Fluorescent Spectroscopic Comparison of Phenotypes of Different Strains of *Saccharomyces cerevisiae* with Point Mutations of the *BRR6* Gene

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The *BRR6* gene codes for a protein that, while not a member of the Nuclear Pore Complex (NPC) within each cell's nuclear envelope, is significant to the process of the cell to properly form a NPC during the mitotic division process. In *Saccharomyces cerevisiae*, a budding yeast species will be studied through different types of fluorescent microscopy to visualize and gather phenotypic data. The data will be gathered and compared between three lab grown strains with different fluorescent components. The point mutations of the *BRR6* gene have produced a temperature sensitive mutant allele that will cause the NPC to be deformed. The results of this experiment highlight expected trends of NE proliferations and poorly formed NPCs between wild-types and the *brr6-1* mutant strains.

INTRODUCTION

As a eukaryotic cell, *Saccharomyces cerevisiae* has a greater capacity to regulate gene expression than prokaryotes. The presence of a nuclear envelope (NE), the doubly layered membrane separating the genetic material from the rest of the cell, to control the transport of proteins and genetic materials between the nucleus and the cytoplasm through nuclear pore complexes is central to that ability to control its genotype. Nuclear pore complexes (NPC's) are conserved structures in the nuclear envelope that mediate the bidirectional nucleocytoplasmic transport, and consists of many individual nuclear pore proteins collectively referred to as nucleoporins (Nups)(3). The large 8 fold symmetrical macromolecular NPCs span the inner and outer membranes of the NE and facilitate all transportation between the nucleus and the cytoplasm and its function is greatly dependent on its structure. In *Saccharomyces cerevisiae* the function of the NPCs has been examined, but little is known about the biogenesis of these transport channels. Due to the closed mitotic process of cell division *Saccharomyces cerevisiae* employs the formation of NPCs must occur de novo during the interphase (1). This process must require some type of fusion between the inner and outer nuclear membranes to form the resulting transport channel (1). Exploring the genetic properties of the biological formation of the NPCs will allow for a better understanding of how their function is dependent on their structure. In turn this will lead to further understanding of the function of NPCs in humans as the morphological properties (structure) is conserved between these yeast and mammalian cells (1). de Bruyn Kops and Guthrie (2001) have identified an essential gene that encodes for a 197 amino acid protein that they have named Brr6p (2). They and other researchers have found the protein is an essential integral membrane protein specific to the NE-endoplasmic reticulum (1). It has been shown that the *BRR6* gene is temperature sensitive. *BRR6* was originally identified in a screen for cold sensitive mutant defective in mRNA export, but was later then shown to be heat sensitive as well. Data analyzed by Hodge *et al.* indicates that the *BRR6* gene is likely to have an important role in the regulation of the cell's lipid homeostasis within the Endoplasmic reticulum, and therefore impacts the Nuclear Pore Complex biogenesis, nuclear transport mechanisms, and nuclear mRNA metabolism. And while not a member of the NPC it can impact those stated mechanisms of the NPC to affect the division processes. Cells carrying the allele are subject to defects in nucleoporin localization and nuclear transport when the gene has been disrupted or mutated. Specifically a mutant of the *BRR6* gene, *brr6-1*, affects transport of mRNA and a protein responsible for nuclear export signaling (2). Complete depletion of *brr6-1* alters nucleoporin distribution and the structural form of the NE, suggesting that the protein Brr6p is required for the spatial organization of the Nups. This is observed through electron microscopy of cells carrying *brr6-1* mutants, which showed NE abnormalities and proliferations of the endoplasmic reticulum. The temperature sensitivity extends event to the normal optimal growth temperature for the cells. At 37^{o} C the *brr6-1* cells display mRNA export and nucleoporin defects. The two nucleoporins identified were Nsp1 and Nup188.

