**The interconnection between oxidative stress, genomic instability, mitotic asymmetry, and chronological lifespan in *Saccharomyces cerevisiae***

**by**

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Candidate for a B.S. in Biology

Submitted to the Department of Biology in partial fulfillment of the

requirements for the completion of the Ethel Waddell Githii Honors Program

at SPELMAN COLLEGE

April 2012

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**ABSTRACT**

Cellular aging in Saccharomyces cerevisiae can lead to genomic instability and impaired mitotic asymmetry. Here, we focus on the role of oxidative stress on genomic instability and mitotic asymmetry. We treated yeast cells from a collection of natural isolates with hydrogen peroxide, and monitored the frequencies of loss of heterozygosity (LOH) in response to hydrogen peroxide concentration. We found that the increase of hydrogen peroxide-dependent genomic instability occurs before a drop in viability. This leadoff is negatively correlated with chronological lifespan, with an R-squared of 0.54 and a p-value of 0.024, and positively correlated with a measure of endogenous mitotic asymmetry with an R-squared of 0.43 and a p value of 0.054. This indicated that better resistance to exogenous hydrogen peroxide is associated with a longer chronological lifespan and better mitotic asymmetry. Moreover, we previously observed that elevation of genomic instability generally lags behind the drop in viability during chronological aging. Hence, hydrogen peroxide treatment and chronological aging lead to opposite timing of genomic instability with regards to viability. This contrast argues that the effect of oxidative stress on genome integrity is well prevented up to the dying-off phase during chronological aging. Overall, our results demonstrate strong associations between oxidative stress, genomic instability, and mitotic asymmetry within the context of aging.

**Acknowledgements**

I owe the completion of this thesis to my advisor, Hong Qin. I thank him for constant support, commitment, and encouragement during this process. I would also like to thank research technicians Erin Jackson, Jenney Rodrigues, and Nilin Gupta and my undergraduate research colleagues Meghan Parker, Megan Maghee, and Brittni Wilson. This project was supported by the National Science Foundation and the Howard Hughes Medical Institute.

I would like to extend a special thanks to the Spelman College Biology Department and the Ethel Waddell Githii Honors program for enriching my learning experience. Lastly, I will forever be grateful for my parents and sisters who have offered their unwavering support and have provided a diverse lifestyle from which I have drawn much of my scientific inspiration. I hope that I have made you all proud and that I can use my knowledge and experience to make a positive contribution.

**Introduction**

Aging in a population describes the change in viability over time and is a feature of all eukaryotic organisms. Benjamin Gompertz, a British mathematician circa the early nineteenth century, established some key principles that shape current understanding of aging in biological systems. Gompertz and his contemporaries proposed that aging could be explained from a phenotypic and statistical standpoint. The phenotypic component contends that there are physical changes that accompany aging individuals. The statistical aspect asserts that the probability of dying increases with age (Park 1998).

Aging, whether described phenotypically or statistically, can be attributed to the interplay of key age-dependent changes in genomic integrity, fitness, metabolic homeostasis, and stress response. From an evolutionary standpoint, populations strive to survive and reproduce. If organisms are able to withstand selective pressures posed by the environment then they become more suitable for survival within a particular place and time. One consequence of aging is the decrease in fitness. Thus with age, a loss of function can reduce an organisms reproductive potential and ability to survive.

This loss of fitness can be explained by a decrease in genomic integrity and can lead to age-related consequences. The free radical theory of aging is an accepted mechanistic explanation for aging in eukaryotic organisms. This theory suggests biological systems age because of the accumulation of free radicals or ROS that react with macromolecules and disturb key pathways that are vital to maintaining the overall integrity of organisms (Yu). ROS are atoms or ions harboring unpaired electrons within an open shell configuration. Free radicals are natural products of metabolic reactions in the mitochondria and are required for cell communication and signaling (Rahman, Weinberger). This ROS accumulation is called oxidative stress, although ROS species are not exclusively oxygen-based. Oxidative stress can upset metabolic homeostasis, which typically regulates cell growth and stability (Ristow 2011). The positive correlation between age and the increased probability of developing certain diseases can serve as evidence of detrimental effect of the loss of genomic integrity (McMurray) **Figure 1.** Increased genomic instability as a consequence of aging can has been studied in multiple organisms including *C. elegans* and mice. However, the distinctly simple features of *Saccharomyces cerevisiae* have made this organism a widely used model for studying the aging process (McMurray, Gravel).

