Chronological Life Span of

Saccharomyces cereviseae Folate Biosynthesis Mutants

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Acknowledgments

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Abstract

Folate levels have been shown to influence lifespan in nematodes, which can only utilize exogenous folate. *Saccharomyces cereviseae*, budding yeast, is able to synthesize endogenous folate through a biosynthetic pathway. This study was performed to investigate the effect of folate biosynthesis genes on chronological life span in budding yeast. Based on the prior nematode studies, it was hypothesized that mutations in folate biosynthesis genes would significantly increase the life span of the organism over that of the wild type. However, for the most part, chronological life span of two mutant strains (*abz1* and *abz2*) was different than the wild type strain early in the aging process. Yeast mutant in *ABZ1* were less viable at week 1, but more viable at week 2. Yeast mutant in *ABZ2* were more viable at both weeks 1 and 5. The third mutant, *mis1*, showed no difference from the wild type in chronological life span, at least for the time tested. These studies suggest that two folate biosynthesis genes (*ABZ1* and *ABZ2*) do contribute early, and one (*MIS1*) does not contribute to chronological lifespan.

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List of Abbreviations

CLS - Chronological Life Span

OD_{600} - Optical Density (600nm)

YPD - Yeast Peptone Dextrose growth media

ABZ1, *abz1*, ABZ1p – Wild type gene, mutant gene, and wild type protein for a folate biosynthesis gene in budding yeast

ABZ2, *abz2*, ABZ2p - Wild type gene, mutant gene, and wild type protein for a folate biosynthesis gene in budding yeast

MIS1, *mis1*, MIS1p - Wild type gene, mutant gene, and wild type protein for a folate biosynthesis gene in budding yeast

FOL1 - Wild type gene for a folate biosynthesis gene in budding yeast

FOL2 - Wild type gene for a folate biosynthesis gene in budding yeast

Introduction

Life span and life expectancy studies in model organisms have indicated that both genes and environment play roles in determining an organism's life span (Murphy *et al*. 2003; Funakoshi *et al*. 2011; Kapahi *et al*. 2004; Powers 2006; Virk *et al*. 2012). Genes that regulate life span can be grouped into: 1) those that limit life span when mutated, and 2) those that increase life span when mutated. The first longevity gene, *age-1*, was found in *Caenorhabditis elegans* (Friedman and Johnson 1988). This and other age-related genes, such as *daf-2, clk-1* function in the insulin-like signaling pathway (Christensen 2006), but others like *sod-3* and *sir-2* function in averting cellular damage by reactive oxygen species (Hemkimi & Guarente 2003).

Environmental influences, such as sanitation, climate, nutrition, and many other factors, play a role in determining the life span. One environmental influence on life span extension that has been found in numerous organisms is calorie restriction (Weindruch and Walford 1988). Calorie restriction induces physiological changes that can also be quantified, such as stress resistance. Furthermore, mutant genes that mimic calorie restriction have also been shown to influence lifespan (Powers *et al*. 2006).

One recent study has discovered another aspect that affects lifespan. Virk *et al*. (2012) isolated a long-lived nematode species, *Caenorhabditis elegans*, and eventually discovered that this worm's increased lifespan was due to its diet of a mutant bacterium that produced little to no folate. The bacterium could not produce folate because the *aroD* gene, which controls the production of a key intermediate in the folate pathway was mutated. Some folate was produced due to the intermediate, shikimic acid, already being present in the growth media (Virk *et al*. 2012). In addition, the same effect was observed

when the worms were fed a folate-deficient diet or when folate synthesis was inhibited by a drug in the mixed culture of nematodes and bacteria. The lifespan of the *C. elegans* in this study were increased by 10% up to 50% (Virk *et al*. 2012). This is particularly surprising since several studies have concluded that folate has antioxidant properties, which are associated with reducing mutations and increasing lifespan (Gliszcyńska-Świglo 2007; Xu *et al.* 2010; Ahire *et al.* 2013), and because it is an essential nutrient required for optimal health (Weinstein *et al*. 2003).

Folate is a water-soluble B vitamin that is utilized as a co-factor by numerous enzymes that function in synthesis of DNA nucleotides. Further, deficiencies in folate are known to cause neural tube defects during embryonic development and megaloblastic anemia in human children and adults. It has been further implicated in prevention of colon cancer and adverse effects on men with prostate cancer (NIH 2012).

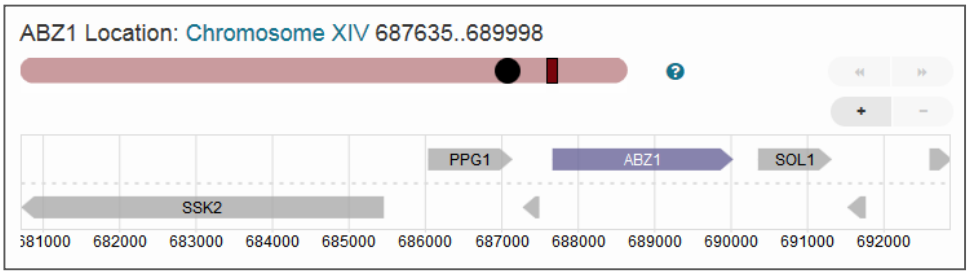
With what seems as conflicting viewpoints indicating that lower folate levels can increase lifespan and folate is an essential nutrient with antioxidant properties, it was of interest to examine whether the life span extension that was observed in the *C. elegans* model due to folate limitation is also true in the other organisms, such as the model organism *Saccharomyces cereviseae*. *S. cereviseae* is an excellent genetic model organism that has many community based genetic and biochemical tools that could enable further insights into these confusing phenomena. In addition, the use of this model organism, which produces high levels of folate (Hjortmo *et al*. 2008), would allow investigation of whether lack of endogenous folate production would affect life span. Furthermore, budding yeast can be used to investigate both replicative lifespan, which is a measure of the number of cellular divisions a single cell can perform, and chronological

lifespan, which is a measure of how long a non-replicating cell can survive (Fabrizio *et al.* 2003).

