The Role of Yeast Nuclear Membrane Proteins Brr6 and Brl1 in Cell Wall Integrity and Cell Separation

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The ability of yeast to replicate and divide properly through mitosis is crucial for the maintenance of species stability. Previous works in the budding yeast *Saccharomyces cerevisiae* has suggested that the peripheral nuclear membrane proteins Brr6 and Brl1 may play a role in the regulation of membrane fluidity in response to both environmental change and cell cycle progression (Hodge *et al*., 2010). In addition, recent observations have connected these proteins to the regulation of the mitotic exit network (MEN) and cytokinesis. To further study this potential link, we explored the cell growth and division properties of the temperature-sensitive conditional mutants *brl1-1* and *brl1-2*, as well as the cold-sensitive mutant *brr6-1*. First, we used an overexpression plasmid with *SIC1*, a cyclin-dependent kinase inhibitor that suppresses the mitotic arrest phenotype of several MEN mutants (Luca *et al*., 2001). This plasmid was transformed into the *brl1* and *brr6* mutant strains as well as into known conditional mutants of *mob1*, a MEN component. Comparison of growth phenotypes in these mutant strains at the permissive (23°C) and restrictive (34°C) temperatures showed no significant correlation between Brr6, Brl1, and the phenotypes expected for a functional MEN component. Zymolyase digestion analysis performed to assess the role of Brr6 and Brl1 in cytokinesis and final cell separation revealed a strong correlation between growth of the mutant alleles at the restrictive temperature and the presence of a weakened cell wall structure. Debris accumulated after as little as 1 hour of exposure to levels of zymolyase that did not compromise wild-type cell integrity. Overall, results suggested that Brr6 and Brl1 played an intriguing role in the regulation of cell wall integrity and the ability of the cell wall to locally degrade during cell separation independent of the MEN.

INTRODUCTION

A model example for cellular reproduction is found in the cell cycle of the budding yeast *Saccharomyces cerevisiae*. The mapping of its entire genome has allowed scientists to approach genetic experiments of the yeast already equipped with relevant knowledge. Consequently, scientists use yeast as a model in general experimentation concerning cellular processes in order to further study evolutionary conservation between yeast and mammals. The study of cell division in yeast may reveal a greater understanding as to how mammalian cell division defects occur and the possible development of cancer. In this project *Saccharomyces cerevisiae* is used to study and categorize the cellular functions that regulate reproduction and overall cell integrity.

Saccharomyces cerevisiae prepares to replicate in the early stages of its cell cycle through cellular growth and energy storage. Later in the cycle, the cells proliferate through a tightly-regulated process known as mitosis in which the parent cell produces and identical copy. During interphase, the offspring cell forms a bud on the parent cell, grows, and then eventually divides in order to finish the mitotic cycle. The yeast division cycle illustrated by Fig.1 consists of four phases: Gap 1 phase (G1), Synthesis phase (S phase), Gap 2 phase (G2), and Mitosis (M phase). G1 phase grants daughter cells ample time to grow in size and prepare for S phase, in which DNA synthesis begins. Chromosomal duplication occurs in S phase, and the cell then continues into G2 phase. In G2 the cell gathers energy in preparation for mitosis as it completes DNA synthesis. G1, S, and G2 phases constitute the interphase stage of cell division.

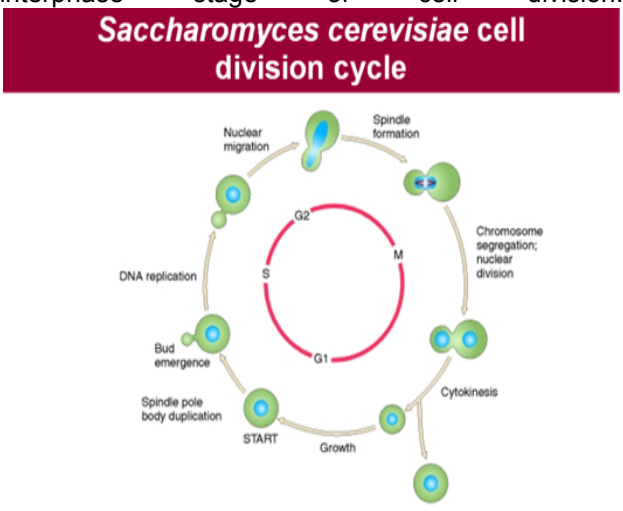

Fig 1. The budding yeast *Saccharomyces cerevisiae* begins cellular division following G1 phase. It undergoes a closed mitosis in which the nuclear membrane remains intact throughout the cell division process. It is important to note that the daughter cells are joined to their respective mother cells through connections of chitin that are normally broken down towards the end of cytokinesis.

Mitosis (M phase) is a stage in which nuclear division of the mother cell occurs, resulting in the formation of a genetically identical daughter cell. Throughout this process, the nuclear membrane remains intact while the external membrane remains surrounded by a chitin cell wall as the cell completes the processes of interphase, prophase, metaphase, anaphase, telophase, and cytokinesis. Interphase, comprised of the G1, S, and G2 phases, prepares the cell for entry into mitosis through the emergence of the daughter bud as well as DNA replication.

Following nuclear migration, a phenomenon by which the nucleus shifts closer to the early bud, the yeast cells undergo a closed mitosis in which the nuclear membrane remains intact, thus allowing for nuclear division to occur within this membrane. The M phase is initiated by prophase, in which spindle pole bodies, initially attached to the nuclear membrane, begin to move to opposite sides of the nucleus and organize microtubules in order to develop the nuclear spindle. Chromatin, a combination of DNA and packaging proteins that both compose nuclear material, also condenses into highly-structured, tightly-packed chromosomes. These are composed of two connected copies of each chromosome, known as sister chromatids, due to the completion of S phase. In metaphase, microtubules of the nuclear spindle attach to the chromosomes which then arrange at the equatorial plate equidistant to the mother and daughter spindle pole bodies. Since cell division in yeast takes place in the mother-bud neck, the spindle must be relocated to the neck for mitosis to proceed (Adames *et al.,* 2001).

