2006

ERC Accumulation and Premature Aging: An Investigation of the Deletion of ASH1 in the Budding Yeast Saccharomyces cerevisiae

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ERC Accumulation and Premature Aging: An Investigation of the Deletion of *ASH1* in the Budding Yeast *Saccharomyces cerevisiae*

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Molecular Biology Senior Thesis
Fall 2005-Spring 2006

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ABSTRACT

When the budding yeast *Saccharomyces cerevisiae* divides, it gains a generation while the daughter cell produced by division begins at generation zero. This asymmetric process by which daughters experience a "resetting of the molecular aging clock" is of great interest, especially since cells of many organisms—including human stem cells—reproduce in this manner. One of the theories surround the aging process in yeast is the extrachromosomal rDNA circle (ERC) aging theory. ERCs are generated spontaneously in mother cells as they age; they accumulate exponentially in older cells. Daughter cells from young mothers benefit from asymmetric aging, but as mothers grow older, they start producing daughters that prematurely senesce. Here's where ERCs come in: It is thought that ERCs may be a cytoplasmic senescence factor that is passed from the mother to the daughter as the mother ages, possibly due to the mother's growing inability to maintain the cellular pathways responsible for asymmetric processes as she ages. *ASH1* is a gene that codes for an asymmetrically-distributed protein that halts the expression of *HO* endonuclease—an enzyme critical to mating type switch—in daughter cells. In her senior thesis, Sheila Vasantharam showed that deleting this negative transcription factor led to a significant decrease in lifespan compared to the wild type strain. I am primarily interested in the mechanism behind how this works. I have found that wild type cells and *ash1Δ* mutants start off life with similar lifespans, but that the wild type strains seem to replicate more quickly than their mutant counterparts as time progresses, possibly hinting at a possible connection between *ASH1* and cell cycle regulation. ERCs have been detected in the mutant *ASH1* strain, but not the WT strain, indicating support for ERCs being a senescence factor. Finally, preliminary microarray analysis has hinted that a deletion in *ASH1* causes premature aging in that strain of cell. Several cell cycle-related genes have been found to be downregulated in *ash1Δ* compared to WT. Also of interest is the upregulation of a Ras inhibitor gene, *RPI1.* As of yet, however, the mechanism for how the deletion of *ASH1* causes premature aging and the leading to premature senescence is still unclear.

1 INTRODUCTION

Saccharomyces cerevisiae, the budding yeast, is an excellent model organism for the study of aging. Yeast aging is similar to that of mammalian cells (and by extension, human cells) in that both yeast and mammalian cells are mortal, and as they age, they grow larger, divide more slowly, and show a loss of fertility, also known as replicative senescence (reviewed in Kennedy, Austriaco, and Guarente, 1994). Yeast ages asymmetrically—that is, while the mother cell is said to have gained a generation through mitotic division, the resulting daughter cell begins life at generation zero, contrary to the commonsense notion that a mitotically-produced daughter cell would be the same age as the mother cell that spawned it. This represents a "resetting of the molecular aging clock" in yeast. However, asymmetry in aging is not limited only to yeast; bacterial cells, cells from *C. elegans*, and cells from *Drosophila* age asymmetrically as well (reviewed in Jan and Jan, 1998). Morever, the asymmetric division of cells has also been observed in human stem cells (Faubert, Lessard, and Sauvageau, 2004), fingering the importance of studying mechanisms of asymmetric molecular aging in regards to human health and therapeutic development. However, it has been observed that any asymmetry between mother and daughter breaks down as the mother ages (Kennedy, Austriaco, and Guarente, 1994). Due to this asymmetry in yeast, it is easy to distinguish mother and daughter cells during mitosis due to phenotypic or strictly morphological differences between the two, the most important being size: the buds on the mother cell (the daughters) are smaller than the mother cell itself. However, daughter cells produced towards the end of a mother's life have phenotypes atypical of a cell beginning at generation zero: whereas daughters of young mothers (DYM) tend to separate from their mothers before they

are the same size, daughters of old mothers (DOM) tend to not separate from their mothers *until* they are similar in size (Kennedy, Austriaco, and Guarente, 1995). Aging in yeast is not simply a matter of size or a loss of asymmetric processes, however; other changes exist as well (Table 1, adapted from Jazwinski, 2001).

Table 1: Age-related Changes in Haploid *S. Cerevisiae*

Feature	Direction of Change
Cell shape	Altered
Granular appearance	Develops
Surface wrinkles	Develop
Loss of turgor	Develops
Cell fragility	None
Cell lysis	Occurs
Loss of refractility	Occurs
Cell wall chitin	Increase
Vacuole size	Increase
Cell cycle time	Increase
Response to pheromones	None/decrease
Mating ability	Decrease
Cell cycle arrest at G1/S boundary	Occurs
Senescence factor	Appears
Mutability of mitochondrial DNA	Decrease
UV resistance	Increase, then decrease
Resistance to methylation agents	Decrease
Telomeric length	None
Random budding	Increase
Gene expression	Altered
rRNA levels	Increase
rDNA circles	Increase
Cellular rRNA concentration	Decrease
Protein synthesis	Decrease
Ribosome activity	Decrease
Transcriptional silencing	Decrease
Nucleolar fragmentation	Appears
Migration of silencing complexes to nucleolus	Appears

An assortment of theories attempt to explain the aging process in *S. cerevisiae*, including a link between aging and telomeric length, a number of genetic influences, and the existence stochastic triggers/senescence factors (reviewed in Sinclair, Mills, and Guarente, 1998). Eglimez

and Jazwinski (1989) first proposed the existence of a diffusible "senescence factor" that can be passed from mother to daughter through the physical connection the daughter and mother share until the point of separation; as the mother ages, more and more of this senescence factor—initially segregated from the daughter by asymmetric processes—could potentially leak to the daughter and cause the prematurely-aging phenotypes observed in DOM cells. But what are these senescence factors? Extrachromosomal rDNA circles (ERCs) have been proposed as a possible senescence factor in yeast (Sinclair and Guarente, 1997), but this has not been firmly established. ERCs originate from the rDNA locus on yeast chromosome XII, which comprises 100-200 tandem copies of a 9.1 kb repeat sequence. Upon chromosomal replication in S phase, ERCs are excised from the chromosome. ERCs tend to accumulate exponentially in cells that have completed many replicative cycles due to the amplification of pre-existing ERCs during replication (Figure 1). There is evidence that implies that a reduction in the number of ERCs in a cell prolongs the lifespan of a yeast cell, but plays no part in delaying the onset nor in decreasing the frequency of genomic instability later in life (McMurray and Gottschling, 2003). Further evidence for the senescence-inducing abilities of ERCs comes from Falcón and Aris (2003), who showed that episomal DNA plasmids—namely *ARS*-plasmids, 2-micron (2μ) circles, and ERCs—may impact the lifespan of yeast. Falcón, Rios, and Aris have since published a paper stating that 2μ circles do not accumulate in old cells, nor do they have any effect on the lifespan of yeast (2005), but since *ARS*-plasmids and ERCs *do* accumulate in old cells, they may still play a role in shortening the lifespan of yeast.

In her thesis, Sheila Vasantharam (2003) investigated the possibility of asymmetrically-distributed regulatory proteins or transcription factors as senescence factors resulting in the

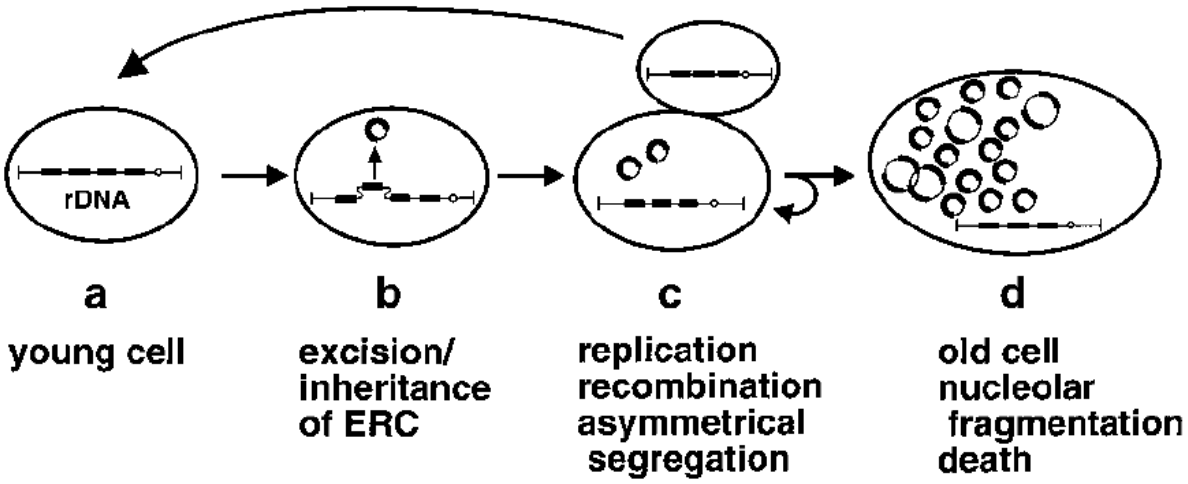


Figure 1: The ERC model of yeast aging (from Sinclair and Guarente, 1997)**.** (A) Every yeast cell contains an array of repeating rDNA sequences on chromosome XII responsible for the generation of ERCs via homologous recombination between the repeated sequences. Young cells (in particular, DYM cells) start life at generation zero, with no ERCs. (B) ERCs can begin to accumulate in a cell either by generation via homologous recombination or through inheritance (once the asymmetric segregation of ERCs breaks down). Excised or inherited ERCs can be amplified very quickly by the cell's replication processes. (C) ERCs can be replicated during S phase, and are normally segregated to mother cells. (D) ERCs accumulate exponentially, resulting in the fragmentation of the nucleolus, the halting of cell division, and replicative senescence. It is hypothesized that senescence occurs due to the extreme strain ERCs place on the cells, so the onset of senescence is dependent on the concentration of ERCs.

resetting of the daughter cell's aging clock as a result of asymmetric division. Among the factors she studied was a gene called *ASH1*, a negative transcription factor that controls the asymmetric synthesis of *HO* endonuclease—an enzyme crucial for mating-type conversion in yeast (Sil and Herskowitz, 1996). She found that the deletion of *ASH1* led to the decrease of the average life span among all cells of that genotype (Figure 2). Whether this was the result of decreased capacity for division (e.g. prematurely reaching the senescent state) or whether this was the result of a reduced rate of division in the *ash1Δ* mutant (i.e. whether the *ash1Δ* mutant divides more slowly, thus increasing the length of the amount of time it takes to complete one cell cycle), it is clear that the deletion of *ASH1* led to a significant decrease in the number of generations a mother cell lives through. Due to the speculation that ERCs may be a possible senescence factor in yeast, and also given the already-established evidence for the significant

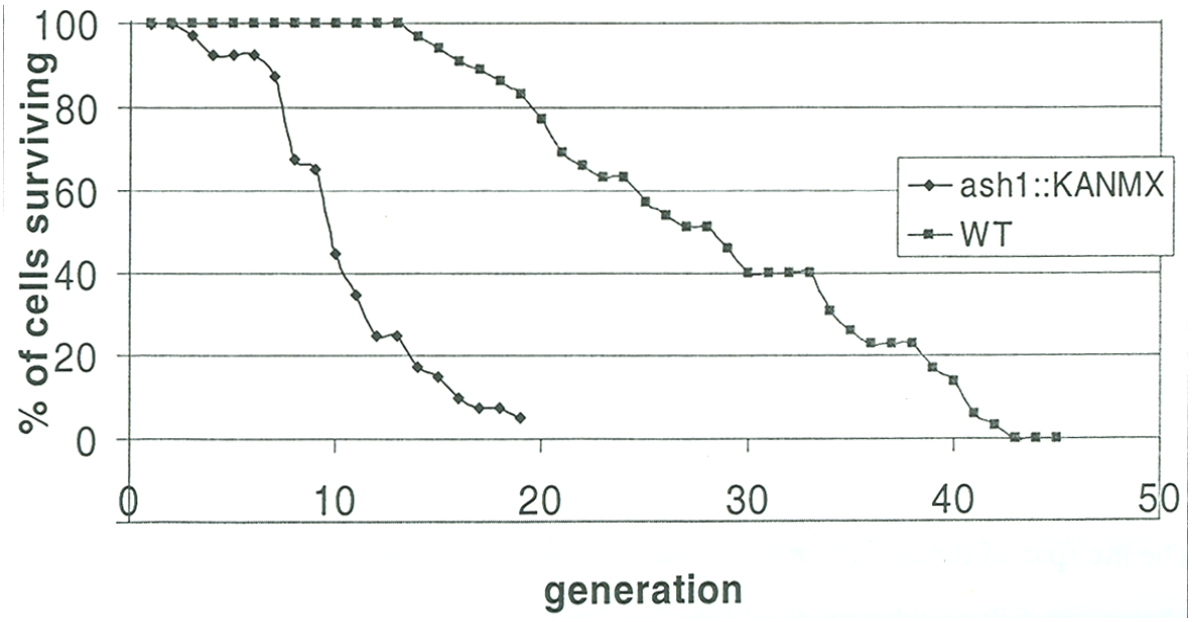


Figure 2: *ash1::kanMX* (*ash1Δ*, or YLH 229) lifespan compared to the lifespan of a *W303* WT strain (YLH 208) (from Vasantharam, 2003). The lifespan of YLH 229 and YLH 208 was determined by isolating 40 cells from a single colony and segregating them on an unused area of the plate they were grown on. Buds were separated from mother cells as soon as they were produced. The number of replication events was counted to give the number of generations of each individual cell. The percentage of cells surviving at every generation represents the sample size of 40 cells.

