Research Article

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Chitin levels and cell death in *Saccharomyces cerevisiae smi1-Δ* cells exposed to antifungal treatment

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Abstract

The *SMI1* gene plays an integral role in the coordination of a cell wall integrity (CWI) pathway in *Saccharomyces cerevisiae*. Thus, *smi1*-Δ mutant cells present cell wall defects due to their reduced capacity to synthesize cell wall components. Responding to weakened cell walls, yeast cells produce increased amounts of chitin as a compensatory mechanism. It was hypothesized that *smi1*-Δ cells will present lower cell death rates, increased chitin levels, and therefore present an increased tolerance to antifungals. Wildtype-a (WT-a) cells, and *smi1*-Δ cells were treated with imidazole to study antifungal effects on cell viability and chitin production in *smi1*-Δ *S. cerevisiae* cells. To examine the effects of an antifungal stressor, cell death was quantified using hemocytometry and chitin levels were visualized using fluorescence microscopy. Imidazole-exposed *smi1*-Δ cells exhibit significantly lower cell death percentages. *smi1*-Δ cells have significantly higher cell wall chitin levels than WT-a cells and display further increases with antifungal treatment. These findings indicate that CWI pathway disruption in *smi1*-Δ cells may lead to an increased antifungal-tolerance. Examining interrupted CWI pathway compensatory mechanisms in mutant *S. cerevisiae* cells allows for a greater understanding of yeast tolerance to drugs, and aids in the development of more potent fungal infection medications.

Introduction

Saccharomyces cerevisiae is a yeast species that is frequently used as a model eukaryotic organism (Stewart, 2014). The yeast cell wall plays an integral role in protecting the cell from external stressors and maintaining cell shape during growth and proliferation (Levin, 2005). The *SMI1* gene in *S. cerevisiae* is responsible for maintaining cell wall integrity (CWI) and assembly by mediating cell cycle progression as seen in Figure 1A (Martin-Yken et al., 2003; Samakkarn et al., 2021). Lack of the *SMI1* gene has been suggested to interrupt the formation of (1,3)-beta-glucan thus impacting the deposition of chitin in the cell wall (Martin et al., 1999).

Yeast cell walls are composed of (1,3)-beta-glucan, (1,6)-beta-glucan, mannoproteins, and chitin (Basmaji et al., 2006). As a compensatory mechanism to damage to the cell wall, yeast cells are reported to increase chitin production and accumulate chitin in an attempt to reduce cell death (García et al., 2017; Ohno, 2007). This rescue mechanism in

response to stress is controlled by the CWI pathway (Sanz et al., 2017).

According to a 2021 paper by Samakkarn et al., mutant cells with *SMI1* knockout mutations experience difficulty with cell division and reproduction. Martin-Yken et al. found that *smi1-Δ* mutant cell walls are greatly affected by extracellular stressors such as extreme temperatures, high levels of ethanol, and other factors that disrupt the cell wall (2016). The *smi1-Δ* mutant cells are reported to have weakened cell walls due to decreased levels of (1,3)-beta-glucan synthesis and a loss of mannoproteins from the cell wall (Basmaji et al., 2006; Martin-Yken et al., 2016). In a 1999 paper by Martin et al., it was reported that *smi1-Δ* mutant cells have elevated chitin levels compared to *S. cerevisiae* cells with functioning *SMI1* genes.

Sanz et al. find that rescue mechanisms, such as increases in chitin in the cell wall to compensate for the decreased amount of (1,3)-Beta-glucan, as seen in Figure 1B, have the potential to decrease the effectiveness of an antifungal treatment (2017). Antifungals are used for the treatment of fungal infections (Chen & Sorrell, 2007). There are four primary types of antifungals: allylamines, azoles, polyenes, and echinocandins (Chen & Sorrell, 2007). Azole antifungals work by blocking the creation of ergosterol, a large and important component of fungi cell membranes (Deswal et al., 2020). Imidazoles are a type of azole containing two nitrogens in their azole ring (Sheehan et al., 1999). In this study, an imidazole antifungal treatment will be used to create a stressful condition for *S. cerevisiae* cells with the goal of determining how the interrupted CWI pathway compensatory mechanisms function

