THE ROLE OF CHROMATIN REMODELERS FOR LONG-TERM SURVIVAL OF *SACCHAROMYCES CEREVISIAE* IN THE QUIESCENT PHASE

by

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A THESIS

Presented to the Department of Chemistry and Biochemistry
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2019

An Abstract of the Thesis of

Erica Pledger for the degree of Bachelor of Science
in the Department of Chemistry and Biochemistry to be taken June 2019

Title: The role of chromatin remodelers for long-term survival of *Saccharomyces cerevisiae* in the quiescent phase

Approved: ____

Jeff McKnight

Chromatin remodelers are a group of enzymes that catalyze the movement of nucleosomes in order to allow other biomolecules access to DNA during processes such as transcription, replication, and DNA repair. Previous studies revealed that Chd1 and Isw1 chromatin remodelers in *Saccharomyces cerevisiae* are necessary for establishing regular nucleosome organization. In the absence of Chd1 and Isw1, a regular pattern of nucleosome positioning is abolished. However, the loss of nucleosome organization has surprisingly little effect on genome-wide transcription or on viability. Here, we test the hypothesis that nucleosome organization may have a more vital function during processes that require global alterations in transcription, such as quiescence. Yeast cells respond to low nutrient conditions by entering the quiescent phase, during which they exhibit significant decreases in transcriptional, translational, and metabolic activity. We isolated quiescent cells of chromatin remodeler knockout strains and monitored their survival in nutrient-starved conditions. Our results indicate that long-term viability in the quiescent phase is significantly reduced in strains lacking Chd1, Isw1, and Isw2, thus suggesting a role for chromatin remodelers during quiescence.

Acknowledgements

I would like to thank Professor Jeff McKnight and the rest of the McKnight lab for their guidance and support throughout these past two years. I feel lucky to have been part of such an inspiring and dynamic group of scientists. I would especially like to thank graduate student Vi Truong for being a thoughtful and diligent mentor, as well as for making the yeast strains. I would also like to thank Professor Ocean Howell for serving on my thesis committee. Finally, I am grateful for my friends and family. I owe it to my closest friends for making my undergraduate experience so special. And of course I cannot express enough gratitude for the constant encouragement of my parents and cats, who value my education and happiness above all else.

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Introduction

The plasticity of life on Earth is unmistakable – all organisms, including humans, are constantly responding to changes in their surroundings. Whether it be in response to changes in temperature, nutrient availability, or the presence of a pathogen, living beings are hardly ever static. The ability to efficiently react to the environment is vital for survival and proliferation. As a consequence, organisms have evolved methods of turning on or off molecular "switches" upon detection of a stimulus. While there are many layers to the regulation of such responses, one of the fundamental mechanisms for such control occurs at the level of transcription.

Transcription is the first step in gene expression, in which the DNA is used as a template to synthesize a messenger RNA (mRNA) molecule, which subsequently gets translated into protein. However, DNA is tightly condensed in order to fit inside the limited space of the nucleus. As a result, the compact organization of DNA imposes a barrier to transcription. Much in the same way yarn is neatly packaged by wrapping it around a spool, DNA is wound around octamers of histone proteins to form an array of structures called nucleosomes (6). The combination of DNA and histone protein is referred to as chromatin. Nucleosome positioning plays a key role in the regulation of gene expression because nucleosomes block transcription machinery from accessing the DNA. As shown in Figure 1, a gene that is contained within a nucleosome cannot be transcribed until the nucleosome is moved.

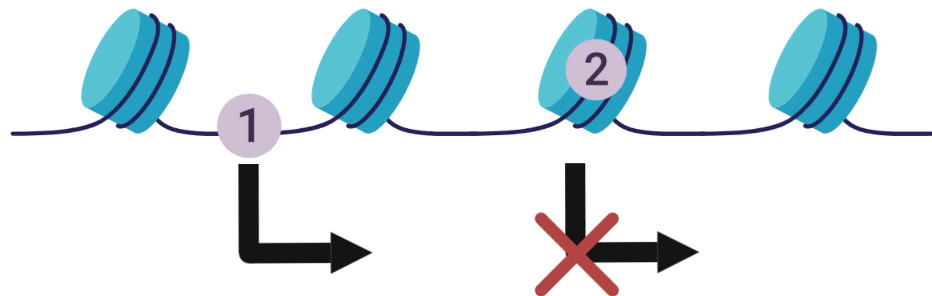

Figure 1. Gene expression regulated by nucleosome positioning

DNA is wrapped around octamers of histone proteins (blue) to form nucleosomes. Gene 1 is not contained within a nucleosome and can therefore be transcribed. In contrast, Gene 2 is not transcribed because it is contained within a nucleosome. Graphic created using BioRender.com