shortening of yeast lifespan in *ash1Δ* mutants from Vasantharam (2003), I hope to show, first of all, that ERCs accumulate more rapidly in *ash1Δ* mutants than in the wild-type strain, and, secondly, that this causes the shorter lifespan observed in the mutant. This hypothesis would be tested by measuring the amount of ERCs in each strain relative to each other, as well as by comparing ERC levels between both the old and young cells within each strain. The molecular mechanism behind how deleting *ASH1* causes the shortened lifespan as well as the eventual distribution of ERCs to the DOM cells would still remain unclear, but counting bud scars (artifacts of cellular division) could help to elucidate a possible mechanism.

ASH1 mRNA is localized to the daughter cell at the end of anaphase. This localization effectively prevents them from synthesizing *HO* endonuclease (Bobola *et al.*, 1996) and is dependent on five genes—*SHE1* through *SHE5* (Long *et al.*, 1997). *SHE1* encodes Myo4, a type

V myosin motor protein, which colocalizes with *ASH1* mRNA at the tips of each daughter cell (Bertrand *et al.*, 1998; Takizawa and Vale, 2000). Localization is not limited to haploid daughter cells, however. Even in diploid daughter cells, Ash1p (the product of the *ASH1* gene) seems to be localized to the daughter cell nucleus despite the lack of a need to suppress mating-type switching via *HO* endonuclease suppression, which is necessary in haploid cells only (Sil and Herskowitz, 1996). The presence of *ASH1* localization to the diploid daughter cell nucleus suggests that *ASH1* could have functions other than the suppression of *HO*, and could possibly have other targets as well. One of the possible alternate functions of *ASH1* may have to do with pseudohyphal growth in yeast cells (Chandarlapaty and Errede, 1998), but as of yet, no other alternate targets or functions have been identified. Given these observations and postulations, an analysis of the regulatory pathways that *ASH1* is involved in could shed some light on the mechanism by which deleting *ASH1* causes shorter lifespan, regardless of whether or not ERCs are the cellular senescence factor. This will be accomplished using microarrays.

Microarrays have been around for quite some time, but as a result of genome-sequencing endeavors as well as advancements in microarray technology they have become extremely useful as a powerful tool for intra- and inter-genomic analysis (reviewed in Duggan *et al.*, 1999). Microarrays contain microscopic wells containing the cDNA of every gene in a genome. By isolating and amplifying mRNA from two different cell types and conjugating them to fluorescent dyes, researchers are able to generate gene expression profiles that show which genes are up- or downregulated in an experimentally-defined cell type relative to a reference cell type (Figure 3). By comparing the old cell samples to the young cell samples (as the reference), I will be able to see which genes are "turned on" or "turned off" as the cells

from the WT and *ash1Δ* strains age and be able to compare them against each other.

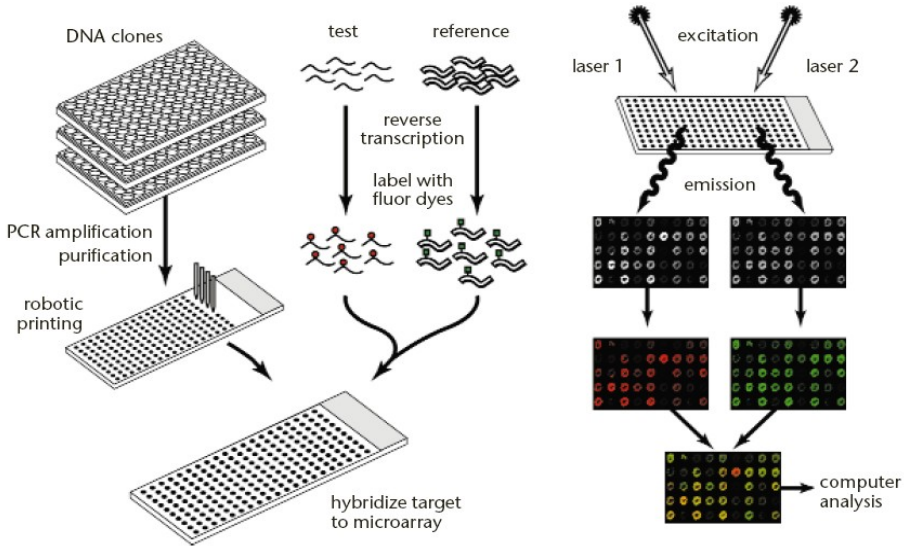


Figure 3: cDNA microarray schema (from Duggan *et al.,* 1999). Cloned and amplified cDNA is affixed to microarray slides. Amplified mRNA from experimental and reference cells is labeled with fluorescent dyes (Cy3 for the experimental set, and Cy5 for the reference set) and hybridized to the wells in the array. The array is scanned with two lasers which excite the dye particles, causing the emission of either red (Cy3) or green (Cy5) light, which is then normalized as a ratio (red:green). A ratio of 1 is indicative of equal hybridization and therefore gene expression, whereas a ratio >1 indicates increased levels of gene expression and a ratio <1 indicates decreased levels of gene expression in the experimental relative to the reference sample. Analysis is carried out using computational methods.

2 MATERIALS AND METHODS

2.1 *Yeast and* E. coli *Strains*

Two yeast strains are being used throughout that course of this project: YLH208 (wild-type) and YLH229 (*ash1Δ*). The wild-type yeast strain YLH208 has a W303R background with the genotype *WT ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*. YLH229, the mutant strain used in this project, has as its parent YLH208, and has the genotype *W303R ash1::kanMX4*. Both strains were taken from freezer stock, streaked on YPD agar plates, and incubated overnight at 30°C to produce single colonies.

Yeast plasmids grown in E. coli are used as a probe for Southern hybridization to detect ERC species. The *E. coli* strain BLH 20 was obtained from Tao Weitao and contains a yeast rDNA plasmid (pYrDNA), which should hybridize with the DNA from the ERCs. BLH 20 was taken from freezer stock, streaked on LB+amp agar plates, and incubated overnight at 37°C to produce single colonies.

2.2 *Probe DNA Mini-Prep (BLH 20)*

From the LB+amp agar plate described above, a single colony is selected and inoculated into ~3 mL liquid LB+amp medium in a test tube. BLH 20 plasmid isolation is done using either the QiaPREP Miniprep kit (Qiagen) or the UltraClean 6 minute Mini Plasmid Prep Kit (Mo Bio Laboratories). To ensure that the purified plasmid is correct, a restriction digest using the EcoRI restriction enzyme using the uncut plasmid as a control is performed.

2.3 *Age Sorting of Yeast Cells*

Age sorting is carried out to separate old and young cell samples of each strain of yeast. A protocol adapted by Laty Cahoon from a protocol employed by Chen *et al.* (2003) is

employed to accomplish this task (Figure 4).

2.3.1 *Biotinylation of Mother Cells*

A single colony from each YLH208 and YLH229 is inoculated into a tube containing liquid YPD medium and grown overnight at 30°C with shaking. The resulting cells are centrifuged, washed, and resuspended in PBS. An aliquot is taken from this resuspension, and a hemacytometer is used to count the number of cells in the aliquot. 1 mg of biotin is then added to the resuspension and incubated for an hour at room temperature using an inverter. After incubation, the biotinylated cells are then washed with PBS and added to a Fernbach flask containing 500 mL of YPD and 500 μL of chloramphenicol in order to grow a population of young cells from these biotinylated mother cells. The flasks are incubated at 30°C overnight with shaking.

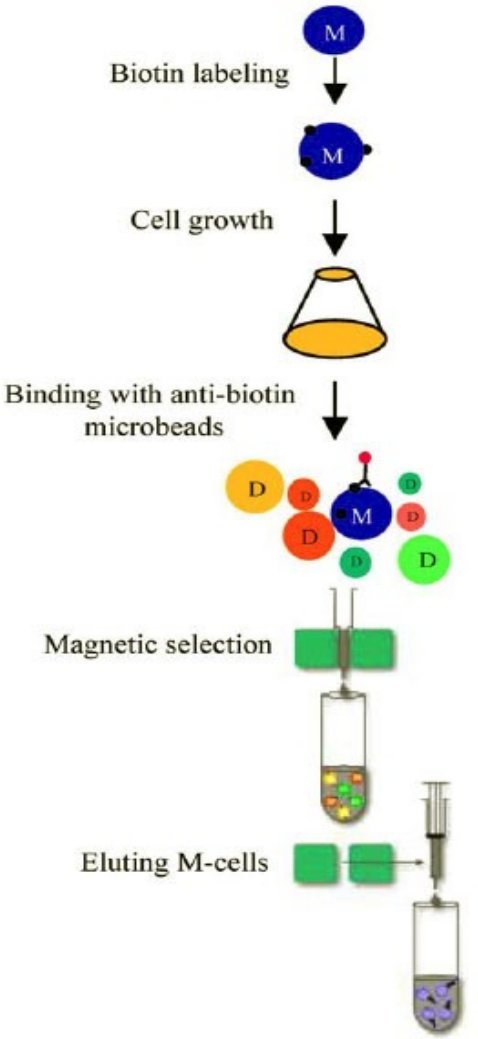


Figure 4: The schematic flowchart for the age-sorting of yeast cells (adapted from Chen *et al.*, 2003)**.** Mother cells are biotin labeled, grown and allowed to reproduce, producing unlabeled daughter cells. Anti-biotin conjugated magnetic beads bind to biotinylated mother cells, and a magnet immobilizes these cells, allowing daughter cells to elute. Finally, the mother cells are collected by forcing them out of the column.

2.3.2 *Attachment of Magnetic Microbeads to Mother Cells*

The contents of each Fernbach flask are then emptied into Sorvall centrifuge bottles and centrifuged. The pellet is resuspended in cold PBS and the resuspension is transferred to 15 mL conical centrifuge tubes and washed. Aliquots are put into Eppendorf tubes, spun down,

and resuspended in PBS, at which point MACS anti-biotin magnetic microbeads (Miltenyi Biotec) are added to each Eppendorf. Incubation is done on ice, and each tube is flicked every 15 min (in the course of 1 hr, or 1.5 hr at the most) to mix the MACS into a cell slurry. After incubation, the cells are washed and resuspended in 10-20 mL of PBS depending on the density of cells in solution.

2.3.3 Magnetically Separating Old and Young Cells

Old (biotinylated) and young (non-biotinylated) cells are separated on LS columns on a MACS separator (Miltenyi Biotec). The magnet in the separator immobilizes the biotinylated cells in the column while allowing the young cells (daughters grown from mother cells in each Fernbach flask) to elute. Each column is first primed with PBS, and then is loaded with the cell resuspension of interest (the maximum column load is 2.5×10^8 cells). Once all of the young cells have eluted, the column is removed from the magnet. The biotinylated cells (still stuck in the column) are forced out by filling the column with PBS and using the plunger to push the cells through.