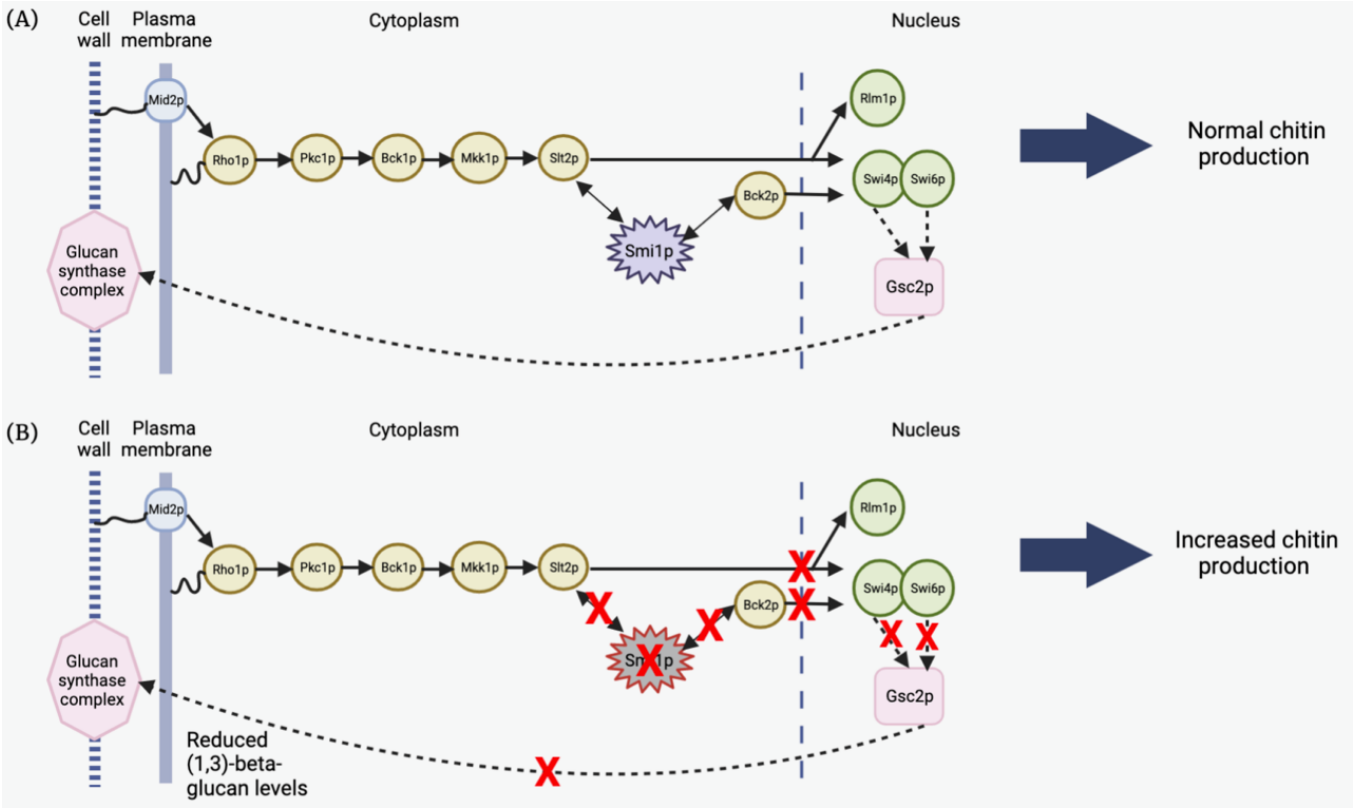

Figure 1. The role of Smi1p in mediating the CWI pathway and the resulting effect on chitin production. (A) Functioning pathway with Smi1p acting as a mediator in the cell cycle integrity pathway. Smi1p activates Swi4p/Swi6p and Rlm1p resulting in the regulation on Fks1p. The production of (1,3)-beta-glucan is in part regulated by Fks1p. Chitin levels in the cell are normal in this functional pathway. **(B)** Dysfunctional pathway in *smi1-Δ* mutant cells due to the loss of Smi1p. Fks1p is not activated and leads to a reduction in the production of (1,3)-beta-glucan required to help maintain CWI. These cells, with decreased levels of (1,3)-beta-glucan, instead, increase chitin production. *(Figure adapted from Basmaji et al., 2006; Heinisch et al., 1999; Sanz et al., 2017).*

in the *smi1-Δ* mutant cells compared to the normal CWI pathway in WT-a (homozygous mating type a strain) cells.

Fungal infections are a global health concern and have the potential to be lethal to humans (Hasim & Coleman, 2019). Using *S. cerevisiae* as a model eukaryotic organism, the effects of an antifungal stressor on the cell wall can be examined to determine how to better treat fungal infections. Additionally, understanding how yeast cell strains with cell wall mutations, such as *smi1-Δ* mutants, function in the presence of an antifungal could lead to the future development of cell wall mutant targeted drugs. No previous literature has explored the effects of antifungal treatment on chitin accumulation and cell death in *smi1-Δ* cells.

From previous literature, it is known that *smi1-Δ* mutant cells have elevated chitin levels compared to *S. cerevisiae* cells with functioning *SMI1* genes (Martin et al., 1999). Thus, it is expected that there should be higher chitin in the *smi1-Δ* mutants relative to the WT-a cells for all concentrations of antifungal treatment. When exposed to the antifungal treatment, it is expected that the *smi1-Δ* mutant cells should see more of an increase in chitin than WT-a cells as they are predisposed to having a damaged CWI pathway and regulation and thus have the mechanisms in place to compensate for further stress to the cell wall. The highly functioning rescue mechanism in addition to the already increased level of chitin present in the *smi1-Δ* mutants suggests that there could be a potential decrease in the effectiveness of the antifungal treatment (Martin et al., 1999; Sanz et al., 2017).

It was hypothesized that after the introduction of antifungal treatment, *smi1-Δ* mutant cells would have higher chitin levels and exhibit lower cell death compared to the WT-a strain. Thus, it was predicted that *smi1-Δ* mutants should be more tolerant of antifungal treatment than the WT-a strain. To test this hypothesis, the effect of imidazole treatment on cell death and chitin levels in the WT-a and *smi1-Δ* cells was measured. First, an appropriate dosage and incubation time of imidazole antifungal was selected by testing a range of dose concentrations and measuring decay in absorbance using spectrophotometry readings taken over time. Once a dosage and incubation time was chosen, cells were exposed to imidazole, and stained with trypan blue, and the cell death percentage was determined by counting live and dead cells using hemocytometry. Finally, to examine the effects of imidazole on chitin accumulation, cells were dosed with imidazole, stained with Calcofluor white, and chitin visualized with fluorescence microscopy.

Methods

All materials and equipment were provided by the BIOL 340 laboratory (Moussavi 2023).

Preparation of cell cultures

Yeast extract-peptone-dextrose (YPD) media preparation and the inoculation and growth of liquid cell cultures for all experiments were conducted as detailed by Moussavi (2023). Cells received YPD 24 hours post-inoculation and incubated at 30 °C for 90 minutes using an Excella E24 Incubator Shaker to reach the stationary phase prior to all experiments.

Imidazole sample preparation

Solid imidazole (ACROS, USA) was weighed and dH_2O diluted the antifungal to a stock concentration of 25,600 µM. The stock solution was stored at 4 °C and was serially diluted to the appropriate experimental working concentrations (40-12,800 µM) using dH_2O as required.

