2013

Using Backcrosses and Tetrad Analysis to Investigate Cell division Cycle Mutants in Kluyveromyes lactis

Sara Good
University of Redlands

Follow this and additional works at: https://inspire.redlands.edu/cas_honors

Part of the Biology Commons, Cell and Developmental Biology Commons, and the Genetics and Genomics Commons

Recommended Citation

Creative Commons Attribution-Noncommercial 4.0 License
This work is licensed under a Creative Commons Attribution-Noncommercial 4.0 License
This material may be protected by copyright law (Title 17 U.S. Code).
This Open Access is brought to you for free and open access by the Theses, Dissertations, and Honors Projects at InSPIRe @ Redlands. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of InSPIRe @ Redlands. For more information, please contact inspire@redlands.edu.
Using Backcrosses and Tetrad Analysis to Investigate Cell division Cycle Mutants in *Kluyveromyces lactis*

Sara Good
Honors Thesis
Research Advisor: Dr. Linda Silveira
May 21, 2013
Abstract

The cell division cycle is the process by which all cells grow and reproduce. Mechanisms underlying the control of this complicated process have been studied at great length, but are not completely understood. Proper timing and sequencing of events in the cell cycle are maintained by gene products (Morgan, 2007). These Cdc (cell division cycle) proteins perform specific cellular functions that will promote transitions in the cell cycle depending on the current conditions of the internal and external environment. It is important to isolate and identify the function of CDC genes in order to understand how a normal cell cycle works, and how to fix it if it malfunctions. An ideal subject for studying the cell cycle in eukaryotes is budding yeast.

Currently, our lab has identified ten cdc mutants using a temperature sensitive (ts) and cdc screen in the budding yeast K. lactis that fall into eight complementation groups. Several additional strains of K. lactis have been identified as potentially carrying cdc mutations in new CDC genes not yet identified. The project discussed in this thesis sought to confirm that these strains were cdc mutants through a new cdc screen. Afterwards, a backcross of the newly identified cdc mutants was performed to create diploids for a recessiveness test and tetrad analysis. Two cdc mutant strains, RCY1110 and RCY1124, were found to carry recessive genes. After several tetrad analyses of these two strains, each was found to only contain a single mutated gene. The ts gene in RCY1110 displayed possible linkage to URA3, while the ts gene in RCY1124 displayed possible linkage to ADE1. Cells from the RCY1110 strain have no bud upon arrest, while cells from the strain RCY1124 exhibit a large bud upon arrest. In future, these mutant strains can undergo complementation testing before the final step of identifying the mutated gene with cloning by complementation. Several other strains including RCY1120 and RCY1123 were found to exhibit a Cdc' phenotype, but analysis was discontinued as they were
found not to be ts on solid media. One strain, RCY1126, was concluded to deviate from the usual bud morphology, but could not be categorized as a cdc mutant because there was no uniform arrest of the cells. RCY1126 and the apparent non-ts strains would be interesting strains to study in a future investigation.

**Introduction**

In order for life to continue on producing future generations, there needs to be a method of reproduction that will pass on genetic information. The smallest unit of life that makes up all organisms is the cell. As it is a form of life, the cell must also have a way to reproduce and continue handing off its DNA to future cells. The way in which cells complete this task is through the efficiently organized cell division cycle (Morgan, 2007).

The cell cycle is broken up into several phases so as to maintain the proper sequence of essential cell-cycle events (Alberts *et al.*, 2002). In the gap phases, G1 and G2, the cell grows and monitors the external and interior environment to determine when conditions are right for the cell to replicate its chromosomes and divide. If during G1-phase the environment is deemed favorable, then the cell transitions into S-phase, which is in-between G1 and G2. In this phase, the DNA of the cell is replicated to produce two sets of identical DNA molecules. After more monitoring at G2, the cell transitions to M-phase, where mitotic division occurs in a series of steps (Alberts *et al.*, 2002).

Mitotic division begins with prophase when, in most eukaryotic cells, the nuclear membrane of the cell breaks down and the genetic information inside condenses into sister chromatids (Alberts *et al.*, 2002). During metaphase, microtubule spindle fibers from the mitotic spindle attach to each pair of sister chromatids and align them in the middle of the cell. After the
enzyme separase destroys the cohesion that holds the sister chromatids together, anaphase begins with each individual sister chromatid being pulled to one side of the cell or the other by the spindle fibers. During telophase, the mitotic spindle is broken down and the sister chromatids on either side of the cell are packaged into two new nuclei and become uncondensed. Cytokinesis is the physical splitting of the old cell into two identical daughter cells (Alberts et al, 2002).

The cell cycle is a thoroughly regulated process that heavily relies on a control system to coordinate events to occur at the right time and in the correct order (Morgan, 2007). This control system ensures that problems with machinery during any phase of cellular replication are fixed prior to the transition to subsequent phases. Positive transition regulators are what induce the forward movement of the cell through each phase. However, if there are any problems detected along the way then the cell arrests at checkpoints controlled by negative checkpoint regulators. In mammalian cells, there are three transitions in the cell cycle that contain checkpoints regulated by these negative signals. The Start checkpoint precedes the G1 to S-phase transition and will not allow progression to subsequent phases of the cell division cycle if there are deficits in cell growth. In many cells, the G2 to M-phase checkpoint will not allow the cell to enter mitosis if the DNA is not properly replicated. The last checkpoint occurs right before anaphase where the spindle is checked for damage before the segregation of sister chromatids. If any problems are detected at checkpoints, a functioning cell will arrest cellular division until these problems are resolved. It accomplishes this by inhibiting the positive transition regulators, which are the enzymes responsible for continuing the cell division cycle (Morgan, 2007)

The enzymes responsible for initiating most of the events in cell division are called cyclin-dependent kinases (CDK’s), which are regulated by cyclins, cyclin-dependent kinase inhibitors (CKI’s), and phosphorylation (Morgan, 2007). As a general rule, CKI’s and
phosphorylation deactivate CDK's, while dephosphorylation and cyclins activate them. Cyclins and CKI's bind to CDK’s and activate or block the active site respectively. Phosphorylation effectively blocks the active site of CDK’s (Alberts et al, 2002). After a cell passes a checkpoint, certain CDK’s are activated in order to initiate specific events at each cell division phase. The basic mechanism of action for an activated CDK is to phosphorylate targets so that they can perform the specific functions needed at each stage of the cell cycle. In this way, CDK’s are responsible for changing the cell cycle state (Alberts et al., 2002).

The G1/S cyclin-CDK complexes are activated in late G1 so as to commit the cell to a cycle of division (Morgan, 2007). This carries the cell past the Start checkpoint and allows for immediate activation of S-phase cyclin-CDK systems. These complexes activate proteins involved with S-phase processes; mainly those involved in DNA replication and centrosome duplication. Activation of M-phase cyclin-CDK complexes occurs at the termination of S-phase and the G2 to M-phase checkpoint is passed by the cell. In mammalian cells, the proteins activated by M-phase CDK’s help the cell reach metaphase by inducing the assembly of spindle fibers. M-phase CDK’s promote the metaphase-to-anaphase transition by phosphorylating the subunits of an ubiquitin-protein ligase called the anaphase promoting complex (APC). The phosphorylated APC is now able to bind to the protein Cdc20 in order to become activated. The activated APC destroys the securin holding sister chromatids together and allows for their segregation during anaphase. The APC then binds to the protein Cdh1 so that it can remain active past late mitosis and into G1. During this time the APC ubiquitinates both M-phase and S-phase cyclins, leading to their destruction. This inactivates M-phase and S-phase CDK’s allowing for the dephosphorylation of their target substrates and permitting the cell to exit mitosis (Morgan, 2007).
Mutations to any of the genes involved in cell cycle transitions can result in an arrest of the cell division cycle, which usually leads to cell death. Mutants that have malfunctioning genes involved with the cell cycle are called cdc (cell division cycle) mutants if that mutation causes the cell to arrest growth (Hartwell et al., 1970). One very useful organism for studying cdc mutants is budding yeast, because it is a eukaryote and is easy to grow and manipulate. Also, finding cell cycle mutants in budding yeast is much easier to do than in human cells because of the way that this organism grows and divides. Budding yeast are so named because as they progress through the cell cycle, a daughter “bud” emerges and continues to grow until the completion of mitosis. Conveniently, the size of the bud correlates to the cell’s location in the cell cycle. Because of this fact, a cdc mutant in budding yeast will arrest with a certain bud size that will indicate what stage of the cell cycle it is stuck in (Alberts et al., 2007; Hartwell et al., 1970).

The goal of this experiment is centered on the isolation and identification of these cdc mutants in the budding yeast K. lactis. It is the overall purpose of this project to contribute to a better understanding of the cell division cycle in all eukaryotes. The general method for accomplishing this goal will be to identify the problems seen in cdc mutants, and find the essential cell functions of gene products involved with the cell cycle that are not yet known.

These cdc yeast mutants we isolate will have problems in one of four areas as described by Pringle et al. (1997). The first class of cdc mutant involves proteins involved with the control of cell cycle progression. This first class of mutants will display a Cdc’ phenotype because without functional regulators of the cell cycle, most cells in the mutant culture will arrest with the same bud size. The second class of cdc mutant involves proteins that are needed to physically complete events at different phases of the cell cycle. Without this cellular machinery to move the
cell forward through the cycle, the cells in the culture will again arrest to display a Cdc phenotype. The third class of cdc mutant causes cells to arrest because they mimic such signals as pheromones or nutrition deficiency and trigger a checkpoint. The fourth class of cdc mutant involves genes that encode proteins responsible for bud formation or cytokinesis. In this last case, cells in a culture will arrest with the same bud morphology because they fail to complete the same morphological event (Pringle et al., 1997).

It is important to note that while the problems in these cdc mutants may have occurred in an enzyme working at one phase of the cycle, the phenotype seen at arrest will sometimes be indicative of another stage of the cycle (Pringle et al., 1997). This highlights the difference between the execution point and the termination point. The execution point is the point in the cell cycle when the mutant protein was needed, while the termination point is the stage when the cell-division cycle stops and the cells will often swell to abnormal sizes (Hartwell et al., 1970). As mentioned previously, budding yeast display certain phenotypes that indicate what phase of the cell cycle they are in. When a culture of cdc mutants arrests, the size of the bud on the cells will indicate the termination point where the cells have been arrested in the cell cycle. A small bud indicates that the cell is in S-phase, a large bud indicates that the cell is in G2/early M-phase or late M-phase, and no bud indicates that the cell arrested in G1. In order to distinguish between pre and post anaphase, the nucleus can be stained for. Depending on whether a single or double nucleus is seen, it will be known if the cells have completed anaphase (Hartwell et al., 1970).

As stated previously, this experiment involves a search for and identification of essential components of the cell cycle that are mutated. Once we isolate these mutated genes, we can determine their function in cell cycle progression of healthy cells. This work is instrumental in understanding problems that occur in cancerous cells. The major problem in cancerous cells is
that the control system seen in healthy cells has been lost due to mutation (Pray, 2008). This causes cell cycle progression to occur at accelerated rates and at times when it is not supposed to.

The two sources of tumor cell creation are generally from mutations in either tumor suppressor genes, or proto-oncogenes (Pray, 2008). Both of these types of genes are involved with either apoptosis (programed cell death) or with the regulation of the cell cycle. When certain mutations occur in those genes involved with either the apoptosis pathway or the cell cycle, this results in a cancerous cell incapable of controlling its growth. As this paper focuses on the effects of a malfunctioning cell cycle, the apoptosis pathway will not be addressed.

In the cell cycle, tumor suppressor genes encode proteins that are responsible for halting cell division when it is not needed. When these genes are mutated, usually due to recessive mutations, cells are incapable of stopping replication (Pray, 2008). Proto-oncogenes, eventually referred to as oncogenes, encode proteins in the cell cycle that are responsible for the progression of the cell division cycle. When they are mutated, usually dominant mutations, they are continually activated and cellular replication continues when it shouldn’t (Pray, 2008). Once cell cycle regulators are identified, their anomalous activity in tumors can be investigated so as to help develop more effective and specific cures for cancer patients.

