the restrictive temperature. The tubes were placed at a slant in a 36°C shaking waterbath at 270 RPM for 7 hours. A portable thermometer was used to monitor the temperature of the waterbath. At the end of the 7 hours, 200 proof ethanol was added to the tubes to a final volume of 50% to fix the cells, and the tubes were stored in the refrigerator (Weinert and Hartwell, 1993; Bohmer, 2009).

Next, bud morphology was scored for all strains. Two cultures at a time, each liquid culture was vortexed and 1.5 mL was transferred to a microcentrifuge tube. The cells were centrifuged at 15,000 RPM for 1 minute in a microcentrifuge. The supernatant was removed, and the cells were washed with 1 mL of water before being resuspended in 750 μL of water (half the original volume). The cells were then placed on ice and sonicated with a Branson Sonifier 450 (Danbury, CT) duty cycle 30 and output control 3 for 20 pulses with a microtip to separate cells from one another for easier visualization. Six μL of sonicated
cells were put on a slide and topped with a coverslip. The cells were visualized under a phase microscope at 1000x for scoring. For each strain, about 100 cells were scored as either unbudded, small budded, large budded, or unique budded (Figure 4). A 70% uniform bud morphology indicated a cdc mutant.

Screen 2: A second round of cdc screening was performed as above on the strains RCY1110, RCY1122, RCY1123, RCY1125, RCY1126, RCY303, GG1888, RCY1129, RCY1130, RCY1131, RCY1132, RCY1133, RCY1134, RCY1135. The strains RCY1110, RCY1122, RCY1123, RCY1125, RCY1126 were a continuation of the analysis of cdc mutants previously found by Heitz (2012) and Callaghan (2012). GG1888 and RCY303 remained the negative and positive controls, respectively. RCY1129-RCY1135 are ts mutants found by Heitz (2012) and Callaghan (2012) that were not previously screened for a cdc phenotype. The experiment was performed in the same manner as in Screen 1, except the waterbath temperature was increased to 37.1°C to elicit better arrest.

Screen 3: A third round of cdc screening was performed on RCY1123, RCY1126, RCY1124, GG1888, and RCY303. The same methods as in Screen 1 were used. The waterbath was at 36.8°C.

Screen 4: A fourth round of cdc screening was performed on RCY1134, GG1888, and RCY303. The same methods as in Screen 1 were used. The waterbath was at 36.3°C.

Nuclear Visualization

In order to determine the nuclear phenotype of the large budded cdc mutant RCY1124, 4',6-diamidino-2-phenylindole (DAPI) staining was used. Three μL of the washed and sonicated cells from the cdc screen were placed on a slide and 3 μL of 0.2 μg/mL DAPI
were added. A coverslip was placed on top of the slide and the cells were visualized at 1000X in an Olympus BX41 fluorescence microscope (Center Valley, PA) with an attached camera and a DAPI filter set.

**Backcross**

A backcross was performed as in Wolf *et al.* (2003). RCY1120, RCY1124, RCY1110, and RCY1123 were mated with LSY25 (wildtype) on a malt extract plate. A 0.2 cm² colony of each mutant and LSY25 were taken from YPD plates and mixed in 4 μL of liquid YPD on a malt extract plate. After 3 days at room temperature, 50 μL of sterile water were put onto an SD plate and a muddle of cells from the malt extract plate was made and spread around half of the SD plate. The SD plate selected for successful matings because the mutants are Ura⁻ and the wildtype is His⁻ Ade⁻ so haploids did not survive on the minimal media. Two colonies of each successful mating were spread on opposite halves of YPD plates and allowed to grow up. Each diploid strain was prepared and frozen for storage in a -70°C freezer (see below for freezing procedure).

**Recessiveness Test**

In order to discover if the *cdc* mutation was recessive in each of the mutants, a recessiveness test was performed on the diploid strains that resulted from the backcross. It was expected that if the mutation was recessive, the diploid would survive at the restrictive temperature and the mutant haploid would die at the restrictive temperature. YPD plates were streaked with the diploid on one half of the plate and the *cdc* haploid strain on the other side of the plate. Two sets of plates were made. One set of plates was
incubated at the permissive temperature, and the other set of plates was incubated in a 36.2°C incubator. The plates were scored for growth or no growth.

**Incubator Troubleshooting**

After observing that all haploid strains were surviving at 36.2°C, we investigated if it was the incubators or the haploid strains were misbehaving. Three different incubators were set at temperatures between 37.3°C and 38.8°C. The temperature was read with the same external portable thermometer for each incubator. Three rounds of test plates were created in which each haploid, LSY25, and RCY303 were plated on YPD plates and incubated for two days then scored for growth. Before the third round of test plates, all strains were broken out of the freezer to ensure that we were not testing diploids or revertants.

A final test was performed on RCY1120, RCY1123, GG1888, and RCY303. Each strain was grown up in 4 mL of liquid YPD. They were all similarly cloudy after 24 hours on the spinning culture wheel. The OD$_{600}$ of GG1888 was taken and the conversion factor 1 OD$=2	imes10^7$ cells was used to calculate a 23,500 fold dilution, which was performed to attain 300 cells per plate. Each of the 4 strains was diluted in sterile water 23,500 fold in a serial dilution of 1:235 followed by 1:100. Fifty μL of each dilution were spread on YPD plates. The plates were incubated at room temperature for 3 days then replica plated using a sterile velvet. The restrictive temperature plate was replica plated first, followed by the permissive temperature plate. The set of permissive temperature plates were incubated at room temperature for one week. The set of restrictive temperature plates were incubated
in a 38.9°C incubator for one week. The colonies were compared for growth at each temperature.

**Sporulation of Diploids from Backcross**

Four mL of sporulation media were put into three sterile test tubes and inoculated with 4 colonies of each diploid from the SD plates containing diploids from the backcross. The tubes were incubated at room temperature on a spinning culture wheel for 5 days, allowing the cells to undergo meiosis, forming ascospores. Each tube was scored for the percent of cells sporulated by putting a drop of the culture on a slide with a coverslip and viewing the cells at 400X. Approximately 100 cells were scored as sporulated or not sporulated (data not shown). The tubes were then stored in a 4°C refrigerator.

**Tetrad Dissection and Analysis**

The sporulated cultures were removed from the fridge and 0.5 mL was removed from one tube and placed in a sterile microcentrifuge tube. Tubes were centrifuged for 1 minute at 15,000 RPM. The supernatant was removed and 0.5 mL of sterile water was added. The tube was then vortexed and centrifuged again for 1 minute at 15,000 RPM. Washing was repeated twice more, then the cells were resuspended in 500 μL of sterile water. Beta-Glucuronidase (glusulase) (Sigma, St. Louis, G7770) stock solution was diluted 20 fold in sterile water and 100 μL of the dilution was added to 100 μL of the cell suspension. Cells were incubated with the glusulase for 10-12 minutes to loosen the asci around the ascospores. The cell/glusulase mixture was then diluted 10 fold in sterile water.
water, and 35 μL were dropped on one end of a YPD plate and the drop was allowed to run down the middle of the plate.