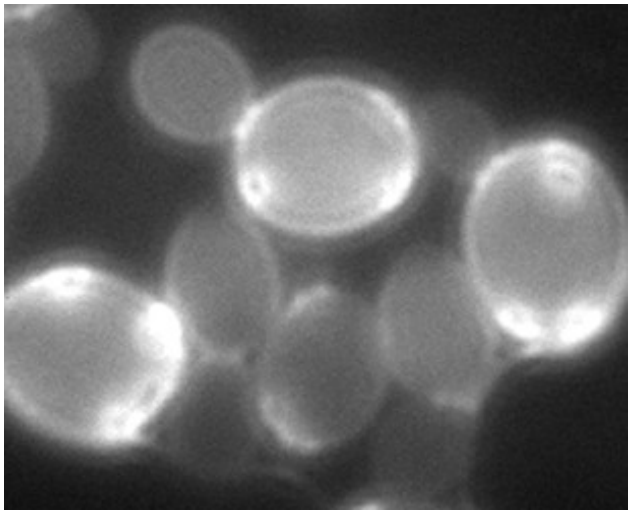


Figure 5: *S. cerevisiae* bud scars (from the Andrews Lab website at University of Toronto)**.** Bud scars are thick accumulations of chitin formed on the outer surface of a yeast cell where a budding daughter was once attached (arrows). Counting bud scars can give us an indication of how many cellular divisions the cell has gone through, and consequently the number of generations the mother cell has lived through.

2.4 Bud Scar Counting

Because chitin bud scars are artifacts of mitotic division in yeast, they can be used to count the number of times a cell has divided, and thus to assess the age of a cell

in generations (Figure 5). To do this, 2-3 μL of each magnetically-separated cell sample is mixed with 5 μL of calcofluor dye supernatant. The volume is then brought to 10 μL total with dH_2O. The mixture is vortexed and allowed to sit for 5 min before spinning down and resuspending the pellet in 10 μL of dH_2O. This resuspension is placed on a slide, focused under bright field conditions, and then examined for bud scars using the DAPI filter and 100X oil objective. 20 cells are chosen at random for each sample and the average number of bud scars is found. This can be performed up to 2 days after magnetic separation if the cells are kept at 4°C.

2.5 Making DNA Plugs from Age Sorted Cell Samples

Agarose DNA plugs are used to prevent the shearing of genomic DNA (Versini *et al.*, 2003). These plugs are made from the age-sorted genomic DNA of YLH208 and YLH229. The cell samples are washed and resuspended in EDTA. The resuspension is then treated with Zymolyase and incubated at 37°C to digest the yeast cell wall and to bring the DNA into solution. While the incubation is taking place, 1% low melting point agarose (in 0.125 M EDTA) is made and cooled to 40-45°C. This agarose is then added to the Zymolyase resuspension and allowed to become firm at 4°C for about 15 min. After the plug has solidified, it is placed into LET buffer (0.5 M EDTA, 7.5% β-mercaptoethanol) and incubated overnight at 37°C with shaking in order to protect against the inactivation of the catalytic site of Zymolyase due to disulfide formation. The next day, the LET buffer is discarded and the plug is rinsed with EDTA. It is then placed in NDS buffer (0.5M EDTA, 1% sarkosyl, proteinase K) and incubated at 50°C with shaking for 24-48 hr. After incubation, the NDS

buffer is discarded and the plugs are once again rinsed in EDTA. An optional overnight washing of the plugs in 0.5 M EDTA can then be performed. Plugs are stored at 4°C in 0.5 M EDTA until they are ready to be used in electrophoresis.

2.6 Electrophoresis of DNA Plugs

A 0.8% agarose gel (in 1X TAE) is used to electrophorese the DNA plugs. Each well in the gel is partially filled with chopped-up plug; the remainder of the well is then filled with 1% low melting point agarose (in 1X TAE). The gel is then run at 1-2 V/cm for 22 hr, and subsequently imaged. Using AlphaImager 3.3d (Alpha Innotech), relative intensities of the bands are found in order to give relative quantities of how much DNA is in each lane, since it is extremely difficult to measure out the same amount of DNA contained in each lane from the plugs.

2.7 Southern Blotting

Southern blotting is used to verify the presence of ERCs after electrophoresis. The probe (BLH 20) is first biotinylated and then hybridized to the blot to enable the detection of ERC species.

2.7.1 Biotinylation of the Probe

Traditional Southern blotting uses a radiolabeled probe which can be potentially dangerous due to radioactivity. By biotinylating the probe and immunoreacting it with an antibody conjugated to horseradish peroxidase (HRP) in lieu of radiolabeling, one can obtain an image of the Southern blot without the need to worry about radiation. Biotinylation of the BLH 20 probe is done using the North2South Biotin Random Prime Kit (Pierce) according to

the instructions included with the kit. Quantification of probe DNA is done using a spectrophotometer to measure the OD_{260} of the sample. Concentration (in ng/μL) is given by the following equation:

$$[probe] = OD_{260} \cdot 37 \cdot \left(\frac{D}{1000} \right)$$

where D is the dilution factor.

2.7.2 Capillary Transfer of DNA onto a Membrane

After electrophoresis is complete, the gel is incubated in an alkaline denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation in order to break the hydrogen bonds holding the two strands of DNA together; DNA must be single-stranded for the probe to hybridize to its complementary sequence. The denaturation reaction is neutralized by rinsing the gel in dH2O and placed in neutralization buffer (1 M TRIS, 1.5 M NaCl) for 45 min with gentle agitation. After neutralization, the gel is moved to a capillary transfer apparatus (Figure 6). The DNA travels upward through the gel and onto a membrane

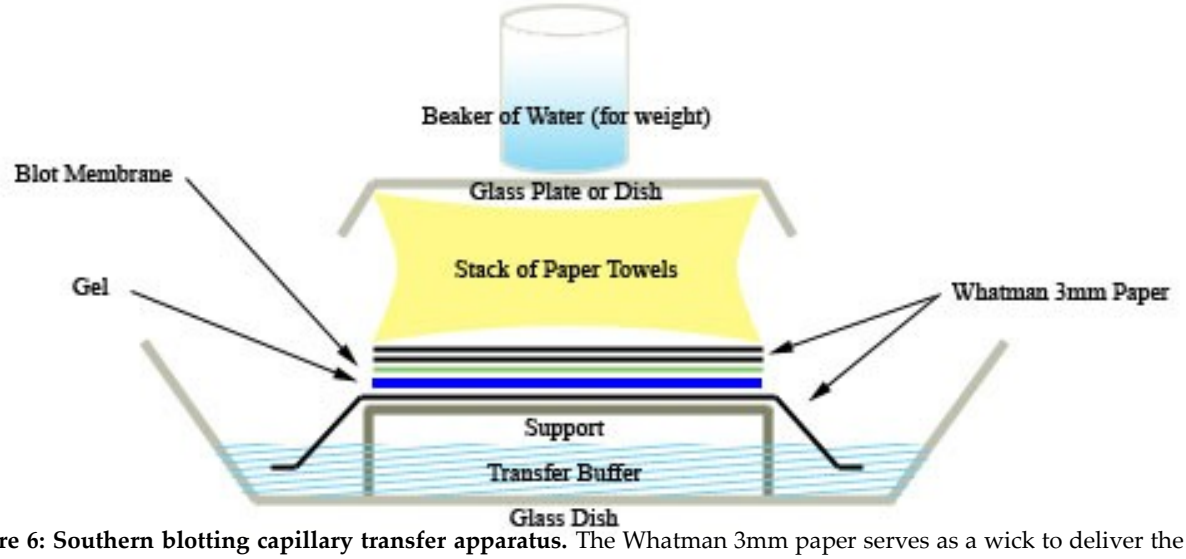


Figure 6: Southern blotting capillary transfer apparatus. The Whatman 3mm paper serves as a wick to deliver the 10X SSC transfer buffer up through the gel. As the SSC is pulled up through the gel, the membrane, and the stack of paper towels, the single-stranded DNA in the gel (generated by treating the gel with alkaline solution) is pulled upwards along with it. It is then deposited on the membrane. The glass plate on top of the paper towels serves as a support for the beaker of water, which compresses the stacked components together to facilitate capillary transfer.

(GeneScreen by NEN LifeSciences) with the aid of 10X SSC transfer buffer via capillary action over the course of 8-24 hr. The DNA is fixed to the membrane by UV crosslinking with the Stratalinker UV oven (Stratagene) with a 120000 μJ setting. The membrane can be dried and stored at room temperature until needed, preferably under vacuum.

2.7.3 Hybridization of Probe to the Blot Membrane

The blot membrane with its fixed DNA is dampened by placing in 6X SSC and then rolled and placed into a hybridization bottle. For every cm^2 of membrane, 0.1 mL of prehybridization solution (6X SSC, 5X Denhardt's reagent or 1X BLOTTO, 0.5% SDS, 1 μg/μL poly(A), 100 μg/mL denatured salmon sperm DNA) is added to the bottle and the bottle is placed in the hybridization oven (VWR) for 1-2 hr at 55°C. Before adding the probe to hybridization solution, it must be denatured by boiling for 5 min. The prehybridization solution is then decanted from the hybridization bottle and replaced with hybridization solution containing probe (0.1 mL/cm^2 membrane). The bottle is then incubated for 2-8 hr at 55°C in the hybridization oven.

2.7.4 Chemiluminescent Detection of Probe

After the probe has been hybridized to the blot membrane, a chemiluminescent HRP-streptavidin conjugate detection system is used to detect the probe on the membrane. HRP catalyzes a reaction between luminol and hydrogen peroxide, whereby the reactive peroxide is turned into water by replacing the nitrogens in luminol with oxygen from the peroxide (Figure 7). This reaction causes the emission of a 428 nm wavelength of light, which is detected by exposing a CCD in a camera (sans lens filter) to the signal. This is all accomplished by using

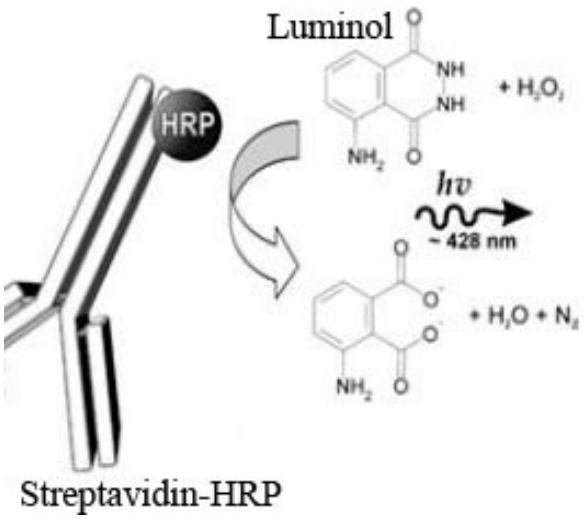


Figure 7: The chemiluminescent reaction on a molecular level. Streptavidin, conjugated to HRP, binds to biotin on the labeled probe. In the presence of lumino and hydrogen peroxide, the above reaction takes place, which ultimately results in the emission of light at around 428 nm.

the Chemiluminescent Detection Module (Pierce), according to the procedure outlined in the kit.

2.8 Microarray Analysis of ASH1

In order to elucidate the difference in gene expression profiles of each type of cell used in this project, RNA must first be isolated from each cell type. After purifying the RNA, it is amplified, coupled to a dye, and subsequently hybridized to a microarray (authored by GCAT) slide, which is scanned and analyzed.

2.8.1 RNA Preparation

RNA is prepped from the age-sorted cell samples from both YLH 208 and YLH 229. First, the cells are washed with PBS and the cell pellet is saved after centrifugation. These cells are then kept on ice until they are ready to be used. 600 μL of acid-washed glass beads (Sigma) are added to 2 mL screw cap tubes, one for each cell type. The pellet (on ice) is resuspended in 600 μL RLT buffer (Qiagen RNeasy Mini kit) and 6 μL β-mercaptoethanol and is then transferred from Eppendorf tubes to the screw cap tubes containing the beads. These tubes are then loaded into the Mini-Bead Beater (Biospec) on setting 3 at 50x100 rpm for four 30 sec bursts (2 min total) in order to grind the cells. At this point, the cells are once again put on ice, and the lysate is transferred to a new 1.5 mL Eppendorf tube. The lysates are then centrifuged

at full speed (>10000 rpm) for 2 min, after which the supernatant is transferred to a new tube. One volume of 70% ethanol is added to the tube and mixed by pipetting gently in order to precipitate the RNA. The sample is then loaded onto an RNeasy column (Qiagen RNeasy Mini kit) and is centrifuged to elute any liquid through the filter which binds the precipitated RNA. The column is then washed with 350 μL of RW1 buffer (Qiagen DNase I kit) and the flowthrough is discarded.