Anaphase oversees the initial formation of the daughter cell as nuclear division continues. Sister chromatids are first separated, pulled by the attached microtubules of the nuclear spindle towards the spindle pole bodies on either side of the dividing cells. The completion of anaphase marks the genetic separation of mother and daughter cells as well as the formation of the cleavage furrow, an impression between the two cells that marks the location of cell separation (Bloecher *et al.,* 2000). In telophase, the final stage of mitosis, the separation of mother and daughter nuclei begins. The chromosomes are also unfolded back into chromatin. At this point mitosis has successfully occurred; however, the cells remain connected, indicating that cell division is not yet finished. M phase officially terminates following the process of cytokinesis by which the full separation of the mother and daughter cells is accomplished. Successful cytokinesis requires the separation of both nuclei as well as chitin cell wall structures. Chitinase is required to degrade the chitin attachment points between the mother and daughter cells.

The cell division cycle is a complex process integral to all biological life forms. The various steps of mitosis and cell growth rely heavily on cellular conditions and environments in order to properly regulate cell division and maintain normal cell growth processes. In order to properly initiate these sequential phases, the cell possesses various checkpoints to ensure successful and efficient division. If cell structures are damaged or unprepared for the next phase of cell division, protein effectors signal either apoptosis (programmed cell death) or if irreparable damage is present a state of arrest, in which the cell is granted time to complete repairs, follows. Biological sensors measure varying levels of molecular subunits in order to ascertain whether or not the cell can proceed to the next stage as well as to ensure homeostasis (Pereira *et al* 2000). These sensors include hormones, phosphoryl groups, proteins, and other particles that initiate different cellular functions. Notable checkpoints occur before and after DNA replication and during and after mitosis. Mitotic checkpoints occur with spindle formation as well as with the end stages of mitosis, including telophase and cytokinesis. These end stages are regulated by the mitotic exit network or MEN (Amon *et al.,* 1994). The MEN, the checkpoint of interest in this project, is a series of proteins that are responsible for ensuring a successful cytokinesis and transition from M phase to the completion of cell division (Luca *et al.,* 2001).

Conditional mutations allow wild-type and mutant cells to be studied under different environmental conditions. For instance, conditional mutants may die at low temperatures while cells at high temperatures survive. Previous research regarding the MEN reveals that in the presence of conditional mutations of *MOB1*, a MEN component, yeast cells arrest at restrictive temperatures and exhibit a stringing and clustering phenotype. The *MOB1* mutant cells undergo nuclear division, but fail to fully separate. Even though the cells remain connected, the cell cycle repeats itself, and another daughter cell is produced. This process repeats itself indefinitely resulting in strings and clusters of cells (Fig 4). This observation suggests that there are defects indicative of failed activation of the MEN due to cytokinesis failure. Interestingly enough, however, previous research has indicated only a partial failure has taken place (Yellman *et al.,* 2006). Specifically, certain MEN components are able to retain their function despite the above-described compromise to *MOB1* activity. However, over expression of *SIC1*, a cyclin-dependent kinase inhibitor suppresses these *MOB1* stringing phenotypes in several MEN mutants (Luca *et al.*, 2001).

In order to explore this stringing phenomenon, we decided to experiment with two

proteins that may be integral to proper MEN function and overall cell integrity. Previous work in *S. cerevisiae* has suggested that two peripheral nuclear membrane proteins, Brr6 and Brl1, may affect membrane fluidity in response to both environmental changes and cell cycle progression (Hodge *et al*., 2010). In addition, conditional mutants of Brr6 and Brl1 exhibit similar clustering and stringing phenotypes to those observed in *MOB1* mutant experimentation. These findings potentially link Brr6 and Brl1 to the regulation of cytokinesis and, more specifically, entry into the MEN. In order to study this potential link, we explored the cell growth and division properties of heat-sensitive conditional mutants *brl1-1* and *brl1-2*, as well as the cold-sensitive mutant *brr6-1*.

Based on Luca's research (2001), we transformed a *SIC1* overexpression plasmid into *brl1* and *brr6* mutant strains and into known mutants of the MEN component *mob1. SIC1* encodes for Cdk inhibitor production which is responsible for the prevention of premature S-phase entry. Incorporation of the plasmid resulted in increased levels of this Cdk inhibitor. In this regard, the cells may exhibit healthier phenotypes due to proper entry into the MEN. Strains containing this plasmid were then examined with cells of the same strain that did not have the plasmid. *SIC1* incorporation allowed a comparative study of our wild-type and *MOB1* conditional mutant controls, *brl1-1* and *brr6-1,* at varying temperature conditions with respect to MEN entry. Growth experiments were also conducted in order to study any relevant growth differences with or without plasmid incorporation at varying temperatures. The previously-mentioned strains used in the overexpression experiment were also used in the growth experiment including another strain: *brl1-2.* Both of these experiments were conducted in order to identify phenotype differences with and without the plasmid at different temperatures. In doing so, we hoped to discover a possible relationship between Brr6, Brl1, and the components of the MEN.

In order to further study the possibilities of existing connections between cells in the cytoplasm, previous data obtained through immunofluorescence microscopy was analyzed to categorize mutant division defects and resultant clustering phenotypes. Wild-type yeast at 23°C was observed to be actively dividing. This is exhibited by green fluorescence, which represents Cdc3 at septin junctions (Fig 2). The red staining was done with anti-tubulin antibodies and therefore indicates the presence of microtubule structures seemingly devoid of defect, although they appear to pass across multiple cells in mutant strains. 4',6-diamidino-2-phenylindole (DAPI) staining appeared blue and indicated the presence of nuclear material of which each cell appeared to contain its own. This is significant because it suggests that nuclear separation has successfully occurred even though the cells remain connected by other means in mutant strains. Observation of mutant strains *brr6-1, brl 1-1, and brl 1-2* at restrictive temperatures suggested that cells were actively dividing, but remained connected in the cytoplasm by microtubule structures and externally by chitin. DAPI staining data suggested that cell division had successfully occurred due to the presence of individual nuclei localized in the center of individual cells.