Nsp1, a nucleoskeletal-like protein, is a component of the central core of the NPC, and has known contributions to nucleocytoplasmic transport and to the maintenance of the permeability of the NPC. Nup188 is a subunit of the inner ring of the NPC and has roles similar to that of Nsp1. These nucleoporins displaying the mislocalization are that of cytoplasmic filament region and not components of the nuclear basket region of the NPC. This finding led to studies being extended to additional nucleoporins in the *brr6-1* cells. Another cytoplasmic filament protein Nup82 was identified and showed extreme mislocalization (1). Other genes, in addition to BRR6, have been identified to have similar functions that have been more thoroughly explored including APQ12. This gene codes for the protein product apq12, which is an essential integral membrane protein of the NE and the ER and is necessary for efficient NPC formation, yet it is not necessary for viability. Hodge *et al.* shows that an overexpression of the allele APQ12 will partially suppress the growth defects of the *brr6-1* mutant cell, demonstrating a commonality between the two genes if one can correct or prevent the mistakes caused by the other (1).

The previous work on this research project by Hampden-Sydney College students explored the more recently identified Nup82 as well as another nucleoporin, Nup60, a component of the central core of the NPC. This Nucleoporin is very similar to Nsp 1 in that it is in the central core and it is involved with nucleocytoplasmic transport and permeability mechanisms. The research completed by students Christian Lehman, J. Drake Bishop, and Alan Fish led to a better understanding that the mutant *BRR6* genes do affect the two nucleoporins mentioned. Our results have led to a possible theory that the cells with the mutant *brr6-1* alleles are still able to enter the mitotic cellular division process, but that once the cells enter mitosis the process is halted at some point during the division of the nucleus, resulting in cells being frozen in a state of mitosis and the nuclear membrane abnormalities. The proposed research is going to employ various types of fluorescent staining to examine the subcellular distribution of the nucleoporins specifically Nup60 and Nup82, to observe and compare the results of the overexpression of *BRR6* in strains prepared by the Cohen-Fix lab of the National Institutes of Health, the Cole lab of Dartmouth College's Geisel School of Medicine, and the Biology department of Hampden-Sydney College. Additionally this research will extend to another nucleoporin, Nsr 1, a nucleolar protein, involved with binding nuclear localization sequences and ribosome biogenesis because of its location on the NPC and its functions in localization. The various strains of *Saccharomyces cerevisiae* prepared by the three labs contain different biological fluorescent tags, allowing us to visualize components of the inner and outer domains of the NPC as well as filaments, which were noted as an affected protein by Hodge *et al.* (2010).

MATERIALS AND METHODS

Strains and Equipment: The strains used for this research were grown and prepared using standard methods provided by the Cole Lab of Dartmouth's Geisel School of Medicine. For growth experiments the strains were grown in standard media overnight and then diluted back appropriately. The live-cell or fixed-cell microscopy was preformed using a Nikon TE2000-E microscope fitted with a Nikon -100 Plan Apochromat oil objective (NA 1.4), Orca-ER CCD camera (Hamamatsu, Bridgewater, NJ) and Velocity 3D Image Analysis (Perkin Elmer, Waltham, MA) and a Zeiss Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software.

Dilution Series Experiment Protocol: Cell cultures were made of the following strains: FY86, W303, 2488-1-3C, 2489-1-13A, and *brr6-1* Nup82, by growing them overnight in 5mL of YPD liquid media. Using a phytometer the optical densities of the strains for 1mL was calibrated to 0.3 (OD_{600}=.3) by dilution with YPD.

Temperature Microscopy Protocol: Cell strains were grown up in 5mL of YPD liquid media overnight on a rotator. Split the cell culture, 2.5mL into two tubes, and dilute to 5mL in YPD and grow for 12 hours one set at room temperature, 25^{o}C, and the other set at 16^{o}C. The cells can then be examined with the fluorescence microscope.

Immunofluorescence Protocol: Cultures of the cell strain were grown in 5mL of YPD media overnight. 2.5mL of the cells were transferred to a new tube and diluted back to 5mL with SC complete media. The cells were fixed by the addition of 500uL of 37.3% formaldehyde and allowed to sit for 1 hour on a