S. cereviseae has five genes involved in folate biosynthesis (Fig. 1, SGD 2014). Two genes, *FOL1* and *FOL2* are essential for the organism to survive, but three genes, *ABZ1, ABZ2,* and *MIS1,* are non-essential. Since the two genes, *FOL1* and *FOL2,* are essential, the yeast cannot survive with these mutations. The non-essential genes, *ABZ1, ABZ2,* and *MIS1,* if mutated are not lethal to the yeast. ABZ1p functions as a synthase and is required for para-aminobenzoic acid synthesis. ABZ2p acts as a lyase and is necessary for the third step in para-aminobenzoic acid synthesis. Para-aminobenzoic acid is an important intermediate in the process of producing folate. Lastly, MIS1p functions to interconvert tetrahydrofolate between its different oxidation states (SGD 2014).

The purpose of this study was to determine whether genetic deficiencies in folate biosynthesis would affect the chronological life span of *Saccharomyces cereviseae*.

A



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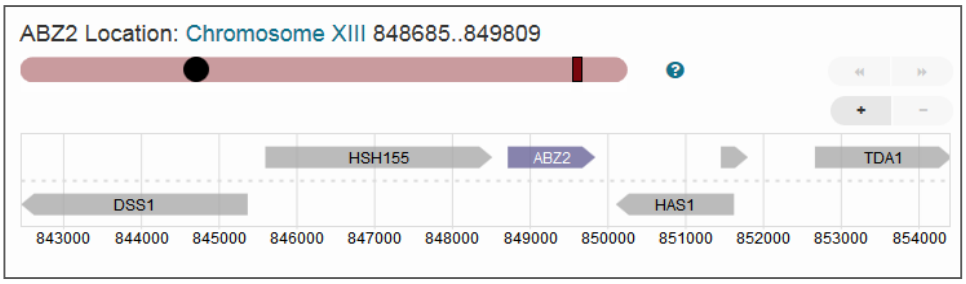

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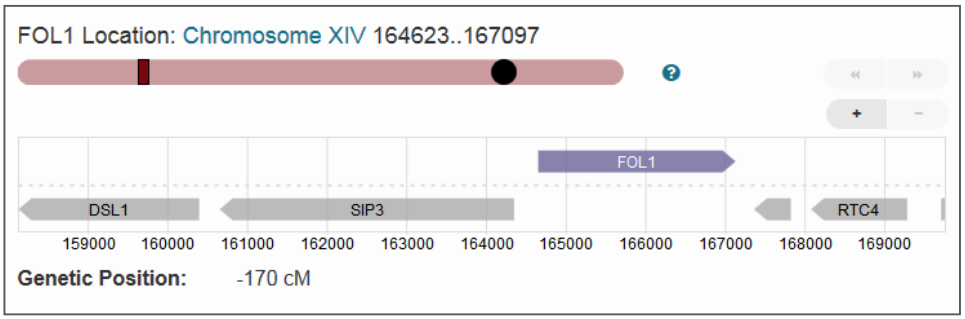

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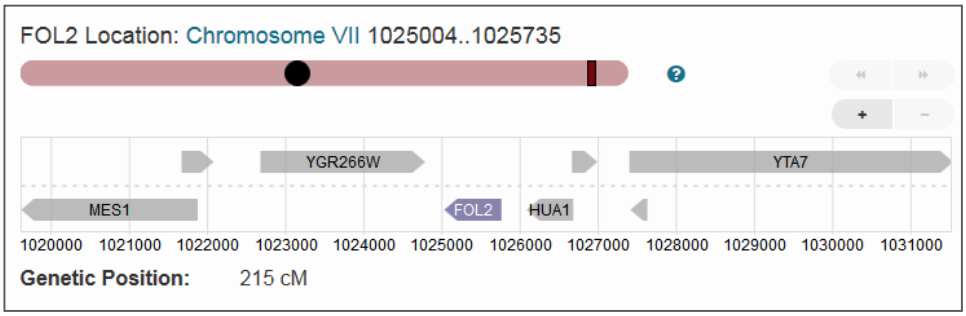

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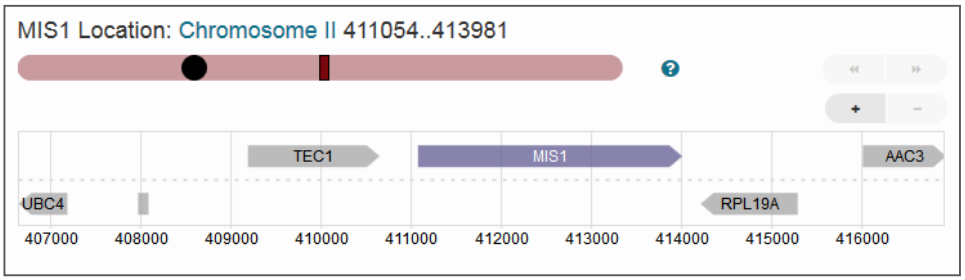

Figure 1. Budding yeast folate biosynthesis genes. Chromosome location, gene size, orientation, and neighboring genes is shown for A. *ABZ1*, B. *ABZ2*, C. *FOL1*, D. *FOL2*, E. *MIS1* (SGD 2014).