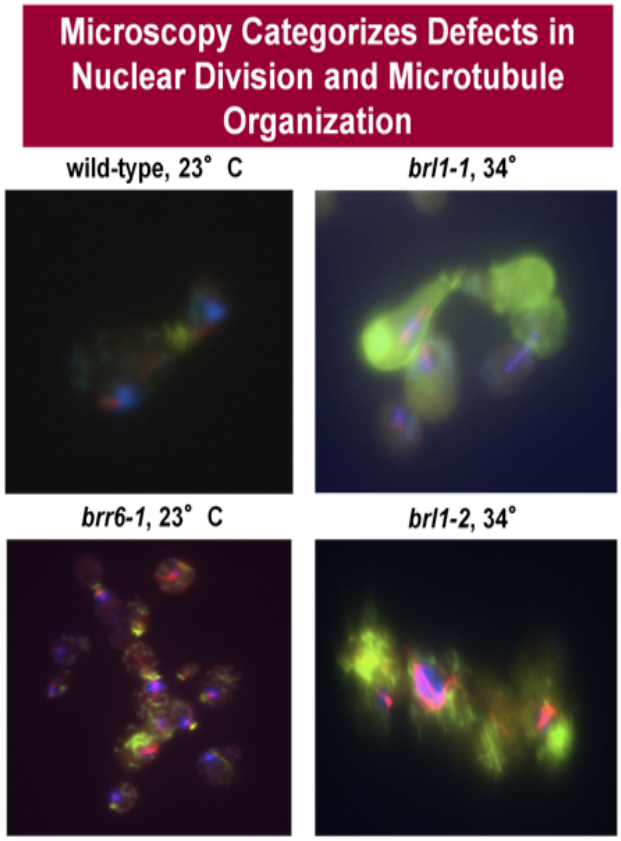

Fig 2. Immunofluorescence microscopy focused on comparative cellular division properties between wild-type and conditional mutatnt stains. Red staining indicates microtubules while green staining labled Cdc3 at septin junctions, precisely marking the exact area where cells were linked. DAPI staining was used to indicate the locations of nuclei. All cells appeared to contain individual nuclei regardless of whether or not complete cell separation had taken place, but microtubules were found to be present across multiple cells in mutant strains exhibiting stringing and clustering phenotypes.

This research suggests that mutant mother and daughter cells do not fully complete cytokinesis and fail to physically separate under restrictive temperature conditions at 34°C for *brl1-1* and *brl1-2,* and 23°C for *brr6-1*. Following the arrest of cell division, the mother and daughter cells appear to continue budding and remain connected, resulting in

the previously-described stringing phenotype in which cell junctions between mother and daughter cells remain connected by microtubules. Overall, this data suggests a failure to separate cells which further suggests that the MEN may be affected by Brr6 and Brl1 mutation.

Taken together, the data allowed us to design experiments that studied the possibility of MEN failure. After these stringing and clustering mutant phenotypes were observed, zymolyase, an enzyme that cleaves chitin, was used because of its similarities to chitinase, the enzyme used by yeast cells to naturally cleave chitin. Yeast cell walls contain chitin, as do the thick connections remaining between arrested cells. Enzyme treatment experimentation was intended to discover whether or not these mutant phenotypes could be corrected as well as to determine if cytoplasmic connections remained between arrested mother and daughter cells. Zymolyase treatment was administered to wild-type, *brr6-1,* and *brl1-1* cells grown at 23°C and 34°C with the intention of cleaving these chitin junctions. Cell phenotypes were then quantified for phenotype and overall appearance with regard to cellular health before treatment, one hour of treatment, and two hours of treatment. These observations were important with regards to a possible role between brl1, brr6, and cell wall maintenance

In order to further study these chitinous connections, DAPI staining experiments were conducted to identify whether or not nuclear connections remained in arrested cells. DAPI staining in conjunction with fluorescent microscopy allowed nuclear material to be visually assessed. DAPI and DIC images of wild-type, *brr6-1,* and *brl1-1* were taken. *Brr6-1* cells grown at their restrictive temperature of 23°C and *brl1-1* cells grown at their restrictive temperature of 34°C were used in this experiment to study the mutant clustering phenotypes. This study was important because it allowed us to observe the failure or success of nuclear division. We can then identify if the cell had arrested during mitosis or after, just before the initiation of cytokinesis.

METHODS

Strains, media, and equipment

Brr6-1, brl1-1, and *brl1-2* mutant yeast strains were grown on YPD plates, composed of yeast extract, peptone, and dextrose; they were also grown in liquid synthetic complete media, asolution of yeast nitrogenous base, complete amino acid supplements, and dextrose. All YPD media supplies were acquired from BD Biosciences (Sparks, MD) with the exception of dextrose, which was purchased from Fisher Scientific (Pittsburgh, PA). Synthetic complete medium components were purchased from Formedium Ltd. (Norwich, UK). Throughout experimentation period, yeast strains were grown at various temperatures requiring different incubators. 23°C strains were placed in a roller drum and left to grow at room temperature in the laboratory. 30°C strains were grown in an Isotemp incubator Model 655D from Fisher Scientific (Pittsburgh, PA). 34 and 37°C strains were grown in an Excella E24 shaking table incubator (New Brunswick Scientific, New Brunswick, NJ).

Microscopy To view fluorescent images of the yeast strains, cells were centrifuged and washed with sterile water or an appropriate media twice. Next, the cells were re-suspended in 100µL of water or appropriate media. 5µL of the mixture was then pipetted onto a microscope glass slide and covered by a cover slip. Immersion oil was then placed on the cover slip in order to properly view the cells under the 100X objective. Microscopic observation was performed on an Olympus 1x70 inverted phase contrast fluorescence microscope purchased from Olympus, Inc. (Center Valley, PA). The microscope was fitted with a Photometrics Coolsnap cf camera (Photometrics, Inc., Tuscon, AZ). The photo software used was the Metamorph 6.3 package (Molecular Devices, Sunnyvale, CA).