rotator. A 12 well slide was prepared by adding 30uL of 0.3% polylysine to each well for 15 minutes then washing each well with water twice. The cells were spun down in a centrifuge at 3000rpm for 2 minutes at room temperature. The cells were washed twice with 0.1M KPI and once with 0.1M KPI/1.2M Sorbitol solution. The cells were then spun down and the supernatant was poured off. The cells were then resuspended in 1mL of the KPI Sorbitol solution. 25uL of the cells were transferred to each well of the plate and allowed to sit for 30 minutes. The liquid was aspirated off and 25uL of DTT solution was added to each well and incubated for 10 minutes at room temperature. The DTT was then aspirated off and replaced with 25uL of Zymolyase solution and incubated for 20 minutes. The cells were then washed with 6 times, 25uL of each solution for 5 minutes each in the following order 0.1M KPI, 0.1M KPI/0.1%NP40, 0.1M KPI, 1X PBS/0.1%BSA, PBS/BSA/0.1%NP40, PBS/BSA. A dilution of the primary antibody was then prepared depending on the antibody being used. 25uL of the primary antibody was transferred to each well and allowed to incubate overnight at 4^{o}C. The cells were then washed again with the PBS/BSA, PBS/BSA/NP40, PBS/BSA sequence. The slide was then allowed to dry completely. The mounting solution was added and the slide and coverslip were sealed. The slide was then examined using the Nikon Fluorescence microscope.

DAPI Hoechst Live Stain: Cell strains were grown overnight in 5mL of YPD liquid media. 2mL of the cell cultures were transferred to 4mL of SC complete liquid media and allowed to grow for 2 hours. 1mL of the cells culture was transferred to a 1.5 mL Eppendorf tube and 05.mg of Hoescht DAPI dye was added to the tube and allowed to incubate at room temperature for 15 minutes.

RESULTS AND DISCUSSION

The temperature solid media growth experiment was carried out with the FY86, W303, 2488-1-10, 2489-1-13, brr6 Nup82, and *brr6-1* Nup82 strains, as seen in figure 1. This was a dilutions series of each strain grown on YPD at four different temperatures to assess growth abilities. At 30^{o}C and 37^{o}C all of the strains were able to grow on the solid media, see figure 2. At 23^{o}C only *brr6-1* Nup82 showed a slight inability to grow. On the 16^{o}C plate W303, 2489-1-13A, and *brr6-1* Nup82 all showed a severely diminished ability to grow (see Figure 3).