As such, nucleosome positioning is strictly regulated within cells. Nucleosomes are placed with respect to the transcriptional start sites of genes: close to the start site, there is a nucleosome-free region, which is closely followed by a well-positioned nucleosome referred to as the "+1 nucleosome" (15). Subsequent nucleosomes are uniformly spaced, however their placement becomes less distinct with increasing distance from the first nucleosome (11) (Figure 2). This well-ordered pattern of nucleosome alignment is found in species ranging from yeast to humans (11). Chromatin structure at the level of regularly-spaced nucleosomes is referred to as the "beads-on-a-string" model (16). However, nucleosomes are far from static. In fact, they are highly dynamic structures that are constantly being moved in order to allow other molecules access to the DNA. The proteins that control the positions of nucleosomes and thereby regulate gene expression are called chromatin remodelers.

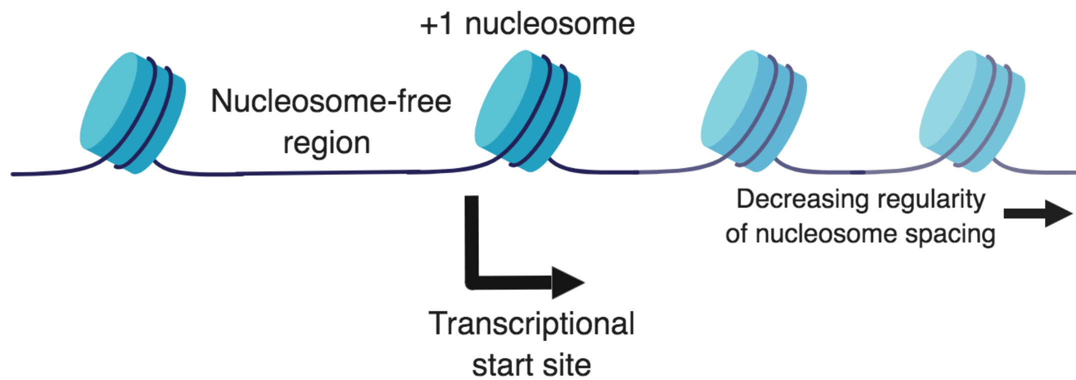

Figure 2. Nucleosomes are regularly positioned with respect to the transcriptional start site

A nucleosome-free region is found upstream of the transcriptional start site, which is immediately followed by the +1 nucleosome. Subsequent nucleosomes are evenly spaced, although the regularity of their positioning decreases with increasing distance from the +1 nucleosome. Graphic created using BioRender.com

Chromatin remodelers are a diverse group of enzymes that harness the energy of adenosine triphosphate (ATP) hydrolysis to slide, eject, or modify nucleosomes (9). They are highly conserved across many species, meaning the structure and function of chromatin remodelers have remained largely unchanged throughout evolution (10). This is not surprising considering their important role in many biological processes that require access to the DNA, including transcription, replication, and DNA repair (9). Chromatin remodeler malfunction is also implicated in a wide variety of diseases, with mutations in human chromatin remodelers occurring at high frequencies in many cancers (9).

Here, we focus on two major families of chromatin remodelers that are ubiquitous among many eukaryotes: CHD and ISWI. Remodelers within these families contain unique domains that allow them to recognize specific nucleosomes (5). We use the model organism *Saccharomyces cerevisiae,* more commonly known as baker's

yeast, to study the function of these enzymes *in vivo*. Yeast possess one form of the CHD remodeler, called Chd1, and two forms of the ISWI remodeler, called Isw1 and Isw2 (13, 12). Homologs of Chd1, Isw1, and Isw2 are also found in humans; hence our results have broader implications on understanding the biology of complex multicellular organisms as well (9). *In vitro,* these three yeast chromatin remodelers have been shown to form arrays of nucleosomes with regular spacing (10). Tsukiyama and colleagues first characterized an *in vivo* phenotype of cells lacking Chd1, Isw1, and Isw2 in 1999 (12). Their results revealed that the triple mutant was significantly more sensitive to environmental stresses such as high temperature in comparison to wild type (12). Since then, many studies have contributed to elucidating the roles of Chd1, Isw1, and Isw2 using a variety of techniques.