SIC1 overexpression plasmid transformation Yeast strains were grown to midlog phase overnight at 23°C. Cells were washed twice in H_2O and washed once in 1x TE/Lithium acetate solution (LiAc). The cells were then resuspended in residual TE/LiAc solution and then incubated for 15 minutes at 30°C. While incubation was occurring, salmon sperm DNA (10mg/ml) was boiled for 5 minutes. The salmon sperm was immediately transferred to ice in order to snap-cool the DNA. The cells were dispensed 50µl per Eppendorf tube, and 5µl of boiled salmon sperm DNA was added to them. Roughly 100ng of *SIC1* plasmid DNA and 300µl of 40% polyethylene glycol/1x TE/LiAc were added to the cells. The tubes were then vigorously vortexed and incubated for 30°C for 30 minutes followed by 42°C for 20 minutes (heat-shock phase). Following heat shock, the cells were centrifuged at 10,000rpm for 15 seconds, the supernatant was removed; the cells were then resuspended in 100µl of H_2O. The 100µl were spread on synthetic complete plates lacking leucine and incubated at 30°C for two to three days.

SIC1 overexpression growth assessment All media were purchased from Sigma-Aldrich

Corporation, St. Louis, MO. All yeast strains were grown in liquid media. Cells were then centrifuged at 10,000rpm for 30sec to produce a pellet. Next, using a Beckman DU 530 UV/Vis spectrophotometer (Beckman–Coulter, Brea, CA), cells were diluted to an optical density near 0.3 ($3.85x10^6$ cells/ml) in order to begin the experiment with similar concentrations of cells. The cells were washed twice with water; next, a series of ten-fold dilutions were made of the wild-type, *brl1-1, brl1-2, fly198,* and *brr6-1* strains on synthetic complete medium plates without leucine. The cells were dropped on the plates in specified areas of known strain and concentration. Then the strains were grown at 23°C, 30°C, 34°C, and 37°C. The plates were grown for 24 to 48 hours, and images were taken using a scanner and a computer (Fig 7).

Zymolyase treatment of mutant strains Wild-type, *brr6-1,* and *brl1-1* were grown at 23°C and 34°C in YPD liquid medium. First, initial cell counts of phenotypes were taken before zymolyase treatment (Zymo Research Corporation, Orange, CA). Zymolyase was used to cleave chitin connections between cells. A 1ml sample of overnight cell culture for each strain was placed in YPD and centrifuged at 14,000 rpm for 1 min. The supernatant was completely removed. The resulting cell pellet was introduced to 120µL YD digestion buffer and 5µL R-zymolyase. The solution was then vortexed and incubated at 37°C for two hours. 5µL of the solution as placed on a microscope slide for observation after 1 hr of zymolyase treatment. After 2 hrs, 5µL of the solution was placed on a microscope slide and observed again for present phenotypes (cell counts n>200).

Visualization of chitin structures with calcofluor white
For this experiment, yeast strains were grown overnight in synthetic complete medium. After 24 hrs, the cells were washed twice with sterile water. In order to prepare a 0.1% calcofluor white solution, 0.1g of calcofluor powder, (Sigma-Aldrich, St. Louis, MO), was added to 100ml of sterile water. Cells were centrifuged to produce a pellet. The supernatant was discarded, and the pellet was re-suspended in 50µL of the calcofluor white solution. All preparations were performed in a dark since calcofluor white is a light-sensitive compound. The cells remained suspended in the calcofluor white solution for 12 min before they were observed by fluorescence microscopy in the manner described above.

Visualization of nucleic acid structures with DAPI For this experiment, 1/10 volume of 37% formaldehyde was added to 200µL of yeast cells in liquid medium. The cells were fixed at 23°C for 2 hrs and washed twice with phosphor-buffered saline (PBS). The cells were then resuspended in 300µL PBS. Next, 70% Ethanol was added to the mixture, which was placed on ice for 30 minutes. The cells were spun down and resuspended in PBS. 5µL of this solution was placed on a microscope slide along with a mixture of mounting medium containing DAPI (100ng/ml). A cover slip was placed on the slide before observing the cells on an Olympus 1x70 inverted phase contrast fluorescence microscope.

RESULTS

With respect to the overall project, it is important to note two crucial pieces of information regarding a typical *S. cerevisiae* cell division cycle (Fig1). The first is that the nuclear membrane remains intact throughout the entire process of cell division. The second is that bridges of chitin, a long chain polymer common to fungi cell walls, connect mother and daughter cells as well as form cell walls around the entire cell. These bridges of chitin are cleaved at the mother/bud junctions later during cytokinesis to allow final cell separation in healthy yeast cells.
In order to view these chitin structures present in wild-type and mutant yeast strains, we conducted a calcofluor white staining experiment in which the chitin present in the yeast cell walls is fluorescently tagged. This experiment was conducted on the wild-type *brr6-1* and *brl 1-1* mutant yeast strains at permissive and restrictive temperatures. Calcofluor white outlines the cell wall as well as reveals the concentrated areas of chitin which indicate junctions between mother and daughter cells (Fig 3). Another yeast strain, *brl1-2,* was also used but did not yield any substantial differences in data when compared to *brl1-1.*

Stained wild-type cells, as expected, showed healthily budding and dividing phenotypes at 23°C. Interestingly, stains of mutant strain *brr6-1* at its restrictive temperature (23°C) showed stringing phenotypes where chitinous junctions existed between multiple cells. The same phenotype was observed for *brl 1-1* at its restrictive temperature of 34°C (Fig 4).