For each column, 140 μL of RDD buffer and 21 μL of DNase I (Qiagen DNase I kit) is mixed by gently pipetting. 80 μL of this solution is applied to the center of the column and incubated for 15 min at room temperature. After this incubation is complete, the rest of the RDD+DNase solution is added to each column and a second round of incubation takes place. Next, 350 μL of RW1 buffer is added to the column. The columns are then centrifuged at 10000 rpm for 15 seconds to elute the liquid buffers. The columns are then washed with 500 μL RPE buffer and transferred to new nuclease-free tubes. 30 μL of RNase-free water is applied to the center of each column and allowed to incubate for 1 minute. The columns are centrifuged at 10000 rpm for 1 minute to elute the water (which now contains the RNA). This step is repeated once more for a total elution volume of ~60 μL.

2.8.2 RNA Amplification and Dye Coupling

Microarrays require a certain amount of input RNA in order to generate a good signal, so the RNA in the previous step must be amplified to reach that level. The miniprepped RNA is amplified and coupled to Cy3 and Cy5 dyes using the procedure and materials contained in the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion).

2.8.3 *Fragmentation of Dye-Coupled RNA*

Prior to hybridization, dye-coupled RNA must be fragmented so that it can hybridize to the mRNA-derived 70-mers contained in each well of the microarray. This will be accomplished using the Ambion Fragmentation Kit. Both dyed RNA samples belonging to each individual microarray experiment are pooled into the same tube and fragmented at the same time. The pooled sample is either vacufuged or has nuclease-free water added to it to bring the volume to 9 μL. 1 μL of Fragmentation Buffer is then added to the pooled sample, and after finger vortexing to mix, is heated at 70°C in a thermal block for 15 min. After heating, 1 μL of Stop Solution is added, mixed again by finger vortexing, and placed on ice under foil to prevent photobleaching of the dyes. This mixture is then vacufuged down to 2-3 μL. It can be saved until it is time to assemble the microarray.

2.8.4 *Hybridization of Dye-Coupled RNA to Microarray Slides*

Each slide used for microarray analysis is first placed into a tube containing 50 mL warm (55°C) microarray prehybridization solution (3X SSC, 0.1% SDS, 0.1 mg/mL denatured salmon sperm DNA). The tube containing the slide is incubated for 30-90 min at room temperature on a rocking platform. After incubation, the slide is dipped briefly in MilliQ water and placed into a dry 50 mL centrifuge tube. The tube is centrifuged at 2 Krpm for 3 min to dry the slide. A clean coverslip for each microarray experiment is needed for assembly (see below).

Once prehybridization is complete, and after the dye-labeled RNA has been fragmented, the assembly of the microarray can take place. 25 μL of hybridization buffer (taken from an aliquot containing 38.2 μL DIG Easy Hyb, 1.8 μL of 10 mg/mL denatured salmon sperm DNA, and 0.14 μL of 1 μg/μL oligo dA) is introduced to the tube containing the

fragmented RNA and mixed by finger vortexing. The tube is then heated for 1 min at 90°C (in the thermal block) and subsequently placed on ice for only 30 sec. The coverslip (obtained above) is placed on the slide and lifted slightly as 25 μL of the labeled RNA is pipetted onto the array grid underneath. When each slide is complete, it is placed into a microarray holder (Corning) with 10 μL water in each well. The holder is wrapped in foil and allowed to incubate at 37°C overnight.

The next day, the slide must be washed. The slide is removed from the holder and placed into a 50 mL centrifuge tube containing 1X SSC/0.1% SDS (preheated to 55°C). The tube is agitated until the coverslips dislodge from the slide. The coverslips are removed from the tube, after which the tube is placed on a rocking platform for 5 min under foil. The 1X SSC/0.1% SDS is decanted and replaced with fresh 1X SSC/0.1% SDS at room temperature, after which the tube is again agitated on a rocking platform for 5 min under foil. The wash is then repeated with 0.5X SSC (preheated to 55°C). The final wash is done with 0.1X SSC at room temperature, agitated as previously described for only 2 min. After washing, the slide is dipped briefly in MilliQ water and transferred to a dry tube to be dried via centrifugation for 5 min.

At this point, the microarray is scanned using the GenePix 4000B microarray scanner with GenePix 5.1 software (Molecular Devices). GenePix 5.1 is also used to find spots on the microarray, take pictures, and gather intensity data for gene expression profile analysis (saved as .gpr files).

2.8.5 Generating Lists of Up- and Downregulated Genes

All analysis of microarray data is done using GeneSpring software (Silicon Genetics).

The .gpr files are imported into GeneSpring and compiled into a single microarray experiment. The program then creates a default interpretation of the data, which can be tweaked by pooling the two *ash1Δ* data sets and comparing them to the WT data. With the gracious help of Dr. Hoopes, I obtained lists of up- and downregulated genes between *ash1Δ* and WT. The first step in generating these lists is to filter the results of the experiment by expression levels to get the complete sets of up- and downregulated genes. This was done using the Filtering tool with the following parameters: for upregulated genes, the minimum and maximum expression levels were 2 and 25.2, respectively; for downregulated genes, these were 0.01 and 0.5. By filtering out any genes that do not meet the criteria, a list can be made. By examining the list and referencing the *Saccharomyces* Genome Database, the functions of the genes in the list can be determined. Here, I only include genes with verified ORFs.

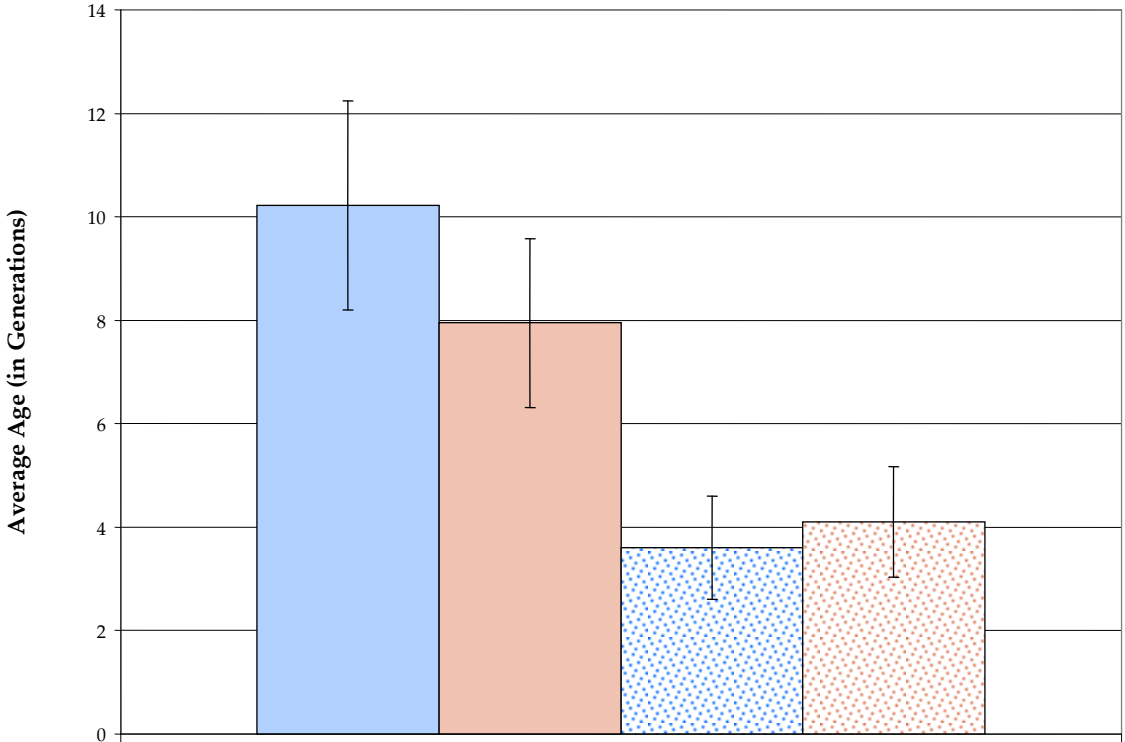
3 RESULTS

3.1 ash1Δ Undergoes Cellular Division Less Quickly Than WT

Vasantharam (2003) found that the *ash1Δ* strain, YLH 229, reached replicative senescence sooner than the WT strain, YLH 208 (Figure 1). In order to gain a more complete picture of the difference in aging between the two strains, I grew colonies of both strains and simultaneously age-sorted them so that the cells in each strain had the same amount of time to grow and divide. I then examined calcofluor-stained samples from each cell type (i.e. old and young cells from both YLH 208 and YLH 229) under a microscope and used a DAPI filter to count the number of bud scars, which fluoresced. Initially, I had only counted the old YLH 208 and YLH 229, but later, I went back and counted fresh samples of both young and old cell types the second time around. Therefore, the total sample size of old YLH 208 and YLH 229 was n = 40, while the total sample size of the young form of each strain was n = 20. Old YLH 208 cells had an average age of 10.2±2.0 generations, while young YLH 208 had an average age of 3.6±1.0 generations. Old YLH 229 cells were 8.0±1.6 generations old on average, while their young counterparts were 4.1±1.1 generations old (Figure 8).

3.2 ERCs Are Detected in Young and Old Cells of ash1Δ*, but Not in WT*

To investigate the possibility that ERCs are the senescence factor first suggested by Egilmez and Jazwinski, I set out to compare ERC levels between old and young cells of each strain (e.g. young YLH 208 vs. old YLH 208 and young YLH 229 vs. old YLH 229), and between old cells of both strains (e.g. young YLH 208 vs. young YLH 229 and old YLH 208 vs. old YLH 229). I hypothesized that if the number of ERCs in the old cell strains is greater than those their young counterparts, and that if the number of ERCs in the old YLH 229 is greater



	Average Age (in Generations)	Sample Size
208 OLD	10.2±2.0	40
229 OLD	8.0±1.6	40
208 YOUNG	3.6±1.0	20
229 YOUNG	4.1±1.1	20

Figure 8: Comparison of the average ages of both old and young YLH 208 and YLH 229. Generational age was determined by staining age-sorted cells with calcofluor and counting bud scars under a microscope for n = 20 cells of each type. Note the growing age gap between young samples compared to old samples.

than those in old YLH 208, then ERCs could be the senescence factor and cause the shortened lifespan of the mutant observed by Vasantharam (2003). To test this hypothesis, I had to detect ERCs via Southern blotting, but before that could even happen, I had to first prepare a probe for detection and run a gel with genomic DNA on it.

I mini-prepped BLH 20 to obtain the plasmid grown in it which contained the repeat sequence array from chromosome XII, from which ERCs are generated. To verify the plasmid was correct, I performed a restriction digest using EcoRI with the uncut plasmid as a control. Each sample was loaded into an ethidium bromide-stained 0.8% agarose gel and run at 80V for

60 min (Figure 9). The estimated size of the BLH 20 plasmid of interest is 12-13 kb—3-4 kb plasmid DNA and 9 kb rDNA (Hoopes, *pers. comm.*). The bands in the digest lane add up to approximately 14.3 kb, making it likely that it was in fact the plasmid I needed. Next, I labeled the probe with biotin and quantified it on the spectrophotometer (78 ng/μL).

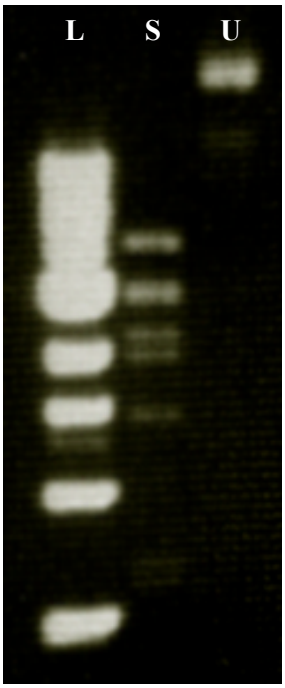


Figure 9: Diagnostic restriction digest of BLH 20 with EcoRI. BLH 20 was mini-prepped using the QiaPrep Mini kit (Qiagen) and then digested using the EcoRI enzyme. The digested sample (**S** for single digest) was then run for 1 hr at 80 V on an ethidium bromide-stained 0.8% agarose (in 0.5X TBE) gel with a 1 kb ladder (**L**) and the uncut plasmid (**U**) as a control. The digested plasmid gives an estimated size of about 14 kb—the actual size is somewhere around 12-13 kb (Hoopes, *pers. comm.*).