One such study lead by Gkikopoulos and colleagues was a launching point for our project. They showed *in vivo* that Chd1 and Isw1 were necessary for establishing regular nucleosome positioning in yeast (3). Mutants lacking both remodelers had aberrant nucleosome positioning in the coding regions of genes, although the nucleosome-free region located before the transcriptional start site was largely unaffected (3). Shockingly, despite the loss of regular nucleosome positioning, there were relatively little alterations to genome-wide transcription patterns in the absence of all three chromatin remodelers (3). This result was especially surprising since mouse and fly mutants lacking only one chromatin remodeler experience a severe reduction in long-term survival and suffer from developmental defects (7, 2). Considering the high energetic cost of maintaining correct nucleosome spacing, it seemed counter-intuitive that yeast strains with irregular nucleosome positioning were not only viable, but also

experienced only minor changes in transcription regulation. Hence we hypothesize that the action of chromatin remodelers to maintain regular nucleosome positioning may play a more significant role during processes that require global alterations in transcription rather than regular conditions.

This lead us to investigate quiescence, or G_0, which is a resting, non-proliferative state outside of the cell cycle. Cells enter the quiescent phase when exposed to a growth-limiting stress, such as nutrient depletion, and re-enter the cell cycle upon detection of favorable environmental conditions (Figure 3). About 60% of the biomass on Earth is thought to be comprised of quiescent microorganisms, and thus no doubt represents an important cell state to study (4). Moreover, many cells in the human body that are vital for tissue regeneration and healing are quiescent, such as adult stem cells, progenitor cells, fibroblasts, and lymphocytes (14). However, quiescence is poorly understood compared to other cell cycle states due to the inherent lowered activity of cells in this state, making it more difficult to perform biological assays (4).

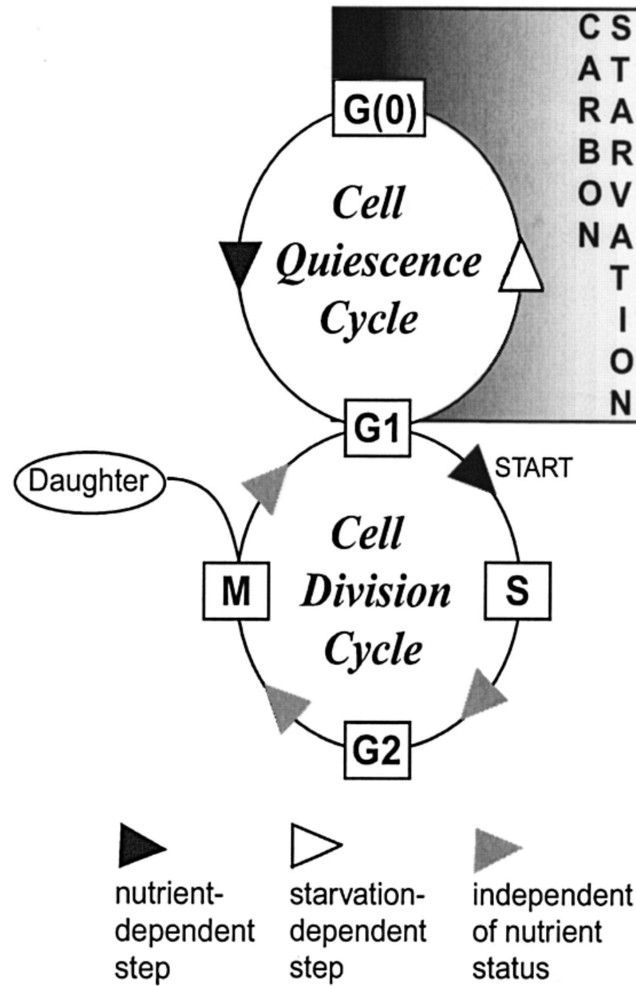

Figure 3. The cell cycle and entry into quiescence

During the normal cell cycle, cells go through a 4-step cycle comprising of the M, G1, S, and G2 phases. Cells grow during the two G phases, replicate their DNA during the synthesis (S) phase, and divide during mitosis (M). If a cell senses that nutrients are unavailable, it may exit the cell cycle and enter the quiescent phase (G0). When nutrients become available again, the cell may re-enter the cell cycle and continue dividing. Figure taken from Gray et al (4).