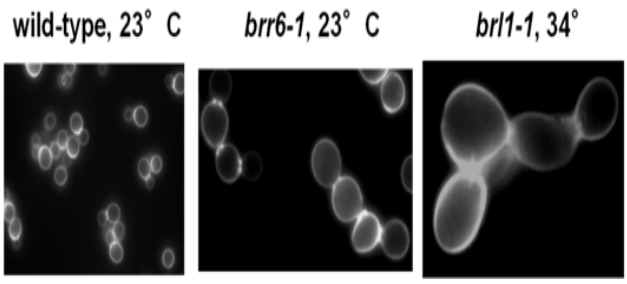

Fig 3. Calcofluor white experiments on wild-type (FY86), *brr6-1* and *brl1-1* strains at permissive and restrictive temperatures revealed concentrated areas of chitin indicating junctions between dividing cell walls. For the *brr6-1* strain the restrictive temperature was 23°C while *brl1-1* and *brl1-2* strains showed restrictive growth at 34°C. Observing the location and quantity of these junctions aides the determining of cytokinesis defects inherent to each strain. Wild-type cells exhibited normal cell division patterns while the *brr6-1* strain showed an increased number of failures to complete the cell division process at its restrictive temperature. The *brl1-1* showed similar defects to *brr6-1* in terms of an apparent cytokinesis failure, albeit at a different restrictive temperature. *Brl1-2* cells (not shown) were similar to those seen for *br11-1*.

The Calcofluor white experiments characterized cellular division defects inherent to the mutant yeast strains. The stringing phenotypes observed from these experiments suggested that the cells successfully underwent nuclear division, yet somehow failed to physically separate. Following this failure to separate, the cells then continued to divide resulting in new bud formation and further clustering or stringing. As the process repeated itself, the stringing phenotype became more apparent as multiple cells failed to separate. These experiments indicate that mother and daughter cells are in fact held together through chitinous junctions, but they do not provide insight as to whether or not the cells are held together through cytoplasmic connections as well.

To study the possible connection between Brr6 and Brl1 and the MEN, we conducted an experiment in which the wild-type and mutant strains were transformed with a *SIC1* overexpression plasmid. Sic1 is a cyclin-dependant kinase inhibitor and serves as a checkpoint before the initiation of S-phase. Previous data has shown that overexpression of this protein corrects the stringing and clustering phenotypes seen in *mob1* conditional mutants at the restrictive temperature (Luca *et al*., 2001). This motivated us to use a *mob1-77* conditional mutant strain (FLY198) strain as a control. The experiment required observation of wild-type, FLY198, *brl1-1, brl1-2,* and *brr6-1* cells at 23°C and 34°C with and without the *SIC1* overexpression plasmid. Cell counts were represented by percentages of certain phenotypes of each strain. These observations were then compiled into the summary seen in Fig 4.

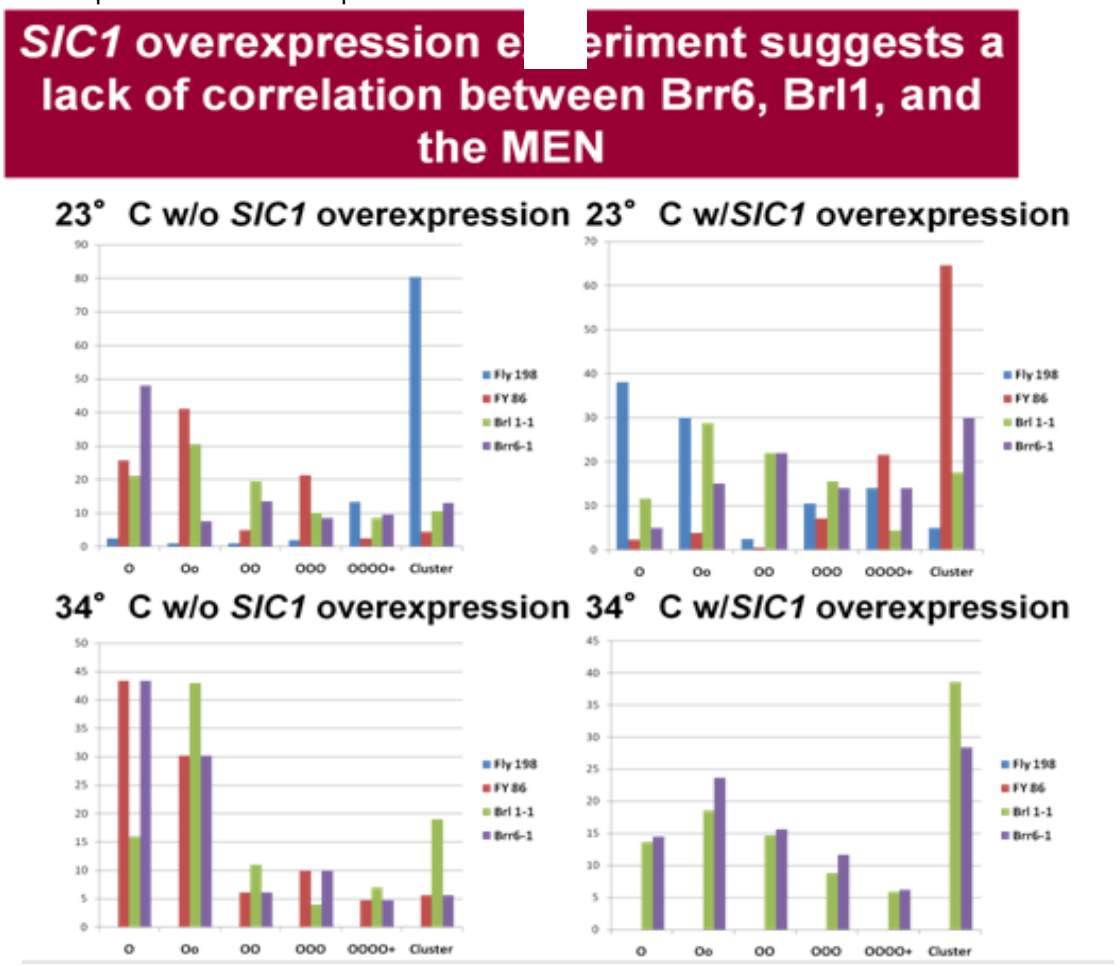

Fig 4. To see if a connection existed between Brr6, Brl1, and the regulation of the MEN, the ability of overexpression of the cyclin-dependent kinase inhibitor Sic1 to suppress the observed cell separation defects w~s assessed. The FLY198 strain is a conditional mutant of the MEN component *mob1* whose cell separation defects were previously shown ɔ be corrected at 23°C by *SIC1* overexpression (Luca el al., 2001). Cell counts were conducted in order to assess the degree of aggrega on found among cells in each strain at permissive and restrictive temperatures (N>100). While FLY198 cell separation was indeed correc ɛd by *SIC1* overexpression at 23°C, no significant differences were observed between *brr6* and *brl1*mutant strain aggregation patterns with o without excess *SIC1.*