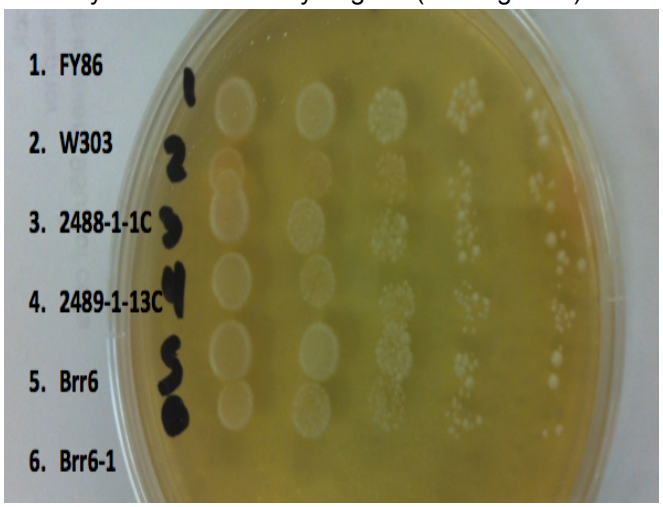

Figure1. Labeled plate with normal growth of all strains

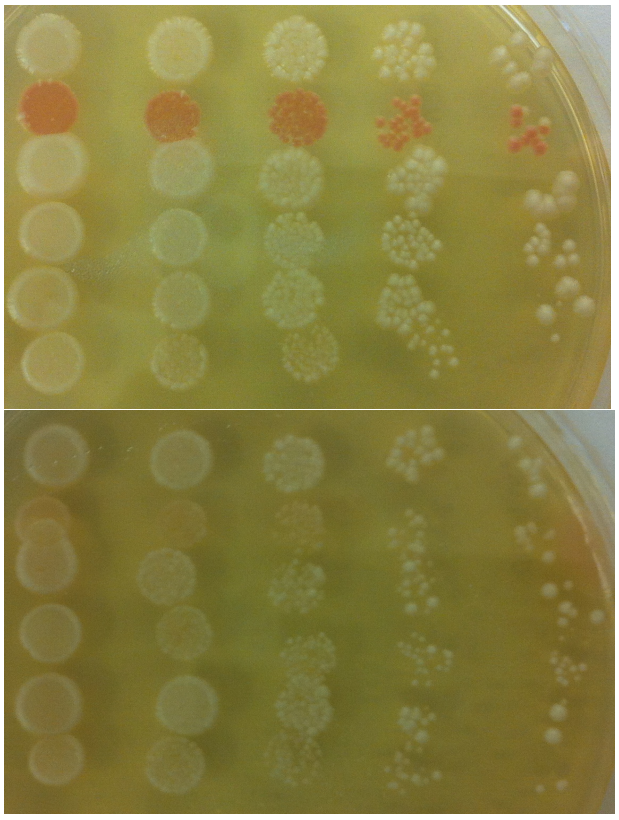

Fig 2. 30^{o}C (top) 37^{o}C (bottom) show all cells grew for all dilutions in the range of what is considered the optimal growth temperature for the yeast.

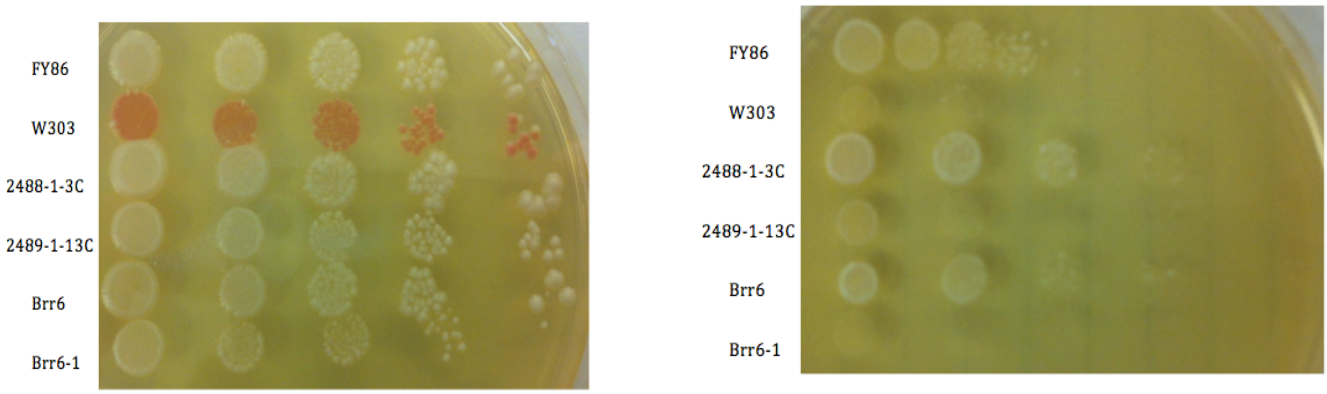

Figure 3: 23°C (left) 16°C (right) show the trend that *brr6-1* mutants are temperature sensitive.

A microscopy temperature experiment was completed to take a mitotic survey of cell phenotypes between the Cole lab strains of Brr6 versus *brr6-1*. This was done using microscopy techniques for strains that were grown at both 27°C and 16°C. In the figures below you can find examples of a typical survey between the two strains.