Entry into the quiescent phase triggers major changes in cell morphology and activity (4). For example, quiescent yeast cells will accumulate glycogen, trehalose, and triacylglycerols as metabolizable carbon sources, which significantly increases their density (4). Furthermore, quiescence is characterized by a major reduction in

transcriptional and translational activity: global mRNA levels in quiescent cells are roughly 30 times lower than in rapidly dividing cells and the rate of protein synthesis is reduced to approximately 0.3% (8, 4). Genome-wide transcriptional shutoff during quiescence entry has also been shown to be accompanied by extensive nucleosome repositioning (8). Quiescent cells exhibit a unique repressive chromatin structure that inhibits gene expression by placing nucleosomes in the nucleosome-free regions near transcriptional start sites (8).

Based on the distinct relationship between transcriptional shutoff and chromatin organization in quiescent yeast cells, we hypothesized that chromatin remodelers are likely to play a role during the entry, maintenance, and exit from quiescence. Thus this study aims to understand the functions of Chd1, Isw1, and Isw2 for cell survival in the quiescent phase. We monitored the long-term viability of quiescent yeast cells lacking these remodelers in nutrient-depleted conditions. Our results indicate that mutant strains lacking Chd1 and Isw1 or all three remodelers died off significantly faster than wild type in the quiescent phase, hence suggesting a necessity for chromatin remodeling during quiescence.

Materials and Methods

Yeast Strains

Yeast strains lacking different chromatin remodelers – called "knockouts" – were made by transformation, which is a process by which exogenous DNA is integrated into a cell's genome. This process involves (1) forcing the cells into a "competent" state in which they are more permeable to foreign DNA, (2) transforming the cells with DNA containing a gene of interest, and (3) selecting for cells that were successfully transformed. Wild type (WT) cells were grown to log phase and washed. The cells were then incubated for 1 hour at 42°C in a solution containing an integration cassette and lithium acetate (LiOAc), which makes their cell walls more permeable to foreign molecules. Integration cassettes are synthetic DNA molecules containing antibiotic resistance genes that can replace specific sites in a cell's genome. The cassette is flanked by sequences of homology to the specific gene of interest – namely, the genes for Chd1, Isw1, and Isw2. Through a process called homologous recombination, the cassette gets incorporated at this location while simultaneously removing the gene and replacing it with an antibiotic resistance gene. The cells were plated on agar plates containing the appropriate antibiotic(s), thus only allowing the growth of successfully transformed cells. Table 1 summarizes the genotypes and cassettes used for each strain. Yeast strains 322 and 334, and 325 and 335 are biological replicates, meaning their genotypes are identical so they serve to confirm that the observed differences between the mutants and wild type are biologically relevant.

Strain #	Genotype	Cassette
YS001	Wild type (WT)	NA
YS322	Δ*isw1*Δ*chd1*	*isw1::KANMX; chd1::NATMX*
YS325	Δ*isw1*Δ*isw2*Δ*chd1*	*isw1::HPHMX; isw2::KANMX; chd1::NATMX*
YS334	Δ*isw1*Δ*chd1*	*chd1::KANMX; isw1::HPHMX*
YS335	Δ*isw1*Δ*isw2*Δ*chd1*	*isw1::KANMX; isw2::HPHMX; chd1::NATMX*

Table 1. Genotypes of yeast strains

Note the nomenclature of yeast genetics – the double colon deletes a gene deletion. The gene that comes before the double colon is knocked out and replaced by the gene that comes after. For example, *isw1::KANMX* denotes a strain in which the *isw1* gene is knocked out and replaced with a gene that confers resistance against the antibiotic kanamycin. The Δ symbol denotes a knockout.