Synergy H1 preliminary imidazole dosing

To determine the appropriate imidazole concentration dosage and incubation time, separate WT-a cell samples received dosages of an equal volume of 0, 40, 80, 120, 160, or 12,800 µM of imidazole before aliquoting each sample into a 96-well plate, with 5 replicates per imidazole-dosed sample. For the preliminary experiment, a large concentration range was selected because an appropriate concentration to dose *S. cerevisiae* was not found in the literature. Three wells were dosed with each imidazole concentration sample as an absorbance blank. The Synergy H1 equipment was operated according to the Synergy H1 Operator's Manual **(**BioTek, USA).

The spectrophotometer recorded the absorbances at 600 nm every 30 minutes at 30°C overnight with medium-speed shaking. The data was recorded in an Excel spreadsheet to study the rates of absorbance change to select an appropriate dosing concentration. A detailed schematic of the experimental procedure is included in Appendix A.

Hemocytometry determination of cell death proportion

To study the proportion of dead to live cells with antifungal treatment, WT-a and *smi1-Δ S. cerevisiae* strain liquid cultures received dosages of 0 (control), 120 and 12,800 µM imidazole and were incubated in the shaking incubator for 90 minutes at 30 °C. 5X of the cell culture volume of 0.4% trypan blue (Bio-Rad, USA) was added to each sample. Hemocytometry was conducted according to Moussavi (2023). Five replicates of each sample were counted. Live and dead cells were counted using the 40X objective lens to visualize trypan blue, which penetrates dead cells with compromised membranes to stain them (Chan et al., 2020). Excel was used to conduct ANOVA and two-sample t-test statistical analyses of the dead cell proportion between the strains and GraphPad was used to graph the results. A two-sample t-test was used because some replicates were omitted due to a cell count of fewer than 30 cells in some of the hemocytometer squares, and thus a paired t-test could not be performed. A detailed schematic of the experimental procedure is included in Appendix B.

Fluorescence microscopy study of chitin levels

To quantify the amount of chitin production with antifungal treatment between WT-a and *smi1-Δ S. cerevisiae,* cultures received dosages of 0 (control), 120 and 12,800 µM imidazole and were incubated in the shaking incubator for 90 minutes at 30 °C. Samples were prepared according to Moussavi (2023), and cells were resuspended in 100 µL PBS before staining with 0.1X the sample volume of Calcofluor white (CFW), which binds to and stains chitin in the cell wall (Ram & Klis, 2006). Three aliquots of each sample were visualized, and two images were taken per aliquot using a 100x oil immersion lens on a fluorescence microscope. ImageJ was used to quantify chitin fluorescence intensity by selecting 10 randomly selected cells from each image and background correcting the images to control for variation in exposure. Excel was used to conduct ANOVA and paired t-tests to determine the significance of the results. Results were graphed using GraphPad. A detailed schematic of the experimental procedure is included in Appendix C.

Results

Preliminary imidazole analysis indicates 120 and 12,800 µM as appropriate dosages

Spectrophotometry was used to determine the appropriate dosage and incubation period of imidazole antifungal treatment that results in a significant reduction of WT-a *S. cerevisiae* cell growth. Absorbances were recorded every 30 minutes for 20 hours, then were analyzed and the results were displayed graphically to examine the changes in cell density over time for each imidazole dosage between 0-12,800 µM (Figure 2). Because there was little change in absorbance after 2 hours, and a much longer incubation period would not be feasible for the experiment due to time constraints, absorbance changes in the first 2.5 hours were analyzed in more detail.

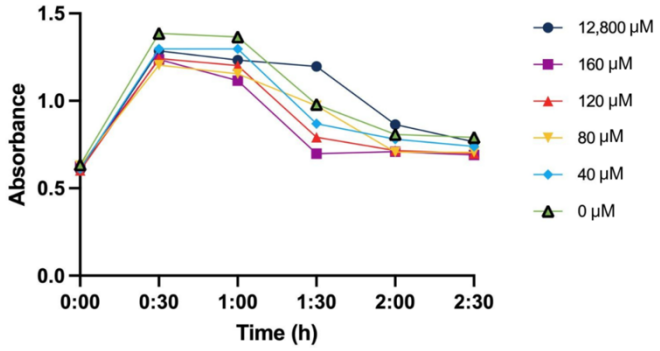

Figure 2. Preliminary experiment to determine the appropriate antifungal dosage and incubation time for future experiments, using spectrophotometry detailing absorbance over time (h) for imidazole doses of 0, 40, 80, 120, 160, and 12,800 µM in WT-a strain *S. cerevisiae* cells. Each treatment group had 5 technical replicates (n=5).

Figure 2 demonstrates that between 1-1.5 hours all samples showed a decreasing absorbance of varying rates, indicating that there was a decrease in

culture growth and an increase in cell death during this period. Thus, 90 minutes was selected as the appropriate incubation period post-antifungal dosing for later studies. The slopes, indicating a change in absorbance over time, were calculated for each sample between 1-1.5 hours and compared to select an appropriate dosage concentration for future experiments. Within this period, samples dosed with 0, 40, 120 and 160 µM showed very similar slopes, however, the highest slope was -0.82 absorbance/h for the 120 µM-dosed samples. The control sample with no imidazole addition had the smallest slope of -0.78 absorbance/h. Both of these slopes were fairly similar, indicating that there may have been some other factor influencing the growth of these cells in the spectrophotometer, however, dosage at 120 µM further reduced the absorbance.

When comparing the absorbance of the 0 µM and 120 µM samples at 0.5 and 1 hour, the absorbance is much lower with the 120 µM sample, indicating that while the slopes are fairly similar between 1-1.5 hours, the cells in the 120 µM sample did not proliferate as much (Figure 2). The sample with the highest dose of imidazole, 12,800 µM, had the smallest slope during 1-1.5 hours of -0.07 absorbance/h. However, despite showing a lower reduction of growth between 1-1.5 hours, the cells in this sample did not proliferate as much as the 0 µM control initially, which could be due to the effects of the antifungal treatment on cell growth. The greatest slope for the 12,800 µM sample decreases between 1.5-2 hours. The 80 µM samples had a similar curve with a steeper decrease in absorbance between 1.5-2 hours, as shown in the 12,800 µM sample.