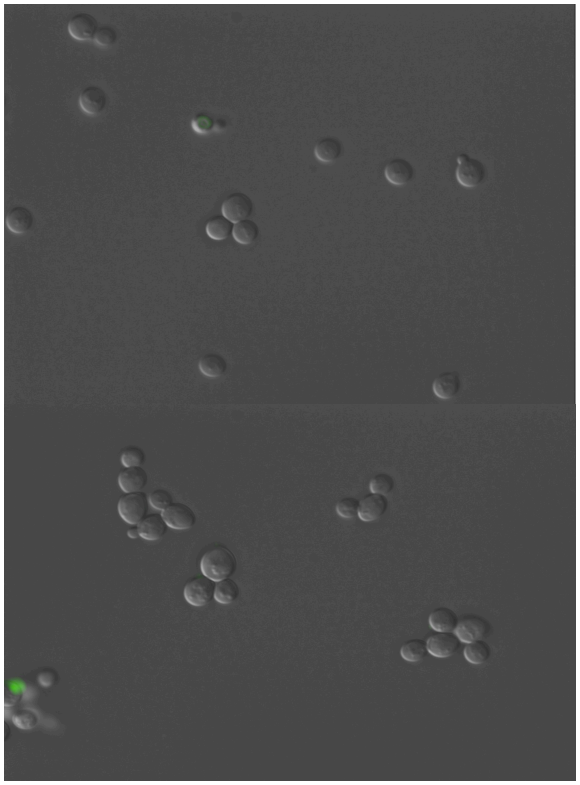

Figure 4: Brr6 cells (left) and Brr6-1 cells (right) typical survey at 27°C

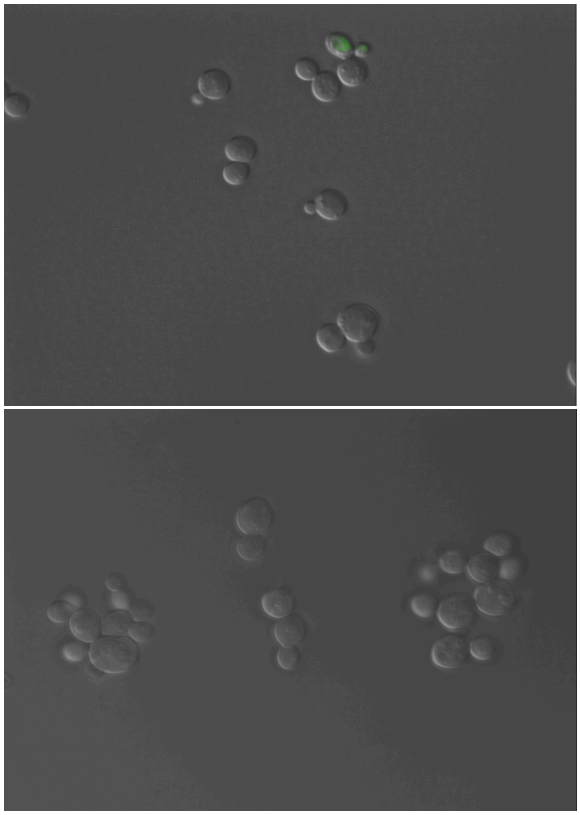

Figure 5: Brr6 cells (left) and Brr6-1 cells (right) typical survey at 16°C

In strains where the Nuclear Envelope is not biologically tagged with a fluorescent protein an Immunofluorescence procedure can be used as a quick, although some times less accurate, way of visualizing the NE. The other option being to cross breed the strain lacking the fluorescent protein with a strain that has one, although this process with result in better fluorescence of the NE the process can take weeks to months. Figure 4 shows a comparison

between a cell's NE immunofluorescence versus a cell's NE being tagged with a fluorescent protein.

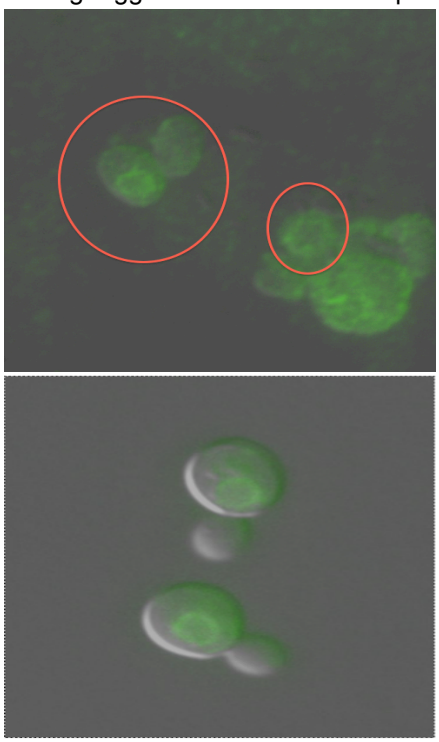

Figure 6: NE fluorescence as a result of Immunofluorescence procedure (top) and NE fluorescence as a result of NE labeled with GFP (bottom).