In order to electrophorese, DNA plugs for each cell sample were created as previously described. The volume of young cells was larger than the volume of the old cells due to the nature of the sorting protocol, so I made a larger volume of young DNA plugs than old DNA plugs. After the plugs were made and inserted into the gel (1% agarose in 1X TAE), I ran it at what I thought was 1 V/cm for 22 hr. (It turned out that I ran the gel at only 1 V as opposed to 1 V/cm, which for this particular gel would be 15 V.) After imaging this first gel, I realized there had been very little migration (barely noticeable in each lane) due to the very weak electrical voltage I applied to the gel. I soaked the gel overnight in water, then soaked the gel overnight again but with 1X TAE this time around in an effort to get rid of the ethidium bromide introduced to the gel during the staining process. Next, I ran the gel again, this time properly at 1 V/cm, and the following day I imaged it (Figure 10a). The old YLH 208 DNA did not appear to electrophorese, at least at any visually quantifiable level, possibly due to a low volume of DNA in that plug. Also, with the 1% agarose gel, it appeared that some of the RNA (blobs at the bottom of each gel) may have

run out the bottom. Because of this, I amended the protocol to use a 0.8% gel for the next time (to make it more difficult for the DNA and RNA to electrophorese), and I also set out to make larger plugs. I then repeated the experiment with the larger plugs and the 0.8% agarose gel (Figure 10b).

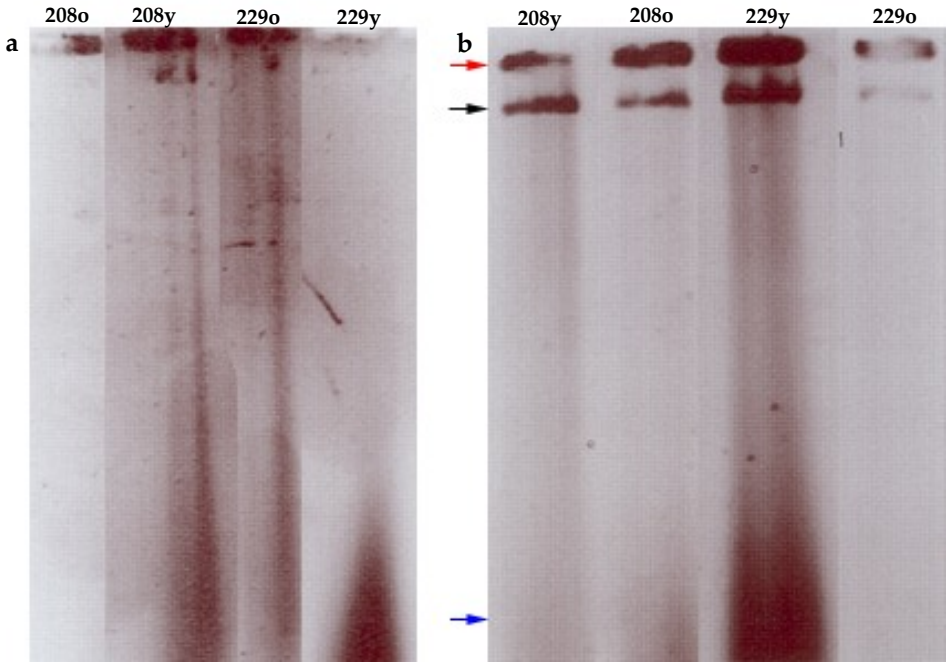


Figure 10. Electrophoresis of DNA plugs. DNA plugs were prepared with age-sorted cells trapped in 1% low melting point agarose, which prevents the DNA from shearing. **(a)** Genomic bands are nearly nonexistent in this gel in every lane, and the old YLH 208 lane did not seem to have any noticeable amounts of DNA in it. Also, RNA might have run out of the bottom of the gel. Gel was 1% agarose in 1X TAE stained in ethidium bromide, and was run for 22 hr at 1 V/cm. Exposure: 15/30 sec. (Discoloration of picture is mainly due to the editing well positions to conserve space.) **(b)** Genomic bands (black arrow) are more pronounced, and there is some migration away from the well (red arrow). Blue arrow indicates RNA. Gel was 0.8% agarose in 1X TAE stained in ethidium bromide, and was run for 22 hr at 1 V/cm. Exposure: 1.5 sec. (Well positions altered.)

Being satisfied with the resulting image of the gel, I transferred the DNA to a blot membrane via capillary transfer. Before I could hybridize the probe to the membrane, however, I had to run a series of dot blots to determine the correct concentration of probe to use. I made 1:10, 1:100, and 1:1000 dilutions of BLH 20, dotted them (5 μL/dot) on a small piece of blot membrane, and then carried out the hybridization step (with 2 μL of probe added to the hybridization solution). I imaged the blot during the chemiluminescent detection step without a lens filter, but with the UV lamp on. This yielded no result. Thinking something may have

been wrong with my probe, I created a control probe (packaged with the Pierce kit) and performed a dot blot under the same conditions as before, which yielded the same results. However, I then realized that the UV light was interfering with the camera's ability to detect the photons emitted from the luminol in the presence of the conjugated HRP. I repeated the control experiment without imaging the dot blot with the UV lamp on and there was a signal. At this point, I did not have any more probe, so I had to make more. (The concentration of the new probe was 10 ng/μL.) I then repeated the BLH 20 dot blot using the same conditions as the second control experiment and received a signal in only the undiluted dot after exposing the blot to the camera for 3 min (Figure 11). I then calculated the probe/target DNA ratio necessary to get a signal of the same intensity as this dot blot. I quantified the BLH 20 and found it had a concentration of 110 ng/μL, which meant that the dot of undiluted BLH 20 contained 550 ng of DNA. The total amount of probe in the hybridization solution was 20 ng, giving a ratio of 1 ng probe/27.5 ng DNA.

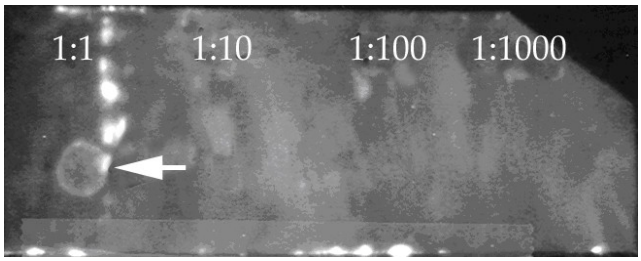


Figure 11: Dot blot of BLH 20, with altered conditions from previous runs. Dot blots are made by first making dilutions of the DNA that you want to blot, then by pipetting them directly onto a blotting membrane. The membrane is then prehybridized and hybridized like normal. Because of their small size, it is not necessary to use a regular hybridization bottle; a 1.5 mL Eppendorf tube may be used instead. The BLH 20 dot blot was imaged *without* UV light (a very important realization on my part). This membrane was hybridized with 2 μL of new probe (10 ng/μL concentration). The undiluted concentration of BLH 20 is 110 ng/μL, which gave an excellent signal. Exposure time: 3 min.

The volumes of the plugs I made gave me the estimated numbers of cells in each plug, and multiplying that number by 1.6×10^{-5} ng DNA/cell, I arrived at an estimated 640 ng of genomic DNA in the gel total. So, to get the desired signal, I had to use 23.3 ng of probe, or 2.3 μL. I liberally added 3 μL of probe to 27.2 mL hybridization solution and hybridized the

Figure 12: Southern blot of the gel in Figure 10b (original well order). Lane for young YLH 208 is to the left of the lane for old YLH 208 (off the picture). Arrows pointing to spots on lane 229o could be indicative of ERCs, but there are also blotches in places where there were blank lanes (see arrow next to lane 208o), so these may be dubious. Signal is coming from wells, but nowhere else, suggesting that ERCs did not migrate from the well. That signal could represent ERCs or the rDNA repeat sequence. Exposure: 3 min.

membrane for 3 hr, reasoning that since this membrane was much larger than the last, I should play it safe and incubate for a longer period of time. The result is shown in Figure 12.

I got a signal from the wells that contained old YLH 208 and old YLH 229, but from nowhere else. I expected to at least see signals in all four wells since the probe would hybridize both the ERCs and the repeat sequence array in the cells themselves. Based on the results of Versini *et al.* (2003), I also expected to see bands of ERCs in the lanes, corresponding to the ERCs being electrophoresed out of the cells, but there was nothing substantial. There are very slight hybridization signals in the old YLH 229 band, but these could be part of the background, as there are many other orphaned bright specks on the image as well.

To try to get a better blot, I made several modifications to the existing protocols for age-sorting and the creation of DNA plugs. First, during the age-sort, I increased the concentration of cells in each plug to bring up the DNA content, hoping that the increased concentration of cells would translate into the increased concentration of ERCs. Second, while making plugs, I increased the concentration of Zymolyase five-fold because it seemed that the cells were not efficiently lysing, which would hamper the electrophoretic migration of ERCs. Lastly, to

investigate the possibility that the ERCs may migrate between the genomic band on the gel and the well, I loaded two complete sets of samples on a gel, ran the gel at 2 V/cm for 22 hr, cut out half of the gel, and let the remainder run at 1 V/cm for 22 more hr (Figure 13).

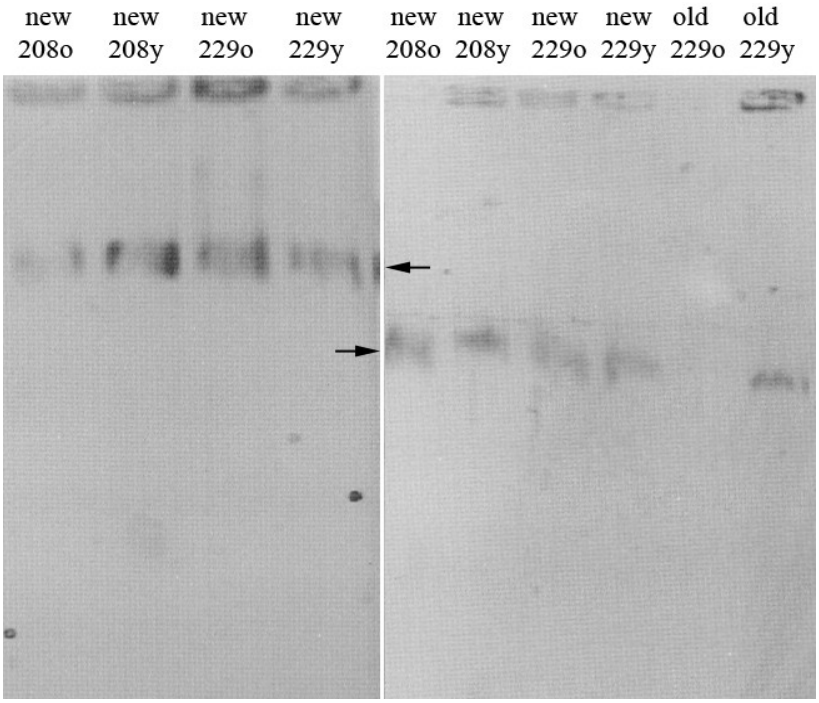


Figure 13: Are there ERCs migrating between the wells and the genomic DNA? The left side of the gel was run for 22 hr at 2 V/cm to get more separation between wells and genomic DNA. The right side of the gel was run for an addition 22 hr at 1 V/cm. "New" or "old" designate the plug that I was using; I had made new plugs to replace the nearly depleted old ones. There was a very small portion of the old 229o plug, but it wasn't enough to generate bands in the lane. Genomic bands are marked with black arrows. The genomic bands from the right side of the gel start to become faint, presumably because the DNA is beginning to separate itself out on the gel. Exposure times: 3 sec (left) and 2 sec (right). ERCs could exist both above and below the genomic DNA band.

The 2 V/cm portion of the gel showed a good separation between the genomic DNA and the wells, while the 1 V/cm portion of the gel showed signs that the genomic DNA was beginning to separate rather than migrate together. I blotted the 2 V/cm portion of the gel (hybridized in 25 mL hybridization solution with 7.5 μL probe) and there was no hybridization signal in either the wells nor in the lanes, even after a 12 min exposure.