An example of a cell cycle regulator that is dysfunctional in several cancer types, such as lung and breast cancer, is cyclin D. Cyclin D is active during G1 and is involved with regulation of CDK4 in humans (Bernards, n.d.). It is a known oncogene that is found to be overproduced in tumors from patients with breast cancer (Wang et al., 1994). This is an example of why it is beneficial to use yeast to study cancer because they also use cyclins in their cell cycle. So finding new cell cycle components, which is what this experiment seeks to do in yeast, has already proven to help us better understand components of cancer in humans.
Our experiment is not the first to look for new cell cycle components. It is based on a similar project performed by Leland Hartwell et al. in 1970. Hartwell used ethyl methanesulfonate (EMS) and nitrosguanidine (NG) to mutate the budding yeast *Saccharomyces cerevisiae* in order to find temperature sensitive (ts) cell cycle mutants. Temperature sensitivity is a very effective method for finding mutants in essential genes because it is a good way to observe a loss of function only under certain restrictive conditions. If mutations in essential genes render them dysfunctional at all temperatures, then this will result in cell death, which makes it impossible to isolate and study mutant cells. For a temperature sensitive mutant, cell death will only occur under certain conditions. At a permissive temperature, both wild type cells and mutated cells should grow. At a restrictive temperature, the wild type cells should grow because their protein products are still functional; however, the ts mutants will not grow because their mutated proteins will denature (Hartwell *et al.*, 1970). Mutants that are temperature sensitive give us an easy way to isolate essential genes because the colony growing at the permissive temperature will contain the mutated essential gene that resulted in cell death at the restrictive temperature.

Hartwell wanted to use temperature sensitivity to find cell division cycle (cdc) mutants because this would be an effective method for finding genes that were essential to this process. This is because without an essential gene, the cell would be expected to arrest at a certain stage of the cell cycle only when exposed to the restrictive temperature. As mentioned previously, bud morphology can be used as a visual representation of where the cell is arresting (Pringle *et al.*, 1997). So for any particular strain that Hartwell studied, he would expect uniform bud morphology in cdc ts mutants. Uniform bud morphology occurs for a population of cells when, at a restrictive temperature, they all arrest with the same bud size (or lack of a bud at all). He
would not see this uniform morphology in cells with non-\textit{cdc} essential mutations because the whole population would not be stopped at a checkpoint or any particular stage of the cell cycle. So in a \textit{cdc} screen, a strain that arrests with all cells having uniform bud morphology are mutated in a component of the cell division cycle, while a strain mutated in a different type of essential gene will show all bud sizes (Hartwell \textit{et al.}, 1970).

Although this method of screening is highly effective for finding the essential cell cycle genes, it should be noted that it likely won't find all genes involved with this process. For instance, it won't find genes that have paralogs to compensate for the \textit{ts} gene's function at the restrictive temperature. It also would not be a good method for finding genes that are not essential to the cell cycle because no arrest will occur at the restrictive temperature. It is also unlikely that components of the cell cycle would be found if they are essential to multiple steps of the cell cycle. This is because the cells would arrest at different checkpoints depending on which stage they were in when placed at the restrictive temperature. This would not return a high arrest of any particular bud morphology and so this type of screen would not identify these mutants as \textit{cdc}. Lastly, some genes of the cell cycle might be mutated more easily than others.

Evidence for this last point is that Hartwell experienced some bias in the mutant alleles he was able to recover using a temperature sensitivity screen. He witnessed a non-random distribution in the genes he was able to turn \textit{ts}. Because of this bias, Hartwell admits he may have missed out on some genes involved in the cell cycle by not being able to mutate them to be temperature sensitive. Strains that are mutated in the same gene are characterized as being in the same complementation group. Hartwell expected that the mutation of \textit{S. cerevisiae's} genome would give him many complementation groups and yield a random distribution of mutated genes within these groups. After extensive screening, he found that on average there were 4.6 \textit{cdc}
mutants per complementation group. This average means that Hartwell continued to isolate the same genes after mutagenesis and was not finding any new complementation groups. Continuing the screen would therefore not do much good. In the case of a random distribution of mutants, one would expect that most complementation groups would have close to the average number of members, with few having a lot more or a lot less. However, although Hartwell found an average of 4.6 cdc mutants per complementation group, there were many complementation groups that only contained a single mutant, and other complementation groups that held 13 or more mutants. The fact that many complementation groups only contained one mutant, suggests that Hartwell’s methods were likely only capable of isolating a subset of all the cdc genes and there were some that were not found at all. Thus, there are most likely more essential genes involved in the cell cycle to find (Hartwell et al. 1973).

For Hartwell’s cdc screen, he looked for 80% uniform bud morphology in each of the 1500 strains of confirmed ts mutants. He was able to isolate 148 cell cycle mutant strains after cdc screening and then sorted them into 32 complementation groups (Hartwell et al., 1973). The complementation groups were based on each strain having the same gene mutated and thus showed the same termination point at arrest. Hartwell was the first to differentiate between the observed termination point and the execution point where the mutated protein was actually needed. The types of morphologies at arrest that Harwell observed at the termination points were unbudded or shmooed, indicating a mutation prohibiting progression past Start, large buds, indicating mutations affecting progression through G2/M-phase, metaphase to anaphase, or mitotic exit, long chains of rebudding cells incapable of cytokinesis, and one mutant culture displaying a tiny bud, indicating a mutation affecting progression through G1 or S-phase (Hartwell, 1970).
Hartwell demonstrated that finding \textit{cdc} mutants is very easy to do in yeast. This is true for many reasons. Yeast is a model eukaryotic organism that is easy to grow and has the perfect genetics for finding the type of mutants that we are interested in. These would be recessive, temperature sensitive cell division cycle mutants. The fact that some species can be grown as either haploids or diploids means it is easy to perform backcrosses and tetrad analysis on them. Haploids allow for an easy view of the mutant phenotypes and can be backcrossed to produce heterozygous diploids. The resulting diploid cells can be used to determine if the mutation is recessive by comparing their ability to grow at the restrictive temperature to that of the \textit{ts} haploid cells. This is an important finding needed prior to sorting strains into complementation groups. The diploids can also be used in tetrad analysis to determine if the \textit{cdc} mutation is due to a single gene which is important to future cloning by complementation.

Another benefit of yeast is that many of their genes have homologous genes in other organisms, so finding functions of cell cycle genes in yeast can apply to the cell cycle in humans as well. One example of this is the \textit{RAS1} and \textit{RAS2} genes in \textit{S. cerevisiae} and the \textit{HRAS} mammalian gene. The \textit{S. cerevisiae} and human \textit{RAS} genes are known to be homologous because when \textit{RAS1} and \textit{RAS2} were made dysfunctional, \textit{HRAS} was able to save the cells (Kataoka \textit{et al.}, 1985). This gives evidence to the fact that similar functions are performed between the cell cycles of yeast and humans (Botstein \textit{et al.}, 1997). Another discovery of homologous genes between eukaryotes was found by Lee and Nurse (1987) between humans, \textit{S. cerevisiae}, and the fission yeast \textit{Schizosaccharomyces pombe}. \textit{S. pombe's cdc2} gene and \textit{S. cerevisiae's CDC28} displayed a 62\% similarity in the comparison of their protein sequence, and both proteins turned out to be Cdks (Mendenhall & Hodge, 1998). Lee and Nurse were able to use cloning by complementation with \textit{cdc2} to isolate the human homologue \textit{CDC2Hs}. Important to this paper
was the discovery that **CDC2Hs** was able to save *S. pombe* mutants with *cdc2* deleted. This indicates that the two protein products shared similar functions between species. Sorger and Murray (1992) later confirmed that Cdc28 was undergoing the same phosphorylation as the Cdk *cdc2* as a regulatory mechanism for progression into mitosis. This showed that a very similar mechanism was involved with the entry of cells into mitosis between the two species. However, for *S. pombe* this mechanism imposes a DNA damage checkpoint (Enoch & Nurse 1990), while for *S. cerevisiae*, the same mechanism is used for a morphogenetic checkpoint (Lew & Reed, 1993). These discoveries are beneficial because they show that yeast cells and human cells contain similar components in their cell cycle. This gives more incentive to study yeast because it will be easier to find components of the eukaryotic cell cycle using these single celled organisms.

The fact that Hartwell found so many cell cycle mutants using *S. cerevisiae* brings up the question of why we should continue looking for genes involved in the cell cycle. It could be assumed that we would find exactly what Hartwell did, and that this experiment had no purpose. For many reasons, this is not the case. Using the same effective *ts* screen, we changed both the species of yeast and the method of mutagenesis that would bring about our mutant strains.

The species of budding yeast we used in our experiment is *Kluyveromyces lactis*. The most important difference between *K. lactis* and *S. cerevisiae* is that *S. cerevisiae* has more redundancies in its genome while *K. lactis* contains the least duplicated genome of five yeast species (Dujon *et al.*, 2004). The redundancy in *S. cerevisiae*’s genome resulted from a duplication of the entire yeast genome in an ancient ancestor species (Wolfe & Shields, 1997). As Wolfe and Shields (1997) show, this duplication likely happened in *S. cerevisiae* after it and *K. lactis* split lineages. Over time, some of the repeated genes were deleted. However, a few
redundant genes that remain in *S. cerevisiae* were pointed out in the paper by Fitch *et al.* (1992). The genes they discovered were several B-type cyclins that regulate Cdk activity during mitosis. These genes included *CLB1, CLB2, CLB3,* and *CLB4* which all perform similar functions in spindle formation yet peak in expression at slightly different times (Fitch *et al.,* 1992). Having multiple genes that perform similar functions in the cell cycle makes screening for *ts* mutations harder to do in *S. cerevisiae* than in *K. lactis.* This is because if their protein products all have similar functions, then when you mutate one gene you might not lose its function completely. This is because another unaffected gene can compensate for the mutated gene’s job. Fitch *et al.* (1992) showed this phenomenon when they created double mutants *clb2* and *clb1 or clb3* and *clb4,* and the cells were still viable. This meant that they may have missed even more B-type cyclins that were helping to perform these known genes’ functions. So with *S. cerevisiae,* all paralogs of some genes would have to be mutated at the same time to see a loss of function and get an effective screen for temperature sensitive mutants. Because *K. lactis* does not have an extensive genome redundancy, once an essential gene turns temperature sensitive, a loss of function is much more likely. This is why using *K. lactis* in our experiment holds so much potential for finding cell cycle components not found in *S. cerevisiae* by Hartwell.

Another benefit of using *K. lactis* is that it may prove useful for finding new genes in the cell cycle that mutate more easily to become temperature sensitive than genes in *S. cerevisiae.* It is very difficult to mutate a gene so it will function at one temperature, but lose all function at another. It is more likely that the mutation will cause complete loss of function. The fact that *K. lactis* is a different species of yeast should mean that the proteins involved in its cell cycle are slightly different in structure from those in *S. cerevisiae.* These differences might be enough to turn yet undiscovered cell cycle genes *ts* after mutagenesis. As stated above, Hartwell may have
missed some *cdc* genes in *S. cerevisiae* because the genes he did recover after mutagenesis were not appearing in a random distribution. He wasn’t able to mutate certain genes to be temperature sensitive, and thus could not find them with his screen.

Besides the species of yeast that is used, perhaps another issue involved in making genes temperature sensitive is the method of mutagenesis. Using a different type of mutagenesis is another good way to create new temperature sensitive cell cycle genes because it targets genes that could not be turned temperature sensitive by an alternate mutagenesis. This experiment employed UV mutagenesis, which was shown to cause many more types of mutations than EMS in both *E. coli* and yeast because it has less specificity for a particular base pair substitution (Coulondre & Miller, 1977; Prakash & Sherman, 1973). This means that there are some genes that would be impossible for Harwell to find because the G-C to A-T base pair substitution caused by EMS and NG might not have the right effect on some genes. In other words, the mutation might cause complete dysfunction or no dysfunction in the protein product rather than making it temperature sensitive (Kaback *et al*., 1984). Because of this, it is difficult to find all essential genes in an organism using the same type of mutagenesis to induce temperature sensitivity. For example, Kaback *et al.* (1984), found 32 *ts* mutants mapped to chromosome I in *S. cerevisiae* after extensive EMS and NG mutagenesis and *ts* screening. They found that all 32 *ts* mutants fell into the same complementation groups for the essential genes *CDC15*, *CDC24* and *PYK1*. Not only is this a surprisingly low number of genes for a whole chromosome, the authors also were aware that *ts* mutants had been found in other studies for the gene *MAK16* on chromosome I. Today it is known that chromosome I contains at least 14 essential genes that would render the cell inviable if they were mutated (Saccharomyces Genome Database, 2013). This means that Kaback *et al.* were unable to identify 11 genes with their method of
mutagenesis, giving evidence that the ability of EMS and NG to find novel genes was dwindling. This was believed to be due in part to the limits of EMS and NG to induce temperature sensitivity with the specificity of its base pair change of G-C to A-T (Coulondre et al., 1977; Kaback et al., 1984). UV irradiation can cause the same G-C to A-T mutations; however, it is also capable of A-T to C-G, G-C to T-A, and A-T to T-A mutations, as well as tandem double mutations (Coulondre et al., 1977). With its broader mutation spectrum, UV irradiation might cause different alterations in these cell cycle genes that would cause their protein product to be temperature sensitive. So by using UV mutagenesis, we increase our chances of finding new cdc mutants because the temperature sensitive screen with this mutagen has not yet been saturated.