The spores of each tetrad were then viewed through a Micro Video Instruments Inc. Tetrad Dissection Microscope (Avon, MA) and separated from one another using a micromanipulator. Once separated, the spores were allowed to grow up into colonies. The colonies were then used for testing to determine if the cdc mutation was in a single gene.

Six analysis plates were created. The plates used were two YPD plates and one each of SD+Histidine+Adenine+Uracil, SD+Histidine+Adenine, SD+Histidine+Uracil, and SD+Uracil+Adenine. The supplements were added to the SD plates by spreading 300 μL of 0.2 g/100 μL histidine and uracil solutions and 0.3 g/100 μL adenine solution. Each plate was streaked with the four spores of each tetrad. All plates were incubated at room temperature, except for one YPD plate which was placed in a 37.5°C incubator. A 2:2 segregation pattern for each tetrad was expected for temperature sensitivity as well as for the selectable markers. The plates were scored for growth.

A chi-squared test was performed by hand to determine if there were significant indications of linkage between the ts mutations in each of the strains and the selectable markers. A physical distance calculation was performed to determine the theoretical distance of the cdc gene from the selectable marker using the equation RF=(NPD + (1/2) TT)/total tetrads x 100 (Hartwell et al., 2008), and that 2.9 kb= 1 cM (Cherry et al., 1997).

**Freezing New Segregants**

Strains were prepared for long-term storage by putting 800 μL of sterile 15% glycerol solution in a 2 mL vial. The yeast was then scraped off of a YPD plate with a sterile
toothpick and suspended in the glycerol solution. A cap was put on the vial and it was stored in a -70°C freezer (Guthrie and Fink, 1991).

Results

cdc Screen

A cell-division cycle mutant screen was performed in order to confirm the results of the previous cdc screen that was completed by Heitz (2012) and Callaghan (2012). The screen performed by Heitz and Callaghan yielded six cell division cycle mutants as defined by 70% uniform bud morphology upon arrest at the restrictive temperature. Representations of each bud size and shape are shown in Figure 4. RCY1110, RCY1120, RCY1124, RCY1123, RCY1125, and RCY1126 were rescreened for the Cdc* phenotype. RCY303 was used as the positive control and GG1888 was used as the negative control.

Figure 4: Bud Morphologies. Examples of unbudded, small budded, large budded, tiny budded, elongated bud, and lemon-shaped cells (left to right). Cells were imaged with phase contrast at 1000X magnification.

In cdc screen 1, RCY1120 was confirmed as an un/tiny budded (71.4%) cdc mutant and RCY1124 was confirmed as a large budded (69.7%) cdc mutant. RCY1125 showed varied bud morphologies, though it was previously found to be 60% small budded by Heitz (2012) and Callaghan (2012), so it was decided to rescreen it in cdc screen 2 in case we did not achieve good arrest in the first screen (Figure 5). RCY1119 would not grow so we were never able to screen it, but later realized that results about RCY1119 in a prior thesis were...
misreported and it should have read 'RCY1110' (Callaghan, 2012). RCY1119 was actually *E. coli*, so analysis was stopped and RCY1110 was added into the second screen (Table 2).

In the second *cdc* screen RCY1110 was found to be 59.6% unbudded (Figure 5), previously 68% unbudded by Heitz (2012) and Callaghan (2012). Though 59.6% is under the 70% cutoff, we decided to continue analysis because the same phenotype was observed and the positive control only showed 64% uniform arrest, when we expect it to be at least 70%. RCY1123 was found to be 76% unbudded (Figure 5) though it was previously 87% small budded by Heitz and Callaghan (2012). It was rescreened in screen 3 to investigate the phenotype discrepancy. In screen 3, RCY1123 was found to be 80% un/tiny budded. RCY1124 was arrested again so that DAPI staining could be performed because the old arrested culture was lost. It still held to be a large budded mutant. Finally RCY1126, or "Frankenyeast," had very strange bud morphology with elongated (Figure 6) and very enlarged buds (Figure 5), but it did not have 70% uniform bud morphology. The cells were very clumped together and distinguishing individual morphologies was difficult so no trends were observed, but a few cells did have the elongated and enlarged buds (Table 2).

In Screen 2 we also performed preliminary *cdc* screens on temperature sensitive mutants identified by Heitz and Callaghan (2012). RCY1122 through RCY1135 were identified as temperature sensitive, but had not yet been screened for the *Cdc-* phenotype. RCY1134 was found to be 68% unbudded, RCY1132 was found to be 61% unbudded, RCY1133 was found to be 58% large budded, and RCY1135 was found to be 58% unbudded (Table 2). Though these numbers are below the 70% cutoff, the arrest on RCY303 in this round was only 64% large budded, so these mutants are potentially *cdc* and should be
screened again. The other ts mutants showed varied morphologies or were farther below the 70% cutoff.

A fourth screen was performed which included RCY1134 because it was so close to the 70% cutoff in Screen 2. RCY1134 was found to be 80% un/tiny budded in Screen 4, confirming a Cdc+ phenotype (Figure 7).

Table 2: Summary of cdc Screen Results.