Hypotheses

In the chronological life span assay, the null hypothesis is that the mean survivorship of the mutant *S. cereviseae* strains will be equal to the mean survivorship of the wild type *S. cereviseae* at all time points.

$$H_0: \bar{X}_{\text{mutant}} = \bar{X}_{\text{wild type}}$$

The alternative hypothesis is that the mean survivorship of the mutant *S. cereviseae* strains will not be equal to the mean survivorship of the wild type *S. cereviseae* at all time points.

$$H_A: \bar{X}_{\text{mutant}} \neq \bar{X}_{\text{wild type}}$$

In the CLS assay, an additional null hypothesis is that the mean survivorship at a certain time point of each mutant strain of *S. cereviseae* will remain equally viable through out the entire CLS assay.

$$H_0: \bar{X}_{\text{Surviorship at time A}} = \bar{X}_{\text{Survivorship at time B}}$$

The alternative hypothesis is that the mean survivorship of each strain would not remain equally viable through out the entire CLS assay.

$$H_0: \bar{X}_{\text{Surviorship at time A}} \neq \bar{X}_{\text{Survivorship at time B}}$$

Methods

Strain Information and Genotypes

Mutant strains were obtained from the Invitrogen Yeast Gene Deletion Collection (Invitrogen). Frozen stocks were used to streak strains onto fresh YPD plates and grown at room temperature for 3 days. Mutant strains used were mutated for *ABZ1*, *ABZ2*, and

MIS1. These strains were generated from the originating strain BY4742 in the with the genotype: *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*. Wild type yeast, BY4741, genotype: *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, was a kind gift of Dr. Brian Robertson.

Chronological Life Span (CLS) assay

To conduct the CLS assay, strains (wild type and strains mutant for ABZ1, ABZ2, and MIS1) were grown for three days in glass culture tubes in Yeast Peptone Dextrose (YPD) medium at 30ºC, 200 RPM, and then 1µl of inoculum was transferred into wells on a 96 well plate containing 150µl of YPD (Fig. 2). Cultures were maintained without shaking at 30°C temperature in high humidity. This is the "aging plate." Starting on week 3, 40µl of YPD was added to each well every week after inoculum was removed. Six replicates (6 wells) of each mutant strain and the wild type were used in this experiment.

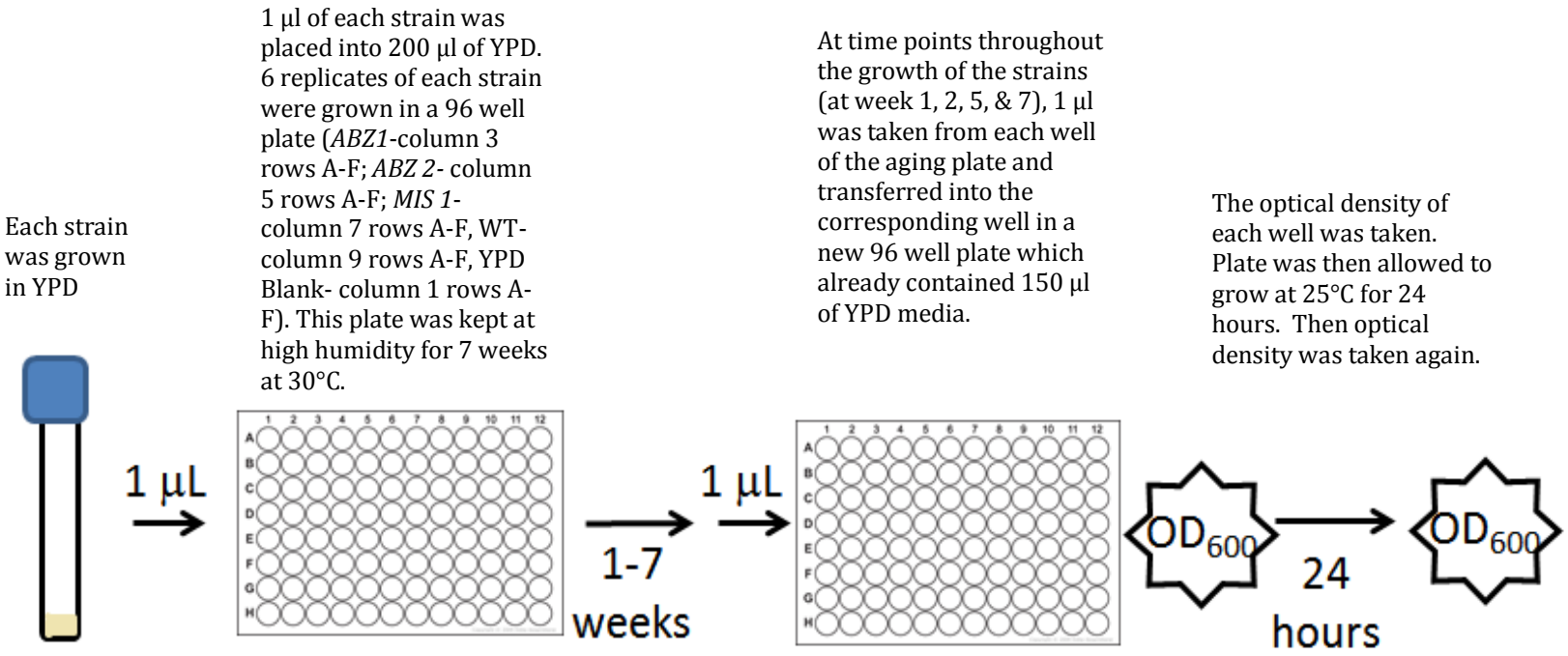

Figure 2. Workflow for Chronological Life Span Assay (CLS Assay).

At time points throughout the "aging" period, 1µl of each strain was taken out of each well and transferred into a new 96 well plate containing 200µl YPD in each well. The optical density (OD_{600}), was measured using a Molecular Devices M2E plate reader with medium only used as a blank. Samples were grown at 25°C for 24 hours, shaken gently, and the OD_{600} measured again using medium only as a blank. Assays and measurements were performed at weeks 1, 2, 5, and 7-week (Powers *et al* 2006). The Student's t-test was then used to compare the growth of each mutant strain to the wild type strain at each type point in order to determine if the differences in growth between the mutants and the wild type were statistically significant. The two-tailed Student's t-test was also used to compare the OD of each time point for each sample in order to determine if viability over the aging period changed for each strain.