This project was different from Harwell's because it used both a new species of yeast and employed a different form of mutagenesis. In our lab's project, there is the potential to find cell cycle components that have already been identified by Hartwell. However, this does not mean that the project would not still reveal new findings about the cell cycle. The fact that we are looking at *K. lactis* means that even if we find similar cell cycle components to those found in *S. cerevisiae* we will still be gaining a more complete picture of the cell cycle as not all mechanisms in the cell division cycle are the same between different species.

An example of this is the observation that S-phase feedback phosphorylation control is quite different in terms of importance between *S. cerevisiae* and *S. pombe* (Sorger & Murray, 1992). This mechanism involves the regulation of M-Cdks prior to entry into mitosis. As a feedback control in some species, the M-Cdks will be phosphorylated and deactivated if problems arose during S-phase. The dephosphorylation and activation of M-Cdks will allow for entry into mitosis. In *S. cerevisiae*, phosphorylation of the Cdk Cdc28 is not imperative to maintaining S-phase feedback control if problems with DNA replication arise. Without
phosphorylation of Cdc28, the cells will still arrest when S-phase problems are created (Sorger & Murray, 1992). Conversely, preventing phosphorylation of the homologous cdc2 in *S.pombe* disrupts this control system and progression into mitosis happens even when there are problems with DNA replication (Enoch & Nurse, 1990). Even during a normal cell cycle, when no problems with DNA replication exist, Gould & Nurse (1989) found that replacing a tyrosine with a phenylalanine at the site of phosphorylation in cdc2 caused cells of *S. pombe* to advance into mitosis prematurely. Sorger & Murray (1992) found that a parallel tyrosine to phenylalanine substitution at the phosphorylation site in Cdc28 did not cause cells of *S. cerevisiae* to prematurely enter mitosis. This gives evidence that *S. cerevisiae* has alternate methods than *S. pombe* for halting entry into mitosis if there are problems with its DNA and in normal cell cycles (Sorger & Murray, 1992).

Another difference in a cellular mechanism between yeast species was observed by Bardin et al. (2000). They found that a spatial sensing mechanism in the mitotic exit network (MEN) pathway involved in mitotic exit was very important to the proper segregation of the nucleus in *S.cerevisiae*. In the fission yeast *S. pombe*, this special sensing mechanism is not displayed even though MEN proteins are still used for septation (reviewed in McCollum & Gould, 2001). Both the S-phase feedback control and MEN examples are indications that studying the same cell cycle components in different species is very important to gaining complete understanding of the overall process. While *S. cerevisiae* and *K. lactis* are both budding yeast, it will be interesting to note differences in their cell cycle mechanisms that share similar cellular components. This experiment has the potential to identify these similar components.

Before the work described in this paper, our lab had identified ten *cdc* mutants and sorted them into eight complementation groups. All of these mutants were seen to arrest with at least
70% of the cells displaying large buds. For our labs characterization of cdc mutants, the 70% cut off for uniform bud morphology was reduced from Hartwell’s cut off point of 80% (Hartwell et al., 1970). This is because 80% is a more stringent cut off that might miss several cdc mutants that have a slightly lower tendency to arrest. There were four genes identified in our lab’s large budded mutants that caused their Ts' phenotype. These genes included CDC14 and CDC15, which are involved with mitotic exit, TUB2, which encodes β-tubulin, and CDC16, which is a subunit of the APC (Fonseca, 2008; Molinos, 2008; Munson, 2003; Oldroyd, 2002; Pope-Rolewski, 2011; Wallace, 2003; Yang, 2008). So far, CDC14 and CDC15 are the only genes confirmed to be mutated in our ts strains. Hartwell was able to sort 148 cdc mutants into 32 complementation groups that included unbudded, one tiny bud, and a few large bud morphologies (Hartwell, et al., 1973). Unlike Hartwell, we haven’t yet seen the tiny bud morphology or the unbudded, or chains of cells and our complementation groups only have between one and two members meaning our screen has not been saturated. This gives us incentive to keep looking from more cell cycle mutants. This thesis describes the investigation of 14 potential new mutants. These mutant strains are RCY1110, RCY1120, RCY1122, RCY1123, RCY1124, RCY1125, RCY1126, RCY1129, RCY1130, RCY1131, RCY1132, RCY1133, RCY1134, and RCY1135. All of these strains are derived from the parent strain GG1888 and were subject to UV mutagenesis by Callaghan (2012) and Heitz (2012), except for RCY1110, which was isolated by Dror (2010). All were found to be new ts mutants after a temperature sensitive screen using the restrictive temperature of 36°C.

RCY1110, RCY1120, RCY1123, RCY1124, RCY1125, and RCY1126 were all subject to a cdc screen by Callaghan (2012) and Heitz (2012) and they classified RCY1110, RCY1120, RCY1123, and RCY1124 as new cdc mutants (in addition to the ten previously confirmed) with
at least 68% uniform bud morphology. They were found to arrest with 68% of R CY1110 cells having no bud (Figure 1), 70% of R CY1120 cells having no bud, 87% of R CY1123 cells having a small bud, and 77% of R CY1124 cells having a large bud (Heitz, 2012). These are very interesting findings because Hartwell found several unbudded and large budded mutants and none that were small budded. In Hartwell’s 1970 screen he did isolate a mutant, cdc1, that he characterized as having both unbudded and tiny budded cells upon arrest (Hartwell et al., 1970). Paidhungat & Garrett (1997) also found this same trend in cdc1 cells’ arrest morphology. It should be noted that the tiny bud seen in cdc1 was very difficult to distinguish as it was so small. The small budded phenotype investigated by this paper shows a much larger bud (while still appearing smaller than a large bud) and can be clearly distinguished from unbudded cells. As a next step we could compare our unbudded and large budded mutants to Hartwell’s, and attempt to discover the identity of the gene mutated in the small budded mutant. R CY1125 (Figure 2) was only found to be 60% small budded by Callaghan (2012) and Heitz (2012), so was not designated a cdc mutant because it did not meet the cut-off for size uniformity, even though this was relaxed somewhat relative to Hartwell’s screen. R CY1126 (not pictured) was found to be a “frankenyeast” with odd bud morphology and enlarged cell size. R CY1122 and R CY1129 through R CY1135 have yet to undergo a cdc screen. Nuclear morphologies were not scored for any of the 14 mutant strains (Callaghan 2012; Heitz, 2012).
As a next step, our experiment attempted to first confirm that RCY1110, RCY1120, RCY1123, and RCY1124 are indeed *cdc* mutants with the same bud morphologies as seen by Callaghan (2012) and Heitz (2012). Because there was a skew in its bud size distribution, we also rescreened RCY1125 to see if we could obtain greater than a 60% uniform small budded morphology. RCY1126 was also rescreened to be sure that the "frankenyeast" wasn't actually a
A cdc mutant. One study that reported a similar "monster" cell phenotype had created a disruption in the telomere cap of *K. lactis* with a mutated version of the *TER1* gene. This led to misregulation of the appropriate length for the telomeres and aberrant cell phenotypes (Smith and Blackburn, 1999).

If we can confirm the arrest with small buds of RCY1123 or RCY1125, this will be an exciting finding as there aren’t many known cdc mutants with this phenotype. The closest phenotype to a small bud is the unbudded/tiny budded cells seen with mutant *CDC1* or *CDC50*.

The biochemical activities of both Cdc1 and Cdc50 have important effects on phospholipids in different areas of the cell (Losev et al., 2008; Bryde et al., 2010). Cdc1 is localized to the endoplasmic reticulum (ER) and acts on a lipid substrate that accumulates at toxic levels when this lipid phosphatase is mutated (Losev et al., 2008). Mutant *cdc1* strains have been observed to have cells with no buds or tiny buds upon arrest by different studies. These cells were also described as having a variety of issues. These issues include malfunctions in such essential cellular functions as macromolecule synthesis, spindle-pole body duplication, actin polarization, regulation of cellular Ca\(^{2+}\) and Mg\(^{2+}\) concentrations, and Golgi apparatus inheritance (Paidhungat & Garrett, 1998; Rossanese et al. 2001). Losev et al. (2008) suggested that the accumulation of the phospholipid substrate for Cdc1 is the beginning of the cascade of events that lead to cell cycle arrest. The phospholipid accumulation is thought to increase the release of Ca\(^{2+}\) from the ER into the cytoplasm. This might result in the defects seen in actin polarization and in turn result in a cell incapable of proper Golgi apparatus distribution between mother and bud. The cytoskeleton defects in *cdc1* mutants may also account for why progression through the cell cycle is hindered, and these cells develop either no bud or a very tiny one.
The *cdc50* mutants display a similar phenotype to *cdcl* as cells are described to have small buds upon arrest (Moir *et al.*, 1982). Moir *et al.* (1982) compared the two strains using a linkage test and found that they fell into different complementation groups. Bryde *et al.* (2010) concluded in a study that Cdc50 is involved in the flipase activity of translocating phospholipids to the proper leaflet of the plasma membrane. This action is important for maintaining the growth and asymmetrical nature of the membrane (Bryde *et al.*, 2010). Perhaps the loss of Cdc50 action results in cells incapable of complete bud growth because of faulty phospholipid translocation at the site of bud growth. With further investigation of our small budded mutants, we might prove that a new gene has been found involved with similar functions as *CDC1* or *CDC50*.

The remaining *ts* mutants investigated in this thesis are RCY1122, RCY1129, RCY1130, RCY1131, RCY1132, RCY1133, RCY1134, and RCY1135. These strains underwent their first *cdc* screen. It was also a goal of this project to score nuclear morphologies using DAPI stain in order to better pinpoint where in the cell cycle each strain was arrested.

Confirming the *cdc* mutants found by Callaghan (2012) and Heitz (2012) as well as finding new ones in the other *ts* strains, was the first major step in the process of finding new cell cycle components. The next step will be to determine whether we have found new genes involved with the cell cycle, or if we have uncovered already known components. Any *cdc* gene isolated will be subject to several complementation tests to determine if it falls into any of the eight already known complementation groups. Since all mutants in this study were derived from the same parent strain (GG1888), they will all contain the same nutritional marker, *ura3*, and will be of the same mating type, a. Complementation testing requires that the haploids used in mating be of different mating type and contain different nutritional markers. As a solution, in this project
a backcross was performed on each confirmed *cdc* mutant to a wild type strain containing different nutritional markers and of opposite mating type. This created heterozygous diploids for each strain. When the diploids were forced to sporulate, some of the *cdc* segregants randomly inherited the opposite mating type and different nutritional (auxotrophic) markers from the original haploid mutant used for mating. Since these segregants still contain the mutated gene we are after, they can be used in complementation testing against a mating type mutants.

Diploids that resulted from the backcross were subject to tetrad analysis. This gave us segregants on which to perform a future cloning by complementation test, which would tell us whether we have a novel gene. If we want any future cloning by complementation test to work, then the mutant *ts cdc* gene must be recessive. Cloning by complementation involves putting plasmids containing genes from the entire yeast genome into the cells of our mutant strains. The yeast strains that survive at restrictive temperatures will be those that were saved by the wild type form of the mutated gene they carry. Isolating the plasmid in these cells will allow us to discover if we have found a new cell division cycle component. However, this can only work if the mutated allele is recessive, so this gives us more reason to perform a backcross other than for complementation testing. The reason for performing a tetrad analysis before cloning by complementation is to ensure that the mutation is only caused by a single gene. If this step is not taken, there is no way to tell if the cells that don’t survive in the cloning by complementation test are dying because they didn’t receive the right plasmid, or just have multiple genes mutated that lead to a *Ts’* phenotype. In the later situation, the temperature sensitive cell has two essential *ts* genes, one or both of which could be *cdc*. This means that taking up a single plasmid that complements one of the *ts* genes, will not account for the second *ts* gene. These mutants would
not be found using cloning by complementation because they would still die at the restrictive temperature without the ability to correctly complement both ts genes.

This experiment culminated with new confirmed ts cdc mutant strains of Kluyveromyces lactis. Each mutant was tested using a backcross and tetrad analysis to establish that they carried only one ts allele that was recessive. The final goal for this lab will be to determine if these genes encode novel cell division cycle products, or if they correspond to one of our previously identified mutants. Achieving either of these results will further enhance the current knowledge base surrounding one of life’s most important processes: the cell division cycle.

Materials and Methods

Strains and Media

Liquid YPD (rich media) and solid YPD and SD (minimal media) plates were prepared in accordance to the procedures of Ausubel et al., 1993. Malt plates used for the backcross were created following the protocol by Zonneveld & Van der Zanden, 1995. A solution of 60% glycerol was diluted to 15% and used to freeze diploids from the backcrosses (Ausubel et al., 1993). SD plates with a volume of ~30 mL were altered for the tetrad analysis by spreading them with 0.3mL of 100X solutions of adenine, histidine, and uracil (Guthrie & Fink, 1991). Prior to tetrad dissection, liquid acetate sporulation media was used to induce meiosis (Ausubel et al., 1993).