<table>
<thead>
<tr>
<th>cdc Screen Number</th>
<th>Strain</th>
<th>New Screen/Rescreen</th>
<th>Results</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RCY303</td>
<td></td>
<td>54.8% large-budded</td>
<td>Positive control. Expected to be higher.</td>
</tr>
<tr>
<td></td>
<td>GG1888</td>
<td></td>
<td>38% unbudded 29% small budded 33% unbudded</td>
<td>Negative control. Expected.</td>
</tr>
<tr>
<td></td>
<td>RCY1120</td>
<td>First Rescreen</td>
<td>71.4% unbudded/tiny budded</td>
<td>Confirmed. Previously 70% unbudded/tiny budded by Heitz and Callaghan (2012).</td>
</tr>
<tr>
<td></td>
<td>RCY1125</td>
<td>First Rescreen</td>
<td>38% unbudded 42% small budded 19% large budded 1% unique budded</td>
<td>Found 60% small budded by Heitz and Callaghan (2012). Repeat in screen #2.</td>
</tr>
<tr>
<td></td>
<td>RCY1119</td>
<td>First Rescreen</td>
<td>E. coli</td>
<td>Error in previous thesis. Disregard RCY1119.</td>
</tr>
<tr>
<td></td>
<td>RCY1124</td>
<td>First Rescreen</td>
<td>69.7% large budded</td>
<td>Confirmed. Previously 76.8% large budded by Heitz and Callaghan (2012).</td>
</tr>
<tr>
<td></td>
<td>RCY303</td>
<td></td>
<td>64% large budded</td>
<td>Positive control. Expected to be higher.</td>
</tr>
<tr>
<td></td>
<td>GG1888</td>
<td></td>
<td>34% unbudded 33% small budded</td>
<td>Negative control. Expected.</td>
</tr>
<tr>
<td>Strain</td>
<td>Screen Type</td>
<td>First Rescreen</td>
<td>Second Rescreen</td>
<td>Observations</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>GG1888</td>
<td>31% large budded</td>
<td>Negative control. 2% tiny budded</td>
<td>Previously 68% un budded by Heitz and Callaghan (2012). Proceed with backcross because same phenotype observed and control has low arrest.</td>
<td></td>
</tr>
<tr>
<td>RCY1110</td>
<td>First Rescreen</td>
<td>59.6% un budded</td>
<td>Previously 87% small budded by Heitz and Callaghan (2012). Proceed with backcross, but screen again.</td>
<td></td>
</tr>
<tr>
<td>RCY1123</td>
<td>First Rescreen</td>
<td>76% un budded</td>
<td>45% un budded</td>
<td>Not cdc. Found 60% small budded by Heitz and Callaghan (2012). Varied morphologies observed in Screen 1.</td>
</tr>
<tr>
<td>RCY1125</td>
<td>Second Rescreen</td>
<td>43% small budded 12% large budded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCY1122</td>
<td>New Screen</td>
<td>Varied morphologies</td>
<td>Inconclusive</td>
<td></td>
</tr>
<tr>
<td>RCY1129</td>
<td>New Screen</td>
<td>53% un budded</td>
<td>Inconclusive</td>
<td></td>
</tr>
<tr>
<td>RCY1130</td>
<td>New Screen</td>
<td>Varied morphologies</td>
<td>Inconclusive</td>
<td></td>
</tr>
<tr>
<td>RCY1131</td>
<td>New Screen</td>
<td>52% multibudded/lemon</td>
<td>Inconclusive</td>
<td></td>
</tr>
<tr>
<td>RCY1132</td>
<td>New Screen</td>
<td>61% un budded</td>
<td>Potential cdc mutant. Screen again.</td>
<td></td>
</tr>
<tr>
<td>RCY1133</td>
<td>New Screen</td>
<td>58% large buds</td>
<td>Potential cdc mutant. Screen again.</td>
<td></td>
</tr>
<tr>
<td>RCY1134</td>
<td>New Screen</td>
<td>68% un budded</td>
<td>Potential cdc mutant. Screen again.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>New Screen</td>
<td>58% unbudded</td>
<td>Potential cdc mutant. Screen again.</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-------------</td>
<td>-------------------------------------</td>
<td></td>
</tr>
<tr>
<td>RCY1135</td>
<td>New Screen</td>
<td>58% large budded</td>
<td>Positive control. Expected to be higher.</td>
<td></td>
</tr>
<tr>
<td>RCY303</td>
<td>Second Rescreen</td>
<td>80% un/tiny budded</td>
<td>Confirmed large budded. DAPI revealed single nucleus.</td>
<td></td>
</tr>
<tr>
<td>GG1888</td>
<td>First Rescreen</td>
<td>Misshapen, varied phenotypes. Cells too clumped to count</td>
<td>Not cdc</td>
<td></td>
</tr>
<tr>
<td>RCY1123</td>
<td>First Rescreen</td>
<td>72% large budded</td>
<td>Confirmed large budded. DAPI revealed single nucleus.</td>
<td></td>
</tr>
<tr>
<td>RCY1124</td>
<td>First Rescreen</td>
<td>39.6% unbudded 26.7% small budded 32.7% large budded 0.01% unique budded</td>
<td>Negative Control. Expected.</td>
<td></td>
</tr>
<tr>
<td>RCY303</td>
<td>First Rescreen</td>
<td>75% large budded</td>
<td>Positive Control. Good Arrest.</td>
<td></td>
</tr>
<tr>
<td>RCY1134</td>
<td>First Rescreen</td>
<td>80% un/tiny budded</td>
<td>Confirmed cdc. Previously 68% unbudded in screen 2.</td>
<td></td>
</tr>
</tbody>
</table>

3 (36.8°C)

4 (36.3°C)
Figure 5: Probable cdc Mutants Rescreened for Cdc Phenotype. Cells incubated for seven hours at the restrictive temperature were visualized under a phase microscope at 1000X. Red boxes indicate cells showing example of phenotype at arrest. A) RCY303- Positive control. Known large budded cdc mutant. B) GG1888- Negative Control. Varied bud size. No arrest. C) RCY1110- Unbudded at arrest. D) RCY1120- Unbudded/tiny budded at arrest. E) RCY1123- Unbudded at arrest. F) RCY1123 - Unbudded/tiny budded at arrest. G) RCY1124- Large budded at arrest. H) RCY1126- Abnormal cell morphology. Enlarged cell highlighted. I) RCY1125- Varied bud morphology at arrest.
Figure 6: Elongated bud morphology of RCY1126. Cells incubated for seven hours at the restrictive temperature were visualized under a phase microscope at 1000X.

Figure 7: Un/tiny budded morphology of RCY1134 from cdc screen four. Cells incubated for 7 hours at the restrictive temperature were visualized under a phase microscope at 1000X.

**Nuclear Visualization**

DAPI staining was performed on RCY1124, a large budded cdc mutant. It was found to have a single nucleus, indicating that its DNA has not been segregated (Figure 8).
Figure 8: DAPI Staining of RCY1124. Cells were incubated at the restrictive temperature for 7 hours then visualized as follows: A) Phase microscope at 1000X. B) Fluorescent microscope with a DAPI filter set at 1000X. Red boxes indicate the same group of cells. Using DAPI staining it was determined that RCY1124 has a single nucleus at arrest indicating that its DNA has not been segregated.

Recessiveness Test

In order to discover if the cdc mutation was recessive in each of the mutants, as a recessive mutation is a prerequisite for cloning by complementation, a recessiveness test was performed. Mutant strains RCY1110, RCY1120, RCY1123, and RCY1124 were successfully mated with the wildtype strain LSY25. Each diploid was then plated on a YPD plate next to its corresponding mutant haploid parent and one set of plates was put at the permissive temperature and one set was placed at the restrictive temperature (36°C). If the mutant phenotype was recessive, we would also expect the diploids to survive at the restrictive temperature because they have a wild type copy of the gene to compensate for their temperature sensitive mutation. If the mutant phenotype was dominant, we would expect the diploids to die at the restrictive temperature. After the first round of testing, all diploids, all haploids and LSY25 were growing at the restrictive temperature. Our temperature sensitive control, RCY303, was dying at the restrictive temperature and behaving as expected. The fact that none of the haploid mutants were dying at the
restrictive temperature led us to search for an alternate method that would allow us to see any differences in phenotype between the temperature sensitive haploids and temperature resistant diploids.

**Incubator Troubleshooting**

Test plates were created by streaking out each of the haploids, LSY25 and RCY303 controls on YPD plates. The temperatures of three incubators were then varied with each set of trials and the test plates were scored for growth at each temperature (Table 3). Of the haploids, RCY1110 and RCY1124 behaved as expected and died at all temperatures tried. However, RCY1120 and RCY1123 showed growth even at the highest temperatures indicating that they were no longer exhibiting the Ts phenotype and could not be used for a recessiveness test (Figure 9).