The 120 µM sample was selected as the intermediate imidazole dosage because it caused the largest decrease in absorbance in the shortest unit of time compared to all other tested concentrations. Because the highest dosage of 12,800 µM showed such a similar result to a lower-dosed sample, 80 µM, it was selected as another experimental concentration to test the effects of high antifungal dosing compared to lower dosing on cell death and chitin levels in *S. cerevisiae* cells.

Lower proportion of cell death in imidazole-treated *smi1-Δ* than in treated WT-a

Once appropriate imidazole dosages were determined, the tolerance of each strain to the antifungal treatment was measured by examining cell death proportions. The objective of this experiment was to determine the effect of imidazole on *smi1-Δ* mutant cells relative to WT-a cells.

Figure 3 shows the percentage of cell death for WT-a and *smi1-Δ* cells at 0, 120, and 12,800 µM. Figure 3B shows the results of one-way ANOVA tests that

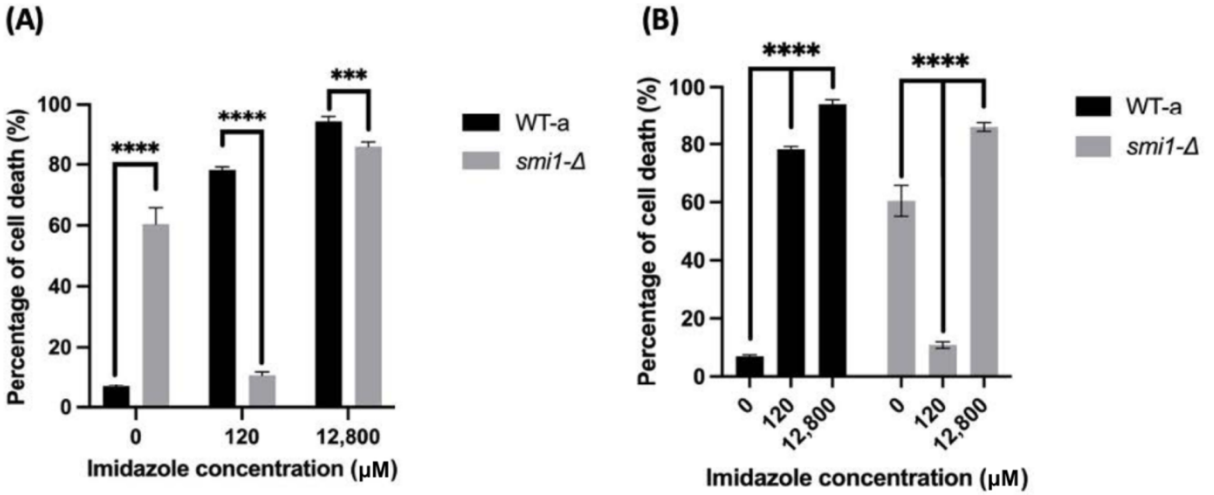

Figure 3. Percentage of cell death for WT-a and *smi1-Δ S. cerevisiae* cells after exposure to imidazole antifungal treatment. Cells were stained with trypan blue to visualize death and counted using hemocytometry. Each treatment group had five technical replicates with three 1 mm^2 squares counted for each (n=15) (*For 12,800 µM in the WT-a strain, n=10 due to <30 cells counted in 5 of the squares). **(A)** Two-sample t-test conducted between WT-a and *smi1-Δ* at each concentration (µM) of imidazole dose. Error bars are represented as ± SEM. **(B)** One-way ANOVA conducted on the WT-a group and *smi1-Δ* group comparing results from different imidazole concentrations (µM) with error bars represented as ± SEM. Significance levels indicated on the graph as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

were conducted on each strain to determine whether changes in imidazole concentration result in significant changes in cell death proportion. The ANOVA test for the WT-a group had a p-value of 3.28E-39 and the *smi1-Δ* group had a p-value of 3.32E-19. These p-values both indicate a statistically significant result and allow for the rejection of the null hypothesis that the mean cell death proportions are the same for each imidazole concentration. From the data presented in Figure 3B, there is evidence to show that changing the concentration of imidazole dosage results in a change in the percentage of cell death. This finding is important because it establishes that imidazole has an effect that results in cell death and that the different concentrations are different enough to show different levels of cell death.

Since the ANOVA tests within each strain displayed in Figure 3B shows significant results, it is a crucial next step to compare the percentage of cell death for WT-a and *smi1-Δ* cells at each of the three concentrations to determine whether there is a significant difference. A two-sample t-test was performed on the cell death proportion of WT-a and *smi1-Δ* for each concentration.

The two-sample t-test conducted on cells that were not dosed with imidazole (0 µM) presents a p-value of 8.89E-08. This p-value indicates a statistically significant result and allows for the rejection of the null hypothesis that the mean cell death proportions are the same for WT-a and *smi1-Δ* with no dose of imidazole. In Figure 3A it is seen that the *smi1-Δ* mutant cells experienced a much higher percentage of cell death at 60.45% than WT-a with 6.94% death. This finding is consistent with the expectation that under normal conditions, the *smi1-Δ* mutant cells have a weakened cell wall and challenges growing and reproducing thus incurring a higher percentage of cell death.

The two-sample t-test conducted on the cells dosed with 120 µM of imidazole has a p-value of 1.06E-27. This p-value indicates a statistically significant result and allows for the rejection of the null hypothesis that the mean cell death proportions are the same for WT-a and *smi1-Δ* at a dosing of 120 µM of imidazole. At the imidazole concentration of 120 µM, the *smi1-Δ* experienced a much lower percentage of cell death at 10.84% than the WT-a cells with 78.18% death (Figure 3A). This finding is consistent with the expectation that *smi1-Δ* mutant cells have a rescue mechanism in place that allows them to survive better than the WT-a cells while being exposed to an antifungal stressor.