Still optimistic that the experimental setup and design was not flawed, and that the snafus I had so far encountered were the result of possibly degraded streptavidin-HRP, I ran yet another gel (with two sets of samples) using identical settings for the 2 V/cm section of the previous gel. To the first half of the gel, I added the streptavidin-HRP from the original Pierce kit we had received in lab nearly a year ago. To the other half, I added the enzyme from the

new Pierce kit we recently ordered. After blotting, my suspicions about the old streptavidin-HRP were confirmed, because the chemiluminescent treatment of the second Southern blot revealed the presence of ERCs (Figure 14). Note that while no ERC bands were found in both age samples of the WT, some repeat sequence array RNA was found in the young YLH 208 lane. We would expect this to be present in the old sample as well, but perhaps the concentration of the DNA in the plugs was too low to detect it. In any case, both *ash1Δ* age samples had bands for ERCs running at the same molecular weight. To quantitatively assess the relative concentration of ERCs in each lane, first I corrected for differences in the amount of DNA loaded into each lane by finding the ratio of genomic band DNA to the DNA in each well, then I made the ratios comparable by normalizing to young YLH 229. Thus, the concentration of ERCs in the old *ash1Δ* strain was 1.1841 times greater than in the young sample of that strain (Figure 15).

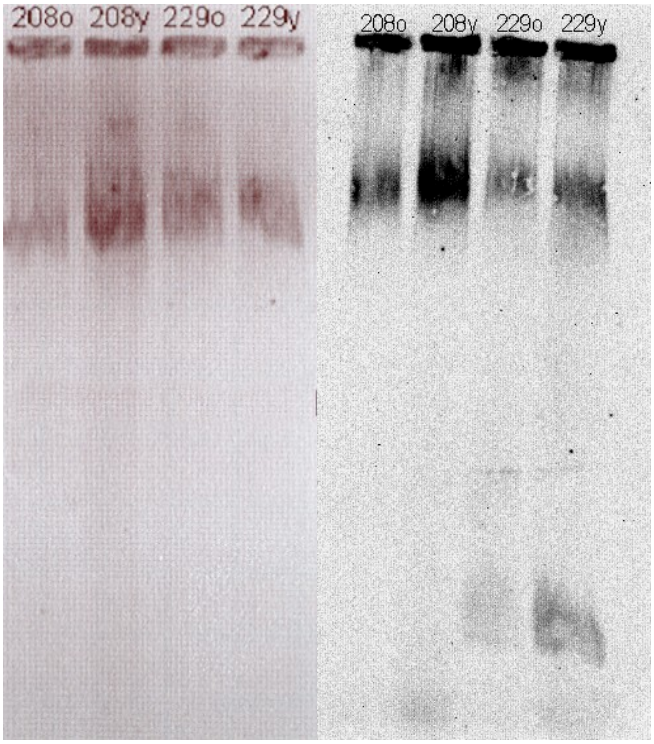


Figure 14. Electrophoresis of newest set of DNA plugs (at left), with respective Southern blot (at right). Detected with a probe volume of 15 μL; exposure time 15 minutes with high degree of contrast and gamma correction. Note the ERC bands denoted by an arrow in the mutant strains; there are no such bands in the WT. Diffuse bands at the bottom may be RNA with repeat sequences being detected by probe.

3.3 Patterns in Preliminary Data Mined from Gene Expression Profiles of ash1Δ and WT cells

To shine some light on the role of deleting *ASH1* in bringing about premature senescence in yeast, I am undertaking a gene expression microarray analysis of how young

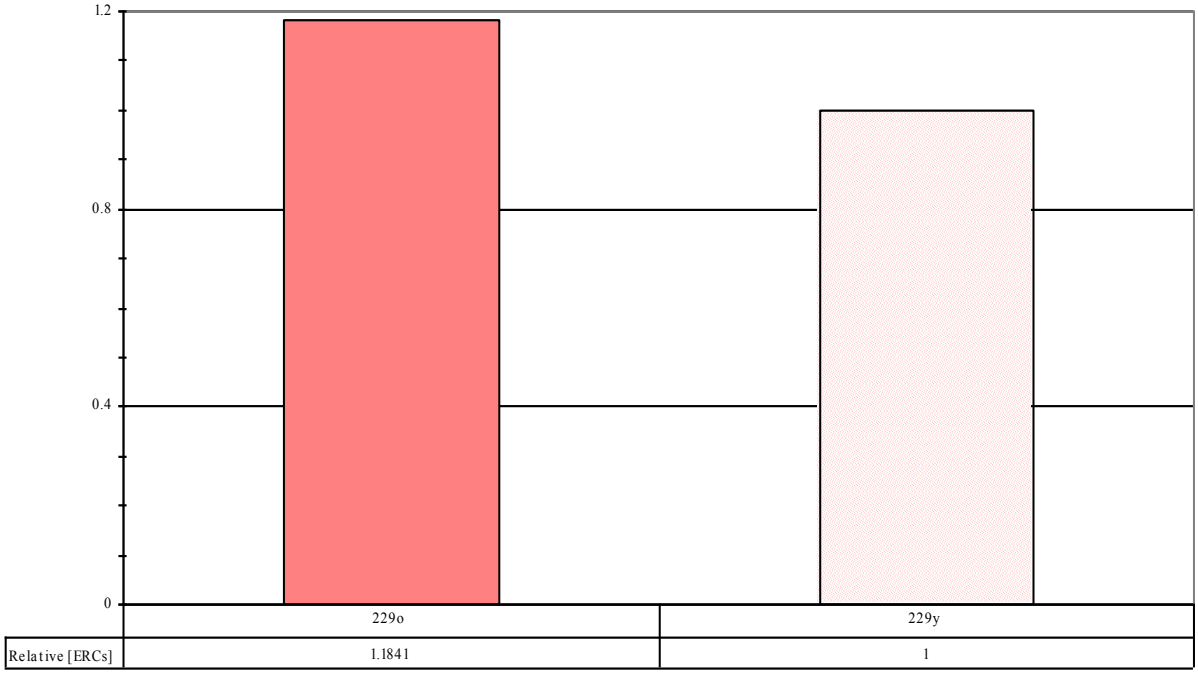


Figure 15. Relative concentrations of ERCs between old and young YLH 229 (*ash1Δ* strain). Old YLH 229 showed a modest increase in ERC levels over the young sample of the same strain. All comparisons made to young YLH 229. Originally, I had planned to compare ERC levels to WT samples, but no ERCs were detected in those lanes.

cells compare to old cells in each cell type. By comparing the gene expression profiles of old and young YLH 229, I hope to find out what genes or types of genes are up- or downregulated in response to premature senescence, or even what may contribute to its onset. These sets of genes could be compared to the results of a parallel investigation I will be carrying out in YLH 208 to see what makes their aging processes so different.

The first step I took with regards to this project was to isolate RNA from each of the four different cell types and amplify them. Before amplification, however, I had to be sure that each of my RNA samples is of high enough quality to get a good hybridization signal when scanning the array. I performed two different quality control experiments to this effect. First, I used a spectrophotometer to not only quantify, but to assess the quality of the RNA. "Good" RNA usually has a A_{260}/A_{280} ratio of 1.8-2.2, but RNA with ratios lower than this range can be

used if the gel electrophoresis reveals high levels of rRNA and lower levels of DNA and tRNA in comparison. This was the other quality control I used (Figure 16). All but one sample (208y) looked good enough to use in a microarray, but that last sample was suspect.

The readings from the spectrophotometer were less encouraging than the results from the gel. All ratios were <1.8-2.2, so I had to consult with Dr. Hoopes to confirm their usefulness. Also, the yields were quite low as well (Table 2).

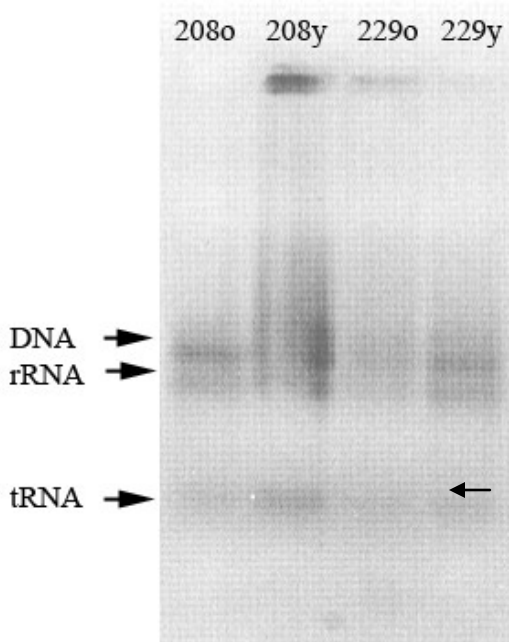


Figure 16: RNA quality control gel. 2 μL of each RNA sample is mixed with nuclease-free water and loading buffer prior to running the gel. Gel is 0.7% agarose in 0.5X TBE. The gel is allowed to run for 1 hr at 75 V. Samples from 208o and 229y are representative of "good" samples—there is very little degraded DNA, a strong rRNA band, and not too much tRNA (which does not give good signals on microarrays). 229o is pretty good as well, except the rRNA band intensity could be higher. 208y is still useable, not is not as good a sample to use as the other three. There is a lot of degraded DNA (at least in comparison to the other three samples) and more tRNA in it as well. Regardless, it still has a band at rRNA, so it can be used with little problem.

The microarrays I used work best with at least 5 μg of RNA, so it was necessary to amplify all RNA samples using the described method. After amplification, Cy3 and Cy5 dyes were used to label each of the four types of cells. I set up the following arrays:

1. Young YLH 229 (Cy3) vs. Old YLH 229 (Cy5)

2. Old YLH 229 (Cy3) vs. Young YLH 229 (Cy5)—a dye-flip control of the previous array

3. Young YLH 208 (Cy3) vs. Old YLH 208 (Cy5)

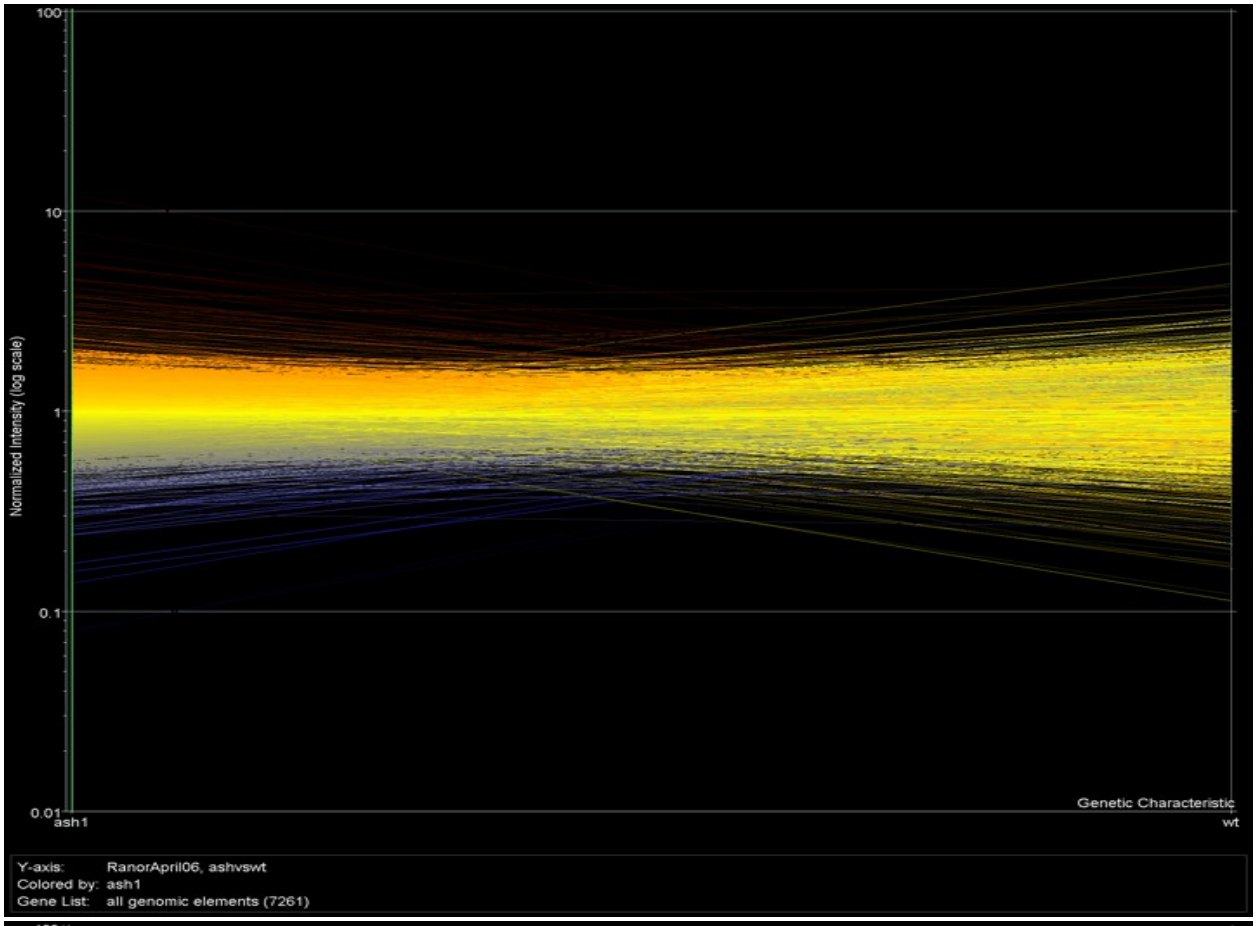
Table 2: RNA yields and $A_{260/A280}$ Ratios