![Incubator Test Plates For Haploid Temperature Sensitive Phenotype](image)

**Figure 9:** Incubator Test Plates For Haploid Temperature Sensitive Phenotype. Star indicates position of LSY25. Continuing clockwise from the star the strains are RCY1124, RCY1110, RCY1120, RCY303, RCY1123. A) 120 Front Incubator 37.8°C, 1120, 1125, LSY25 showed growth. B) 120 Back Incubator 38.0°C, 1120, 1123, LSY25 showed growth. C) 118 Incubator 38.4°C, 1120, 1123, LSY25 showed growth.
Table 3: Growth of Haploid and Diploid Strains in Incubators of Varying Temperatures

<table>
<thead>
<tr>
<th>Incubator</th>
<th>Temperature °C</th>
<th>Growth Results</th>
</tr>
</thead>
</table>
| 118       | 37.5           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Front | 37.4           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Back  | 37.5           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 118       | 38.5           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Front | 37.8           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Back  | 38.0           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 118       | 38.4           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Front | 37.8           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Back  | 38.0           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 118       | 39.0           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Front | 38.5           | Dead: 303, 1110, 1124
             |                | Growth: 1120, 1123, LSY25 |
| 120 Back  | 38.8           | Dead: 1110, 1120, 303, 1124
             |                | Growth: 1123, LSY25 |

A final incubator test trial was performed on RCY1120 and RCY1123 by replica plating each strain and incubating one plate at the permissive temperature and one plate at the restrictive temperature. Strains were broken out of the freezer as well to ensure that contaminants, diploids, or revertants were not being plated accidentally. Plating for single colonies would reveal any differences between cells in the original stock. We anticipated finding some colonies of each that did not survive the restrictive temperature in case we had a mixed population where some reversion or adaptation was allowing some cells to survive. Any colonies that did not survive could then be recovered from the permissive temperature plate and used for a recessiveness test. However, all colonies survived at the
restrictive temperature for both strains and we were forced to abandon RCY1120 and RCY1123 (Figure 10).

![Figure 10: Replica Plating to Determine if Any RCY1120 or RCY1123 Colonies Show a Temperature Sensitive Phenotype. A) GG1888- Negative Control. Shows growth at both temperatures as expected. B) RCY303- Positive Control. Shows growth at the permissive temperature, but not the restrictive temperature, as expected. C) RCY1123- All colonies grew at 38.9°C. No colonies that could be used for a recessiveness test were found. D) RCY1120- All colonies grew at 38.9°C. No colonies that could be used for a recessiveness test were found.]

Recessiveness Test

A successful recessiveness test was performed on RCY1110 and RCY1124 in which both haploid strains died and both diploid strains, RCY1110xLSY25 and RCY1124xLSY25, survived at the restrictive temperature (Figure 11). If the mutated allele were recessive, we would expect the diploids to survive at the restrictive temperature because the ts haploids were mated with Ts strain LSY25. It was observed that the diploids survived at the restrictive temperature, just as the LSY25 wild type control did. The haploid strains
exhibited a Ts− phenotype, as did the RCY303 ts control. This indicates that there is a recessive cdc mutation in both RCY1110 and RCY1124 (Figure 11).

**Figure 11: Recessiveness Test on Diploid Strains RCY1124xLSY25 and RCY1110xLSY25.** This figure shows the ts haploid strains on the left of the plates and the corresponding diploid strains on the right side of the plate. A) RCY1124. B) RCY1110. C) RCY303 and LSY25 Controls. Incubation temperature is noted for each row of plates.

**Tetrad Dissection**

Tetrad dissection was performed on the sporulated diploid cells in order to confirm that the ts mutation was in a single gene (Figure 12). Tetrads of the RCY1110xLSY25 and RCY1124xLSY25 crosses were dissected successfully and plated to test for temperature sensitivity and segregation of selectable markers. We expected to see a 2:2 segregation of the ts:7S alleles to confirm that the mutation was in a single gene, as well as 2:2 segregation of the selectable markers to confirm that the four spores were from the same ascus. All of
our mutant strains are Ura' and LSY25 is His' Ade', so segregation of each of these markers was tested.

Figure 12: Example of a Successful TetrA Dissection from the Cross RCY1110xLSY25. Four spores were separated and grown up into colonies. The red color in two spores is due to the segregation of the ade1-52 allele into the haploid spores.

In the first round of tetrad analysis, two tetrads of RCY1110 were analyzed. All of the spores survived at the permissive temperature, but two spores from each tetrad did not survive at the restrictive temperature (Figure 13). Each of the selectable markers for each tetrad also segregated 2:2, as two spores from each tetrad survived and two did not when not provided with histidine, adenine or uracil, respectively (Figure 14).

Figure 13: Temperature Sensitivity Test of Two Tetrads from the Cross RCY1110xLSY25. Four spores from one tetrad are above the red line, and four spores from the other tetrad are below the red line. A) All spores from both tetrads survive at the 24°C. B) Two spores from each tetrad show the temperature sensitive phenotype and do not survive at 37°C.
Figure 14: Nutrient Requirement Analysis of two Tetrads from the Cross RCY1110xLSY25. Four spores from one tetrad are above the red line, and four spores from the other tetrad are below the red line. A) Growth of all individual spores when necessary nutrients were provided on minimal media. B) Growth of two spores from each tetrad when adenine was not provided. C) Growth of two spores from each tetrad when histidine was not provided. D) Growth of two spores from each tetrad when uracil was not provided.

Refer to Table S1 (Appendix) for complete data of all tetrads dissected. All tetrads of RCY1110 and RCY1124 dissected so far show a 2:2 segregation in the temperature sensitivity test as well as 2:2 segregation in the nutrient requirement analysis test. As shown in Table 4, most tetrads indicate unlinked genes when considering linkage of the ts to any of the marker genes. They show many tetratypes similar to the 1 PD: 4 TT: 1 NPD ratio expected for unlinked genes. The only possible indications of gene linkage can be seen in the ts and ade1 marker ratio of 5:2:0 of RCY1124 and the ts and ura3 marker ratio of 4:1:0 of RCY1110.
Table 4: Tetrad Types for Assessment of Possible Linkage of ts Allele to Selectable Markers

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<tr>
<td>RCY1110</td>
<td>0:5:0</td>
<td>0:5:0</td>
<td>4:1:0</td>
</tr>
<tr>
<td>RCY1124</td>
<td>5:2:0</td>
<td>2:3:2</td>
<td>0:6:1</td>
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A chi-squared analysis was performed to determine the likelihood of linkage in the most promising case for each strain. The null hypothesis that the 4:1:0 ratio = 1:4:1 ratio in RCY1110 was rejected ($\chi^2 = 14.5, p<0.001$) (Figure S2). The null hypothesis that the 5:2:0 ratio = 1:4:1 ratio in RCY1124 was rejected ($\chi^2 = 15.3, p<0.001$) (Figure S2). Genome distance calculations gave values of 29 kb for ura3 linkage in RCY1110 and 40 kb for ade1 linkage in RCY1124 (Figure S1) based on the average physical distance per map unit in S. cerevisiae.

Discussion

The purpose of this project was to analyze previously identified ts/cdc mutants to ensure that they had a recessive mutation and that the mutation was in a single gene, as both are requirements for cloning by complementation later in the project (Heitz, 2012; Callaghan, 2012). A secondary objective was to perform preliminary cdc screens on mutants previously identified as ts but not yet screened for a Cdc* phenotype (Heitz, 2012; Callaghan, 2012).