The two-sample t-test conducted on the cells dosed with 12,800 µM of imidazole has a p-value of 0.00085. This p-value exemplifies a statistically significant result and allows for the rejection of the null hypothesis that the mean cell death proportions are the same for WT-a and *smi1-Δ* at a dosing of 12,800 µM of imidazole. The WT-a cells with a cell death percentage of 94.14%, experienced a more similar cell death percentage to the *smi1-Δ* cells at 85.85% death than at the other concentrations (Figure 3A). Since these death percentages are both relatively higher and much closer together, this result may suggest that this concentration is trending towards a lethal dosage for both strains.

smi1-Δ exhibits hyperaccumulation of chitin in the presence of an antifungal

Using the previously determined imidazole concentrations, chitin accumulation in each strain, when exposed to the antifungal treatment, was measured by visualization and quantification of calcofluor white stained cells. The effect of an antifungal stressor on *smi1-Δ* chitin levels has not been studied and therefore is a main objective of this experiment; in addition to the confirmation of background understanding of chitin levels in *smi1-Δ* mutants compared to wild-type strains.

The data (Figure 4A) suggest significant differences between WT-a and *smi1-Δ* groups in each antifungal treatment group, with asterisk brackets indicating p-values. Paired t-tests were conducted between WT-a and *smi1-Δ* mean fluorescence intensity at each concentration of imidazole, showing a p-value of 0.037204 at 0 µM, 0.000998 at 120 µM and 0.000375 at 12,800 µM. The significant p-values present in the imidazole-treated groups compared to the control group (0 µM imidazole) indicate that a greater difference in chitin levels is present

between WT-a and *smi1-Δ* cells when exposed to an antifungal.

Based on these paired t-tests (Figure 4A), the results are consistent with the hypothesis; with *smi1-Δ* presenting significantly higher chitin levels than WT-a cells at each treatment level of imidazole. The increased significance values from these t-tests in 120 µM and 12,800 µM imidazole treatment groups suggest further statistical analyses to uncover the relationship between antifungal exposure and chitin accumulation within both the WT-a and *smi1-Δ* strains.

When an additional stressor such as an antifungal treatment is added on top of the disrupted CWI pathway in *smi1-Δ* cells, chitin is predicted to hyper-accumulate in order to further compensate for this. The results from the paired t-tests shown in Figure 4A suggest this may be true, however, ANOVA tests were required to determine significance.

Secondary statistical tests were conducted on both WT-a and *smi1-Δ* groups (Figure 4B) in addition to the paired t-tests (Figure 4A) to determine if significant changes present themselves in each strain at different antifungal concentrations. From the graph (Figure 4B), increases in chitin are visible in the *smi1-Δ* group, with mean values jumping from 60.2202 at 0 µM, up to 77.4588 at 120 µM and 74.8976 at 12,800 µM. The ANOVA test conducted on the *smi1-Δ* fluorescence intensity data shows a