Results and Discussion

Folate restriction, induced either by genetic or pharmacological means, has been shown in prior studies to increase lifespan in nematodes (Virk *et al*. 2012). The effect of folate restriction on the chronological lifespan of the model organism budding yeast has not been investigated. Budding yeast have five folate biosynthesis genes: *ABZ1, ABZ2, FOL1, FOL2,* and *MIS1* (Fig. 1), and three result in viable strains when these genes are mutated (*ABZ1, ABZ2, MIS1*). The viable mutant strains were available as part of the Yeast Gene Deletion Collection and were the subject of this study to investigate the effect of folate restriction on chronological lifespan and stress resistance.

Chronological Life Span Assay

For this study, chronological lifespan for viable yeast mutants in folate biosynthetic pathways was assayed using a previously developed method (Powers *et al*. 2006). Each mutant strain was aged and the viability of the aged strains measured and compared to wild type at weeks 1, 2, 3, 5, and 7 (Fig. 2). First, wild type yeast was viable to varying degrees in the CLS assay over the full seven-week assay (Fig. 3-5, Table 1). Viability, as measured by the OD_{600} change in a fresh inoculum following aging, was reduced over time from 0.939 ± 0.041 (mean ± SE) at week 1 to 0.048 (± 0.036) by week 7 (Table 1, Fig. 3-5). Viability changes in wild type yeast were statistically significant except when comparing week 1 to week 2 (Table 2).

strain	Week 1 Viability	Week 7 Viability
Wild type	0.939 ± 0.041	0.048 ± 0.036
abz1	1.026 ± 0.006	0.094 ± 0.022
abz2	1.002 ± 0.012	0.123 ± 0.036
mis1	0.971 ± 0.014	0.046 ± 0.024

Table 1: Viability of each strain at weeks 1 and 7. Mean change in OD_{600} with standard error. n=6. df=5. α=0.05. Critical value, $t_{0.05\,(2),\,5} = 2.571$. The change in viability between weeks 1 and 7 for wild type and all mutant strains is significant with a confidence of >95%.

	Time points	t test	critical value	calc. > crit.			Time points	t value	critical value	calc. > crit.
abz1	1 to 2	6.14392	4.773	Yes		***mis1***	1 to 2	0.21754	4.773	No
	1 to 5	31.8701	4.773	Yes			1 to 5	17.1668	4.773	Yes
	1 to 7	40.6203	4.773	Yes			1 to 7	19.5248	4.773	Yes
	2 to 5	32.3398	4.773	Yes			2 to 5	26.2929	4.773	Yes
	2 to 7	39.6064	4.773	Yes			2 to 7	28.7803	4.773	Yes
	5 to 7	0.70798	4.773	No			5 to 7	1.67848	4.773	No
	Time Points	**t test**	**critical value**	**calc. > crit.**			**Time Points**	**t value**	**critical value**	**calc. > crit.**
abz2	1 to 2	0.09474	4.773	No		**wild type**	1 to 2	0.37828	4.773	No
	1 to 5	20.8255	4.773	Yes			1 to 5	38.8519	4.773	Yes
	1 to 7	23.1356	4.773	Yes			1 to 7	23.8954	4.773	Yes
	2 to 5	606.270	4.773	Yes			2 to 5	50.3293	4.773	Yes
	2 to 7	23.8402	4.773	Yes			2 to 7	703.492	4.773	Yes
	5 to 7	2.29653	4.773	No			5 to 7	51.6040	4.773	Yes

Table 2: Survivorship of each strain with itself at different time points. n=6. df=5. α=0.05 (with Bonferroni Adjustment actual α of each reading is $\alpha/24=0.05/24=0.0021$ (24 is the number of t tests preformed)]. Critical value, $t_{0.0021\ (2),\ 5} = 4.773$. "Yes" in the "calc > crit" column indicates that the null hypothesis is rejected, and the difference in growth from one time point to the next is significant. See appendix for raw data.