All strains of Kluyveromyces lactis used in this experiment are listed in Table 1 along with their specific purpose in this experiment.
Table 1. Strains of *K. lactis* used for experimentation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG1888/LSY17</td>
<td><em>MAT a, ura3</em></td>
<td>Zonneveld and Van der Zanden (1995)</td>
<td>Original wild type strain from which all mutant RCY strains were derived. Used as a negative control for <em>ts</em> and <em>cdc</em> screening.</td>
</tr>
<tr>
<td>RCY303</td>
<td><em>MAT a, ura3, ts/cdc</em></td>
<td>L. Silveira, I. Wallace, A. Oldroyd —derived from GG1888 using EMS mutagenesis</td>
<td>Confirmed <em>ts</em> and <em>cdc</em> mutant used as a positive control in these screens.</td>
</tr>
<tr>
<td>GG1846/LSY25</td>
<td><em>MAT a, his7, ade1</em></td>
<td>Zonneveld and Van der Zanden (1995)</td>
<td>Wild type strain of opposite mating type than RCY mutants. Used to create diploids for backcross and tetrad dissection.</td>
</tr>
<tr>
<td>RCY1110^</td>
<td><em>MAT a, ura3, ts</em></td>
<td>Dror (2009)—derived from GG1888 using EMS mutagenesis</td>
<td><em>cdc</em> screen, <em>ts</em> rescreen, backcross, and tetrad analysis</td>
</tr>
<tr>
<td>RCY1120**</td>
<td><em>MAT a, ura3</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen, <em>ts</em> rescreen</td>
</tr>
<tr>
<td>RCY1123**</td>
<td><em>MAT a, ura3</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen, <em>ts</em> rescreen</td>
</tr>
<tr>
<td>RCY1124^</td>
<td><em>MAT a, ura3, ts</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen, <em>ts</em> rescreen, backcross, and tetrad analysis</td>
</tr>
<tr>
<td>RCY1125^</td>
<td><em>MAT a, ura3, ts</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen</td>
</tr>
<tr>
<td>RCY1126^</td>
<td><em>MAT a, ura3, ts</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen</td>
</tr>
<tr>
<td>RCY1134^</td>
<td><em>MAT a, ura3, ts</em></td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td><em>cdc</em> screen</td>
</tr>
<tr>
<td>RCY1122; RCY1129-RCY1133, RCY1135</td>
<td>MATa, ura3, ts</td>
<td>Callaghan (2012) and Heitz (2012)—derived from GG1888 using UV mutagenesis</td>
<td>cdc screen</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>RCY1187</td>
<td>MATa/MATa, his7/HIS7, ade1/ADE1 ura3/URA3, ts cdc/TS CDC</td>
<td>Good (2013) and King (2013)—Diploid from cross of LSY25 and RCY303</td>
<td>Frozen for future analysis</td>
</tr>
<tr>
<td>RCY1188</td>
<td>MATa/MATa, his7/HIS7, ade1/ADE1 ura3/URA3, ts cdc/TS CDC</td>
<td>Good (2013) and King (2013)—Diploid from cross of LSY25 and RCY1120*</td>
<td>Frozen for future analysis</td>
</tr>
<tr>
<td>RCY1189</td>
<td>MATa/MATa, his7/HIS7, ade1/ADE1 ura3/URA3, ts cdc/TS CDC</td>
<td>Good (2013) and King (2013)—Diploid from cross of LSY25 and RCY1110*</td>
<td>Tetrade dissection and analysis; Frozen for future analysis</td>
</tr>
<tr>
<td>RCY1190</td>
<td>MATa/MATa, his7/HIS7, ade1/ADE1 ura3/URA3, ts cdc/TS CDC</td>
<td>Good (2013) and King (2013)—Diploid from cross of LSY25 and RCY1123*</td>
<td>Frozen for future analysis</td>
</tr>
<tr>
<td>RCY1191</td>
<td>MATa/MATa, his7/HIS7, ade1/ADE1 ura3/URA3, ts cdc/TS CDC</td>
<td>Good (2013) and King (2013)—Diploid from cross of LSY25 and RCY1124*</td>
<td>Tetrade dissection and analysis; Frozen for future analysis</td>
</tr>
</tbody>
</table>

*Proposed ts mutant Callaghan (2012) and Heitz (2012). Found not ts in this paper.
*Proposed cdc mutant by Callaghan (2012) and Heitz (2012). Found not cdc in this paper.
^Proposed cdc mutant by Callaghan (2012) and Heitz (2012). Confirmed cdc in this paper.
* Initial cdc screen in this paper found these strains not to be cdc.
^ Initial cdc screen in this paper found this strain to be cdc.

Cdc Screening

The strains RCY1110, RCY1120, RCY1122-RCY1126, and RCY1129-RCY1135, were all removed from the -70°C freezer and streaked onto YPD plates. These were left to grow at room temperature for four to five days. When single colonies were observed, 4mL of liquid YPD for each strain were inoculated with about half a colony then left to grow on the spinning culture
wheel for 24 hours. An optical density reading for a 15 fold dilution sample of the cloudiest culture was taken at 600nm (OD_{600}) using the Jasco V-530 UV/VIS Spectrophotometer (Easton, MD). Using the conversion factor 2.0 \times 10^7\text{cells/OD} the approximate number of cells per mL of YPD was calculated with a reading between 1OD/mL and 3OD/mL indicating log phase growth of a strain. The cloudiest culture exhibited the most growth so a reading for this sample between 1OD/mL and 3OD/mL, after accounting for the dilution factor, indicated that all cultures were in logarithmic growth phase.

Once logarithmic growth was reached, this meant that the cells were actively dividing and so were moved to a shaking water bath set to the restrictive temperature of 37°C. The temperature was measured using the Fisher Traceable® Full-Scale Thermometer (Waltham, MA). After 7 hours at the restrictive temperature, all strains were removed and 200 proof ethanol was added to a final concentration of 50% (v/v) to arrest cellular division. The strains were stored in a refrigerator until a cdc screen could be performed.

To prepare each strain for a cdc screen, the cells in ethanol were vortexed and then 1.5mL was removed and placed into an microcentrifuge tube. Each was centrifuged at a relative centrifuge force (RCF) of 21,000xg for 1 minute in a microcentrifuge. The supernatant was removed, the cells were resuspended in 1mL of water, and each tube was recentrifuged at an RCF of 21,000xg for 1 minute. After removing the final supernatant, the cells were resuspended in 750\mu L of water and sonicated for 20 pulses using a microtip on the Branson Sonifier 450 (Danbury, CT) at 30% cycle duty and an output control of 3. Following sonication, 6\mu L of the cells were put on a slide, covered by a cover slip, and then observed using a phase microscope at 400x and 1000x magnification.
To score each strain, at least 100 cells were observed for their bud morphology. The categories included: unbudded/tiny budded, small budded, large budded, and unique. Unique was defined as any cell that displayed either multiple buds or buds that were irregular in shape and deviated from the isotropic growth pattern of an evenly enlarging bud (Lew & Reed, 1993). If any strain showed more than 70% arrest with any of the bud morphologies un/tiny budded, small budded or large budded, then they could be considered a cdc mutant. In this experiment, GG1888 acted as a negative control because it is not a cdc mutant and would show varied percentages of each bud morphology. RCY303 served as the positive control for the cdc screen because it is a known large budded cdc mutant.

**DAPI staining**

One strain underwent 4',6-diamidino-2-phenylindole (DAPI) staining. RCY1124 was prepared for a slide and cover slip using the same procedure used for the cdc screen. Once the cells were sonicated, equal volumes of 0.2μg/mL DAPI stain and cell sample were mixed on the slide. Images of nuclear localization were taken using the attached camera on the Olympus BX41 fluorescence microscope (Center Valley, PA) at 1000x magnification.

**Backcrossing cdc ts Mutants to LSY25**

In order to perform a backcross, the mutant ts cdc haploid strains RCY1110, RCY1120, RCY1123, RCY1124, and the control RCY303 (all being a mating type and ura3) were individually mated to the wild type haploid strain LSY25 which is of opposite mating type (α) and is ade1 his7. Prior to mating, each strain was removed from the -70°C freezer and then streaked onto YPD plates. These were left to grow up for two days at which time single colonies
were distinguishable. A pinhead size amount of each mutant strain (about 10^7 cells) was mixed with an equal amount of LSY25 in 4μL of liquid YPD on a malt plate.

After three days of growth at room temperature, SD plates were divided up to accommodate a section for each backcross. In each section, 50 μL of sterile water was added. The entire portion of growth on the malt plate for each cross was then transferred to an SD plate and mixed into the sterile water. A sterile spreader was used to spread the mix throughout the whole section. No nutrients were added to the minimal media plates as diploids of the cross should be capable of making their own adenine, histidine and uracil.

After two days of growth, a single colony from each cross was streaked onto a YPD plate and left to grow up for 2 days. At this time, a red colony or sectors of red would indicate a return to the haploid state as the wild type parent strain LSY25 was ade1. A full toothpick head of diploid cells from each strain was added to 800μL of 15% glycerol in a cryotube, and then stored in a -70°C freezer. The remaining diploid cells on the SD plates were saved for the recessiveness test and sporulation.

Rescreen for Ts* Phenotype

To be sure of our results, each of the haploid strains RCY1110, RCY1120, RCY1123, RCY1124, RCY303 and LSY25 underwent a rescreen for temperature sensitivity prior to performing the recessiveness test on any diploids. Each haploid strain was streaked from the original -70°C freezer stock onto three YPD plates that were then placed into three different incubators. The incubators were set to the three restrictive temperatures: 37.4°C, 38.0°C and 38.4°C. After two days, the growth of each haploid strain was measured.
As a second test, half a colony each of the haploid controls RCY303 and LSY25, and the haploid mutants RCY1120 and RCY1123 from the incubator test plates were used to inoculate 4mL of liquid YPD. These were left to grow on a spinning culture wheel for 2 days, then the cloudiest culture (exhibiting the most growth) had its optical density read using the Jasco V-530 UV/VIS Spectrophotometer (Easton, MD). With a 7.038 OD/mL reading, a 1:235 dilution followed by a 1:100 dilution was performed on each strain to reach the goal of 300 cells/50μL. To separate YPD plates, 50μL of each diluted strain was spread and left to grow for 24 hours. After 24 hours, two replica plates per strain were made using sterile velvet. One of each set was left to grow at room temperature, while the other was left to grow at 38.9°C in an incubator. After 3 days, the plates were observed for growth.

Recessiveness Test

In order to determine if the gene responsible for the cdc mutation was recessive, confirmed ts cdc mutants underwent a recessiveness test. The strains involved were the haploids RCY1110, RCY1124, RCY303, and LSY25 as well as the diploid crosses RCY1110xLSY25 and RCY1124xLSY25.

All haploids were removed from the -70°C freezer and streaked across half of a YPD plate. The two controls, RCY303 and LSY25, were streaked onto two halves of the same plate. A single colony of diploids from the backcross was streaked on the opposing half of the YPD plate containing the corresponding mutant haploid strain. Two identical sets of plates were made using this procedure. One set was left at room temperature and the other was left at the restrictive temperature in an incubator set for 37.5°C (the temperature where the mutant haploids were first observed to die). After 3 days, the growth on the restrictive and permissive plates was compared.
Sporulation of Diploid cells from Backcrosses

From the SD plates that ended the backcross, four colonies of each of the crosses RCY1110xLSY25, RCY1124xLSY25, and RCY303xLSY25 were used to inoculate 4mL of liquid sporulation acetate media. These were left to sporulate for five days on a spinning culture wheel. Each cross was transported to a refrigerator for storage prior to tetrad dissection.

Tetrad Dissection and Analysis

After spinning in the liquid sporulation acetate media, each of the three crosses contained tetrads of haploid spores held together in asci. To break apart the asci, the cells were exposed to a 5% Sigma (St. Louis, MO) glusulase solution from Helix pomatia (G7017). In preparation, 500µL of sporulation acetate media containing each cross was added to a microcentrifuge tube and centrifuged at an RCF of 21,000xg for 1 minute in a microcentrifuge. After removing the supernatant, each cross was washed and recentrifuged three times with 500µL of sterile water then resuspended in 500µL of sterile water. After combining 100µL of the suspended cells with 100µL of the 5% glusulase solution, each cross was left to incubate at room temperature for ten minutes. At this time, observation of the tetrads under a microscope would indicate if half of the asci had been loosened. When about half of the asci appeared to be loosely associated with the haploid spores, the cells were diluted to a 1:10 dilution in sterile water in order to halt the effects of the glusulase.