A cdc screen was performed on previously identified cdc mutants and ts mutants not yet screened for Cdc* phenotype (Heitz, 2012; Callaghan, 2012). Hartwell et al. (1973) disregarded mutants showing less than 80% uniformity, however we relaxed this number.
somewhat due to the Hartwell et al. (1973) observation that different alleles of the same cdc gene may cause lower amounts of uniformity (20-40% lower).

Of the previously identified cdc mutants, cdc mutations were confirmed in RCY1110, RCY1120, RCY1123, ad RCY1124, as each was found to have approximately 70% uniform bud morphology upon arrest at the restrictive temperature (Table 2). This screen showed that RCY1110 was 59.6% unbudded which is under the 70% cutoff, but in the original screen it was only found to be 68% unbudded by Heitz and Callaghan (2012). We decided to continue analysis on RCY1110 because the same phenotype was observed in each screen and the low arrest uniformity could be due to some cells advancing past a point of long delay or the second point of arrest. The arrest of the positive control, RCY303, in Screen 2 was only 64% indicating that low arrest may have also occurred in the mutants under our experimental conditions.

RCY1123 also exhibited strange phenotypic behavior. It was originally scored as a small budded mutant (Heitz, 2012; Callaghan, 2012). This was exciting because there is no known checkpoint in S. cerevisiae that would yield a small budded mutant. This arrest phenotype caused us to think perhaps there was some checkpoint mechanism that would arrest cells at the point in the cell cycle in which they have small buds in K. lactis. However, upon rescreening the first time, RCY1123 was found to be 76% unbudded. Due to the lack of agreement between the two phenotypes, it was screened a third time to be found 80% un/tiny budded (Figure 5). These two phenotypes were grouped together because it appeared as though the cells were trying to form buds but their bud initiation was unsuccessful. It was also difficult to distinguish between unbudded and tiny budded cells due to their orientation on the slide. Sometimes the tiny bud was hidden from view until
the cells rolled over on the slide. Analysis was continued because, though we did not confirm the small budded phenotype, the un/tiny budded phenotype was consistent between our two screens. This variance in phenotype agrees with the inconsistencies observed in the phenotypes of CDC1 mutants and can possibly be attributed to CDC1, or a similar gene, acting at multiple points throughout the cell cycle, especially through depolarization of the actin cytoskeleton (Paidhungat and Garrett, 1998; Rossanese et al., 2001). Other possibilities of genes responsible for a phenotype like that of CDC1 mutants are CDC49 and CDC50 (Moir et al., 1982). CDC50 is involved in cell polarization, actin cortical patch localization, and phospholipid metabolism (Saccharomyces Genome Database; Bryde et al., 2010). No further reports on the nature of CDC49 have been located.

CDC50 and CDC1 are similar in that they are both involved in the actin cytoskeleton as well as in phospholipid metabolism. Rossanese et al. (2001) proposed that the growth defects seen in cdc1 mutants are due to the depolarization of the actin cytoskeleton. cdc50 mutations also cause depolarization of the actin cytoskeleton and when not mutated, CDC50 is responsible for polarized growth after the emergence of the bud (Misu et al., 2003). Interfering with the actin cytoskeleton could affect general cell growth in many ways, resulting in tiny buds upon arrest. The growth problems may also be attributed to defects in phospholipid metabolism. CDC1 is a lipid phosphatase found in the membrane of the endoplasmic reticulum. When CDC1 is mutated, the cells accumulate an unknown phospholipid (Losev et al., 2008). Cdc50 is also a transmembrane protein found in the plasma membrane, nuclear membrane, and endoplasmic reticulum membrane that is involved in phospholipid transport (Bryde et al., 2010). It is associated with P4-ATPases, which are responsible for maintaining phospholipid asymmetry on either side of plasma
membranes (Bryde et al., 2010). P4-ATPases move phospholipids from one leaflet of the membrane to the other to maintain unequal phospholipid compositions, and they also flip phospholipids from the leaflet where they are inserted after they are made to the other leaflet (Bryde et al., 2010; Alberts et al., 2007). Cdc50 interactions with P4-ATPases are necessary for this phospholipid transport to occur. Perhaps it is this similarity in biochemical function of each of these proteins that is responsible for cell cycle disruption and arrest with tiny buds.

RCY1125 and RCY1126 were not confirmed as cdc mutants due to a lack of uniform bud morphology upon arrest (Table 2). RCY1125 showed a trend towards more unbudded and small budded cells, so it appears that there is some kind of disturbance in the cell cycle. However, there were about equal numbers of unbudded and small budded cells, therefore we could not classify it as 70% uniform. RCY1126 also did not meet the 70% uniformity cutoff because the cells were too clumped to count, but it showed strange phenotypes of swollen cells and elongated buds (Figure 5; Figure 6). We discontinued analysis on these mutants for this project, but they may be worth investigating by others in future projects. The swollen cells of RCY1126 may be a normal cdc mutant phenotype because, unlike other ts mutants, cell cycle mutants continue to grow even after the cell cycle has stopped. Swollen cells could also indicate that this particular mutant has uncoupled its cell growth and cell cycle, which are normally closely regulated to maintain uniform cell size. A number of genes have been shown to be involved in the coupling of cell size to the cell cycle. Zhang et al. (2002) identified genes that, when mutated, caused cells to be abnormally large (uge mutants) or abnormally small (whi mutants). In a genome-wide search, 20 whi mutants and 29 uge mutants were identified. The major functions of these
gene products in the cell include transcription regulation, signal transduction, and cell cycle regulation (Zhang et al., 2002). Elongated buds often occur when the cell is able to pass Start and initiate bud growth, however M-Cdks are not turned on. When the bud first emerges, apical, or outward growth, occurs. Without the M-Cdks active, the cell never makes the switch to isotropic, or bud enlargement growth (Lew and Reed, 1993). Lew and Reed (1993) proposed that the Clb/Cdc28 kinase was responsible for depolarizing the cortical actin patches in the bud allowing for outward growth. Finally, "monster" phenotypes have been observed in cells with uncapped telomeres (Smith and Blackburn, 1999). These "monster cells" appear large or elongated or remain in chains (Smith and Blackburn, 1999).

In the first three cdc screens, RCY303, our positive control did not show as consistent a large budded phenotype as was expected. RCY303 is a known well-behaved cdc mutant so we would expect to see at least a 70% uniform bud morphology in each screen. However the consistency was 54.8%, 64%, and 58% for screens 1, 2, and 3, respectively. This indicates that we may not be achieving the level of arrest that we should be with any of the cdc mutants. In order to try to correct this problem, the temperature on the waterbath was turned up from 36°C to 37°C between screen 1 and screen 2 in hopes of achieving better arrest, which it did slightly, but still not to the 70% level expected. It is possible that the temperature in the waterbath fluctuates, as we have not tried to constantly monitor and record the temperature over the seven hours. Good arrest for RCY303 was finally achieved in screen 4 (75% large budded), though the temperature was intermediate to where it was between the first and second screens (36.3°C).
Of the ts mutants that were screened for a cdc phenotype for the first time, RCY1134 showed a 68% unbudded phenotype, RCY1132 showed a 61% unbudded phenotype, RCY1133 showed a 58% large budded phenotype, and RCY1135 showed a 58% unbudded phenotype (Table 2; Figure 5). RCY1134 was screened again in screen 4, yielding an 80% un/tiny budded phenotype, confirming that it is a cdc mutant (Figure 7). RCY1132, RCY1133, and RCY1135 should also be screened again to confirm the cdc phenotype because they all fell well above the wildtype distribution level and this was a screen where the arrest on RCY303 was only 64% indicating that better arrest can possibly be achieved in all strains, as it was in RCY1134.