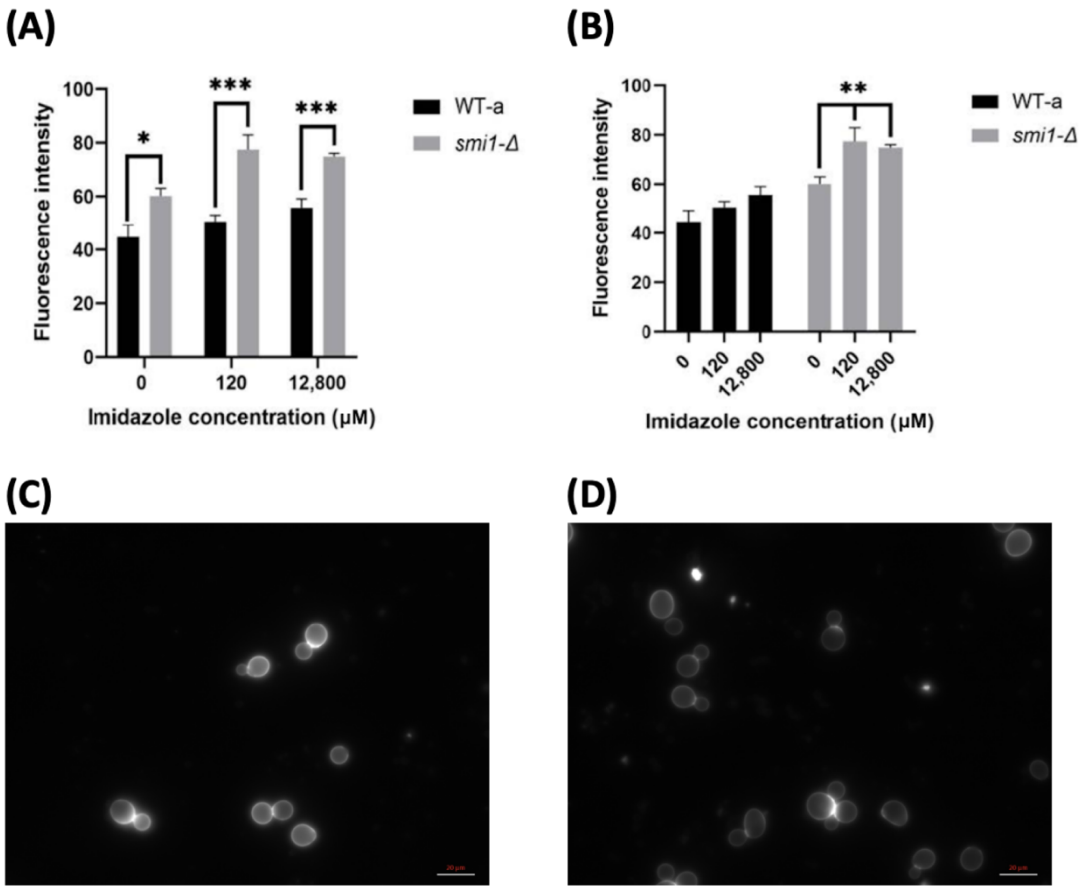

Figure 4. Effect of antifungal treatment on fluorescence intensity of chitin levels in WT-a and *smi1-Δ S. cerevisiae* cells. For (A) and (B) data are quantified using ImageJ and are represented as mean fluorescence intensity of Calcofluor White-stained cells (n=10) per image (n=5) taken with the fluorescence microscope. **(A)** Paired t-test conducted between WT-a and *smi1-Δ* at each concentration (µM) of imidazole dose. Error bars are represented as ± SEM. **(B)** One-way ANOVA conducted on the WT-a group and *smi1-Δ* group comparing results from different imidazole concentrations (µM) with error bars represented as ± SEM. Significance levels indicated on the graph as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Fluorescence microscopy of **(C)** *smi1-Δ* cells and **(D)** WT-a cells at an imidazole concentration of 120 µM with 100x lens.

p-value of 0.006495, therefore indicating these changes in chitin levels are highly significant.

The WT-a group shows a small increase in fluorescence intensity as the antifungal concentration increases, with mean values of 44.6698 at 0 µM, 50.6512 at 120 µM, and 55.7940 at 12,800 µM. The ANOVA test conducted on the *smi1-Δ* fluorescence intensity data (Figure 4B) shows a highly significant p-value of 0.006495. The WT-a ANOVA test presents a p-value of 0.077988, therefore indicating that these increases in chitin levels are insignificant.

These results indicate that significant increases in chitin production occur in response to the antifungal treatment in *smi1-Δ* cells, while the WT-a cells exhibit no significant change in chitin production in response to the antifungal treatment, even at extreme concentrations.

Discussion

Three experiments were conducted to examine WT-a and *smi1-Δ* responses to antifungal treatment in terms of lethality and the chitin cell wall. Literature review demonstrated that the relationship between the *smi1-Δ* mutant and antifungal dosing had not yet been investigated. Using spectrophotometry, it was determined that 120 and 12,800 µM were appropriate imidazole dosages to trial on the *smi1-Δ* mutant cells. Hemocytometry with a focus on the proportion of cell death showed that WT-a cells were significantly less tolerant to imidazole dosing than *smi1-Δ*. Fluorescence microscopy and CFW staining indicated that *smi1-Δ* had higher chitin levels in all conditions and a hyper-accumulation of chitin with imidazole dosing compared to WT-a. Together, these results signify that *smi1-Δ* is more tolerant of antifungal treatment than the WT-a cells and that the chitin accumulation may play a role in this mechanism. These results were as predicted and support the proposed hypothesis, that antifungal-treated *smi1-Δ* would elicit a more tolerant response to the treatment with a reduced proportion of dead cells and increased chitin deposition, as a potential compensatory mechanism to the stressor that has been proposed in previous literature (García et al., 2017; Ohno, 2007). However, the relationship between chitin levels and cell death has not been elucidated in the context of this study and is required to fully understand this mechanism to draw more decisive conclusions about compensatory pathways in *S. cerevisiae*. A summary of the key study findings is indicated in Figure 5.

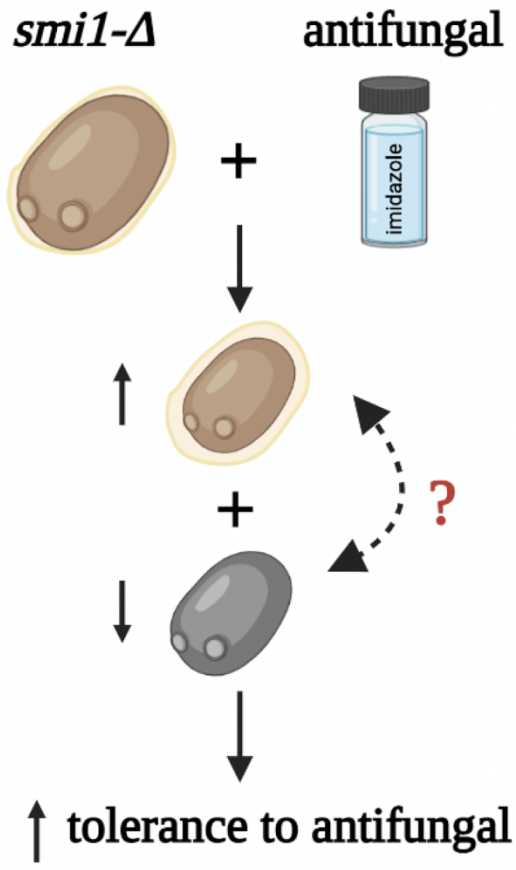

Figure 5. Summary of key findings. The thickness of the yellow border around the cell indicates the chitin level, and the grey cell symbolizes cell death. The missing link in the results is indicated using a dotted line and red question mark and is a future direction of study. Created using BioRender.

The study of cell death in *smi1-Δ* cells in relation to imidazole exposure has not been studied specifically in previous research, however, the results are in agreeance with literature proposing the role of compensatory mechanisms in *smi1-Δ* cells, as well as the role the cell wall plays in cell growth and reproduction (Levin, 2005; Sanz et al., 2017). Under normal conditions (0 µM), WT-a cells exhibit significantly lower levels of cell death than *smi1-Δ*, speculated to be due to the presence of the *SMI1* gene and fully functioning CWI pathway.

When the mid-range dosage (120 µM) of antifungal is present, the results differ from the 0 uM dosing due to the compensatory mechanism proposed to be functioning in the *smi1-Δ* mutant as a result of their damaged cell walls (García et al., 2017; Ohno, 2007). This compensatory mechanism is not at play in the WT-a cells, as they have a functioning CWI pathway, and are not experiencing the same levels of initial stress to the cell wall as the *smi1-Δ* cells. This lack of stress to the cell wall in WT-a cells is proposed to actually lead to higher cell death in comparison because of the missing compensatory mechanism seen in *smi1-Δ* cells.