The Immunofluorescence procedure was carried out with the FY86, 2489-1-13A, 2488-1-3C strains using two different samples of the Rat7 #4 antibody of different dilutions and then a new Nuclear Envelope antibody that is anti-NE and anti-Endoplasmic Reticulum. This last antibody will attach to the nuclear envelope as well as the endoplasmic reticulum. When the cells were examined using the fluorescence microscope all three sets, each a different antibody, showed correct fluorescence in some of their cells. Examining the cells it appeared that the process was not uniform. Whether the antibody process was a success or not seemed to be a 50/50 chance with each cell. On each plate in every well there were examples of cells that came through the immunofluorescence process successfully, there were others that either did not fluoresce correctly or seemed to over-fluoresce and result in an over exposure problem when trying to visualize them under the microscope. A successful FY86 cell with the Rat-7 #4 antibody will show fluorescence of the nuclear envelope in green at a wavelength around 488 nm and fluorescence of DNA in blue at 462 nm. In a 2489-1-13A and a 2488-1-3C cell the previous two will be visible as well as NSR1 a nucleolus protein tagged with RFP that will fluoresce red. With these two strains I was able to examine the shape and placement of these cell organelles simultaneously. A successful cell with the new anti-NE-ER antibody system will fluoresce the same as described above with the only difference being that there is also GFP attached to the endoplasmic reticulum. Under the microscope this will result in a less localized green fluorescence. Below are examples of the wild-type FY86 cells after the immunofluorescence process. This strain will show the NE and the DNA, but is lacking the RFP fluorescent tag.

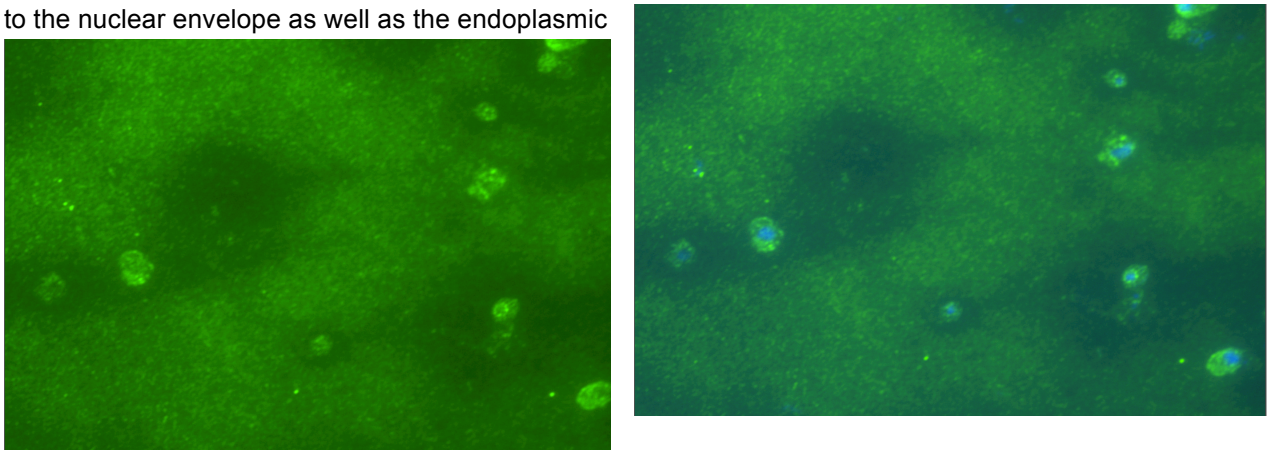

Figure 7: Strain FY86 after immunofluorescence with antibody rat7 to visualize the NE, DAPI to visualize the DNA, but no RFP tagged filaments. under fluorescence for NE (top right), Under fluorescence for NE and DNA (bottom left

The Cohen-Fix lab strain 2489-1-13A is one that contains the cherry red RFP label on the NSR1 protein. This strain is also contains the *brr6-1* mutant, and figure 8 shows a comparison between the

fluorescent labels so the phenotype data can be studied.

Figure 8, below, shows an example of a NE disruption in one of the *brr6-1* mutant strains. When looking at the cell, with the NE or white light only, a bud on the bottom is visible. If the fluorescence for the DAPI (DNA) and RFP (nucleolus) is present it is shown that no nucleolus or DNA is present in the bud's NE. This lack of nuclear organelles in the NE is evidence that the *brr6-1* mutant has affected the cells division process. Below is another example of a NE abnormality in the *brr6-1* strain 2489-1-13A where the cell has not yet produced a new budding yeast cell, but the NE is clearly dividing and the DNA did not separate as it should have.