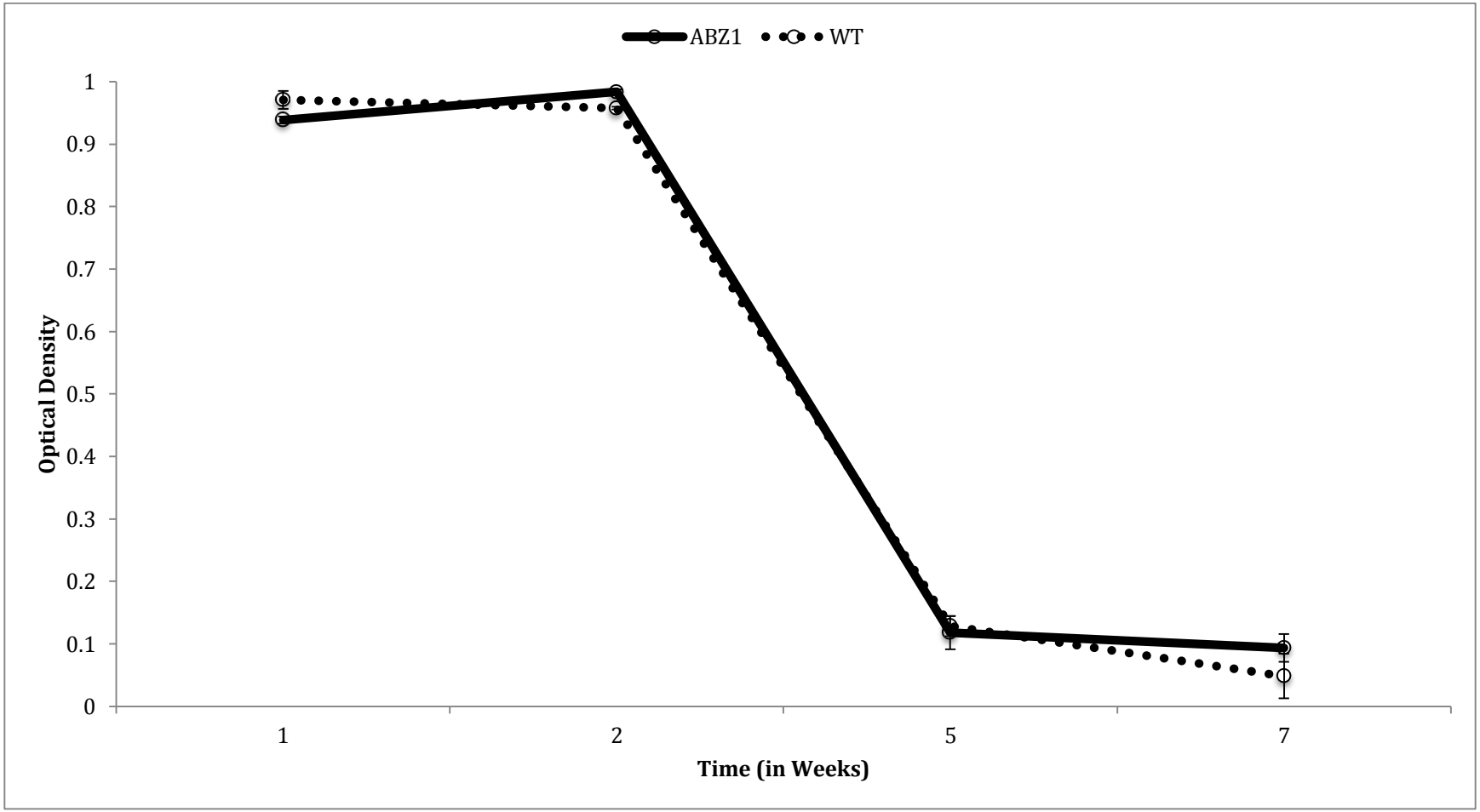

Figure 3. Survivorship of yeast with a deletion in *ABZ1* compared to wild type yeast. Viability is measured by taking the difference in the OD_{600} between post-aging inoculum and after 24 hours of growth. See appendix for raw data.

Next, yeast with a mutation in *ABZ1* were assayed. This yeast was also viable and able to support growth to varying degrees in the CLS over the full assay (Table 1 and Fig. 3). Viability was reduced over time from a mean of 1.026 ± 0.006 (mean ± SE) at week 1 to 0.094 ± 0.022 by week 7 (Table 1). Statistical differences were found when comparing all time points except week 5 to week 7 (Table 2), suggesting the later times (weeks 5 and 7) are significantly different from the earlier aging time points (weeks 1 and 2). When the difference in the wild type lifespan and the mutant life span were compared, mean viability ranged from 1.026 ± 0.006 (mean ± SE) for the mutant and 0.939 ± 0.041 for the wild type for week 1 to 0.094 ± 0.022 for the mutant and 0.048 ± 0.036 for wild type at week 7 (Tables 1 and 3, Fig. 3). Significant differences were observed for the time points at week 1 and week 2 when wild type and the *ABZ1* strain were compared (Table 3). Surprisingly, yeast mutant in *ABZ1* survived less well at week 1, but more at week 2 than wild type yeast (Fig. 3, Table 3).

Week #	Calculated t-value	Critical Value	Calculated > Critical	Null Hypothesis
1	3.541	2.571	Yes	Reject
2	6.271	2.571	Yes	Reject
5	0.337	2.571	No	Do Not Reject
7	1.070	2.571	No	Do Not Reject

Table 3. Student's t-test to compare CLS of yeast mutant in *ABZ1* to wild type yeast. n=6. df=5. α=0.05. Critical value, $t_{0.05\,(2),\,5} = 2.571$. At no time points does mutant growth significantly differ from the wild type. See appendix for raw data.

Chronological life span was next measured for the strain mutant in *ABZ2*. This strain also showed viability and was able to support growth to varying degrees in the CLS over the full assay (Table 1, Fig. 4). Viability of this mutant strain (ABZ2) was reduced over time from 1.002 ± 0.012 (mean ± SE) in week 1 to 0.123 ± 0.036 by week 7 (Table 2), which was statistically significant. The difference in viability was also statistically significant when comparing all time points but week 5 with week 7 (Table 2). When the difference in the wild type lifespan and the mutant life span was compared, mean viability ranged from 1.002 ± 0.012 (mean ± SE) for the mutant and 0.939 ± 0.041 for the wild type for week 1 to 0.123 ± 0.036 for the mutant and 0.048 ± 0.036 for wild type at week 7 (Tables 1 and 5, Fig. 4). When the differences for each week were tested for significance using the t-test, weeks 2 and week 5 were statistically different from the wild type (Table 4). This indicates the strain mutant in *ABZ2* shows an advantage over the wild type yeast in survivorship at these time points.