Growth Curve

WT, Δ*isw1*Δ*chd1*, and Δ*isw1*Δ*isw2*Δ*chd1* were grown at 30°C overnight in liquid YPD. The following morning, all strains were diluted to an optical density (OD_{600}) = 0.1 in fresh YPD. The diluted cultures were grown for approximately 6 hours at 30°C. We periodically measured the OD_{600} of the cultures to monitor their growth.

Isolation of Quiescent Cells

All five strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) media for 5 days. This gave adequate time for the cells to deplete the nutrients in the media and enter a non-proliferative state called stationary phase. We then used the procedure outlined by Allen and colleagues to isolate quiescent cells (1). This method takes advantage of the increased density of quiescent cells to separate them from non-quiescent cells using a density gradient. We created mini density gradients in 2 ml tubes

by centrifuging 1 ml of a 1:10 dilution of Percoll Plus at 10,000 xg for 10 min. The stationary phase yeast cultures were washed, resuspended in water, and gently added to the Percoll Plus density gradients. We then centrifuged the Percoll-cell mixture for 45 min at 400 xg to gradually separate the cells based on their density. Two distinct layers of cells formed – the quiescent cells, which are denser due to their accumulation of storage carbohydrates, sank to the bottom of the gradient, whereas the non-quiescent cells stayed near the top. Thus we were able to remove the top layer and extract just the quiescent cells. These cells were washed and used to perform the long-term survival experiments.

Long-term Survival of Quiescent Cells

The quiescent cells were diluted to an OD_{600} ~ 0.01 in 5 ml of sterile water, which is equivalent to a cell concentration of $1 \cdot 10^5$ cells/ml. We plated 50 µl of a 1:10 dilution on YPD agar plates at 30°C in duplicate, which we calculated to yield roughly 500 colonies per plate. Two days later, we counted the number of colony formation units that grew on the agar plates. We marked the waterlines of the tubes containing the cells in water and incubated them at 30°C for 7 days.

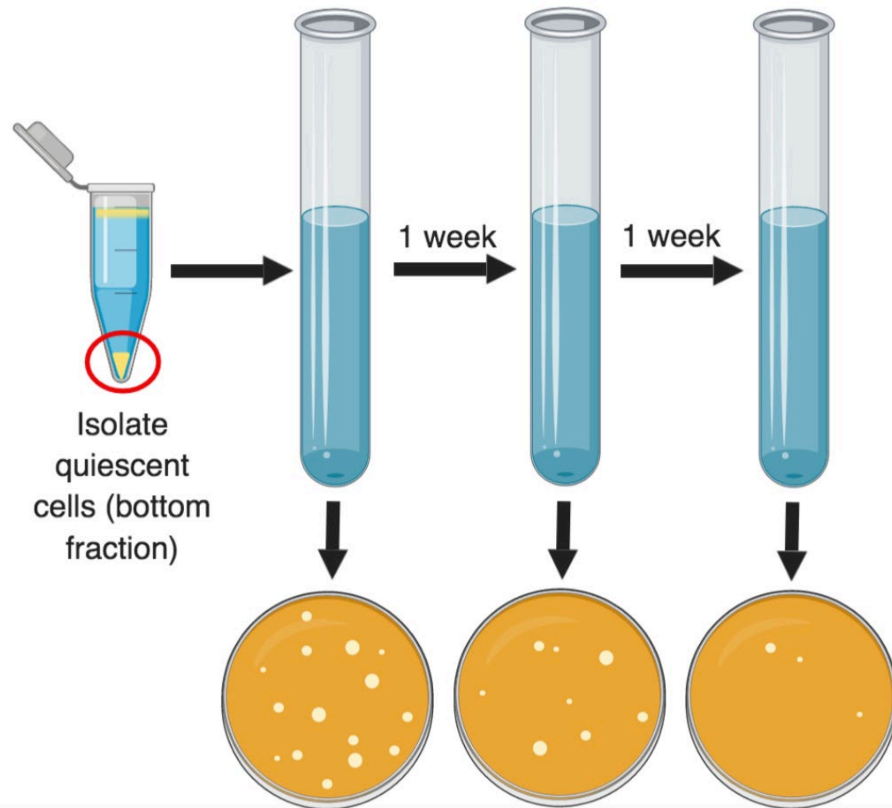

Figure 4. Isolation of quiescent cells and long-term survival of quiescent cells

Quiescent cells were purified using a Percoll density gradient method. The quiescent cells were then transferred to sterile water and plated weekly. Graphic created using BioRender.com