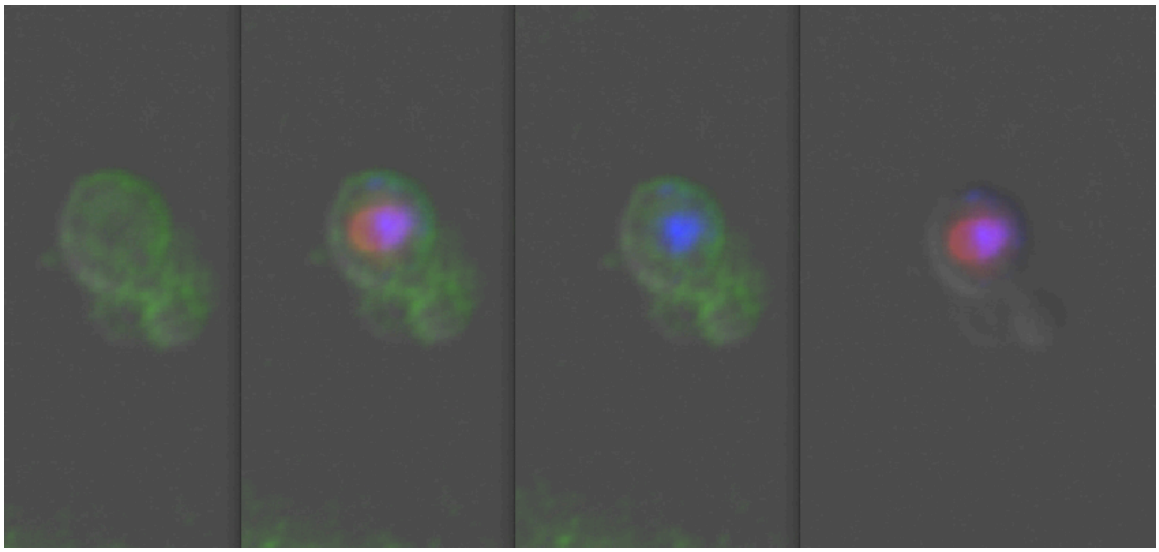

Figure 8: a comparison of the fluorescent components of the mutant strain 2489-1-13A. From left to right, the NE can be visualized, the Nucleolus can be visualized, the DNA, and then the DNA and the Nucleolus in the cell under normal light without the NE.

A live cell stain was also used as an alternative fluorescence technique. This process uses the DAPI Hoechst Live Stain, which will stain the Chitin of the cell wall and the DNA simultaneously for viewing under a DAPI lens on a fluorescent microscope. It differs from the immunofluorescent techniques in that these cells are still alive during the staining process and the microscopy and not fixed with a formaldehyde solution. With the images gathered using this technique data on the phenotype of the cells can be collected. Below are examples of the images acquired using the Hoechst dye.

A problem was encountered while using the live cell Hoechst dye, when the mutant *brr6-1* strains were exposed to the ultraviolet light of the fluorescent microscope of wavelengths around 350nm the nucleus as a result the cell itself would explode and the organelle would flow out into the liquid media on the microscope slide as seen in figure 10.