Using a volume of 35µL, the diluted solution of cells was made to flow as a single streak down the middle of a YPD plate. After allowing the fluid to seep into the media for several minutes, the plate was flipped upside down and positioned over a needle in the Micro Video Instruments, Inc. Tetrad Dissection Microscope (Avon, MA).
To dissect each tetrad, the needle was positioned in the center of the field of view and was manipulated using a joystick on the dissecting scope. Targeting individual asci, the needle was used to pick up the four spores and move them away from the concentrated area of cells to another position on the plate. From here, each individual cell was teased apart from the others and deposited in a position that was immediately next to the other spores, but at a distance that allowed for secluded colony growth. The location of each dissected tetrad was recorded, and the resulting YPD plate was incubated at room temperature until each spore had grown into a visible colony. Plates with enough growth were stored for future analysis in a refrigerator.

Analysis of the tetrads began with division of two YPD plates and four SD plates into separate sections for each colony of a given tetrad. Each colony was streaked onto an individual section of each plate so growth of each spore from the original tetrad could be studied. One of the YPD plates was left to grow at room temperature while the other was left at the restrictive temperature of 37.5°C. The four SD plates were spread with 0.3mL of 100X solutions of adenine, uracil or histidine (Guthrie & Fink, 1991). The first plate contained all three of the nutritional supplements, the second plate contained only adenine and histidine, the third plate contained only adenine and uracil, and the fourth plate contained only uracil and histidine. After streaking each colony, these plates were grown at room temperature. The nutritional supplements were used because LSY25 has his and ade markers while the mutants RCY1110 and RCY1124 have ura markers. After four days, the growth of each of the tetrads was observed. A chi square test was performed for each mutant strain to compare the linkage between the ts gene in each strain and a nutritional marker.
Results

Cdc screen for novel mutants and confirmation of previously characterized cdc ts mutants

Including the controls, a total of 16 strains of *K. lactis* were screened for a Cdc⁻ phenotype. Previously, the mutant strains RCY1110, RCY1120, RCY1123, RCY1124, RCY1125, and RCY1126 had all undergone an initial cdc screen by Callaghan (2012) and Heitz (2012). The strains RCY1122 and RCY1129—RCY1135 had no previous cdc screen prior to this experiment. All mutant strains, including the control RCY303, were identified as ts mutants prior to our cdc screen.

To induce the temperature sensitive phenotype of the mutants, all were placed at the restrictive temperature of 37°C for 7 hours prior to sonication. The cellular phenotypes were observed using a phase-contrast microscope. The generalized findings of the cdc screens can be found in Table 2. All confirmed cdc mutant strains can be observed for their phenotype in Figure 3. The controls in this figure indicate that our method of arrest was effective. The wild type strain GG1888 was seen to display almost equal occurrences of each variant of bud morphology (Figure 3A), while the known cdc mutant RCY303 showed large buds when arrested (Figure3B). In all, there were only four confirmed cdc mutants found from the strains previously screened by Callaghan (2012) and Heitz (2012) (Table 2).

### Table 2. Cdc screen results for strains of *K. lactis* derived from GG1888.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nature of Strain</th>
<th>Cdc</th>
<th>Phenotype Upon Arrest</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG1888/LSY17</td>
<td>Wild type</td>
<td>No (38, 29,33,0)⁺</td>
<td>Varied bud morphology</td>
<td></td>
</tr>
<tr>
<td>RCY303</td>
<td>Cdc control</td>
<td>Yes (25,11,64,0)</td>
<td>Large budded</td>
<td></td>
</tr>
<tr>
<td>RCY1110</td>
<td>*</td>
<td>Yes (59,36,4,1)</td>
<td>Unbudded</td>
<td></td>
</tr>
<tr>
<td>RCY1120</td>
<td>*</td>
<td>Yes (71, 8, 17, 4)</td>
<td>Unbudded/Tiny Budded</td>
<td></td>
</tr>
<tr>
<td>RCY1123</td>
<td>*</td>
<td>Yes (76, 20, 4, 0)</td>
<td>Unbudded/Tiny budded</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Score 1</td>
<td>Score 2</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>RCY1124</td>
<td>Yes</td>
<td>21, 9, 70, 0</td>
<td>Large budded</td>
<td></td>
</tr>
<tr>
<td>RCY1125</td>
<td>No</td>
<td>38, 42, 19, 1</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1126</td>
<td>No</td>
<td>(cells too clumped to score)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1122</td>
<td>No</td>
<td>27, 7, 25, 41</td>
<td>Unique category had unusually swollen cells with a single tiny bud</td>
<td></td>
</tr>
<tr>
<td>RCY1129</td>
<td>No</td>
<td>54, 13, 33, 0</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1130</td>
<td>No</td>
<td>50, 15, 20, 15</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1131</td>
<td>No</td>
<td>10, 8, 29, 53</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1132</td>
<td>Possible</td>
<td>61, 4, 11, 24</td>
<td>Unbudded</td>
<td></td>
</tr>
<tr>
<td>RCY1133</td>
<td>No</td>
<td>29, 10, 58, 3</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>RCY1134</td>
<td>Yes</td>
<td>80, 8, 7, 5</td>
<td>Unbudded/Tiny Budded</td>
<td></td>
</tr>
<tr>
<td>RCY1135</td>
<td>Possible</td>
<td>66, 5, 3, 26</td>
<td>Unbudded</td>
<td></td>
</tr>
</tbody>
</table>

*Percentages at arrest of different bud types (unbudded/tiny budded, small budded, large budded, unique)  
*Previously screened by Callaghan (2012) and Heitz (2012)  
**Confirmed ts by Callaghan (2012) and Heitz (2012) but no previous cdc screen

RCY1110 was confirmed as a *cdc* mutant because it arrested with a 59% unbudded phenotype (Figure 3C) and was previously scored with a 68% unbudded arrest (Callaghan, 2012; Heitz, 2012). Although the percent arrested was low, this was consistent with the initial screen and the arrest on the RCY303 control was not much higher so it was decided that the unbudded designation would be kept. RCY1120 was confirmed as a *cdc* mutant as it displayed a 71.4% arrest with an unbudded/tiny budded phenotype as can be seen in Figure 3D. This is in confirmation of its previous arrest of 70% with the same phenotype (Callaghan, 2012; Heitz,
RCY1123 was screened twice due to inconsistency with previous results. It had been characterized as arresting with a small bud at a rate of 87% (Callaghan, 2012; Heitz, 2012). Our first screen found RCY1123 to arrest with 76% of cells unbudded (Figure 3E) and the second screen saw an arrest of 80% with the unbudded/tiny budded phenotype (data not shown). As such, we confirmed our own results and characterized RCY1123 as a cdc mutant. RCY1124 was previously found to be 77% large budded (Callaghan, 2012; Heitz, 2012). Our screen confirmed these results by finding that 70% of the cells were arrested with large buds, seen in Figure 3F, so this strain was characterized as a cdc mutant.

The last two strains that had been screened for a cell division cycle mutation previously were RCY1125 and RCY1126. RCY1125 was only found to be 60% small budded in its previous cdc screen (Callaghan, 2012; Heitz, 2012). Both of our screens for this mutant found varied bud morphologies with no obvious high rate of arrest at a particular size which can be seen in Figure 3I. RCY1126 was found in both the initial screen and our screen to have strange and deviant bud size and shape and can be visualized in Figure 3H. As no uniformity was found in the bud morphology for either RCY1125 or RCY1126, they could not be characterized as a cdc mutant and analysis was discontinued on these strains.

For the second set of mutant temperature sensitive strains, no initial cdc screen had been performed. For RCY1122, RCY1129—RCY1133 and RCY1135, no uniform bud size at arrest was observed after removal from the restrictive temperature. However, as seen in Table 2, there were two strains that were possible cdc mutants. After removing RCY1132 and RCY1135 from the restrictive temperature, the cells in each culture were scored for their bud morphology. It was found that both cell cultures had over 60% of cells displaying no bud. These two strains could be
found to be \textit{cdc} mutants if future screens found greater than 70% uniform bud morphology among their cells.

Also seen in Table 2 are the \textit{cdc} screen results for the strain RCY1134. Eighty percent of the cells in this culture were unbudded, characterizing this strain as a novel \textit{cdc} mutant. The cells of RCY1134 can be observed in Figure 3G.

![Phase-contrast microscopy images](image)

\textbf{Figure 3. Phase-contrast microscopy of confirmed \textit{cdc} mutant cells arrested with uniform bud morphology and non-\textit{cdc} cells exhibiting no uniform bud morphology.} Magnification is 1000x. A—GG1888 wild type strain (compiled examples of varied bud morphology), B—RCY303 known \textit{cdc} mutant (64% large budded), C—RCY1110 (59% unbudded), D—RCY1120 (71.4% unbudded/tiny budded), E—RCY1123 (76% unbudded/tiny budded), F—RCY1124 (70% large budded), G—RCY1134 (80% unbudded) H—RCY1126 (compiled examples of deviant bud morphology) I—RCY1125 (varied bud morphology)
DAPI staining of RCY1124

RCY1124 was confirmed in this experiment as a large budded cdc mutant. In order to further investigate this phenotype, DAPI stain was used to localize the genetic components in the budding cell. DAPI stain was applied to sonicated RCY1124 cells after this strain was incubated for 7 hours at the restrictive temperature of 37°C. Using a camera attached to a fluorescence microscope, the image in Figure 4B was acquired and can be compared to the complementary phase-contrast microscopy image seen in Figure 4A.

![Image](image-url)

**Figure 4. Localization of nuclear components of the ts cdc mutant RCY1124.** Magnification is 1000x. A—Phase-contrast microscopy of RCY1124 displaying large buds at arrest, B—as in A but using fluorescence microscopy to visualize the DAPI stain in the same cells. Arrows indicate cells (A) and their nuclear location (B).

A comparison between Figure 4A and Figure 4B showed that the location of the genetic information in RCY1124 was located in only one half of the budded cell. This indicated that the DNA had not yet segregated between the mother and daughter bud.
Backcross to find mutants with recessive $cdc\, ts$ mutations and prepare for tetrad dissection

The confirmed cell division cycle mutants from the first $cdc$ screen were moved on to a backcross to determine if their mutations were recessive. The strains used in our backcross included the $cdc$ control RCY303 (a mating type), the wild type strain LSY25 (a mating type) and the newly confirmed $cdc$ mutants RCY1110, RCY1120, RCY1123, RCY1124 (all a mating type). As all of these strains are haploid, the RCY mutants were individually mated to the wild type strain LSY25 on malt plates, and then moved to minimal media plates to check for diploid cell growth. Cells that had remained in the haploid state from either parent strain would not survive on minimal media. The results of the mating can be seen in Figure 5. Figure 5B shows the diploid colonies transferred from the malt plate (Figure 5A), and grown up on the minimal media plate. Once the colonies from the SD plates were transferred to YPD plates, it became apparent that mating was successful because no red colonies were observed. Red colonies would indicate the presence of haploid colonies because LSY25 is $ade1$ so any haploid progeny that inherited this marker would appear red.

Figure 5. Mating of haploid strains to make diploid cells for recessiveness test. A—Malt plate containing mixed mutant strain with wild type strain, B—SD plate showing the diploids from the cross of RCY1123xLSY25. Arrow indicates a colony.
The successful mating of haploid strains meant that we had diploid strains for each \textit{cdc} mutant and could use these diploids to test for the recessiveness of their temperature sensitive gene. A summary of the mating attempts and results can be seen in Table 3.

**Table 3. Summary of backcross of \textit{cdc} mutants and the wild type LSY25.**

<table>
<thead>
<tr>
<th>Strains in Backcross</th>
<th>Successful Mating?</th>
<th>New Diploid Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSY25xRCY303</td>
<td>Yes</td>
<td>RCY1187</td>
</tr>
<tr>
<td>LSY25xRCY1110</td>
<td>Yes</td>
<td>RCY1189</td>
</tr>
<tr>
<td>LSY25xRCY1120</td>
<td>Yes</td>
<td>RCY1188</td>
</tr>
<tr>
<td>LSY25xRCY1123</td>
<td>Yes</td>
<td>RCY1190</td>
</tr>
<tr>
<td>LSY25xRCY1124</td>
<td>Yes</td>
<td>RCY1191</td>
</tr>
</tbody>
</table>

**Rescreen for \textit{Ts}\textsuperscript{*} Phenotype of Some Mutant Strains**

Prior to the recessiveness test with the diploids collected from mating, a rescreen for temperature sensitivity was performed to be sure that all \textit{ts} haploid strains would behave \textit{Ts}\textsuperscript{*}. This test was also used to determine the non-permissive temperature at which each strain would behave \textit{Ts}\textsuperscript{*}. Each haploid version of the strain that took part in the backcross was streaked onto three YPD plates and left to grow at three different restrictive temperatures. Table 4 summarizes the results of this experiment.