*S. cerevisiae* are fungal, eukaryotic organisms whose simple structures and extensively studied. The lifespan of budding yeast can be quantified under experimental conditions over short periods of time. Budding yeast have a replicative lifespan (RLS) and a chronological lifespan (CLS). RLS and CLS are distinguished by the ways in which lifespan is measured. CLS measures the amount of time required for a single mother cell to stop replication. RLS refers to the number of times a cell undergoes the cell cycle (Wei 2008, Park 1998, Qin).

*S. cerevisiae* can also be studied in both haploid and diploid states. Loss of heterozygosity has become a commonly used method for detecting loss of genomic integrity in yeast (McMurray). Heterozygosity on the MET15 locus is achieved via the knockout of one copy of the wild-type allele by a kanamycin resistance marker. LOH can be monitored in *Saccharomyces cerevisiae* only when the heterozygous form of MET15 +/- is converted into a homozygous recessive form (MET15 -/-) following mitotic division.

When yeast is plated on lead containing medium, the colors of the colonies change, in a sectional manner, depending on the timing at which the mutational event occurs. Thus, MET15 -/- leads to fully black colonies. Colonies may have a brown tint, depending on the yeast strain used. Both dominance for the MET15 gene (MET15 +/+) and MET15 +/- yield white color colonies. As a result, only fifty percent of mutational events are observed because the two latter genotypes are phenotypically indistinguishable. The number of cells that did not undergo a mutational event at the MET15 locus was an indication of robustness, with respect to that specific locus **(Qin 2008) Figure 3.**

H2O2 induced damage triggers LOH through mitotic recombination (MR) when double-strand breaks are present on DNA. If this damaged DNA is detected, one allele is replaced the other allele on its homologous chromosome. As a result, the goal of MR is to restore the genotype prior to DNA damage (McMurray Hyperecombinational state, **Hiraoka et al 2000).** Thus, LOH can be used as a sign of genomic alteration on the MET15 locus.

Apart from the technical benefits for using yeast to study aging, most of the known genes related to lifespan are conserved in both humans and yeast; some of which include Sir2 and Tor1. The TOR (Target of Rapamycin) pathway has been shown to be involved in regulating cell growth, mitotic division, as well as nutrient response in both yeast and humans **(Wei 2008).**

Aging is a conserved fundamental biological phenomenon. Evidence of this lies in the fact that restriction (CR) can extend lifespan in both yeast and humans. Wei et al. reported a 10-fold increase in the lifespan of calorie restricted *S. cerevisiae* mutants that had deleted RAS2 and SCH9 genes. Further, the presence of key gene products live Rim15 is shown to promote this lifespan extension in the mutant budding yeast **(Wienberger 2010, Wei 2008).**

A primary argument for this is that many species eat sporadically. Many bear species may hibernate and some plants may live in arid locations and thus endure long periods of time with low nutrient availability. The individuals that were able to survive during those nutrient-free periods were ultimately more fit and able to transfer these protective mechanisms to offspring.

A thirty-six year follow-up study in 2000 non-smoking Japanese-American men also supports the proposal the CR can extend lifespan. It showed that caloric intake that was 15% less than the average extended lifespan **(Wilcox 2004).** This data also aligns with the studies that show a correlation between obesity and premature death. Dietary habits that involve excess caloric intake are associated with shorter lifespan whereas individuals that have moderate eating habits live longer **(Stanfel 2009).**

Cellular signals either delay or promote growth depending on intracellular or external conditions. When nutrients like glucose are not readily available in the cell, quiescence occurs, in which metabolic activity is reduced in order to conserve cellular energy. Thus the progression of the cell’s lifespan is either slowed or halted. Conversely, elevated nutrient levels will speed up metabolic rates and promote cell-cycle progression. This can shorten CLS (Ruckenstuhl 2010).