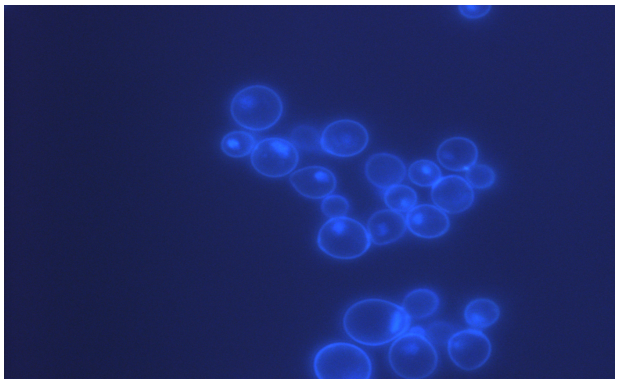

Figure 9. Brr6-1 cells with the Live Hoechst Stain

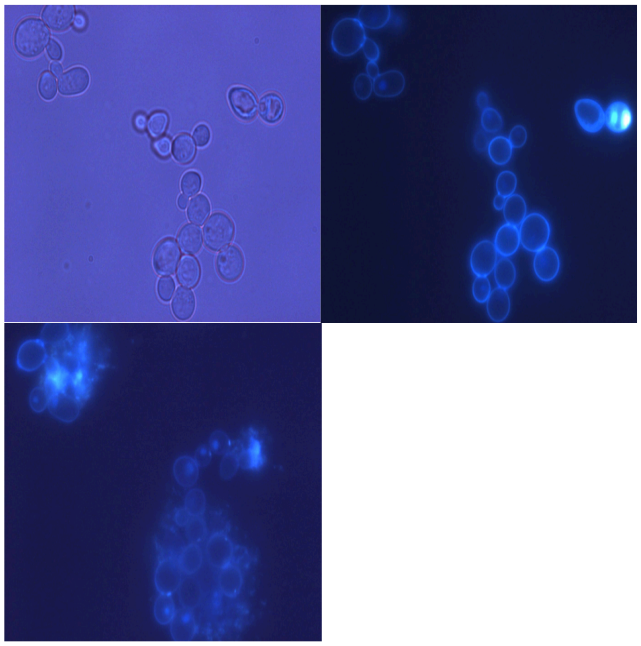

Fig11: brr6-1 cells under normal white light (top left), under ultraviolet light at 350nm (top right) under ultraviolet light at 350nm after 3 seconds of exposure (bottom left)

RESULTS AND DISCUSSION

The results of the solid growth experiment reaffirm that the *brr6-1* mutants are temperature sensitive with a decreased ability to grow at temperature ranging from 23^{o}C, room temperature, and below. The 2489-1-13A is a *brr6-1* mutant that was made and named by Dr. Cohen-Fix; therefore the two *brr6-1* strains were the ones that showed a cold sensitivity. The W303 strain is a wild type strain of the *Saccharomyces cerevisiae*, but it is a strain that was created in a lab to serve as a wild type for experiments; therefore it can have a temperature sensitivity just as a trait due to its background without it having significance to the research.

The temperature microscopy experiment was a mitotic survey between the Cole lab brr6 and *brr6-1* strains both grown at room temperature 27^{o}C and 16^{o}C. The data collected was a count of the cells currently in division and how many budding cells are attached to each cell. This information can give evidence of whether a sample is having difficulty completing the mitotic process or is having a phenotypic reaction to the temperature. The mitotic survey of the *brr6-1* strain at 27^{o}C showed an increase in the number of four bud clusters by almost 100%. This is characteristic of the mutant strain; to have a larger number of cells that are producing buds but not fully dividing and separating into individual cells. From other mitotic surveys it is likely that the buds would not contain full NEs. When comparing the data from the cells grown at 16^{o}C the number of individual cells falls significantly, the number in the brr6 strain is more than three times that in the mutant strain. This observation is also evidence that the temperature sensitivity of the mutant is present, and it is causing the division process to be disrupted or never complete.

The immunofluorescence experiment was a way to visualize the NE and depending on the antibody used the ER in cells that did not previously have a fluorescent protein tagged to the NE. This method, though quicker than the cross breeding of strains, can produce its own complications such as the matching of antibodies that caused trouble for the first two attempts at the procedure and the lack of specificity the antibodies can have when attaching to cellular components. In this case the Rat7 antibody will also attached itself to the ER and cause that to fluoresce. Another issue with this method was the inaccuracy of the results. The last attempt was successful with the three combinations of antibodies but finding cells where it had worked nicely and didn't cause any visualization problems with light exposure was about 50/50. Despite the difficulties the method had it did give useful data. Trends were established between the wild-type FY86 and the *brr6-1* strains; seen in the examples above there were many instances where the NE had begun to divide or had separated when the cell was in the process of mitosis, yet the DNA or the nucleolus had not.

The problem encountered with the Hoechst live cell stain may be related to the pH environment the cells are exposed to. Literature searches have shown papers where scientists have been more successful with the Hoechst Staining process when a mounting solution is used that contains phenylenediamine, a chemical known for being a pH buffer. Although none of the papers found specifically mentioned a problem with cells exploding when being exposed to the ultraviolet light a cautionary next step when reproducing that experiment would be the addition of a phenylenediamine mounting solution. Another possible cause of the cell deaths may be due to the intensity of the fluorescent ultraviolet light. A continuation for this research should include more attempts of the live cell staining process to see if the

fixation of the immunofluorescence method has any effect on the phenotypes of the cells.

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