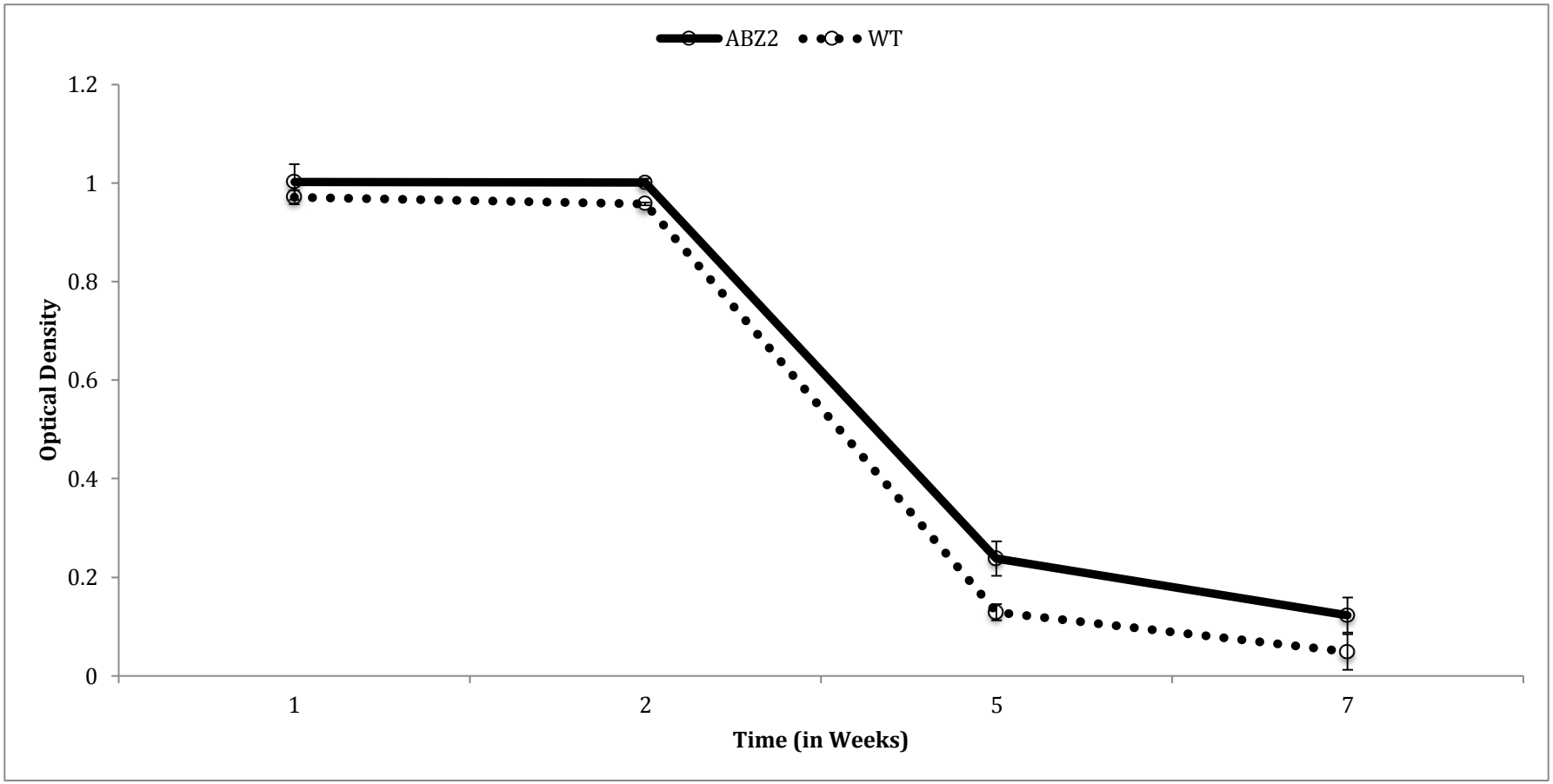

Figure 4. Survivorship of yeast with a deletion in *ABZ2* compared to wild type yeast. Viability is measured by taking the difference in the OD_{600} between post-aging inoculum and after 24 hours of growth. Raw data is available in appendix.

Week #	Calculated t-value	Critical Value	Calculated > Critical	Null Hypothesis
1	1.649	2.571	No	Do Not Reject
2	5.354	2.571	Yes	Reject
5	2.861	2.571	Yes	Reject
7	1.476	2.571	No	Do Not Reject

Table 4. Student's t-test to compare CLS of yeast mutant in *ABZ2* to wild type yeast. n=6. df=5. α=0.05. Critical value, $t_{0.05\,(2),\,5} = 2.571$. The null hypothesis is rejected for the week 1 time point ($0.02 < P < 0.05$). See appendix for raw data.

Lastly, the yeast strain with a mutation in *MIS1* was assayed. Again, the strain showed viability and was able to support growth to varying degrees in the CLS over the entire assay (Table 1, Fig. 6). The viability of *MIS1* mutant yeast decreased over time starting from 0.971 ± 0.014 (mean ± SE) at week 1 and decreased down to 0.046 ± 0.024 by week 7 (Table 2). This change was statistically significant. Both week 1 compared to week 2 and week 5 compared to week 7 show no statistically different survivorship (Table 2). When the difference in the wild type lifespan and the mutant life span was compared, mean viability ranged from 0.971 ± 0.014 (mean ± SE) for the mutant and 0.939 ± 0.041 for the wild type for week 1 to 0.046 ± 0.024 for the mutant and 0.048 ± 0.036 for wild type at week 7 (Tables 1 and 5, Fig. 5). When the difference between the lifespan of the wild type strain and the strain with the *MIS1* mutation was tested using t-test at each week, no significant differences were observed for any time point (Table 2).