	Yield (μg)	Amplified Yield (μg)	Ratio
208 old	1.6	16.4	1.452
208 young	4	7.11	1.324
229 old	3.6	10.21 (2 samples)	1.104
229 young	3.2	14.00 (2 samples)	1.22

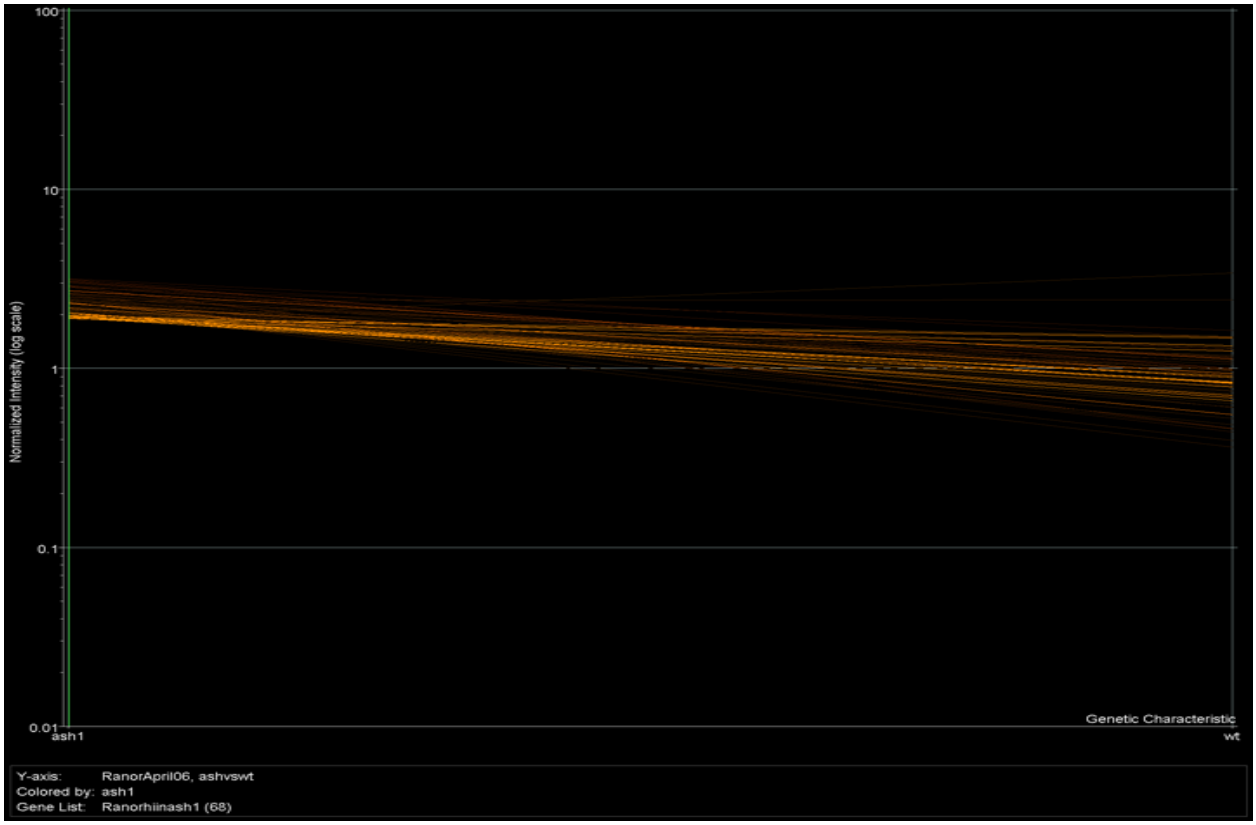
I then hybridized the microarrays as previously described. After hybridization was completed, I scanned them. I had to adjust the PMT values to get a red:green ratio close to 1. The scanned microarrays could not be re-opened for analysis while I was away at Spring Break, so Dr. Hoopes had to rescan them for me.

After the arrays had been properly rescanned, I performed spotfinding and exported the data to .gpr files, which I then imported into GeneSpring. Dr. Hoopes was a great help during this step, as she created the experiments on GeneSpring for me to analyze. Using an *ash1Δ* vs. WT interpretation of the data, I was able to obtain preliminary microarray data on up – and downregulated genes in the mutant strain when compared to the WT strain (Figures 17-19, Tables 3-4 on the following pages).

17


100
10
1
0.1
0.01
Normalized Intensity (log scale)
ash1
wt
Genetic Characteristic
Y-axis: RanorApril06, ashvswt
Colored by: ash1
Gene List: all genomic elements (7261)

18


100
10
1
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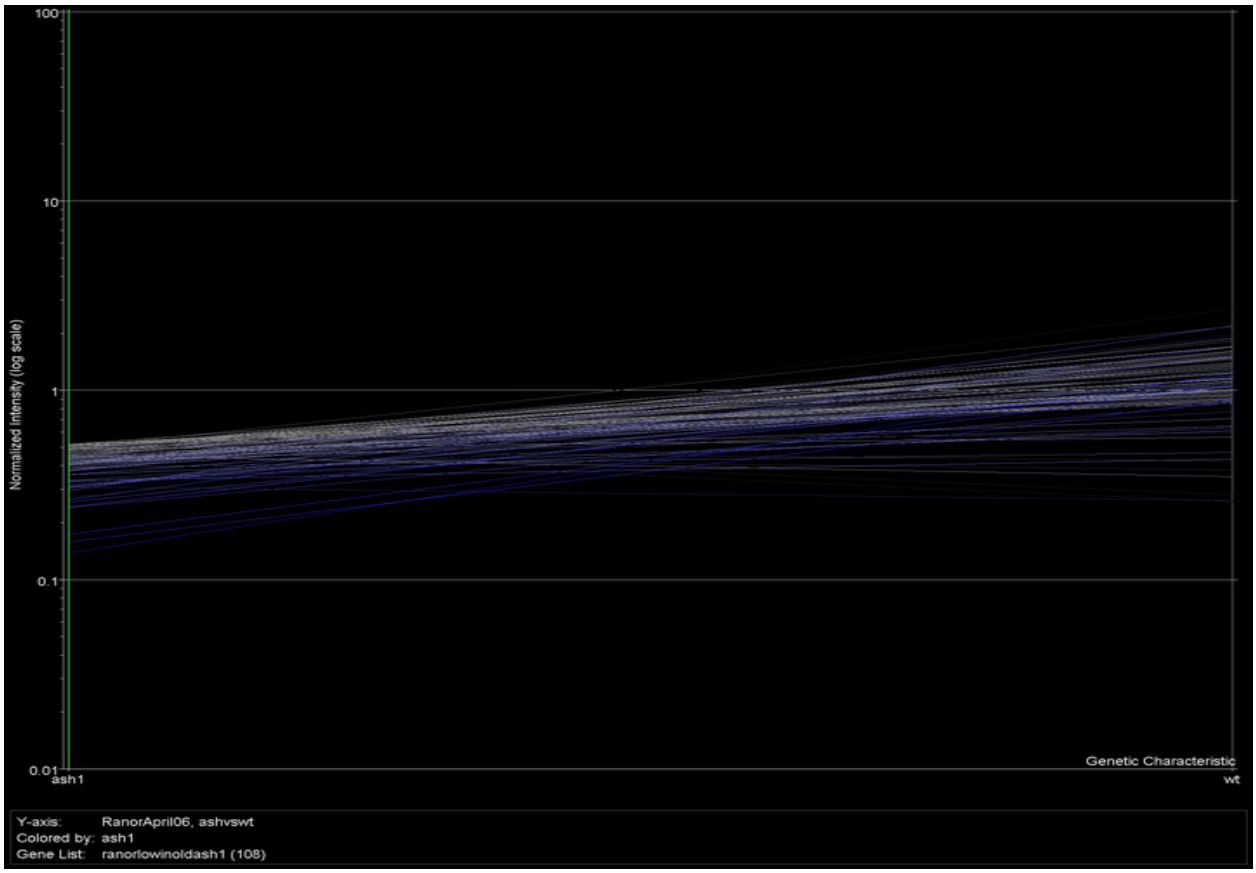
Normalized Intensity (log scale)
ash1
wt
Genetic Characteristic
Y-axis: RanorApril06, ashvswt
Colored by: ash1
Gene List: Ranorhiinash1 (68)

19



Figures 17-19. Gene expression graphs for the *ash1Δ* vs. WT microarray experiments. (17) All expressed genes from both types of arrays. Warmer colors (red-orange-yellow) represent genes upregulated in the mutant strain (**18** shows only upregulated genes), and cooler colors (shades of blue) represent downregulated genes (**19** shows only these genes). Tables 2 and 3 (proceeding pages) show genes up– and downregulated by the deletion of *ASH1,* corresponding to the genes represented by Figures 18 and 19.

Table 3: Upregulated genes in *ash1Δ* compared to WT.

Gene Name	Common Name	Brief Description/Function
YHL009W-B		Ty element transposition
YNL328C	*MDJ2*	protein import into mitochondrial matrix
YFL017W-A	*SMX2*	nuclear mRNA splicing (spliceosome)
YLR315W		kinetochore proteins; unk funct
YDL240W	*LRG1*	cell wall biosynthesis
YKL055C	*OAR1*	aerobic respiration
YHR079C-A	*SAE3*	DSB repair, meiotic recombination
YIL119C	*RPI1*	Ras inhibitor
YKL149C	*DBR1*	RNA metabolism/catabolism; Ty element transposition
YNL245C	*CWC25*	nuclear mRNA splicing (spliceosome)
YER142C	*MAG1*	DNA alkylation
YDL037C		similar to cell surface flocculin
YGL227W	*VID30*	negative regulation of gluconeogenesis
YCR018C	*SRD1*	Transcription regulator
YGL183C	*MND1*	meiotic recombination
YEL004W	*YEA4*	chitin wall biosynthesis
YPL200W	*CSM4*	meiotic chromosome segregation
YKR014C	*YPT52*	targeting to vacuole, endocytosis
YKR004C	*ECM9*	putative: cell wall biogenesis
YDR179C	*CSN9*	protein deneddylation
YLR376C	*PSY3*	error free DNA repair
YLL046C	*RNP1*	ribosome biogenesis
YIL049W	*DFG10*	pseudohyphal growth
YPR193C	*HPA2*	histone acetylation
YLR151C	*PCD1*	peroxisome-related
YPR065W	*ROX1*	negative translational regulator
YMR104C	*YPK2*	protein kinase; pathway: cell wall integrity

Table 4: Downregulated genes in *ash1Δ* compared to WT.