DAPI staining of RCY1124, a confirmed large budded cdc mutant, showed a single nucleus indicating that the DNA had not yet been segregated into the daughter cell (Figure 8). This lack of segregation could be due to a problem with DNA replication, the spindle, or with anaphase promoting complex. An issue with DNA replication in S phase would cause the cell cycle to be arrested at the metaphase-to-anaphase transition when the cell has a large bud, assuming the checkpoint in K. lactis is similar to S. cerevisiae (Morgan, 2007). Some genes involved in the process of DNA replication include CDC17 (DNA polymerase I), CDC2 (DNA polymerase III), and CDC9 (DNA ligase) (Hartwell and Weinert, 1989). If the spindle was not fully formed, or not functioning properly, the chromosomes would not be correctly aligned on the spindle in mitosis, so the spindle checkpoint would inhibit Cdc20, thereby preventing the activation of APC (Hwang et al., 1998). Without APC the cell cannot enter anaphase so the cell will be arrested at the metaphase to anaphase transition checkpoint. Finally problems with anaphase promoting complex, a ubiquitin ligase, would not allow the cells to make the transition from metaphase to anaphase. Normally, when
activated by Cdc20, anaphase promoting complex tags securin for degradation. Once securin is degraded by a proteosome, separase can cleave the cohesion that holds the sister chromatids together so that they can be segregated into the mother and daughter cells (Peters, 2002). These cells may also be defective in the G2 to M transition, however if this were the case we would expect to see elongated buds, as in S. cerevisiae (Lew and Reed, 1993).

The confirmed cdc mutants RCY1120, RCY1124, RCY1110, and RCY1123 were moved onto the backcross portion of the experiment. Each cdc haploid was mated with LSY25, a wildtype strain, to form diploid strains. Performing a backcross was a prerequisite for performing a recessiveness test on the mutant strains as well as a tetrad dissection. One successful round of backcrossing was performed on each mutant, though in the future more rounds of backcrossing should be performed because backcrossing repeatedly has the advantage of removing mutations that may be adding phenotypes that are unrelated to the Ts- phenotype we are selecting for. The use of random UV mutagenesis likely generated mutations that we can and cannot detect through phenotype. With each backcross in which we select only for the Ts- phenotype, we have a 50% chance of removing any other unrelated mutations as they become replaced with wild type alleles. What this allows us to look for are phenotypes that segregate perfectly with the Ts- phenotype, indicating that the ts allele is in fact the cause of that phenotype. We can select out undesirable phenotypes such as slow growth if we notice they do not segregate perfectly with the Ts- phenotype, leaving us a strain that is easier to work with.

The backcross will also yield segregants after sporulation and tetrad dissection that still hold the ts mutation but have an array of different markers and mating types. These
varied strains can be used for complementation testing in which we will cross our mutants to each other. If the strains have ts mutations in different genes, the diploid will hold a functional copy of each gene, therefore the diploid will not show a temperature sensitive phenotype. However, if the two strains have ts mutations in the same genes, the mutation will not be complemented and the diploid will show a temperature sensitive phenotype. This will allow us to identify if we have found cdc mutations in genes previously identified by this project, or if we have generated cdc mutants new to this project.

A recessiveness test was then performed to ensure that when cloning by complementation occurs, the wildtype gene will be dominant to the cdc mutation, thereby saving the transformant and allowing for identification of the mutated gene. When a recessiveness test was performed it was expected that the haploid strains would die at the restrictive temperature, while the corresponding diploid strains would survive at the restrictive temperature because they have a wildtype copy of the gene to compensate for the temperature sensitive mutation. However, in the first round of recessiveness testing, all haploids survived at the restrictive temperature. We were not able to draw any conclusions about the diploids from this test because the haploids were not displaying the temperature sensitive phenotype at 36°C. We decided to turn the incubator up to 37.5°C to make the restrictive conditions more stringent and because the cells were arrested for cdc screening at 37°C. Test plates were created with all of the haploids and controls on them. At approximately 37.5°C, RCY1110 and RCY1124 showed a temperature sensitive phenotype, however RCY1123 and RCY1120 both showed growth (Figure 9). Test plates were continually created as the incubator temperatures were made higher and higher. However with each set of plates the same results were observed- RCY1110 and RCY1124
were dying and RCY1123 and RCY1120 were not showing a visible growth disturbance. We discontinued incubator testing when the incubators got up to approximately 39°C because any temperatures much higher than that will kill wild type strains as well. We hypothesized that perhaps we had revertants or that maybe some colonies would behave temperature sensitive and others would not, so a round of plates containing many single colonies of RCY1123 and RCY1120 were created by replica plating. However, these plates also showed that each colony that grew at the permissive temperature also grew at the restrictive temperature (Figure 10), not yielding any colonies that could potentially be used for recessiveness testing.

The apparent change in the temperature sensitive phenotype of RCY1120 and RCY1123 is perplexing for a couple of reasons. First, previous researchers were able to identify these mutants as ts in the initial screen after mutagenesis on solid YPD media. A potential explanation for the change in behavior of the strains is that they somehow acquired behavior altering secondary mutations or adaptations between when they were discovered and when they were frozen for storage. Another possibility is that, though the YPD plates were made with the same recipe, the components of the plates may have slight differences. For example, the peptone appears less granular now than it used to (L. Silveira, personal communication).

The temperature sensitive phenotype discrepancy is also interesting because mutants that were scored for a Cdc phenotype after arrest at the restrictive temperature in liquid YPD were no longer behaving temperature sensitive on solid YPD. It is possible that this effect is due to the media we are testing them on. Perhaps solid YPD provides more osmotic support than liquid YPD so that the mutant protein is stabilized when on plates,
but shows a ts phenotype in liquid media because there is less osmotic support. A similar phenomenon has been observed in RCY246, which shows a temperature sensitive phenotype on rich media, but not on minimal media (Louie, 2008). It was hypothesized that this result was seen due to the amount of osmotic support provided in each type of media (Louie, 2008). The temperature sensitive phenotype of chitin synthesis mutants has been shown to be suppressed on minimal media due to the higher salt concentration (Bulawa, 1992). Similarly, temperature sensitive nutritional mutants of *S. cerevisiae* have been shown to be osmotic-remedial because the higher salt concentration stabilizes the mutated protein (Hawthorne and Friis, 1964). Perhaps RCY1120 and RCY1123 are a similar case of mutant sensitive to osmotic support levels. Their same tiny budded phenotype suggests that they may have similar behavior when it comes to their levels of temperature sensitivity.