After the one week of incubation, we added sterile water up to the marked waterlines in order to make up for water lost due to evaporation. We then plated the quiescent cells using the same volume and dilution as before. Once again, colony formation units were counted two days later to monitor a change in viable quiescent cells (Figure 4). Survival proportion for each time point was calculated as the average number of colonies divided by the average number of colonies from the initial plating. This process was repeated weekly for 5 weeks, at which point the majority of cells in all five yeast strains were dead.

Results and Discussion

To gain an initial understanding of the phenotypes of chromatin remodeler knockout strains, we first sought to characterize the differences in their growth rates. We grew WT and double and triple knockout strains in YPD media and monitored the changes in their cell density as a function of time (Figure 5). Our results indicate that the WT strains grow significantly faster than the two mutant strains, with WT reaching approximately double the cell density of both Δ*isw1*Δ*chd1* and Δ*isw1*Δ*isw2*Δ*chd1* strains within 6 hours. The two knockout strains exhibit very similar growth curves.

Tsukiyama and colleagues previously showed that Δ*isw1*Δ*isw2*Δ*chd1* strains had a significant growth defect at high temperatures (37°C) (12). They concluded that at least one of these chromatin remodelers is necessary for establishing and maintaining proper nucleosome positioning in order to survive stress conditions (12). We report a similar growth defect in the triple mutant, which is present even in ideal growth conditions (high nutrient content at 30°C). The double mutant has a nearly identical growth curve as the triple mutant, which suggests that Isw2 alone is not capable of rescuing the growth defect. This suggests an important role for chromatin remodelers in contributing to the overall fitness of rapidly dividing cells. Nonetheless, it is surprising that the triple mutant is viable despite its lack of global nucleosome organization, as reported by Gkikopoulos et al. Hence we hypothesized that proper nucleosome positioning may be especially vital during processes that require genome-wide changes in transcription. To this end, we aimed to test the role of Chd1, Isw1, and Isw2 chromatin remodelers for survival in the quiescent phase and ability to re-enter the cell cycle.

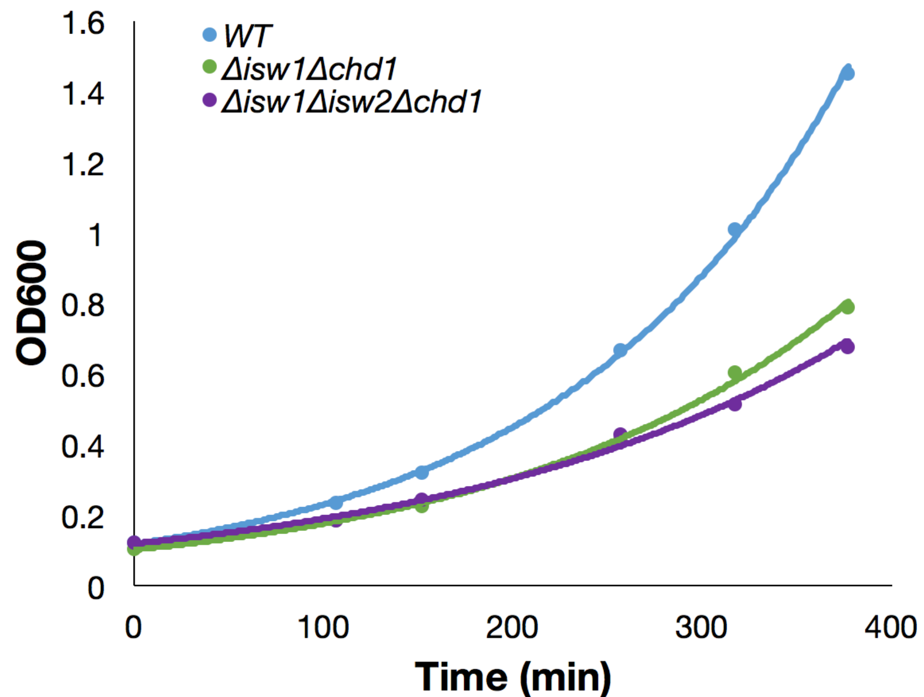

Figure 5. Growth curves of WT, Δ*isw1*Δ*chd1* and Δ*isw1*Δ*isw2*Δ*chd1*

WT cells exhibit faster growth kinetics than both mutant strains.