We observed expected results concerning phenotype correction for the FLY198 strain at 23°C. Here, comparative inspection of cells in these two conditions suggested that the clustering phenotype was corrected by incorporation of the *SIC1* overexpression plasmid. Wild-type cells exhibited normal, budding phenotypes without the plasmid and, like Luca's research, showed a significant increase in the clustering phenotype with the *SIC1* plasmid. *Brl1-1* and *brr6* strains did not show significant aggregation differences with or without the plasmid at either temperature (all phenotypes remained under 30% of the total cell count per strain). Interestingly, FLY198 and wild-type strains did not grow at 34°C with the plasmid. This data suggests that there is not an integral relationship between Brr6 and or Brl1 and the MEN, at least not through the observation of aggregation patterns.

In order to further examine whether or not this relationship between the MEN and Brr6 and Brl1 existed, we conducted an experiment to categorize the overall effects that incorporation of the *SIC1* overexpression plasmid had on general growth patterns. Known dilutions of wild-type, FLY 198, *brr6-1, brl1-1, and brl1-2* were grown with and without the plasmid at 23°C, 30°C, 34°C, and 37°C (Fig 5).

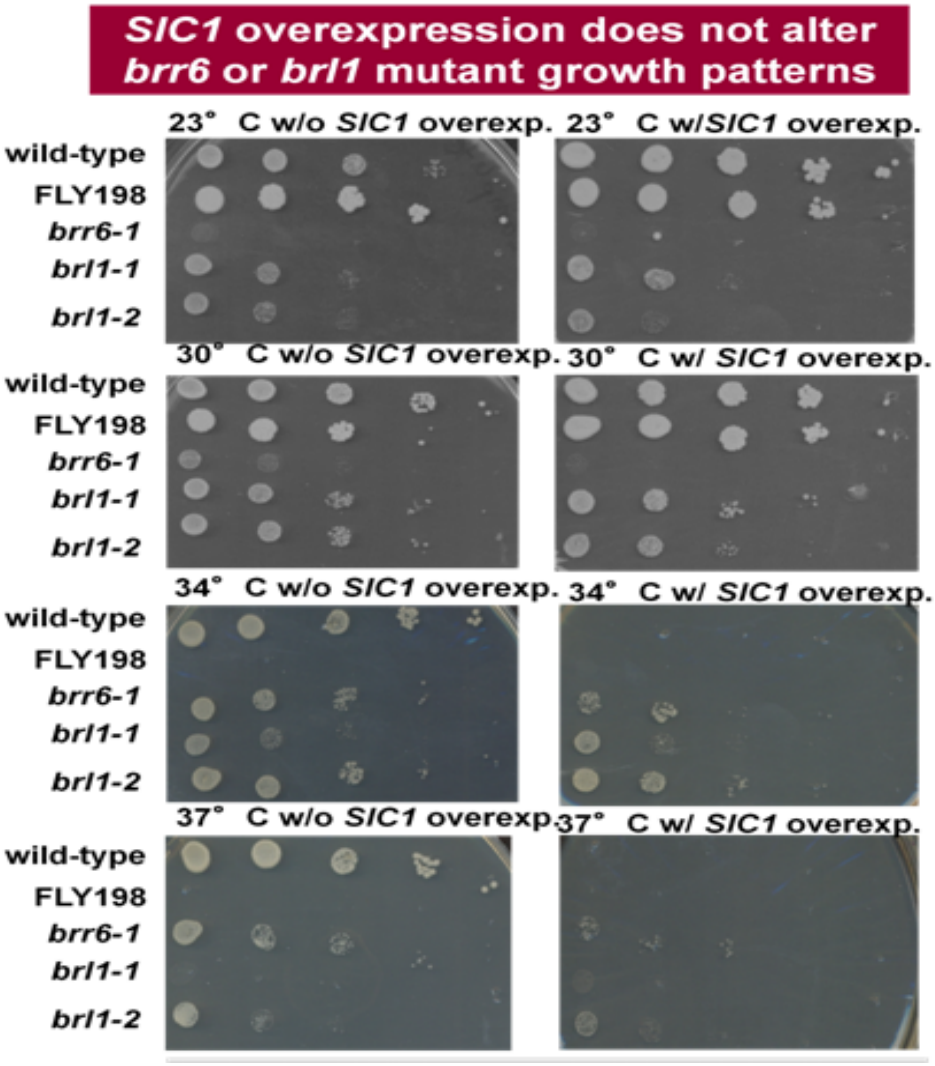

Fig 5. A series of ten-fold dilutions were made from *brl-1, brl1-2,* and *brr6-1* strains with or without the *SIC1* overexpression plasmid on YEPD plates to assess their overall growth abilities in comparison to wild-type and FLY198 controls. While *SIC1* overexpression prevented growth of the wild-type strain at 34 and 37°C, no notable difference were seen between *brr6-1, brl-1,* and *brl-2* strains with or without SIC1 overexpression, suggesting that these genes do not play a regulatory role with respect to the MEN.

Comparative study of the yeast strains in these different conditions suggested that there were no significant differences in the growth patterns for *brl1-1, brl1-2,* and *brr6-1* based on the presence or absence of overexpressed *SIC1.* Interestingly, the intention of testing whether or not mother and daughter cells exhibiting the clustering phenotype were held together simply by chitinous junctions or also through cytoplasmic channels. Wild-type, *brr6-1,* and *brl1-1* cells were treated with zymolyase, an enzyme that is known to cleave chitin. Cell counts (n>200) were conducted at an initial time of 0 wild-type strain did not grow at 34°C or 37°C with the plasmid. FLY198 failed to grow at 34°C and 37°C.