**Table 4. Growth behavior of haploid strains incubated on YPD plates at three different restrictive temperatures.**

<table>
<thead>
<tr>
<th>Plate Temperature</th>
<th>LSY25 (wild type) Behavior</th>
<th>RCY303 (known \textit{cdc} mutant) Behavior</th>
<th>RCY1110 Behavior</th>
<th>RCY1120 Behavior</th>
<th>RCY1123 Behavior</th>
<th>RCY1124 Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.4°C</td>
<td>Growth</td>
<td>No Growth</td>
<td>No/Minimal Growth*</td>
<td>Growth</td>
<td>Growth</td>
<td>No/Minimal Growth</td>
</tr>
<tr>
<td>38.0°C</td>
<td>Growth</td>
<td>No Growth</td>
<td>No/Minimal Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>No/Minimal Growth</td>
</tr>
<tr>
<td>38.4°C</td>
<td>Growth</td>
<td>No Growth</td>
<td>No/Minimal Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>No/Minimal Growth</td>
</tr>
</tbody>
</table>

*Minimal growth was defined as slight growth in the initial streak only*
It was found that two of our ts cdc strains were not behaving like temperature sensitive strains when put at any of the three restrictive temperatures. It can be seen in Figure 6 that the control strain RCY303 was behaving as a temperature sensitive mutant and dying at all of the restrictive temperatures. Similarly, the wild type strain LSY25 was surviving at each of the restrictive temperatures.

![Figure 6](image)

**Figure 6. Test for growth of presumed temperature sensitive mutants at various restrictive temperatures.** The haploid strains on each plate are, clockwise beginning at the arrows, LSY25, Rcy1124, Rcy1110, Rcy1120, Rcy303, and Rcy1123. The three temperatures: 37.4°C, 38.0°C and 38.4°C

In Figure 6, each plate is seen to have minimal to no growth for the two haploid strains Rcy1110 and Rcy1124. This was expected because both of them had been characterized as ts and thus should not survive at heightened temperatures. However, the other two haploid mutant strains Rcy1120 and Rcy1123 were exhibiting growth at all of the restrictive temperatures. These results were unexpected because these two strains were previously characterized as ts.
Since the controls were behaving as expected, it was concluded that there must be a problem in RCY1120 and RCY1123 themselves rather than there being a problem with the incubators.

One possibility is that the strains were a mixture of both temperature sensitive and non-temperature sensitive cells. This could have been due to mistakenly mixing two strains during the freezing process or it could be that some individual cells of RCY1120 and RCY1123 became revertants. In the previous temperature sensitivity test where we were only looking for growth, a mixed population of Ts' and Ts+ cells would have been scored as Ts+ because the Ts' cell phenotype would have been masked by the growth of the Ts+.

To rule out such a possibility, two replica plates of single colonies were made for these two strains and the controls. This would effectively spread out individual cells so that colonies could be individually screened for temperature sensitivity. One plate was left to grow at room temperature, and one plate was left to grow at the extremely restrictive temperature of 38.9°C. As seen in Figure 7A1 and B1, the wild type control strain LSY25 was surviving at both the permissive and the restrictive temperature as expected. Looking at Figure 7A2 and B2, the cdc control RCY303 was dying at the restrictive temperature and surviving at room temperature as expected. Figure 7A3 and B3 as well as A4 and B4 show that RCY1123 and RCY1120 were surviving at both the permissive temperature and the highest restrictive temperature. These findings were especially important because every colony, not just a few, was seen to survive at the restrictive temperature. This indicated that the originally stored strains in the freezer were purely non-temperature sensitive and were not mixtures of wild type and mutant strains. As these findings of survival at the restrictive temperature were consistent with the results seen in Figure 6, analysis of these two strains was discontinued, and they did not move on with the RCY1110 and RCY1124 to the recessiveness test.
Recessiveness Test

Only two mutant strains, RCY1110 and RCY1124 were used in the recessiveness test because they had been confirmed as ts from the previous experiment. In order to test if these mutants were temperature sensitive due to a recessive mutation, the haploid strain of each was streaked onto half of a YPD plate. On the other half of the plate, we streaked a colony of the diploid resulting from the same mutant haploid crossed to LSY25. A control plate was also made with LSY25 (wild-type) streaked on one half, and RCY303 (cdc) streaked on the other. Two identical plates of each pair were made so that one could be grown at room temperature and the other could be grown at the restrictive temperature. We expected that all mutant haploids would die at the restrictive temperature due to having a ts cell division cycle gene. However, if these haploids are displaying temperature sensitivity due to a recessive mutation, then we expected to see their corresponding diploid partner surviving at the restrictive temperature. The results we saw for this test can be seen in Figure 8.
Figure 8. Recessiveness test for temperature sensitivity of ts cdc mutants. Row A—all plates grown at room temperature, B—all plates grown at the restrictive temperature 37.5°C. H^{1124}—haploid strain RCY1124, D^{1124}—diploid cross of RCY1124xLSY25, H^{1110}—haploid strain RCY1110, D^{1110}—diploid cross of RCY1110xLSY25, H^{cdc}—RCY303, H^{wt}—LSY25

As indicated by Figure 8A, all strains were capable of growth at room temperature, which was expected of both haploid and diploid cells. Figure 8B gives evidence for recessive mutations in both RCY1110 and RCY1124. The columns H^{1110} and H^{1124} in row B indicate that the haploids of these strains were unable to withstand the restrictive temperature due to their being ts. However, diploids formed from backcrosses of RCY1110 and RCY1124 both grew at non-permissive temperature (Figure 8B D^{1110} and D^{1124}). These results indicated that the ts mutation seen in RCY1110 and RCY1124 were recessive because the functional alleles from LSY25 were able to mask the temperature sensitivity.

Tetrad dissection and analysis to determine whether Ts' phenotype is due to a single gene mutation

After determining that both RCY1110 and RCY1124 held recessive temperature sensitive cdc mutations, tetrad dissections of these strains were performed. The analysis of these tetrads
would indicate if the temperature sensitive phenotype in these mutants was due to a $ts$ mutation in one or two genes.

Each diploid strain RCY1110xLSY25 and RCY1124xLSY25 was exposed to sporulation acetate media to induce meiosis and the creation of four haploid spores. After loosening of the asci with glusulase, dissection was accomplished on YPD plates. After dissection, analysis of the tetrads for the segregation of the temperature sensitive phenotype was performed by streaking each haploid segregant individually and letting it grow either at the permissive or at the restrictive temperature. We expected to see a 2:2 segregation pattern for temperature sensitivity if this phenotype is due to a single $ts$ mutated gene. A separate test for the segregation of the nutritional markers uracil, adenine, and histidine was performed to ensure that each group of four colonies truly originated from the same tetrad. This would indicate that the segregation pattern seen for the temperature sensitivity was an accurate result.

An example image of a successfully dissected tetrad for RCY1110xLSY25 can be viewed in Figure 9. Evidence that these colonies originated from the same tetrad was already supported by the appearance of spores 1a and 1c. The red color indicates that these spores were most likely carrying the adel allele and are incapable of completing the creation of adenine. The fact that there was a 2:2 segregation of this phenotype is good evidence that these colonies originated from the same tetrad. This conclusion is confirmed in Table A1 (Appendix), where a scoring of the Ts' phenotype and nutritional requirements for all tetrads dissected in this experiment can be found.
Figure 9. Dissected tetrad of RCY1110xLSY25 on YPD. This tetrad is the first one dissected for this cross and is lettered across the top to distinguish colonies derived from individual spores. These colonies grew for 4 days at room temperature.

The scoring of each spore in Table A1 indicates that every group of four spores came from the same tetrad. Also, the ability of each segregant from each tetrad to grow on a plate containing all three molecular markers suggested that no new auxotrophies were created during mutagenesis. This means that all results for the segregation of temperature sensitivity seen in Table 5 and Table 6 are true of each tetrad. So far, only five tetrads of the RCY1110 backcross have been scored (Table 5) and seven tetrads of the RCY1124 backcross have been analyzed (Table 6). These are not very large sample sizes to draw conclusions from. However, since the segregation pattern for temperature sensitivity in both strains has been 2:2 thus far, this is good evidence that the Ts- phenotypes are only due to single mutated genes.

Table 5. Summary of scoring for tetrads dissected from the RCY1110 backcross.

<table>
<thead>
<tr>
<th>Tσ: Tσ Segregation</th>
<th>Number of Tetrads</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:2 Tσ: Tσ</td>
<td>5</td>
</tr>
<tr>
<td>Total tetrads dissected: 5</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Summary of scoring for tetrads dissected from the RCY1124 backcross.

<table>
<thead>
<tr>
<th>Tσ: Tσ Segregation</th>
<th>Number of Tetrads</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:2 Tσ: Tσ</td>
<td>7</td>
</tr>
<tr>
<td>Total tetrads dissected: 7</td>
<td></td>
</tr>
</tbody>
</table>

Using the data in Table 7, the linkage was studied between the ts gene of RCY1110 and RCY1124 and the nutritional markers: URA3, ADE1, and HIS7. The results in Table 7 show the ratio of parental ditype (PD) tetrads to tetratype tetrads (TT) to non-parental ditype (NPD) tetrads for each strain. A PD tetrad is one that has no recombinant spores, a TT tetrad has two
recombinant spores, and a NPD has four recombinant spores. The ratio that is expected for these three types of tetrads is 1:4:1 PD:TT:NPD when there is no linkage between the genes. A change in this ratio that results in a greater number of PD tetrads and a reduced number of the other two types is an indication that the two genes under study are linked.

As seen in Table 7, there are two gene pairs that show the possibility of linkage. The first case is the ts gene in RCY1124 and ADE1 and the second case is the ts gene in RCY1110 and URA3. In both of these cases, there is a substantial skew toward more PD tetrads. A chi square test was performed to determine if linkage in both of these data sets was statistically supported by these data (Appendix Figure A1). As a null hypothesis for both cases, it was assumed that the two loci were unlinked. The model used for the chi square test compared the expected and observed ratios for PD:TT:NPD. It was found that the ts gene from RCY1110 and the URA3 gene were linked as the chi square test gave a p<0.001, and the null hypothesis was rejected. It was also found that the ts gene from RCY1124 and the ADE1 gene were linked as the chi square test for this pair of genes also gave a p<0.001. However, as the sample size of the tetrads dissected for these two mutant strains was very small, more dissections should be performed before characterizing either of these pairs of genes as being linked. Assuming that the linkage is valid, a calculation of the map unit distance between the genes was performed based on the equation given by Hartwell et al., (2008). These values were converted into a physical distance in kb (Cherry et al., 1997). The genetic distance between the ts RCY1110 gene and URA3 on Chromosome E is approximately 10 mu (physical distance of 29kb) based on the current data (Appendix Figure A2). The genetic distance between the ts RCY1124 gene and ADE1 on Chromosome C is approximately 14 mu (physical distance of 40kb) based on the current data (Figure A2).
Table 7. Summary of segregation patterns seen for nutritional markers in RCY1110 and RCY1124

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RCY1110</td>
<td>4:1:0</td>
<td>0:5:0</td>
<td>0:5:0</td>
</tr>
<tr>
<td>RCY1124</td>
<td>0:6:1</td>
<td>5:2:0</td>
<td>2:3:2</td>
</tr>
</tbody>
</table>

*ratio of number of parental ditype tetrads to number of tetratype and non-parental ditype tetrads.

**Discussion**

Isolation and identification of genes essential to the cell division cycle is an important step to building a complete understanding of how cells grow and reproduce. Once these mechanisms are better understood, the reasons behind why they malfunction will become much clearer, which could make problems such as cancer easier to cure. Complete understanding of the cell cycle begins with a search for essential *CDC* genes in mutants that are deficient in an essential cell division cycle function. After isolation of those mutated genes, the next step is to identify the function of that gene. This will contribute to the overall goal of understanding what goes wrong in cells deviating from the normal cell division process.

This project began the process of isolating essential *CDC* genes by first screening previously identified *ts cdc* mutants. The results of the *cdc* screen confirmed some results from the study performed by Callaghan (2012) and Heitz (2012) by confirming the four strains RCY1110, RCY1120, RCY1123, and RCY1124 as *cdc* mutants (Table 2). Additionally, RCY1122, and RCY1129—RCY1135 were screened for a Cdc− phenotype for the first time in this paper. The stains RCY1132 and RCY1135 were found to be borderline *cdc*. Additional screening could further confirm the Cdc− phenotype of these mutant strains. The cells from the strain RCY1134 had 80% uniform unbudded morphology. This was the only novel *cdc* mutant found in this thesis that had never been previously screened.
The cdc screens performed in this paper all followed the guideline of characterizing the bud morphology of ts mutants. In order for a ts mutant to be recognized as a cdc mutant, 70% of the approximately 100 cells counted would have to display the same bud morphology. This was the general cut-off point established by this lab to separate cdc and non-cdc mutants.