Biological aging has become a popular area of study because it has broad biomedical implications. This area of research has aesthetic implications; for many of us want to look and experience the benefits of youth for as long as possible. Ultimately, this area can open brand new realms for understanding phylogenic relationships among species, and contribute to the study of age-related diseases like Alzheimer ’s disease, cancer, and atherosclerosis, and diabetes. Ultimately, these studies can introduce ways to extend human lifespan.

**Materials and Methods**

***Yeast Culturing***

Strains with heterozygous Met 15 +/- were grown overnight at 30°C in 5 mls of YPD using autoclaved glass tubes. Strains used with heterozygous Met15+/- were described previously by Qin et al (Qin). Following incubation, a spectraphotometer was used to determine saturation of yeast in the glass tubes at an optical density of 600 (OD600). The yeast culture was restaged to 0.6 at OD600 in fresh YPD in new autoclaved glass tubes with a final volume of 4 to 6 mls. The restaged culture was grown in a 30 °C shaker for an additional two hours, during which the optical density should have ranged between 0.8 and 0.9. Cells were harvested, transferred to 1.5 ml eppendorf tubes, and centrifuged at maximum speed. Following YPD decantation, cells were washed in an equal volume of double distilled water, vortexed, and centrifuged. Cells were washed two additional times. The eppendorf tubes were immersed into a waterbath sonicator and sonicated for 4 minutes using one power setting.

***H2O2 Treatment***

The protocol used models the H2O2 sensitivity test used in Yu et al (Yu). Ten concentrations, including 0.3%, 0.2%, 0.15%, 0.1%, 0,075%, 0.05%, 0.025%, 0.01%, and 0.005%, and 0% of 2X H2O2 stock solutions were made. For each dilution, a 1.5 ml eppendorf tube was acquired and filled with 4 µl of a 10X dilution of yeast cells, 16 µl of ddH2O, and 20 µl of the appropriate hydrogen peroxide dilution. All treatment was done under sterile conditions with use of a Bunsen burner. Each eppendorf tube was vortexed and wrapped in parafilm. The tubes were incubated in a shaker for 3 hours at 30 °C. The reaction was terminated by adding 960 µl of water (50X dilution and chilled on ice. Eppendorf tubes were sonicated in a water bath for 2 minutes. 250 µl of each concentration were spread onto large MLA plates using sterile glass beads. If small plates are used, 150 µl of each sample of treated cells should be added to each plate. Plates were spread in triplicates for each H2O2 concentration. Plates were placed in a 30 °C incubator for overnight or two additional days depending on observed growth.

***Counting Colonies***

Images of each MLA plate were taken using a ColonyDoc-It Imaging Station. Colonies were assessed for any notable characteristics and counted by color-section patterns using a Bantex Colony Counter. The number of fully black, fully white, half black, quarter black, three-quarter black, quarter-quarter black, and others were documented. Color-section patterns that were less than one-eighth were ignored.

***Data Analysis***

As colonies were counted, all results were documented on formatted charts. Original data was then transferred to an excel format in the following: charts requiring the strain, the absorption value at OD600, the dilution, date, H2O2 treatment (%), number of white colonies, number of black colonies, number of half black colonies, number of quarter black colonies, number of three-quarter black colonies, number of quarter-quarter black colonies, the number of other color-section patterned colonies, and notes.

R 2.11.1 was used to plot mitotic asymmetry in the form of black colonies versus cell viability. R software was also used to determine the middle concentration of black colonies (Cb) versus the middle concentration of cell viability (Cv).

**Results**

The interconnection between oxidative stress, genomic instability, mitotic asymmetry, and chronological lifespan in Saccharomyces cerevisiae was addressed using H2O2 treatment to induce an oxidative stress response. LOH assays on lead-containing plates were used to detect and quantify mutational events during a strains CLS. It was hypothesized that the frequency of loss of heterozygosity events will occur in a dosage-dependent manner of hydrogen peroxide (H2O2).