Gene Name	Common Name	Brief Description/Function
YIL171W	*HXT12*	hexose transport
YER044C-A	*MEI4*	meiotic recombination: DSB inducer
YLR144C	*ACF2*	actin cytoskeleton organization and biogenesis
Q0085	*ATP6*	ATP synthesis
YMR251W	*GTO3*	glutathione transferase; unch
YMR274C	*RCE1*	protein processing: protease, acts on Ras and a-factor C-termini
YGR272C		transcriptional processing
YJR112W	*NNF1*	chromosome segregation: necessary for accurate chromosome segregation
YHL032C	*GUT1*	glycerol metabolism
YFL010W-A	*AUA1*	amino acid transport: involved in ammonia regulation of Gap1, a general amino acid permease
YML060W	*OGG1*	DNA repair, BER (AP site formation), telomere maintenance
YLR176C	*RFX1*	DNA damage checkpoint
YJL157C	*FAR1*	cell cycle arrest in response to pheromone
Q0050	*AI1*	mRNA processing, cellular respiration
YGR188C	*BUB1*	Ser/Thr protein kinase; mitotic spindle checkpoint
Q0275	*COX3*	aerobic respiration
YGL083W	*SCY1*	putative kinase; suppressor of GTPase mutant
YMR060C	*TOM37*	mitochondrial import
YPL174C	*NIP100*	dynactin complex; spindle orientation
YLR457C	*NBP1*	bipolar spindle formation
YBR293W	*VBA2*	amino acid transport; amino acid permease
YKL221W	*MCH2*	membrane transport
YOR129C		putative: component of outer plaque in spindle body
YJL115W	*ASF1*	involved in silencing; nucleosome
YLR183C	*TOS4*	G1/S specific transcription in mitotic cell cycle
YGR109W-A		Ty element transposition
YOR016C	*ERP4*	ER to Golgi transport
YNL289W	*PCL1*	G1 cyclin; involved into entry into mitotic cycle
YDR223W	*CRF1*	negative transcription regulator
YPL267W	*ACM1*	uncharacterized; potential Cdc28p substrate
YDR493W	*FMP36*	found in mitochondrial proteome
YIL169C		Ser/Thr rich protein; unch
YOL101C	*IZH4*	zinc/lipid metabolism
YJR025C	*BNA1*	NAD biosynthesis

Continued on the next page...

Table 3 continued...

Gene Name	Common	Brief Description/Function
YOL104C	*NDJ1*	involved in meiotic chromosome segregation
YLR312W-A	*MRPL15*	mitochondrial ribosomal protein; protein biosynthesis
YKL078W		RNA helicase; ribosome biogenesis
YOL007C		Appears to be a structural component of the chitin synthase 3 complex
YBR070C	*ALG14*	dolichol-linked oligosaccharide biosynthesis
YBR189W	*RPS9B*	ribosomal protein: protein biosynthesis, regulation of transcriptional fidelity
YGR084C	*MRP13*	mitochondrial ribosomal protein: protein biosynthesis
YMR290C	*HAS1*	RNA helicase; rRNA processing
YER146W	*LSM5*	mRNA catabolism, mRNA splicing via spliceosome
YER070W	*RNR1*	ribonucleotide reductase; regulated by DNA replication and DNA damage checkpoints
YGR082W	*TOM20*	protein import into mitochondrial outer membrane
YEL020W-A	*TIM9*	protein import into mitochondrial inner membrane
YDL222C	*FMP45*	cell wall organization and biosynthesis
YDR204W	*COQ4*	CoQ biosynthesis
YAL043C	*PTA1*	tRNA processing; transcriptional termination
YGR174C	*CBP4*	protein complex assembly
YBL038W	*MRPL16*	mitochondrial ribosomal protein: protein biosynthesis
YDR146C	*SWI5*	transcriptional activator of G1 genes, regulated by Cdc28p
YOR247W	*SRL1*	required for cell wall stability; nucleic acid metabolism
YPL187W	*MFα1*	mating factor alpha
YDL192W	*ARF1*	ER to Golgi/intra-Golgi transport
YKL195W	*TIM40*	mitochondrial intermembrane space import
YHR152W	*SPO12*	positive regulator of exit from mitosis
YIL140W	*AXL2*	bud site selection
YML009C	*MRPL39*	mitochondrial ribosomal protein: protein biosynthesis
YOL155C		cell wall organization and biosynthesis
YMR305C	*SCW10*	cell wall protein; may play a role in conjugation during mating
YBR191W	*RPL21A*	ribosomal protein: filamentous growth, protein biosynthesis
YBL016W	*FUS3*	mitogen activated kinase in mating pheromone response
YPL163C	*SVS1*	cell wall protein; resistance to vanadate
YJL034W	*KAR2*	protein import into ER
YKL103C	*LAP4*	vacuolar protein catabolism
YML129C	*COX14*	aerobic respiration; negative regulator of transcription
YKL217W	*JEN1*	lactate transport

Continued on the next page...

Table 3 continued...

Gene Name	Common Name	Brief Description/Function
YMR011W	*HXT2*	hexose transport
YCR010C	*ADY2*	acetate transport required for normal sporulation
YGL055W	*OLE1*	fatty acid synthesis
YNL030W	*HHF2*	required for chromatin assembly; contributes to telomeric silencing
YJL158C	*CIS3*	cell wall organization and biosynthesis
YDR258C	*HSP78*	protein folding; stress response
YLR110C	*CCW12*	cell wall organization and biosynthesis

4 DISCUSSION

Vasantharam (2003) found that deleting *ASH1* led to a significant decrease in lifespan compared to the wild type strain. This shows that *ash1Δ* mutants reach replicative senescence sooner than their wild type counterparts. However, the mechanism behind this was unclear. By counting bud scars of the old and young YLH 208 and YLH 229 strains, I found that the *ash1Δ* mutant possibly undergoes a slower cell cycle than does the wild type due to their similar ages in their youth and their increasingly disparate ages as time wears on (Figure 8). This suggests that *ASH1* may be somehow influence genes that regulate the cell cycle. Given the same amount of time to grow and divide, the *ash1Δ* mutant undergoes less cellular division, which suggests that, somehow, the deletion of *ASH1* causes a lengthening of cell cycle turnover. This is underscored by the fact that there is a greater difference in cellular age between the old cells of each strain than between the young cells of each strain. Therefore, upon biotinylation, it is assumed that the old cells (which are, at this time, still young) are all roughly the same age. When the age-sort is completed and the cells are examined under the microscope for bud scars, the difference in average age widens due to the aforementioned decrease in the rate of cellular division. There are, of course, caveats to consider when viewing these results in this context: 1) while the gap in age between the two strains widens as time progresses, the difference in their respective ages is probably not significant; and 2) these are average ages for each respective cell type, but it would be more convincing if I got these same results starting off with two sets (one for each strain) of synchronous, virgin cells rather than a collection of cells that are around the same age, but are not necessarily the same age. Perhaps it would be interesting to repeat the experiment by biotinylating only virgin cells of each strain.

However, I have not come across any evidence in the literature that ties *ASH1* to the mitotic cell cycle, and it would be edifying and possibly important to see if there is a link between the two.

The preliminary microarray results suggest that the deletion of *ASH1* leads to gene expression changes consistent with prematurely-aged yeast (as described on Table 1). If we cross-reference the types of genes up– and downregulated to the list on Table 1, there is a good deal of overlap in terms of the "direction of change" in which certain genes are regulated (Tables 2 and 3), which implies that deleting *ASH1* leads to premature aging; this suggests that the cause of the shorter lifespan found by Vasantharam (2003) is due to reaching a senescent state after a shorter number of generations than it takes for WT to reach the same state. The assertion that *ash1Δ* cells are prematurely-aged is further supported by the bud scar counting data (Figure 8), which show a lengthening of cell cycle division time. There are, however, genes that are up– and downregulated that seem contrary to the expected "direction of change" as mentioned in Table 1. Could these genes be activated or deactivated as part of redundant or compensatory pathways? Only more careful analysis of microarrays (which is regrettably not an option due to time constraints at this point) will tell. A major regret I have about this project is regarding the mechanism by which deleting *ASH1* actually makes it such that the mutant strains die more quickly. Again, further analysis of these microarrays may reveal this.

A gene of particular interest that was seen to be upregulated in *ash1Δ* cells is *RPI1*, a Ras inhibitor. Ras is a protein with homology to a proto-oncogene in humans (reviewed in Bos 1989). When human Ras is activated, it promotes cell division and proliferation. An inhibitor to

Ras would therefore suppress activation and not lead to out-of-control division. In the context of this experiment, *RPI1* is upregulated in *ash1Δ* cells, which would lead to the suppression of Ras, and consequently to an increase in cell cycle completion time, which is seen in *ash1Δ* cells since they divide more slowly than WT as they become older (Figure 8).

According to Jazwinski (2001), an indicator of aging in yeast is the appearance of a senescence factor. Sinclair and Guarente (1997) postulated that this senescence factor may be ERCs. When measuring the ERC content of both young and old ERCs, I found that ERCs accumulated in old *ash1Δ* yeast cells, and were present in young *ash1Δ* cells as well (Figures 14 and 15). No ERCs were detected in WT cells. Due to the methodology of the age-sorting protocol (Figure 4), young cells are the daughters of old cells, and thus, it can be assumed that the old *ash1Δ* cells gave rise to daughters with ERCs in them due to a breakdown in aging asymmetry. Old WT cells may not have been old enough to generate appreciable levels of ERCs in the first place, which would explain the lack of ERCs in the Southern blot. However, since RNA accumulates at the bottom of each lane after electrophoresis (Figure 14), we should still see these bands in WT cells because they are genomic RNA containing the repeat sequence. The absence of this band in the old WT may indicate that there was not enough DNA in the plug in the first place, which may also account for the perceived lack of ERCs in the Southern blot. However, Dr. Hoopes (*pers. comm.*) informed me that ERCs do not really appear in appreciable amounts in old WT until about 15 generations, so my first interpretation of the data may be correct. In any case, the inheritance of ERCs by young *ash1Δ* cells from old *ash1Δ* cells still lends credence to ERCs being a senescence factor in yeast.

ACKNOWLEDGMENTS

Now that the thesis is complete, I find I have nothing to say. It may have something to do with how tired I am, or how much complaining I did to fellow labbies and students alike during the course of the actual thesis, but I'd like to remain optimistic and think that I have nothing to say because the relief I am feeling is ineffable and has rendered speech an inadequate medium for expression. Yeah. That's it.

But before I decide to remain dumbstruck for the rest of my life, there are a few people I would like to thank, without whom this altogether-too-long-and-life-draining work would be impossible:

First of all, I would like to thank Dr. Laura Hoopes for guiding me with the research and helping out with the microarray stuff when time and computer issues arose. A million thanks for everything—the help, the words of encouragement, the opportunity for working in your lab in the first place—and a million apologies for not being able to perform a more comprehensive and in-depth microarray analysis due to a lack of time. If I had a month more, maybe I could do it. Maybe.

A huge "thank you" to Dr. Tina "Cystina" Negritto for being my second reader and for generally being one of the most amusing professors I have had. I don't know how mo-bio lab last year would have gone if the atmosphere weren't so relaxed (for lack of a better word).

I'd also like to thank Kristina Bajema and Alyssa Cope for being so fun to, um, *complain* (that's a nicer word) to about anything thesis-related. In that same vein, thanks to all my friends—especially my sponsor group from Freshman year—for putting up with my general moodiness at times and for keeping my memory alive when I disappeared into the infinite abyss known to the initiated as "lab."

Even though I have never actually met her, I would like to thank Sheila Vasantharam for constructing the *ash1Δ* strain I used in this project, and for doing her thesis on something mildly interesting to springboard off of when deciding what to do this year.

I would like to also throw out that I love Starbucks, the Motley at Scripps, and the Coca Cola company because without their caffeinated drinks, this thesis would not be done and I would be lying comatose somewhere in lab. Really.

Most importantly, I'd like to thank my family for everything they've done—all the sacrifices they've made (this school is *not* cheap) and all the support they've given me throughout the years (financially and emotionally). I know it wasn't a cakewalk sending me to "the best schools" all my life, and I want you to know that I am infinitely grateful for all of it, even if I rarely, if ever, say it. I hope that I have made you proud. And Alyssa, your big brother thanks you muchly for somehow always managing to call me when I'm alone at lab at night and for telling me stupid things that made me laugh and kept me sane just long enough to make it out of there alive. I hope you know that I am working hard for you, too, because I want you to have the same opportunities that I had growing up, and I want to help provide for that when the time comes. You know, um, if I have money. I love you.

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