As seen in Figure 3, the arrest of RCY1110 was 59% un budded after exposure to the restrictive temperature. This is a low percent arrest to still qualify as a cdc mutant if the cutoff point is usually 70%. The reason analysis continued on RCY1110 is because the presence of the unbudded phenotype at a low percentage has a few possible explanations. RCY1110 could be a leaky ts mutant. This means that when its CDC gene was turned ts, the protein resulting from this gene was potentially maintaining some function even after growth at the restrictive temperature. This would cause us to see more cells slipping past the arrest point and thus a lower percentage of unbudded cells. Another explanation is that we are really seeing the strain arrest at more than one stage of the cell cycle. This would occur if the ts gene product was required at several phases of the cell cycle. This would mean that cells would arrest with different bud morphologies, depending on which checkpoint they arrived at first after loss of the gene function. RCY1110 was found to show a lower arrest both in this thesis and that of Callaghan (2012) and Heitz (2012). While its Cdc− phenotype may be weaker than other mutants, the consistency between the two research groups shows that analysis should continue. It is also noted that in a search for additional alleles of previously isolated cdc mutants, Hartwell found three mutants that displayed greater than 50% arrest but less than his cut-off point of 80%. The original cdc mutants that he isolated did meet the 80% cut-off point, but other mutants in the same genes did not. This shows that some Cdc− phenotypes are weaker than others, depending on the particular allele (Hartwell et al., 1973).
The possible cdc mutants from the second cdc screen, RCY1132 and RCY1135, also displayed a low percent uniform bud morphology upon arrest. The reason analysis was not yet continued on these mutants is because they have only been subjected to one cdc screen so far. RCY1110 was screened in this thesis and by Callaghan (2012) and Heitz (2012), where it was both times found to have a low percent uniform bud morphology. Prior to characterizing RCY1132 and RCY1135 as cdc mutants, additional cdc screens should be performed in the future.

Another factor that could contribute to observing a low percent arrest rate for any of the cdc mutant strains is the fact that our cdc control also displayed a low percent arrest. RCY303 was screened several times and never made it to the 70% minimum uniform large bud morphology. The fact that the known cdc mutant is displaying a lower arrest rate means that any mutant strain with an arrest rate at least as high as RCY303 could be characterized as a cdc mutant. A rescreen of RCY1110, RCY1132, and RCY1135 at the same time as RCY303 would hopefully clarify these results.

Also seen in Figure 3 are those cdc mutants that were able to achieve the 70% arrest rate. These include: RCY1120 with cells that arrest unbudded/tiny budded, RCY1123 with cells that arrest unbudded/tiny budded, RCY1124 with cells that arrest with a large bud, and RCY1134 with cells that arrest unbudded. Both the unbudded and large budded phenotypes are two that Hartwell saw in his original cdc screen in 1970. The interesting phenotype that we saw in RCY1120 and RCY1123 is the tiny bud that was about as prevalent as the unbudded cells. For our cdc screen the cells that arrested with tiny buds were combined in our counts with the cells arresting with no bud. This similarity between these phenotypes probably indicates that these mutants have trouble organizing the components needed to form a bud, and that some (those with
tiny buds) are more capable than others due to a semi functional ts gene product. As previously discussed, this phenotype was detected in one mutant, cdc1, by Hartwell et al. (1970) and in another, cdc50, by Moir et al. (1982). Mutant cells in CDC1 displayed the unbudded phenotype and were occasionally capable of producing a tiny bud. The biochemical activity of CDC1 is its involvement in lipid metabolism as a lipid phosphatase on the ER membrane (Losev et al., 2008). It is hypothesized by Losev et al. (2008) that cdc1 mutants have a toxic buildup of phosphorylated lipids that produce a cascade of events in the cell. These eventually lead to problems with actin polarization and organelle inheritance. Mutants in CDC50 display a small bud as described by Moir et al. (1982). However, it should be noted that the size of the bud is similar to that described as a tiny bud by Losev et al. (2008). CDC50 mutant strains, much like cdc1 mutants, are affected primarily due to the loss of lipid metabolism. CDC50 encodes a membrane protein required for the translocation of phospholipids between leaflets of the plasma membrane. Without the action of this flipase, the membrane loses its asymmetry (Bryde et al., 2010). It could be that cdc50 mutants are unable to continue bud growth because of a defect in making the second leaflet of phospholipids. It would be interesting to discover if similar phospholipid issues to cdc1 and cdc50 mutants are occurring in the K. lactis strains RCY1120 and RCY1123.

The large budded mutant pictured in Figure 3F, RCY1124, is an important mutant to analyze further because such mutants have been found by both Hartwell and by researchers in this project in the past (Hartwell et al., 1970; Fonseca, 2008; Molinos, 2008; Munson, 2003; Oldroyd, 2002; Pope-Rolewski, 2011; Silveira, 2000; Wallace, 2003; Yang, 2008). Large buds are an interesting phenotype because it can indicate arrest at either the G2 to M-phase checkpoint, the metaphase to anaphase checkpoint, or a mitotic exit phenotype. A way to further
differentiate the arrest point is by determining the location of the genetic information using DAPI stain. Figure 4 shows a comparison of duplicate images of RCY1124 using phase-contrast microscopy and fluorescence microscopy to determine nuclear location. The arrows pointing to the single mass of DNA indicates that this mutant strain is arrested prior to segregating its DNA.

The location of the DNA in RCY1124 indicates that it is having problems with one of several areas: DNA replication, attaching the spindle to the chromosomes, or the Anaphase Promoting Complex (APC). These components must all be functional in order for a cell to continue past the metaphase to anaphase checkpoint (Morgan, 2007). Both RCY435, characterized by Molinos, 2008, and RCY597, characterized by Fonseca, 2008, display the same large budded phenotype as RCY1124. It was later found that the mutation in RCY597 was complemented by DNA that encodes β-tubulin and contains a mutation in the TUB2 gene (L. Fonseca, N. Molinos, and L. Silveira, personal communication). This indicates that RCY597 is also stuck at the metaphase to anaphase checkpoint because it is having trouble with the components of the microtubule-composed spindle. It would be interesting to compare RCY1124 to other large-budded mutants that also have unsegregated DNA. These would include the control for this experiment RCY303, RCY597 and RCY435. The mutant gene in RCY303 was previously complemented by DNA including CDC16 by Pope-Rolewski (2011). CDC16 encodes a subunit of the Anaphase Promoting Complex (APC), and cdc16 mutant cells are seen to arrest at metaphase when exposed to a restrictive temperature (Lai et al., 2003). A complementation test would reveal if RCY1124 falls into the same complementation group as any of these three mutants or if our large budded mutant has a different problem causing it to arrest.

Figure 3H highlights a few examples of the cell shapes seen for the mutant RCY1126. Some of the main phenotypes observed in this mutant were swollen cell size, elongated buds,
and extreme deviant shape. The swollen cell phenotype indicates that cell growth and the cell cycle have become out of sync. It is known that for cell size homeostasis to be maintained, a functional cell cycle is needed (Polymenis & Schmidt, 1999). The most important feature of the cell size coordination to the cell cycle is the fact that the cell must reach a "critical cell size" prior to committing to cell division (Hartwell, 1974). A study by Zhang et al. (2002) investigated aberrant cell growth and the actual mechanism behind this critical cell size through the creation of 46 cell size mutants. It was found that a group of mutants characterized as uge would reach the critical cell size but then continue to grow without beginning the cell cycle. Several of these uge mutants had severe decreases in CLN (G1 cyclin) transcription. This decrease in CLN transcription is likely the cause for cell swelling because the Cln protein products have been shown to be responsible for cell size homeostasis (Polymenis & Schmidt, 1999). It is notable that most temperature sensitive mutants continue to grow and swell up in size even though the cell cycle has halted (Johnston, 1977). RCY1126 is ts and the fact that it follows this pattern means it could be mutated in a gene similar to one found by Zhang et al. (2002).

Lew & Reed (1993) provide a possible explanation for why RCY1126 displays elongated buds. This phenotype indicates that cells have passed START and have begun the apical bud growth process. However, in cells with only apical bud growth there is a defect in turning on the M-CDK's that are responsible for the switch to isotropic growth. Isotropic growth is a uniformly distributed growth of the bud. Lew & Reed (1993) found that isotropic growth only occurred when B-type cyclins Clb1 and Clb2 were functional during G2 and were able to activate the kinase Cdc28. This study provides more possible genes that could be malfunctioning in RCY1126.
If the defect seen in RCY1126 turns out not to be a gene involved with critical cell size, or the switch to isotropic growth, there is another possible explanation. The deviant shapes seen for many RCY1126 cells caused Callaghan (2012) and Heitz (2012) to label this mutant as a "frankenyeast". The aberrant cell shapes observed in this strain are similar in phenotype to the "monster" cells characterized by Smith & Blackburn in their 1999 study. They found that mutating or uncapping the telomeres in K. lactis (ter1 mutants) would almost immediately cause the cells to appear "monster" like with hugely distorted cell shape. Their conclusion was that mutated telomeres led to problems in genomic segregation that could lead to these morphological defects. The ter1 mutants were observed to have increased DNA content so a FACS analysis of RCY1126 would also prove interesting if this same trend is seen (Smith and Blackburn, 1999).

With a total of five confirmed cdc mutants, the next step to isolating CDC genes was to perform a backcross. The backcross was used to create diploids of our mutant strains that were then subjected to a recessiveness test for their Ts’ Cdc’ phenotype and tetrad dissection to see if that phenotype was due to mutation in a single gene. Unfortunately, RCY1134 was not included in the backcross or tetrad dissection because it was screened and confirmed as a cdc mutant too late in the course of the experiment. In the future, both of these tests should be used to analyze this strain.

The results of the backcrosses were successful for all strains that we mated to the wild type LSY25 (Table 3). It is important to perform several backcrosses because this will ensure that all mutant progeny share the same ts gene and that there is no other mutated gene accounting for the phenotype. If progeny from our confirmed cdc mutants are backcrossed and continually show the same phenotype, then it can be assumed that no other mutated gene is segregating among them. Another benefit to repeated backcrossing is to effectively separate two unlinked ts
genes if our mutants are found to contain two. Some of the resulting tetrads from such a mutant would have all segregants displaying the Ts' phenotype (referred to as nonparental ditypes). Each of these segregants would only contain one of the two unlinked ts genes, so backcrossing individual segregants to a wild type strain would purify a new strain with only one ts gene. This could be confirmed if several tetrad analyses with the new strain only displayed the 2:2 segregation ratio for a single ts gene.

The resulting cells from our backcrosses underwent a recessiveness test. However, after beginning the recessiveness test on all diploid and haploid strains for our mutants, we found that two of the haploid strains were not behaving ts as they should. RCY1120 and RCY1123 are previously characterized as ts mutants (Callaghan, 2012; Heitz, 2012) and we (Good & King, 2013) confirmed that they have Cdc' phenotypes at 37°C. After retesting this phenotype at multiple possible restrictive temperatures (Figure 6) and finding that they were still able to grow (Table 4), we concluded that they were not maintaining the expected Ts' phenotype. This was confirmed with a replica plate test in Figure 7.

The reason that these mutants are no longer displaying the Ts' phenotype but could be scored as having a Cdc- phenotype could be due to an issue with using different media to induce temperature sensitivity. When we performed the cdc screen, these two strains showed more than a 70% uniform arrest (Figure 3). This indicates that they are indeed temperature sensitive mutants. However, the cdc screen induces the Ts' phenotype in liquid YPD. The rescreen for temperature sensitivity, as well as the recessiveness test, occurred on solid YPD. Although Callaghan and Heitz in 2012 originally isolated RCY1120 and RCY1123 as temperature sensitive mutants on solid YPD, it may be easier to induce this temperature sensitive phenotype using liquid media. This would account for why we are unable to achieve the Ts' phenotype on
solid media. This difference in media phenomenon was observed by R. Louie in 2008 when RCY246 was only found to be temperature sensitive on YPD (containing rich media), but never on SD (containing minimal media). This was hypothesized to be due to the osmotic effects of the high salt content in SD plates helping a ts gene product to maintain its shape (Louie, 2008). The type of mutant that returns to prototrophic, or wild type, growth patterns only under certain osmotic media conditions was defined by Hawthorne and Friis (1964) as an osmotic-remedial mutant. Hawthorne and Friis found it was common for ts mutants to be osmotic-remedial mutants. It is concluded in this thesis that the ts gene products of RCY1120 and RCY1123 are likely hindered by the osmotic effects from the liquid YPD, which would be more likely to induce the Ts' phenotype than solid YPD. Bulawa (1992) saw the same trend in her study of chitin ts mutants. When her YPD plates were supplemented with increasing molarity of sorbitol, she witnessed the ts mutants surviving at the restrictive temperature. When she decreased the YPD media's yeast extract, peptone, and glucose content, the temperature sensitive phenotype was again observed (Bulawa, 1992). It would be interesting to try this same technique with our strains that are no longer appearing ts in conventionally made solid media. Decreasing the salt content of our solid YPD plates would potentially decrease the osmotic effects helping the ts gene products to remain functional. Another temperature sensitivity screen of RCY1120 and RCY1123 on these altered YPD plates might increase the instance of the expected Ts' phenotype. Because both of these strains showed the same uniformly unbudded/tiny budded cells upon arrest, there could also be an issue with this particular morphology that leads to a challenge with maintaining temperature sensitivity.