**Contrasting Switching Pattern of H2O2 and Chronological Aging on LOH**

Qin et al. measured biological aging with a logistical model using the ratio, Tg/Tc. Tg represents the midpoint of the genome integrity, which is measured by LOH. Tc represents the midpoint of chronological lifespan. With respect to the biological survival curve, the midpoint of genome integrity comes after the midpoint of chronological lifespan **Figure 2** (Qin 2008). Thus, the biological survival curve will likely display a greater frequency of strains with Tg/Tc ratios that are one or greater **Figure 6**. The logistical model for the hydrogen peroxide dose-response curve uses the ratio Cb/Cv, which represents mitotic asymmetry. Cb represents the middle concentration of black colonies, which is a measure of genome instability. Cv represents the middle concentration of viability. With respect to dose-response curve, Cb usually comes before Cv in the strains used **Figure 4B**. Thus, there is a greater likelihood of observing a higher frequency of strains with Cv/Cb ratios that are lower than one **Figure 6, Table2**.

**Genome tolerance (Cb) and viability tolerance (Cv) to H2O2 induction varies by strain backgrounds**

A regression analysis revealed that genome and viability sensitivity varies with each strain background. There is a significant association between CLS and the Cb/Cv ratio with a p-value of 0.024. The R-squared value of 0.54 indicates a strong association between these measures. A longer CLS corresponds to a smaller Cb/Cv. A ratio less than 1.0 indicates that Cb comes before Cv, and thus a greater dose of hydrogen peroxide is required to kill the cell. Thus, strains with lower ratios are more tolerant to hydrogen peroxide with respect to viability. A Cb/Cv greater than 1.0 indicates that Cb comes after Cv, and thus cells are more sensitive to hydrogen peroxide treatment. Strain M13 seems to be the most tolerant to hydrogen peroxide treatment.YPS128 seems to have the most sensitive response to hydrogen peroxide treatment whereas M13 seems to be substantially more tolerant to H2O2 treatment **Figure 7**.

**There is a significant correlation between CLS and the relative timing of the H2O2 trigger on LOH**

A regression analysis revealed that there is a significant correlation between L0, which represents the ratio of half black and fully black colonies at time zero, and Cb/Cv. The p-value representing this data is 0.055. This significant association is supported by a relatively high R-squared value of 0.43. A smaller Cb/Cv is associated with a larger L0. A ratio less than 1.0 suggests that a drop in viability follows the middle concentration of black colonies whereas those strains with ratios greater the 1.0 tend to lose their viability before genomic instability is significant **Figure 8**.

**Discussion**

In this study, we report that the biological survival curve and the hydrogen peroxide dose-response curve exhibit contrasting switching patterns. These results suggest that there is opposite timing of genomic instability with regards to viability. The LOH assay allowed us to quantify age-dependent changes in response to hydrogen peroxide dosage in previously used strains **Table 1**.

***Assessment of Materials and Methods***

Early results revealed that cells in their stationary phase were more resistant to oxidative stress. Strains were treated in their log phase, a period of exponential growth, so that differences in responsiveness to hydrogen peroxide would be more apparent. It would be more challenging to compare robustness or tolerance to hydrogen peroxide if all strains were resistance to oxidative stress.

After culturing the samples were sonicated in order to ensure uniform segregation of the cells. Cells were re-sonicated following H2O2 treatment due to the likelihood that cells stuck together during the final incubation period. Without re-sonification, there would have been a higher number of half-black colonies on plates compared to fully black colonies. As shown by a previous protocol.

***Assessment of Results***

The timing at which there is an increase in black colonies is relative to the viability drop. In biological aging, ROS must be low enough such that mutation is suppressed before there is a substantial drop in viability. Conversely, the H2O2 treated cells dosage has more of an immediate effect on the robustness of the cell. Viability drops more rapidly due in H2O2-treated cells because ROS levels are increased via the external elevation of H2O2 and the inhibition of superoxide dismutase activity (SOD) **Figure 1** (Weinberger et. al).

Cells with better mitotic asymmetry have a longer lifespan and better H2O2 viability tolerance.

Future directions include testing gene deletion mutants with H2O2. MSN2/4 has been shown to be vital to the pathway for extending CLS in yeast. This gene product functions by upregulating genes that enable the cell tolerate stress. SOD2 activity, for example, is increased and extends lifespan during this process, but can also reduce CLS if it is expressed excessively (Medvedik 2007).Conversely, the Weinberger model proposes that inhibition of SOD activity can result in the increase of ROS levels and reduce CLS in yeast (Weinberger et al. 2010). If the SOD gene is deleted, we should see similar Cv and Cb patterns to the ones reported in this current project. Under the same experimental conditions, deleting SOD and eliminating its action may also increase superoxide levels.