Both RCY1123 and RCY1120 show a tiny bud phenotype upon arrest at the restrictive temperature. There are a number of potential defects associated with tiny budded mutants, some of which have phenotypes that vary with conditions other than temperature. *PKC1* mutants in *S. cerevisiae*, arrest after DNA replication and with a small bud, however upon addition of Ca^{2+} to the medium, the mutant protein product, Pkc1, regains function (Levin and Bartlett-Heubusch, 1992). Pkc1 is involved in a cell wall integrity signaling pathway and a disruption in the pathway causes the cells to lose osmotic stability, especially at 37°C, where the cells need an osmotic stabilizer to be viable (Saccharomyces Genome Database). Pkc1 interacts with Rho1, and mutations of some alleles of *RHO1* cause unbudded cells, while mutations in other alleles of *RHO1* cause small budded cells (Saccharomyces Genome Database). Another protein, Fks1, also interacts
with Rho1, and when mutated shows a temperature sensitive phenotype. At the restrictive temperature \textit{FKS1} mutant cells arrest shortly after bud emergence, similar to \textit{PKC1} mutants, indicating a likely checkpoint that arrests mutants in response to inadequate cell wall biosynthesis (Suzuki \textit{et al.} 2004; Negishi and Ohya, 2010). A defect in any of the \textit{PKC1}, \textit{RH01}, or \textit{FKS1} pathways that do not completely knock out the pathway may be the cause of observing the un/tiny budded phenotype in liquid but leaving mutants that do survive on the plates. A slight change in nutrient composition of the plates, for example a slight increase in calcium, may also be the cause of the Ts* phenotype.

A similar phenomenon of nutrient sensitivity was described by Paidhungat and Garret (1998) in \textit{cdc1} mutants. \textit{cdc1} mutant growth defects were reversed by addition of Mn$^{2+}$ to the medium. This observation led them to hypothesize that \textit{CDC1} regulates intracellular Mn$^{2+}$ in order to compensate for Mn$^{2+}$ depleted environments. Paidhungat and Garret (1998) propose that Cdc1 is involved in catalyzing Mn$^{2+}$ transport or regulating Mn$^{2+}$ transporters.

\textit{RCY1123} and \textit{RCY1120} were the un/tiny budded mutants identified by this project. They seemed to behave like \textit{cdc1} mutants in their phenotype, especially \textit{RCY1123}, which showed small (Heitz, 2012; Callaghan, 2012), unbudded, and un/tiny budded phenotypes. They may also be exhibiting defects in the \textit{PKC1}, \textit{RH01}, or \textit{FKS1} pathways. In the future it could be worth experimenting with different media for the recessiveness test to elicit a difference in phenotype between the haploids and the diploids. It would be valuable to be able to continue work on these mutants because they are different than the large budded mutants that have already been identified by this project. If we could identify whether more osmotic stress would cause \textit{RCY1123} and \textit{RCY1120} to behave Ts* perhaps we could
continue analysis on them. To investigate this, 2-3 replicate tubes of GG1888, RCY1123, RCY1120, RCY1110, and RCY1124 in liquid YPD could be created. These tubes could then be incubated in a shaking waterbath at around 37°C, which is a temperature at which Cdc\(^{-}\) phenotypes were observed in RCY1120 and RCY1123. If growth is observed in GG1888 but not the other strains, it would suggest that the Ts\(^{-}\) phenotype varies depending on the kind of medium. If this experiment was successful, an extension could be to create half-strength YPD plates, as in Bulawa (1992), which hopefully would offer less osmotic support than full strength YPD and would allow for plate analysis.

After incubator troubleshooting was completed, a successful recessiveness test was performed on RCY1124 and RCY1110, in which it was confirmed that the ts mutations were recessive (Figure 11). At this point, analysis was discontinued on RCY1123 and RCY1120, but RCY1110 and RCY1124 were moved onto tetrad analysis, which would allow us to determine if the mutation was in a single gene. The mutation must be in a single gene so that when cloning by complementation is performed, a single gene on a plasmid will be able to compensate for the cdc mutation. We dissected the tetrads that resulted from the sporulation (meiosis) of the diploids. What we expected to see if the mutation was in a single gene was a 2:2 segregation pattern in the tetrads of the Ts\(^{-}\):Ts\(^{+}\) phenotype. A 2:2 segregation would allow us to distinguish a single ts from multiple ts mutations, which would give more Ts\(^{-}\) spores than expected; 4:0 segregation, or 3:1 or 2:2 segregation. If there were two ts mutations present we would see three different segregation patterns; parental ditype (PD) is when a tetrad yields two kinds of spores, each which looks like one of the parents (tetrad would be 2:2 Ts\(^{-}\):Ts\(^{+}\)), non-parental ditype (NPD) two kinds of spores neither of which look like the parents (tetrad would be 4:0 Ts\(^{-}\):Ts\(^{+}\)), and tetratype
(TT) where all four spores are different (tetrad would be 3:1 Ts⁻:Ts⁺). If the two ts genes were unlinked, a 1 PD: 4 TT: 1 NPD ratio of tetrads would be expected. If the genes were linked, more and more 2:2 tetrads would be observed the more tightly the genes were linked, with parental ditype increasing and tetratype and nonparental ditype decreasing. We could also identify the possibility of needing two alleles at once to give the Cdc⁺ phenotype if we saw fewer Ts⁻ spores than expected; mostly 1:3 with some 2:2 and 0:4 segregation. We would be less likely to see Ts⁻ spores because both mutant alleles must be present in the same spore, making it more likely that the spore would only have one or the other and show a wild type phenotype.

So far, five tetrads of RCY1110 have been dissected and seven tetrads of RCY1124 have been dissected. Every tetrad has shown a 2:2 segregation pattern for the temperature sensitive phenotype (Figure 13; Table S1) indicating that each strain likely only has one ts mutation. This result is supported by seeing that the ade, his, and ura markers of the diploids are segregating 2:2 as well, ensuring that the four colonies were in fact the four spores of the same ascus (Figure 14; Table S1). More tetrad dissections should be performed on RCY1110 and RCY1124 to make sure that the 2:2 segregation pattern of temperature sensitivity holds true for all tetrads dissected and to confirm that we see no indications of multiple tightly linked ts mutations. After that we will be able to confirm with certainty that the mutation is in a single gene for each of these strains.

Additional analysis was performed on the segregants to look for any acquired auxotrophies in the mutagenized haploids besides the intended ts mutation. In order for cloning by complementation to be successful, the haploids must have only a single ts mutation and the original ura mutation of GG1888. Transformants will be selected for on
SD plates because the plasmid will provide a functional URA gene for the haploids. If any additional mutations have been acquired, say in leu, no transformants will be recovered even if they were generated. The tetrad data provides confirmation of only the wanted mutations because any tetrads with auxotrophies besides ura, ade, or his, would not grow on the SD+uracil+adenine+histidine plates, and if the strains had additional ade, ura or his mutations, 2:2 segregation of the ade, ura, and his markers would no longer be observed. So far, each tetrad dissected has shown growth on the SD+uracil+adenine+histidine plate and the ade, ura and his markers have been segregating 2:2, indicating that with respect to auxotrophies (Table S1), both RCY1110 and RCY1124 are candidates for cloning by complementation.