The discrepancy over whether RCY1120 and RCY1123 are really ts is present regardless of the reasoning behind this problem. Because of this, we decided to halt analysis of these two
strains and move on to the recessiveness test with RCY1110 and RCY1124. Both of these strains were found to hold recessive mutations. This is evident from Figure 8, where the mutant haploid strains were unable to survive at the restrictive temperature. However, it is seen that the diploid resulting from the backcross of either mutant to the wild type LSY25, was able to survive at the restrictive temperature. This indicates that when the two were mated, the wild type version of the mutated gene was able to compensate for this gene’s loss of function at the restrictive temperature. If we had seen the diploids also dying at the restrictive temperature, this would have been indicative of a dominant ts mutation because the wild type would not be able to mask the temperature sensitive phenotype.

The finding that RCY1110 and RCY1124 hold recessive mutations is important for future cloning by complementation. This is because cloning by complementation involves mutant cells taking up single plasmids with specific regions of a chromosome. Hopefully these regions contain the functional gene that is mutated in the cells. If the ts mutation in either of these strains were dominant, then even if they took up the correct plasmid containing the functional gene they needed, the dominant Ts' phenotype would not be masked by the wild type gene. This would make it impossible to distinguish cells with the correct plasmid from those containing the wrong plasmid because all cells would die at the restrictive temperature. However, it is expected that our results will find recessive ts mutations since most temperature sensitive mutations that are lethal to the cell are also recessive (Kaback et al., 1984; Hartwell et al., 1970).

The last step of this experiment was to determine if the Ts' phenotypes seen in RCY1110 and RCY1124 are due to a single ts gene. This is again important to confirm prior to cloning by complementation because a single gene on a single plasmid will not be able to mask a temperature sensitive phenotype that is due to two ts genes. The expected results for a mutation
in only one gene will be a 2:2 (Ts\textsuperscript{+}:Ts\textsuperscript{−}) segregation of the Ts\textsuperscript{−} phenotype between the four spores of a tetrad. Due to the inheritance patterns in meiosis, this would mean that two of the spores received the functional allele from LSY25, while the other two received the ts allele from the mutant strain (either RCY1110 or RCY1124). The results in Table 5 and Table 6 show that this 2:2 segregation of ts to wild type is exactly what we saw in the tetrads dissected from RCY1110 and RCY1124. However, the low sample size for each strain means that this segregation pattern should be investigated further with more tetrad dissection. Many more tetrads of each strain must be dissected in order to be certain that the Ts\textsuperscript{−} phenotype is not due to two linked genes.

If upon further dissection we start to see other ratios of segregation, this would be evidence for the Ts\textsuperscript{−} phenotype being the result of two genes. The different ratios arise because now the spores could show a temperature sensitive phenotype by inheriting either of the two ts genes. There are several types of tetrads that would result in this situation. The first is called a parental ditype tetrad (PD), which would show two segregants that are temperature sensitive (like RCY1110 or RCY1124) and two that are not (like LSY25). Although this would also be a segregation of 2:2, in this case it is due to two ts genes segregating separately into the same spore. The way to tell the difference between the parental ditype and the segregation of just a single ts gene, is to look for the presence of other ratios. Parental ditype tetrads with two unlinked segregating ts genes will be accompanied by tetrads with the ratios 3:1 (Ts\textsuperscript{+}:Ts\textsuperscript{−}), called tetratype (TT), and 4:0 (Ts\textsuperscript{−}:Ts\textsuperscript{−}), called non-parental ditype (NPD). After dissecting many tetrads, a ratio of 1:4:1 for PD:TT:NPD will indicate the presence of two unlinked ts genes in a mutant. As mentioned previously the NPD tetrads for a strain can be useful because they indicate that each segregant contains only one of the two ts alleles from the mutant. Isolating just one of those ts alleles is accomplished by backcrossing one of the cdc segregants to a wild type strain.

57
This essentially creates a new mutant that only contains a single ts gene from the original mutant. We have so far dissected three tetrads for RCY1110 and seven tetrads for RCY1124. Future experimentation should involve the dissection of many more tetrads to confirm that there is only a single mutated gene responsible for the temperature sensitive phenotype. If an increase in PD tetrads and decrease in TT tetrads is witnessed, according to the ratio 1:4:1 for PD:TT:NPD, then it can be concluded that there are two ts alleles in our mutants that are linked. If after future tetrad dissections it is concluded that one of the mutants contains two linked ts genes, then these can be separated by isolating the segregants of NPDs, and backcrossing them to a wild type strain.

Every segregant from every tetrad could grow on a minimal media plate that contained the nutritional supplements adenine, uracil and histidine. This confirmed that the mutant strains did not develop any new auxotrophy as a result of mutagenesis. This means that a segregant that can’t grow on a minimal media plate lacking uracil for example is unable to grow because they carry the ura3 marker. If this same segregant was unable to grow on the plate that provided uracil, adenine, and histidine, this would suggest that the mutant strain acquired a new auxotrophy during mutagenesis. It is important to identify any new auxotrophies in our mutants so that experiments performed on them in future can take into account all of their nutritional requirements. In particular, since cloning by complementation typically uses nutritional markers to select transformants, a complete picture of the markers is key.

The tetrad analysis plates containing adenine, uracil and histidine were not only important for determining if the mutants developed new auxotrophies, they were also needed to be sure that each of the four colonies came from the same tetrad. It was possible to have experimental error that resulted in random cells being grouped and analyzed as a tetrad. Table
A1 shows that each nutritional marker for every tetrad segregated 2:2 (e.g. *URA3:ura3*). This validated that all of the segregants we listed as being a part of a tetrad were indeed a part of that tetrad. This was important to clarify prior to evaluating the 2:2 *Ts*:*Ts* data and looking at linkage between the *ts* gene in the mutants and the three molecular markers: *ADE1*, *URA3*, and *HIS7*.

The linkage between the *ts* gene in RCY1110 and *URA3* was investigated due to the skew observed in the expected tetrad type ratio of PD:TT:NPD. For RCY1110, there was a great increase in the number of PD tetrads observed for the *ura3* marker, accompanied by a decrease in the number of TT and NPD tetrads (Table 7). A chi square test was performed using a model that compare the expected and observed ratios of PD:TT:NPD (Figure A1). The test gave a p value of less than 0.001 which led us to reject the null hypothesis that the *ts* gene and *URA3* were unlinked. A distance of 29kb between these two genes was calculated in case of linkage (Figure A2). It must be noted that the conversion factor used for map units to kb units is a value for *S. cerevisiae* because there is no known conversion factor for *K. lactis* at present. This may contribute to some error in calculations if the actual *K. lactis* value is drastically different than that of *S. cerevisiae*.

If the *ts* gene in RCY1110 is indeed linked to *URA3*, this would be an important finding because it would give the approximate location of this unknown gene. Knowing the approximate location gives us possible candidates for the identity of the *ts* gene. Because of the unbudded phenotype displayed by RCY1110 mutants, there are two possible groups that the *ts* gene could belong to. One group includes such genes as KLAOE22595g and KLLAOE22749, which have not yet been characterized and are within approximately 29kb of *URA3* (Génolevures, 2013). The second group of genes would be those that are known to arrest with unbudded cells when
mutated. These include genes involved with nutrient sensing, pushing a cell past Start, or cell polarization. One possible gene from this group that could be the identity of the ts gene in RCY1110 is ARPI (KLLAOE22925g), which is involved with spindle orientation and actin polarization (Génolevures, 2013).

While the ts gene from RCY1110 showed linkage to URA3, the ts gene of RCY1124 was seen to show possible linkage to ADE1. Using the same chi square test model, the null hypothesis that these two genes were unlinked was rejected after the test gave a p value of 0.001 (Figure A1). The distance of the ts gene from ADE1 was calculated to be approximately 40kb (Figure A2). This means that the identity of the ts gene in RCY1124 could potentially be within this range. Like the gene in RCY1110, it could be a gene whose function has yet to be identified such as KLLA0C03960g or KLLA0C04323g (Génolevures, 2013). Since the mutant cells of RCY1124 display a large bud after they arrest, the identity of the ts gene could also be one that is involved with DNA replication, the APC, or the mitotic spindle. One such gene within 40kb of ADE1 is KLLA0C04345g. This gene is similar to CDC7 in S. cerevisiae which is involved with the initiating DNA replication (Génolevures, 2013).

The chi square tests for the ts genes and nutritional markers in both RCY1110 and RCY1123 indicate that linkage exists. However, both strains had a very small sample size of tetrads dissected. Additional dissections in the future will either confirm or deny the linkage observed for these two strains in this thesis.

Whatever is determined about the linkage for the ts genes of RCY1110 and RCY1124, the identity of both genes still must be confirmed through the use of cloning by complementation. However, there is still one final step prior to cloning by complementation. Future researchers could perform complementation testing to determine if RCY1110 and
RCY1124 belong to any of the same complementation groups as mutants previously identified. This thesis can contribute to the complementation testing because the backcrosses performed on these strains likely generated segregants of the alpha mating type. This will allow these mutant strains to mate with other cdc mutants of opposite mating type in the complementation tests. Once the gene for each mutant is identified, further analysis will give evidence for what functions these genes are responsible for in the cell division cycle.
References:


### Appendix

Table A1. Scoring of the Ts' phenotype and nutritional requirements of all tetrads dissected for RCY1110xLSY25 and RCY1124xLSY25

<table>
<thead>
<tr>
<th>Backcrossed Strain</th>
<th>Spore Number</th>
<th>Ts</th>
<th>Ura</th>
<th>Ade</th>
<th>His</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCY1110</td>
<td>1a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCY1110</td>
<td>2a</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCY1110</td>
<td>3a</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3c</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCY1110</td>
<td>4a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RCY1110</td>
<td>5a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RCY1124</td>
<td>1a</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RCY1124</td>
<td>2a</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2d</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RCY1124</td>
<td>3a</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3c</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RCY1124</td>
<td>4a</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCY1124</td>
<td>5a</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5b</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5c</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
+ Indicates phenotype of segregant (depending on column—either Ts', Ura', Ade', or His')
- Indicates phenotype of segregant (depending on column—either Ts, Ura, Ade, or His)

<table>
<thead>
<tr>
<th></th>
<th>5d</th>
<th>6a</th>
<th>6b</th>
<th>6c</th>
<th>6d</th>
<th>7a</th>
<th>7b</th>
<th>7c</th>
<th>7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCY1124</td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RCY1124</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCY1124</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure A1. Chi Square Tests for RCY1110 ts gene linkage to URA3 and RCY1124 ts linkage to ADE1.

**RCY1110**

H₀: ts gene and URA3 are unlinked; ratio PD:TT:NPD 4:1:0 = 1:4:1
Degrees of freedom: 2
Expected: 5(1/6):5(4/6):5(1/6) = 0.83:3.33:0.83 (PD:TT:NPD)
Observed: 4:1:0 (PD:TT:NPD)
\[ X^2 = (4-0.83)^2/0.83 + (1-3.33)^2/3.33 + (0-0.83)^2/0.83 \]
\[ X^2 = 12.1 + 1.63 + 0.83 \]
\[ X^2 = 14.56 \]
p<0.001
Reject H₀—The genes are not unlinked

**RCY1124**

H₀: ts gene and ADE1 are unlinked; ratio PD:TT:NPD 5:2:0 = 1:4:1
Degrees of freedom: 2
Observed: 5:2:0 (PD:TT:NPD)
\[ X^2 = (5-1.167)^2/1.167 + (2-4.67)^2/4.67 + (0-1.167)^2/1.167 \]
\[ X^2 = 12.5 + 1.5 + 1.167 \]
\[ X^2 = 15.3 \]
p<0.001
Reject H₀—The genes are not unlinked

Figure A2. Calculated map distance for RCY1110 ts gene and URA3 and for RCY1124 ts gene and ADE1.

**RCY1110**

Recombination frequency = ((NPD + 1/2TT)/total tetrads) x 100 (Hartwell et al., 2008)
\[ RF = ((0+1/2(1))/5) x 100 = 10 \text{mu} \]
Physical distance conversion: 2.86kb/mu (Cherry et al., 1999)
\[ 2.9\text{kb/mu}(10\text{mu}) = 29 \text{ kb} \]
RCY1124
Recombination frequency = \((NPD + 1/2TT)/\text{total tetrads}\) x 100 (Hartwell et al., 2008)

\[
RF = \left(\frac{0+1/2\times2}{7}\right) \times 100 = 14 \, \text{mu}
\]

Physical distance conversion: 0.35mu/kb or 2.86kb/mu (Cherry et al., 1999)

\((2.9\, \text{kb/mu})(14\, \text{mu}) = 40 \, \text{kb}\)