We isolated quiescent cells from 5-day cultures of WT, two strains of Δ*isw1*Δ*chd1,* and two strains of Δ*isw1*Δ*isw2*Δ*chd1* in order to investigate the fitness of chromatin remodeler knockout strains upon entering quiescence. The two strains of identical genotypes serve as biological replicates to ensure that the observed results were biologically relevant rather than random artifacts. Thus each data point for the two mutant strains represents averaged results from biological replicates in addition to technical duplicates. The quiescent cells were incubated in sterile water (without nutrients) for slightly over a month. The same volume was plated weekly on YPD agar to monitor their survival and ability to exit quiescence. We observed that the mutant strains had a distinct phenotype in comparison to WT (Figure 6). After one week, WT cells (light blue) were all still viable and able to successfully exit quiescence and re-

enter the cell cycle when introduced to nutrients. By stark contrast, all of the mutant strains already had a decrease in the number of viable cells – notably the two strains with less than 40% survival. By the end of two weeks, the survival phenotypes of the mutant strains were still distinct from WT. While about 75% of WT cells were still viable, all mutant strains had fallen below 50% viability. After three weeks, all strains including WT exhibited similar trends in survivability. By the end of 5 weeks, essentially all cells from all five strains were dead.

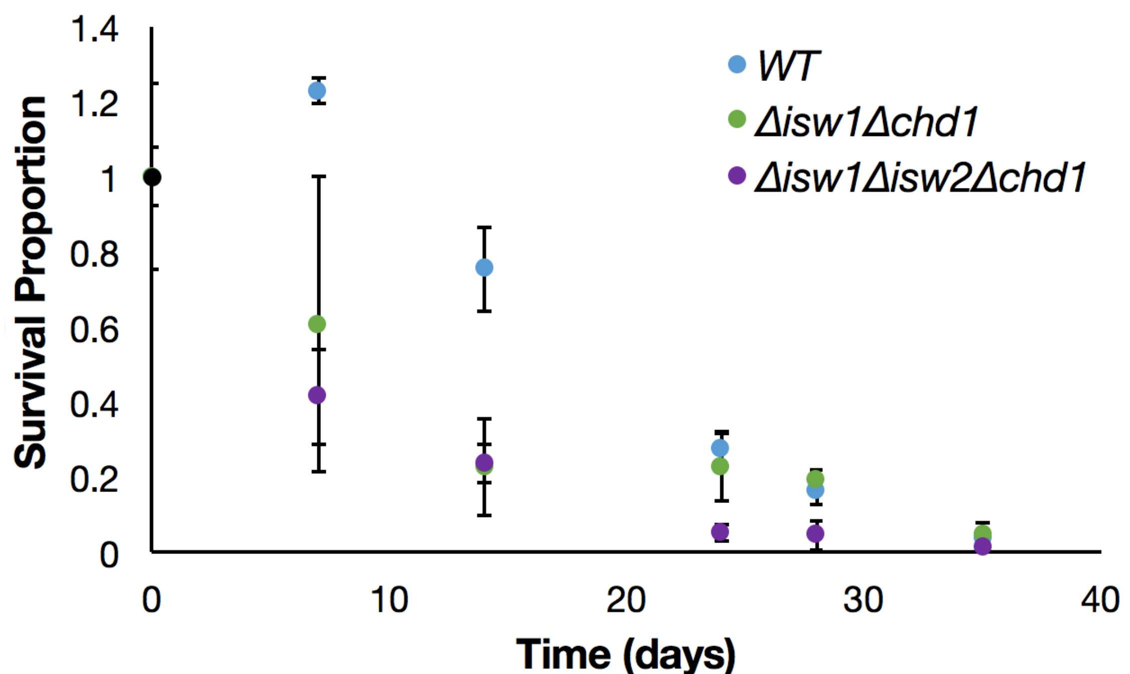

Figure 6. Survival plots of WT, Δ*isw1*Δ*chd1* strains, and Δ*isw1*Δ*isw2*Δ*chd1* strains. WT cells exhibit greater survivability in the quiescent phase in comparison to the mutant strains.