The extreme dosage of imidazole (12,800 µM) used in this study indicates increased levels of cell death in both strains. This may be due to the presence of such a strong dosage overriding any compensatory mechanisms at play in the *smi1-Δ* cells, however, future experimentation is required to determine if this is the case.

The focus on chitin accumulation and production in this study strengthens the background understanding that *smi1-Δ* cells hyper-accumulate chitin due to their disrupted CWI pathway and loss of regulatory chitin deposition mechanisms (Martin et al., 1999; Sanz et al., 2017). This pattern is prevalent in the control group of this study (0 µM imidazole) and further extends to *smi1-Δ* cells treated with 120 µM and 12,800 µM imidazole that exhibit significantly increased chitin levels compared to WT-a cells at the same antifungal concentrations.

The significant p-value from the *smi1-Δ* ANOVA group, paired with the data presenting large increases in mean fluorescence intensities when the antifungal treatment is present, leads to the speculation that the missing role of the SMI1 gene and the functionality of the CWI is a factor at play, as the changes are not seen in WT-a cells of the same treatments. Due to previous research finding yeast cells to accumulate chitin as a compensatory mechanism to weakened cell walls, the results suggest that this increase in chitin in *smi1-Δ* cells may, on top of the already elevated chitin levels, be due to the *smi1-Δ* cells hyperaccumulating chitin in response to the additional stress on their damaged cell walls from their disrupted CWI pathway (García et al., 2017; Ohno, 2007).

Under normal conditions (0 µM imidazole), the *smi1-Δ* cells exhibit higher levels of chitin compared to the wild type, however, simultaneously experience increased rates of cell death. The increased rates of cell death are speculated to be due to the weakened cell wall, which leads to growth and reproduction challenges when dysfunctional (Levin, 2005). In comparison to the WT-a strain with functional cell walls, these higher rates of cell death are expected because the WT-a strain is fully functioning and not undergoing any stress in this environment. When an antifungal stressor is present, the data yield different results because the WT-a strain is now undergoing stress and lacks the compensatory mechanism proposed in the *smi1-Δ* strain (Sanz et al., 2019).

The combination of significant relatively low cell death levels, and increased chitin levels at 120 µM imidazole treatment suggests the proposed rescue mechanism in prior literature may increase tolerance to antifungal stressors in *smi1-Δ* cells (García et al., 2017; Ohno, 2007; Sanz et al., 2019). At 12,800 µM, the same pattern of increased chitin is visible in smi1 cells but not WT-a, however, with cell death levels of over 80% seen in both. This may be due to the extreme dosage of imidazole having a lethal effect on both strains, regardless of the rescue mechanisms at play in the *smi1-Δ* cells.

Future studies conducted would preferably use an antifungal treatment that specifically targets components of the cell wall such as mannoproteins, beta-glucans or chitin (Basmaji et al., 2006). The use of one of these antifungal treatments may have heightened effects on the chitin production in *smi1-Δ* mutants seen in this study, as well as increased production in WT-a cells. For WT-a cells, the targeted antifungal could create direct stress on the cell wall which may cause enough damage to their functioning CWI to stimulate the compensatory mechanism of chitin hyperaccumulation; a response that was not seen in this study. An antifungal treatment that directly targets chitin production could be used to combat the linkage found in this study between decreased cell death and increased chitin levels in

smi1-Δ cells, as it would inhibit the compensatory mechanism discussed in this paper, and specifically target this mutation (Sanz et al., 2017).

Another limitation of this study is the method of chitin intensity quantification using ImageJ. Ten random cells from each image were selected for quantification of fluorescence intensity, however, this random selection process could introduce possible human bias when determining which cells to count. For example, brighter or better-defined cells could be selected over dimmer, less-well-defined cells, thus skewing the results.

The use of hemocytometry as an experimental strategy was a key limitation of the study. This procedure is time-consuming and laborious, which minimizes the volume of samples that can be analyzed. The method relies on manual counting, which can introduce human error and may result in a skewed proportion of dead cells when comparing samples. The trypan blue staining was not visible at a lower objective lens, so the 40X lens was used to visualize the stained dead cells. This introduced the potential for more error because the whole hemocytometer counting surface was not visible, and the lens had to be moved to count in all squares, signifying the possibility that some cells may have been miscounted.

Despite being a very common dye, the trypan blue stain used to identify dead cells has some limitations to its use that may have affected the study results, including a short viable counting period before cell lethality, which can be as short as within 5 minutes post-exposure to the stain (Chan et al., 2020). Particularly because hemocytometry is such a time-consuming process, if the trypan blue stain were to kill the cells, the calculated proportion of dead cells would be misrepresented. However, because the results of cell death proportion were all statistically significant (Figure 3), the dead cells are likely not affected enough by the trypan blue stain to skew the results. Another important thing to note is the Chan et al. (2020) study was on animal cells which do not have a cell wall like yeast, thus trypan blue may have differing effects.

Hemocytometry cell counts can show human bias due to misidentified particles and subjectivity during the counts, and these preceding issues can be reduced with the usage of equipment such as a flow cytometer (Peniuk et al., 2015). The dilutions required to count with a hemocytometer can also introduce errors due to a misrepresented sample concentration (Peniuk et al., 2015). Flow cytometry is a reliable, higher-throughput method that would not require manual counting that may introduce error (Peniuk et al., 2015).

A future direction of this study is to employ flow cytometry to count the cell death proportion in a much wider range of imidazole antifungal dosing concentrations to greater understand yeast lethality, which was found at the highest dosage of 12,800 µM imidazole in both WT-a and *smi1-Δ*. This would aid in the understanding of the limitations of the proposed stressor-induced compensatory mechanism that is pronounced in *smi1-Δ*. This would allow for the understanding of the concentrations where the imidazole dosage changes from mutant-insensitive to lethal to make more precise conclusions about this mechanism and its functionality.