Finally, the tetrads can reveal if the ts genes are closely linked to the his7, ade1, or ura3 selectable markers. The tetrads were analyzed to look for recovery of only parental ditypes for any of the selectable markers (Table 4), which would indicate strong linkage of the ts, and therefore cdc mutation to a selectable marker. Mostly, no indication of linkage was observed, as the ratios observed were similar to the 1 PD:4 TT:1 NPD ratio of tetrads expected for unlinked genes. The only possibilities of linkage indication are between the ts mutation and ade1 mutation of RCY1124 with a ratio of 5:2:0, and the ts mutation and ura3 mutation of RCY1110 with a ratio of 4:1:0 (Table 4). The null hypothesis that the 4:1:0 ratio = 1:4:1 ratio in RCY1110 was rejected ($\chi^2 = 14.5$, p<0.001), indicating that there is significant indication of linkage between ura3 and ts (Figure S2). The null hypothesis that the 5:2:0 ratio = 1:4:1 ratio in RCY1124 was rejected ($\chi^2 = 15.3$, p<0.001), indicating that there is also significant indication of linkage between ts and ade1 in RCY1124 (Figure S2). These $\chi^2$ values should be recalculated once more tetrads have been dissected and there is...
a larger sample size. However, with the data present now we assumed that the \( \chi^2 \) does in fact indicate linkage and proceeded as such. Genome distance calculations were performed to investigate the nature of the linkages and gave values of 29 kb for \( \text{ura3} \) linkage in RCY1110 and 40 kb for \( \text{ade1} \) linkage in RCY1124 (Figure S1). These physical distances provide guidance for predicting possible \( \text{cdc} \) genes located within these distances of the selectable markers in the genome.

Because RCY1110, an unbudded mutant, has a phenotype not yet seen by this lab, we are optimistic that it will lead to identification of a gene not yet found by this lab. Its unbudded phenotype indicates that it is arrested at Start and could be due to a deficiency in nutrient sensing, problems with G1-Cdk activation, or a mutation causing activation of mating signaling which stops the cells before Start because they think they are mating. RCY1124 does have a phenotype that has already been identified by this project, with probable defects in spindle formation, anaphase promoting complex, or DNA replication. However we hope that it will fall into a new complementation group.

Some possibilities of \( \text{cdc} \) genes within 29 kb of the \( \text{ura3} \) gene that may yield the unbudded phenotype of RCY1110 when mutated include KLLAOE22683g which is a potential substrate of Cdc28, KLLAOE22551g which is a ubiquitin-protein ligase, KLLAOE22925 which is involved in the actin cytoskeleton, KLLAOE23189g which is responsible for priming origins of DNA replication in G1, and finally a host of hypothetical proteins including KLLAOE22947g, KLLAOE22749g, and KLLAOE22595g (Génolevures). Some possibilities of genes within 40 kb of \( \text{ade1} \) that may yield the large budded phenotype in RCY1124 when mutated include KLLAOC04037g which is a potential substrate of Cdc28, KLLAOC04345g which is involved in replication for progression, KLLAOC04411g which is
involved in the DNA damage response, KLLAOC04455g which is another possible substrate of Cdc28, and finally hypothetical proteins such as KLLAOC04059g (Genolevures). At this point, all of these possibilities are purely hypothetical and further analysis will be needed to pinpoint which gene is mutated.

After analysis on RCY1110 and RCY1124 has concluded, they will be ready for cloning by complementation, which will allow for identification of the mutated gene. Each mutant will be transformed with a K. lactis DNA library. The transformants will then be shifted to the restrictive temperature, and the transformants with the gene that complements the mutated gene will be identifiable by their survival at the restrictive temperature. Once the gene is identified it can be cloned and sequenced, and we hope to find genes that are brand new in this search.

Our linkage analysis can also help to confirm that a gene identified during cloning by complementation is in fact the cdc mutation and not a suppressor gene. Suppressor genes are genes that could help our mutants bypass the defect resulting from the cdc mutation. Comparing the location in the genome of the complementing gene to a marker that is known to be linked to the cdc mutation can help us decipher this. If the complementing gene is found well outside the range of how tightly we expect the cdc mutation to be linked to the marker, there is a good chance that our complementation testing has yielded a suppressor gene.

Analysis of putative cdc mutants has generated two strains, RCY1110 and RCY1124, that look to be likely candidates for complementation testing and cloning by complementation. We are hopeful that these two mutants will reveal cdc genes not yet identified by this project, or more significantly that they reveal cdc genes not yet identified
by any previous research. The abundance of genomic similarities between yeast and humans makes yeast a valuable tool for identifying new cell cycle factors. We hope to add to the overall knowledge of factors that regulate the cell cycle in humans so as to generate better treatments for diseases that result from a malfunctioning of the cell cycle.
Appendix

Table S1: Growth Results for all Tetrads Dissected. This table shows the results of the temperature sensitivity screen as well as the nutrient requirement analysis for each tetrad dissected for the strains RCY1110 and RCY1124. An "X" indicates growth on the medium noted in the column heading.

<table>
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<th>Tetrad</th>
<th>24°C</th>
<th>36°C</th>
<th>SD+Ade+His+Ura</th>
<th>SD+Ura+His</th>
<th>SD+Ade+Ura</th>
<th>SD+Ade+His</th>
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<td>X</td>
<td>X</td>
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<td></td>
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<tr>
<td>1B</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td></td>
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<td>X</td>
<td>X</td>
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<tr>
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<td>X</td>
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</table>
### Figure S1: Genome Distance Calculation

$$RF = \frac{NPD + (1/2)TT}{\text{total tetrads x 100}}$$ (Hartwell et al., 2008)

2.9 kb = 1 cM in *S. cerevisiae* (Cherry *et al.*, 1997; no information is available for *K. lactis*)

**RCY1110- ts and ura3, 4:1:0**

$$RF = \frac{0 + (1/2)(1)}{5} \times 100 = 10 \text{ cM (2.9 kb/cM)} = 29 \text{ kb}$$

**RCY1124- ts and ade1, 5:2:0**

$$RF = \frac{0 + (1/2)(2)}{7} \times 100 = 14 \text{ cM (2.9 kb/cM)} = 40 \text{ kb}$$

### Figure S2: Chi-Squared Test

**RCY1124**

Null hypothesis: 5:2:0 = 1:4:1  
Expected = 7(1/6): 7(4/6): 7(1/6) = 1.167: 4.67: 1.167  
Observed: 5:2:0  
Degrees of Freedom = 2  

$$\chi^2 = \frac{(5-1.167)^2}{1.167} + \frac{(2-4.67)^2}{4.67} + \frac{(0-1.167)^2}{1.167} = 12.6 + 1.5 + 1.167 = 15.3$$

p < 0.001
Null Hypothesis: 4:1:0 = 1:4:1
Expected: 5(1/6): 5(4/6): 5(1/6) = 0.833: 3.33: 0.833
Observed: 4:1:0
Degrees of Freedom= 2

\[ \chi^2 = \frac{(4-0.833)^2}{0.833} + \frac{(1-3.33)^2}{3.33} + \frac{(0-0.833)^2}{0.833} = 14.5 \]
\[ p < 0.001 \]
References


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