Based upon immunofluorescence microscopy observations, we designed an experiment with the minutes, 60 minutes, and 120 minutes after zymolyase treatment. Interestingly, treatment after 120 minutes resulted in compromised cell walls and the excessive presence of cellular debris among mutant strains while wild-type cells were living and 100% single (Fig 6).

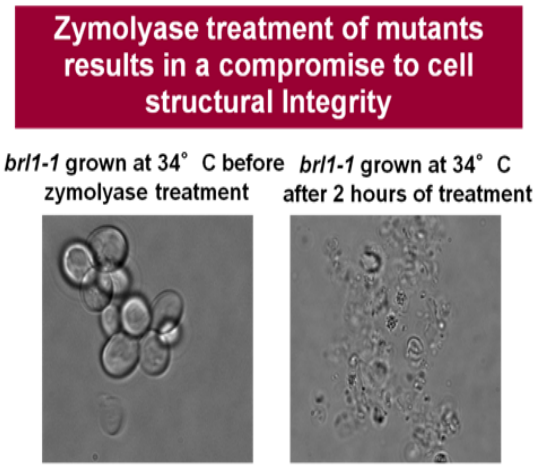

Fig 6. Representative DIC images of the brl1-1 strain after treatment with 0.2 units/µl zymolyase. Before zymolyase treatment, the cells exhibit a healthy phenotype but also the patterns of cell clustering previously observed for the strain under these conditions. After 2 hours of treatment, the cells showed a 95% mortality rate. The visible elements in the image are hollowed-out "husks" of cells as well as other debris. This result suggested that Brl1 may play a role in the maintenance of cell wall integrity.

These DIC images of *brl1-1* grown at 34°C chronicle this unexpected occurrence. Before zymolyase treatment, *brl1-1* cells exhibiting the clustered phenotype appeared to be alive and relatively healthy. The DIC image after 2 hours of zymolyase treatment displays nothing more than debris. This debris appears to be remnants of cell walls, suggesting that hollowed-out cells are all that remained. This drastic compromise to the cellular integrity suggests that Brl1 may play a key role in cell wall integrity.

An examination of the percentages of cells in each group before and after different lengths of zymolyase treatment further illustrates this potential link between mutation in *BRR6* or *BRL1* and the regulation of cell wall separation during mitosis (Fig 7).

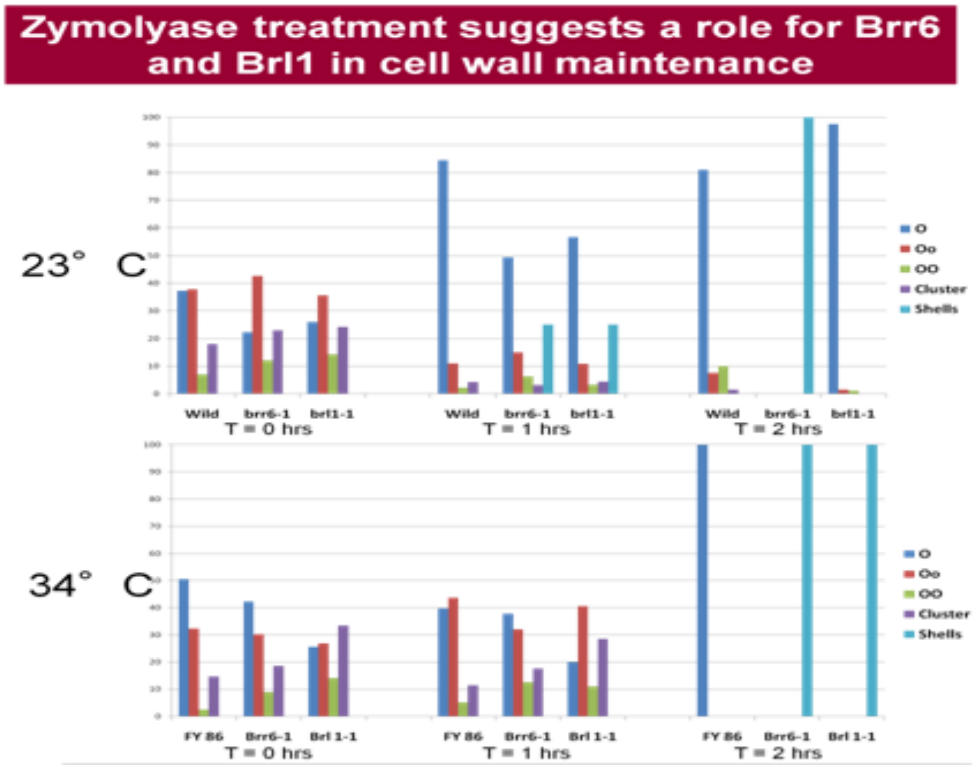

Fig 7. As suggested in Fig 7, 2 hours of zymolyase treatment resulted in the severe compromise of cell integrity of *brr6*-1, *brl1-1,*and *brl1-2* cells at their respective restrictive temperatures (N>100). As wild-type cells were not structurally compromised, this data strongly suggests a

role for Brr6 and Brl1 in the maintenance of cell wall integrity during cell separation.

At 34°C, zymolyase treatment of wild-type cells results in a 100% single-cell phenotype after 2 hours. *Brr6-1* and *brl1-1* data does not suggest any significant changes in phenotype percentage in the first hour. Like before, note that after treatment for 2 hours, only hollowed-out debris remained for both strains.

The 23°C zymolyase treatment data resulted in overall lower percentages of clustered wild-type and *brl1-1* phenotypes. After one hour of treatment, *brr6-1* cells had significantly lower

material not been localized in individual cells, then we could assume that a nuclear separation defect was present as well. Fluorescent and DIC images were captured for wild-type, *brr6-1, brl1-1,* and *brl1-2* cells (Fig 8).