Future plans also involve treating strain with paraquat dichloride (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride) to induce superoxides directly. Superoxide levels when cells are treated with H2O2 and paraquat will also be measured directly using a fluorescent probe.

**Conclusion**

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**Figure Legends**

|  |  |  |
| --- | --- | --- |
| **Strain** | **Description** | **Source** |
| 101S\* Met15+/- | 101S (Parental Strain ) | Qin et. al 2008 |
| M8\* Met15+/- | M8 (Parental Strain) | Qin et. al 2008 |
| M5\* Met15+/- | M5 (Parental Strain) | Qin et. al 2008 |
| M34\* Met15+/- | M34 (Parental Strain) | Qin et. al 2008 |
| YPS163\* Met15+/- | YPS163 (Parental Strain) | Qin et. al 2008 |
| M2-8\* Met15+/- | M2-8 (Parental Strain) | Qin et. al 2008 |
| YPS128\* Met15+/- | YPS128 (Parental Strain) | Qin et. al 2008 |
| M13\* Met15+/- | M13 (Parental Strain) | Qin et. al 2008 |
| M1-2\* Met15+/- | M1-2 (Parental Strain) | Qin et. al 2008 |
| M32\* Met15+/- | M32 (Parental Strain) | Qin et. al 2008 |

Table 1. Yeast strains in this study were derived from Qin et al. 2008.

|  |  |
| --- | --- |
| **Terms and variables** | **Explanation** |
| CLS | Chronological Lifespan is a measure of lifespan that quantifies the number of times |
| RLS | Replicative Lifespan is a measure of lifespan in budding yeast that quantifies the amount of times required for a mother cell to stop undergoing cell division. |
| MR | Mitotic Recombination refers to the exchange of genetic information between homologous chromosomes in a somatic cell (LaFave et.al 2009). |
| ROS | Reactive Oxygen Species are a group of molecules or atoms that have a free radical. ROS are a natural by-product of metabolic processes, but can be elevated under certain conditions and damage macromolecules. |
| LOH | Loss of heterozygosity in a gene that has one expressed and one unexpressed gene. In subsequent generations, the expressed gene becomes non-functional. MR can cause LOH. |
| MA | Mitotic asymmetry refers to the generation of two dissimilar daughter cells following mitotic division. |
| MET15 locus | Locus at which LOH is detected via the knock-out of one allele using a kanamycin resistance marker. |
| Cb | A variable in the H2O2 dose-response curve that represents the middle concentration black colonies on MLA plates. |
| Cv | A variable in the H2O2 dose-response curve that represents the H2O2 concentration at which cell viability decreases by half. |
| Tg | Based on the biological survival curve, Tg represents the time at which there is a 50% decrease in genomic integrity (Qin 2008). |
| Tc | Based on the biolocial survival curve, Tc represents the the midpoint of CLS (Qin 2008). |
| L0 | The initial time at which mitotic asymmetry first occurs. |

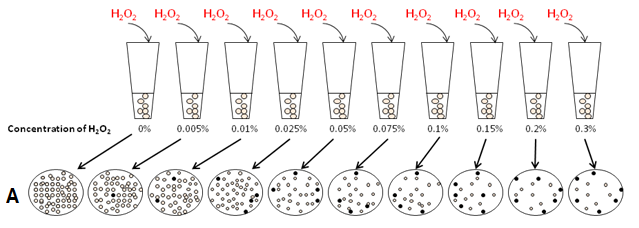
Table 2. Summary of key terms and variables.

Figure 1. H2O2 levels can be modified by a straightforward intervention method to increase ROS. ROS are a natural by-product derived from the breakdown of food. Oxygen from this metabolic process raises ROS levels. This activates SOD, which triggers H2O2 production in low levels. Introducing H2O2 externally activates an opposite pathway. A rise in H2O2 inhibits SOD activity and increases ROS levels. Aging and effects associated with aging is a consequence elevated intracellular ROS levels.