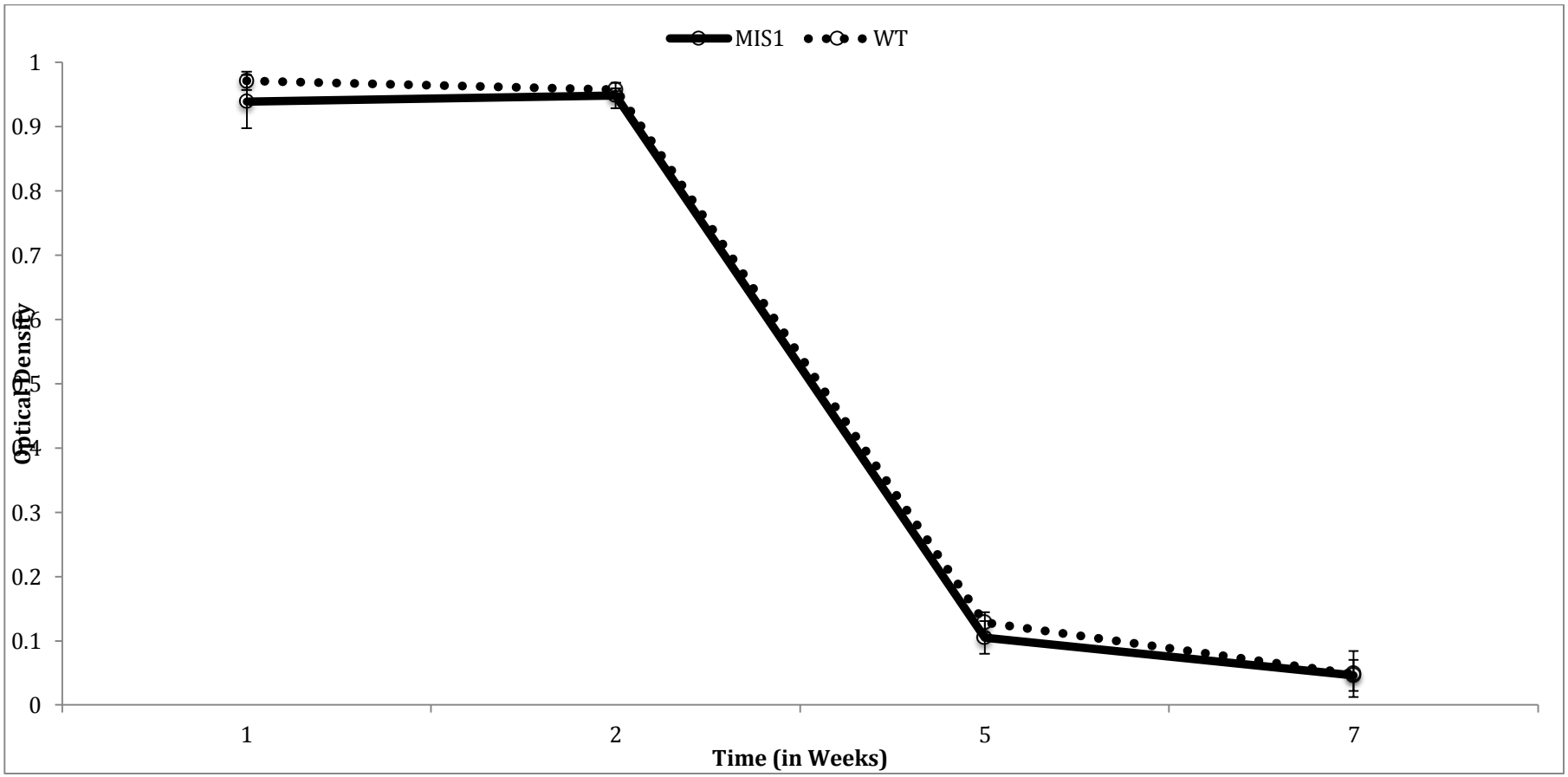

Figure 5. Survivorship of yeast with a deletion in *MIS1* compared to wild type yeast. Viability is measured by taking the difference in the OD_{600} between post-aging inoculum and after 24 hours of growth. Raw data is available in appendix.

Week #	Calculated t-value	Critical Value	Calculated > Critical	Null Hypothesis
1	0.737	2.571	No	Do Not Reject
2	0.439	2.571	No	Do Not Reject
5	0.776	2.571	No	Do Not Reject
7	0.046	2.571	No	Do Not Reject

Table 5. Student's t-test to compare CLS of yeast mutant in *MIS1* to wild type yeast. n=6. df=5. α=0.05. Critical value, $t_{0.05\,(2),\,5} = 2.571$. The null hypothesis is rejected for the week 1 time point ($0.02 < P < 0.05$). See appendix for raw data.

In summary, each strain of budding yeast (wild type and each mutant) do show a significantly different decrease in chronological lifespan when compared to itself over seven weeks (Table 1). Two mutant strains (mutant in *ABZ1* and *ABZ2*) showed a statistically different increase in CLS compared to wild type at week 2 (Tables 1 and 4, Fig. 3-4). One strain also showed the statistically different increase extended through week 5 (*ABZ2*, Fig. 4). One of the three mutant strains (mutant in *MIS1*) exhibited no increased viability at any aging time point. Finally, surprisingly, one strain showed a statistically different decrease in survivorship at week 1 (mutant in *ABZ1*, Fig. 3). Therefore, genetic folate deficiency in the ABZ pathway was advantageous in extending chronological lifespan in early aging and that one mutant further extended CLS, but that effect is limited. These results are surprising and would not have been predicted based on the studies in nematodes. Perhaps, ABZ1p and ABZ2p act in a pathway or form products that normally reduce life span. If true, then reducing their levels would be expected to increase life span. The effect may be early and transient because there may be redundant, less efficient pathways to produce the same products.

Based on previous studies regarding folate effects on longevity (Virk *et al*. 2012), it might be unexpected that chronological life span was not extended for all yeast carrying mutations in folate biosynthesis genes for all time points, particularly since yeast are known to be significant producers of folate (Hjortmo *et al*. 2008). Several explanations that lead to further investigation of this phenomenon in yeast are clear. First, the originating study for this phenomenon used nematodes, which do not produce endogenous folates. The folate levels in the nematode experiments reduced exogenous folate to the nematodes by either feeding wild type nematodes with mutant *E. coli* or by

treating the mixed culture with sulfamethoxazole, which inhibits folate synthesis in the bacteria. The mutant strains of yeast necessarily produce no folate, however, the current study utilized medium that contains low levels of folate by the use of yeast extract-containing medium. Yeast extract, which was used at 10g per liter, is known to contain folate at 20 μg/g (Schertel *et al*. 1965) and therefore necessarily provided minimal levels of exogenous folate to the cultures. Perhaps later lifespan extension, and in all three strains, might be observed if folate-free medium is used.