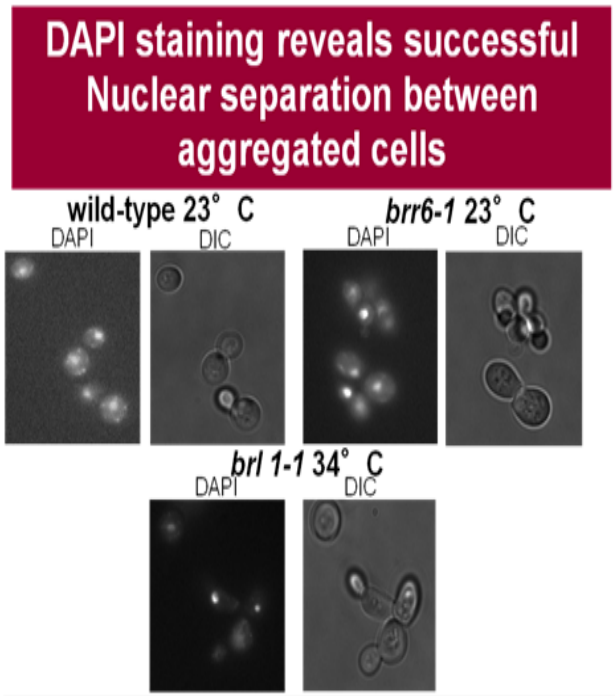

Fig 8. DAPI staining was performed in order to determine whether or not aggregated *brr6* and *brl1-1* cells each contained their own nucleus. In all cases, each individual member of a cell aggregate contained its own nucleus (in many cases, all nuclei cannot be seen in one focal plane), which suggests that all strains successfully underwent mitosis but were unable to completely separate from the parent cell and therefore remained connected through deposits of chitin.

Comparisons between mutants and wild-type revealed that each individual cell in aggregated clusters contained its own, centralized genetic material. This suggests that aggregated cells have successfully undergone nuclear separation but clustered percentages and higher single-cell percentages. After 2 hours of zymolyase treatment, however, only hollowed-out debris of *brr6-1* existed. This suggests that *brr6-1* cells are unable to withstand protracted exposure to zymolyase again hinting at a deficiency in cell wall integrity.

DAPI staining experiments were then conducted to ensure that successful separation of genetic material had occurred in the aggregated as well as healthy phenotypes of mutant and wild-type strains respectively. We observed that each cell had its own localized genetic material. Had nuclear remained attached. We can then assume that the failure to fully separate has occurred somewhere between telophase of mitosis and cytokinesis.

DISCUSSION

These experiments arrived at three important conclusions. First, the *SIC1* overexpression data is not indicative of a direct relationship between Brr6, Brl1, and the MEN. Second, Brr6 and Brl1 may play an important role in the maintenance of cell wall integrity during the process of final cell separation. Third, individual cells in aggregated clusters contain individual DNA and have successfully undergone nuclear separation. These conclusions suggest that cellular division has successfully occurred in these mutant strains, but physical cell separation cannot be achieved due to defects in localized cell wall remodeling and separation that may arise from mutations to *BRR6* and *BRL1.*

This experimentation has allowed an in-depth view of the phenomenon of cell division with the goal of studying defects associated with mutations to Brr6 and Brl1. At the time of cell separation, Brr6 and Brl1 may play an important role in the maintenance of cell wall integrity. Zymolyase treatment experiments suggest this role since a two hour exposure results in the death of *brr6-1* and *brl1-1* cells while wild-type cells remain intact. This data indicates that mutations to the Brr6 and Brl1 proteins may serve as the driving force behind a zymolyase induced cell wall compromise. These proteins may affect cell fluidity and overall maintenance of the cell wall. However, we cannot rule out the possibility that this data may also be the result of an osmotic defect because the dead cells appeared to be hollowed out “husks” of the mutant strains. These hollow cells suggest this type of defect because it appears as if the cell had been placed in either a hyper- or hypotonic environment in

which the cell imploded or exploded. Future experimentation can be geared towards testing this alternate interpretation by conducting zymolyase experiments in a denser solution such as sorbitol so as to create a controlled osmotic environment, or by simply exposing mutant cells grown at the restrictive temperatures to varying osmotic conditions.

DAPI staining experiments suggest that cell division has successfully occurred, but not complete cell separation in mutant strains. Our observations indicate that nuclear material is concentrated in all individual cells. This suggests that the cells have separate genetic identities, but clustered cells remain connected through chitinous junctions. Aggregated phenotypes fail to physically separate, which is indicative of a failure to fully activate the MEN, and continue to divide thus resulting in the stringing and clustering phenotypes of the mutant strains. These mutations of Brr6 and Brl1 may result in the failure of MEN entry or even an incomplete activation of the network. Whatever the case, the cells then fail to fully divide resulting in aggregated phenotypes.

SIC1 overexpression experiments do not reveal a correlation between the Brr6 and Brl1 proteins and the MEN. According to Luca et al., the data collected from these experiments suggests that addition of the plasmid does not result in the correction of stringing and clustering phenotypes. In fact, our data suggests that at 34°C cellular aggregation increases because the cells possibly fail to properly enter into S-phase due to increased presence of Cdk-inhibitors. Also growth experiments do not reveal significant differences in growth tendencies between yeast strains with or without the overexpression plasmid; however, wild-type and FLY198 strains did not grow at 37°C with the plasmid.

Overall, these experiments represent a model study of cellular division properties and tendencies. Although our results do not directly show a correlation between Brr6 or Brl1 to entry into the MEN, we can be sure that they play some regulatory role throughout the cell cycle. Mutations to these specific proteins resulted in cellular death during the zymolyase experiments, which suggests that they may be crucial to cell fluidity and maintenance. DAPI staining showed that nuclear division successfully occurred at restrictive temperatures even though the cells remained connected through chitin junctions and microtubule structures. These experiments show that mutations to these peripheral proteins, Brr6 and Brl1, affect the cell's ability to properly divide. Therefore, we can conclude that Brr6 and Brl1 are important components to the process of cell cycle regulation. Future research will investigate these interesting cellular properties as well as the possible presence of an osmotic defect evident in the zymolyase treatments.

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