Figure 2. The biological survival curve shows that the midpoint of CLS comes before a decline in genomic integrity.

Figure 3. H2O2 was used to detect LOH. A Kanamycin-resistance marker was used to knock-out one copy of the MET15 gene to yield a heterozygous genotype for that locus. In the mother cell, the chromosome with the dashed segment represents the wild type gene for and the chromosome with the black segment is the knock-out gene. During CLS, a mother cell may produce daughter cells without mutations on the target locus, whereby white colonies form. White-colored colonies may also form if mutations occur and yield daughter cells MET15 +/+ genotype. Only 50% of the mutational events are observed because MET15+/- and MET15+/+ are indistinguishable. Fully black colonies are homozygous recessive at the MET15 locus (MET15-/-) and represent LOH as a result of a mutational event that is most likely linked to mitotic recombination.



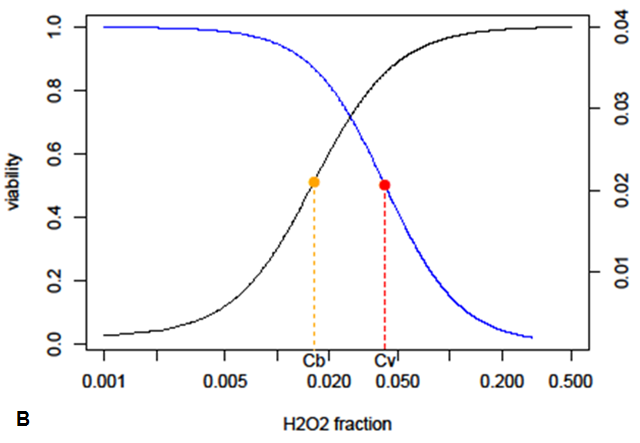


Figure 4. Increasing H2O2 dosage to yeast strains causes a decline in viability (Cv) and an increase in percentage of mutants (Cb). MET15+/- yeast strains were treated with respective concentrations of H2O2. Cells were plated on lead-containing medium. Excess H2S was generated as a result of alteration of MET15 locus in response to induced oxidative stress. The interaction of H2S with Pb2+ on MLA plates formed PbS, which gave colonies their black color. 4A A drop in strain viability corresponds to fewer colonies grown on plates as a result of increase in H2O2 dosage. There is an increase in the percentage of black colonies on each plate, despite a decrease in the total number of colonies. 4B At a Cv of 0.05, the initial concentration of colonies decreased by approximately one-half indicated by the blue curve. At a Cb of 0.02, the percentage of black colonies should have doubled from the initial quantity at a 0% H2O2 dosage. 

Figure 5. MLA plates show black and half-black colonies as a sign of LOH events, an indication of a loss in genomic integrity. Blue arrows point to fully black colonies, which result from LOH in mother cells. Red arrows point to half-black colonies following one or two mitotic events in a mother or daughter cell (Qin et al 2008). 5A shows strain M1-2\* at 0.01% treatment. 5B shows strain M1-2\* at 0.51% treatment.



Figure 6. There was a contrasting switching pattern of pattern of H2O2 and Chronological Aging on LOH. The midpoints of the biological survival curve and the H2O2 dose-response curve were taken to normalize the data. A) The H2O2 dose-response curve suggests that most strains have Cb/Cv ratios that are less than one (represented by the black columns). This implies that Cv generally comes before Cb . Data from the biological survival curve (Qin et. al 2008) suggests that strains have Tg/Tc ratios that are greater than (represented by the gray columns). This implies that Tc comes before Tg.



Figure 7. Genome tolerance (Cb) and viability tolerance (Cv) to H2O2 induction varies by strain backgrounds. Cells with a greater tolerance to H2O2 have a longer CLS. Strain YPS128 has the shortest lifespan and has the least tolerance to H2O2 since Cv comes after Cb.



**Figure 8. There is a significant correlation between CLS and the relative timing of the H2O2 trigger on LOH.** Mitotic asymmetry (L0) describes the production of two different daughter cells following a mitotic division. Cells with a better mitotic asymmetry generally have a longer lifespan and better tolerance to H2O2.