A technical issue that might have affected the outcome was evaporation. At around week 3 it was clear that there was significant media volume loss in the 96-well plate that contained our *S. cereviseae* strains. The 96-well plate was kept in high humidity environment at all times during the 7-week period, but there was still substantial loss of volume due to evaporation. In order to counteract the volume loss after week 2, more YPD media was added to the wells of each strain weekly in order to keep them from drying out. While this remedied the volume loss problem, it may have affected our results seeing as the yeast were receiving a large influx of nutrients every week which could have allowed many yeast to survive that might have died without the additional nutrients.

On the other hand, these results may indicate that folate levels affect only certain strains and in early aging. Future directions for this project could include performing experiments with additional, intermediate and later time points to determine the full range of effects with and without exogenous folate. Also, repeating the yeast experiments with larger volume cultures in medium that contains varying concentrations of folate to

determine if there is a dose response. It is also of interest to test whether folate levels affect the lifespan of other organisms, particularly human cells, where dietary folates are added in excess to foods such as breads, pastas, and cereals.

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Appendix: Raw Data for CLS assay

A) Week 1	ABZ1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.149	1.173	1.024		0.164	1.156	0.992
		0.149	1.176	1.027		0.153	1.152	0.999
		0.161	1.208	1.047		0.151	1.133	0.982
		0.162	1.18	1.018		0.155	1.058	0.903
		0.173	1.178	1.005		0.167	1.147	0.98
		0.168	1.202	1.034		0.184	1.164	0.98
	ABZ2	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.147	1.136	0.989		0.164	1.156	0.992
		0.165	1.167	1.002		0.153	1.152	0.999
		0.178	1.179	1.001		0.151	1.133	0.982
		0.164	1.211	1.047		0.155	1.058	0.903
		0.186	1.143	0.957		0.167	1.147	0.98
		0.151	1.168	1.017		0.184	1.164	0.98
	MIS1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.136	0.989	0.853		0.164	1.156	0.992
		0.138	1.096	0.958		0.153	1.152	0.999
		0.134	0.912	0.778		0.151	1.133	0.982
		0.141	1.141	1		0.155	1.058	0.903
		0.164	1.171	1.007		0.167	1.147	0.98
		0.161	1.198	1.037		0.184	1.164	0.98

ABZ1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
	0.164	1.137	0.973		0.176	1.131	0.955
	0.177	1.167	0.99		0.174	1.138	0.964
	0.153	1.141	0.988		0.171	1.135	0.964
	0.165	1.16	0.995		0.201	1.151	0.95
	0.169	1.152	0.983		0.199	1.154	0.955
	0.18	1.155	0.975		0.194	1.15	0.956

ABZ2	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
	0.164	1.164	1		0.176	1.131	0.955
	0.17	1.171	1.001		0.174	1.138	0.964
	0.185	1.201	1.016		0.171	1.135	0.964
	0.131	0.13	-0.001		0.201	1.151	0.95
	0.17	1.184	1.014		0.199	1.154	0.955
	0.196	1.169	0.973		0.194	1.15	0.956

MIS1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
	0.167	1.14	0.973		0.176	1.131	0.955
	0.161	1.133	0.972		0.174	1.138	0.964
	0.165	1.067	0.902		0.171	1.135	0.964
	0.159	1.173	1.014		0.201	1.151	0.95
	0.161	1.109	0.948		0.199	1.154	0.955
	0.158	1.042	0.884		0.194	1.15	0.956

c) Week 5	ABZ1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.139	0.159	0.02		0.146	0.261	0.115
		0.146	0.318	0.172		0.142	0.226	0.084
		0.154	0.352	0.198		0.142	0.24	0.098
		0.142	0.26	0.118		0.152	0.29	0.138
		0.143	0.271	0.128		0.151	0.29	0.139
		0.153	0.225	0.072		0.165	0.362	0.197
	ABZ2	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.147	0.402	0.255		0.146	0.261	0.115
		0.145	0.265	0.12		0.142	0.226	0.084
		0.144	0.435	0.291		0.142	0.24	0.098
		0.136	0.337	0.201		0.152	0.29	0.138
		0.15	0.514	0.364		0.151	0.29	0.139
		0.166	0.363	0.197		0.165	0.362	0.197
	MIS1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.161	0.307	0.146		0.146	0.261	0.115
		0.158	0.296	0.138		0.142	0.226	0.084
		0.148	0.207	0.059		0.142	0.24	0.098
		0.138	0.222	0.084		0.152	0.29	0.138
		0.155	0.34	0.185		0.151	0.29	0.139
		0.157	0.176	0.019		0.165	0.362	0.197

D) Week 7	ABZ1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.137	0.203	0.066		0.132	0.137	0.005
		0.135	0.206	0.071		0.132	0.127	-0.005
		0.144	0.211	0.067		0.136	0.136	0
		0.137	0.189	0.052		0.134	0.15	0.016
		0.139	0.246	0.107		0.221	0.444	0.223
		0.155	0.353	0.198		0.175	0.226	0.051
	ABZ2	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.139	0.226	0.087		0.132	0.137	0.005
		0.136	0.426	0.29		0.132	0.127	-0.005
		0.132	0.203	0.071		0.136	0.136	0
		0.134	0.212	0.078		0.134	0.15	0.016
		0.172	0.232	0.06		0.221	0.444	0.223
		0.21	0.364	0.154		0.175	0.226	0.051
	MIS1	PreRead	Post Read	Difference	Wild type	PreRead	Post Read	Difference
		0.155	0.2	0.045		0.132	0.137	0.005
		0.142	0.285	0.143		0.132	0.127	-0.005
		0.161	0.21	0.049		0.136	0.136	0
		0.14	0.18	0.04		0.134	0.15	0.016
		0.139	0.185	0.046		0.221	0.444	0.223
		0.213	0.168	-0.045		0.175	0.226	0.051