Our results show strong evidence that Isw1, Isw2, and Chd1 chromatin remodelers do indeed contribute to the ability of yeast cells to maintain viability over long periods of time in the quiescent phase. There are several possible explanations for the observed phenomena. First, the mutant strains may be less equipped to survive in

the quiescent phase, thus dying off more rapidly than WT. On the other hand, the more rapid decay of the mutant strains may be a result of their inability to effectively exit the quiescent phase in response to nutrient availability. It is likely that a combination of these two phenomena result in the observed reduction in survivability of both the double and triple mutant strains.

Future Directions

The results of this investigation reveal a possible role for Chd1, Isw1, and Isw2 chromatin remodelers during quiescence in *Saccharomyces cerevisiae.* Triple mutant and double mutant strains exhibit slower growth kinetics and more rapid decay in the quiescent phase. While this study revealed a distinct survival phenotype for chromatin remodeler knockout strains, further investigation into the precise mechanisms that lead to these deleterious effects are necessary. Future studies will seek to differentiate between the two possible explanations proposed here – that is, whether mutant cells fail to survive in the quiescent phase or if they are less efficient at re-entering the cell cycle upon nutrient availability.

Further studies should be carried out to probe the molecular phenotypes of each of the mutant strains during quiescence. Similar to the experiments performed by Gkikopoulos and colleagues, mapping nucleosome positioning using micrococcal nuclease (MNase) digestion in different mutant strains during quiescence will provide a molecular understanding of the observed differences in survivability. It will be interesting to investigate whether nucleosome landscapes for both the WT and mutant strains differ between proliferating and quiescent cells. Furthermore, MNase digestions on quiescent cells from the mutant strains at different time points in the survival experiment will reveal whether irregular nucleosome positioning worsens with time in the quiescent phase. Lastly, it would be useful to perform MNase digestions on WT cells that exited quiescence upon exposure to nutrients. This would address the question of whether nucleosome positioning is re-established to its original form after a lengthy period of quiescence.

Moreover, given the major role played by chromatin remodelers in regulating gene expression, analyses of the transcriptome will help elucidate the effects of aberrant nucleosome positioning on transcription. RNA-seq is a technique that uses sequencing methods to determine the quantity and sequences of RNA molecules in a sample. Thus it allows for examination of global transcription patterns in cells – that is, we can observe which genes are being turned "on" or "off" and to what extent. RNA-seq of the mutant and WT strains before, during, and after a long period of quiescence will allow examination of differences in gene expression as a result of the presence or absence of chromatin remodelers.

Glossary

Adenosine triphosphate (ATP): A high-energy molecule that is used to drive many biological processes.

Centrifugation: A method of separating particles with different densities by spinning them in solution around an axis at high speed.

Coding region: The portion of a gene that codes for protein.

Domain: In protein structure, *domain* refers to a portion of a protein that possesses a defined function, such as binding DNA.

Gene expression: The process by which information from a gene is used in the synthesis of a gene product, often a protein.

Homologous recombination: The exchange of genetic material between two strands of DNA that contain stretches of similar sequences.

In vivo: Experiments that are tested on whole, living organisms or cells. This is in contrast to *in vitro* experiments, which are tested in a controlled environment outside of a living organism.

Micrococcal nuclease (MNase) digestion: An assay that reveals chromatin structure by digesting DNA regions between nucleosomes.

Optical density (OD_{600}): A method for estimating cell concentration by measuring the absorbance of a sample at a wavelength of 600 nm.

Percoll: A solution composed of silica particles that is used to separate particles based on their density.

Phenotype: The observable physical properties of an organism. This is closely related to *genotype,* which is the genetic makeup of an organism that determines its *phenotype.*

Transcriptional start site: The location on a gene where transcription begins.

Transcriptome: The entire set of transcribed genes in a cell at a given time.

Wild type (WT): The phenotype or genotype that represents the typical form found in a species as it occurs in nature.

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