The greatest limitation of this study is the undefined mechanistic relationship between chitin levels and cell death (Figure 5). This study determined that there was a pronounced increase in chitin and a decrease in cell death in *smi1-Δ* with antifungal stressor treatment compared to WT-a. The *smi1-Δ* mutant shows a decrease in cell wall mannoproteins, contributing to cell wall weakness (Basmaji et al., 2006). Previous literature has suggested that stressors (ex. heat, EtOH) induce the loss of cell wall proteins such as 1,3-Beta-glucan synthase and mannoproteins, and this causes an increase in chitin as a compensatory mechanism (Martin-Yken et al., 2016; García et al., 2017; Ohno, 2007). Until now, this had not been studied in *smi1-Δ* with antifungal stress.

It is speculated that the results found in this study, that antifungal addition to *smi1-Δ* causes increased chitin and reduced cell death to improve stressor tolerance, may occur because of the reduction in cell wall proteins. To study this, WT-a and *smi1-Δ* should be imidazole-dosed and a Western blot

should be conducted to quantify whether there is a change in 1,3-Beta-glucan synthase and mannoproteins that could correspond to the increase in chitin. To expand the understanding of this mechanism and further apply the results found to different mutant yeast, similar *S. cerevisiae* mutant strains with cell wall defects should be analyzed with similar methods to understand mutant tolerance to antifungals.

Conclusion

Understanding the mechanisms at play in yeast cells with cell wall mutations can provide insight into how to specifically target these strains when developing future antifungal treatments. Studying a range of antifungal concentrations to determine a dosage that proves to be lethal to cells, regardless of mutant compensatory mechanisms like the one seen in this study, could lead to the development of antifungal drugs with higher success rates, due to the increased understanding of the mutation and specificity of the treatments. These studies in turn can extend to the understanding of human fungal infections with cell wall mutations and aid in the development of highly specified drugs to combat these mutations.

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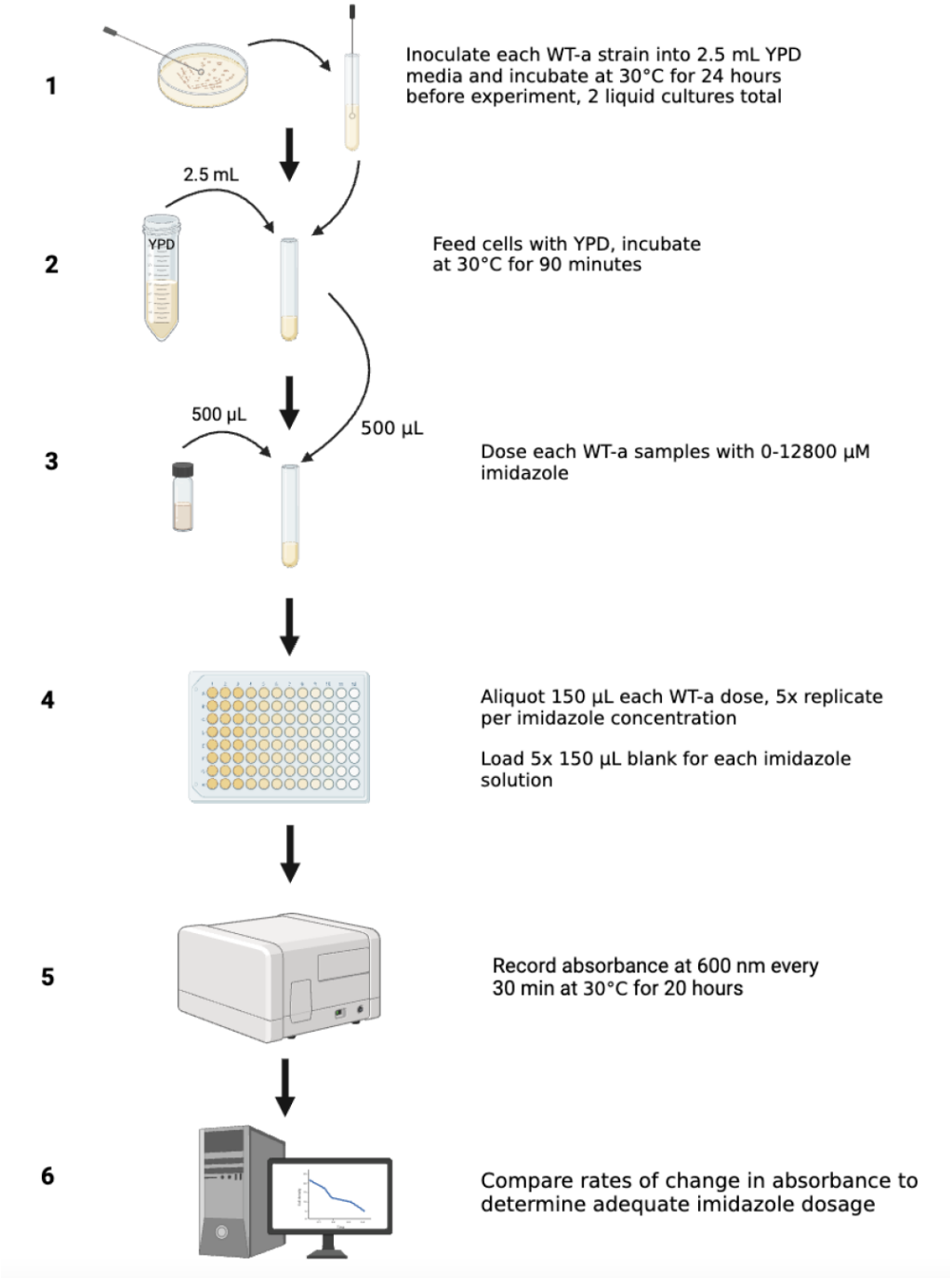
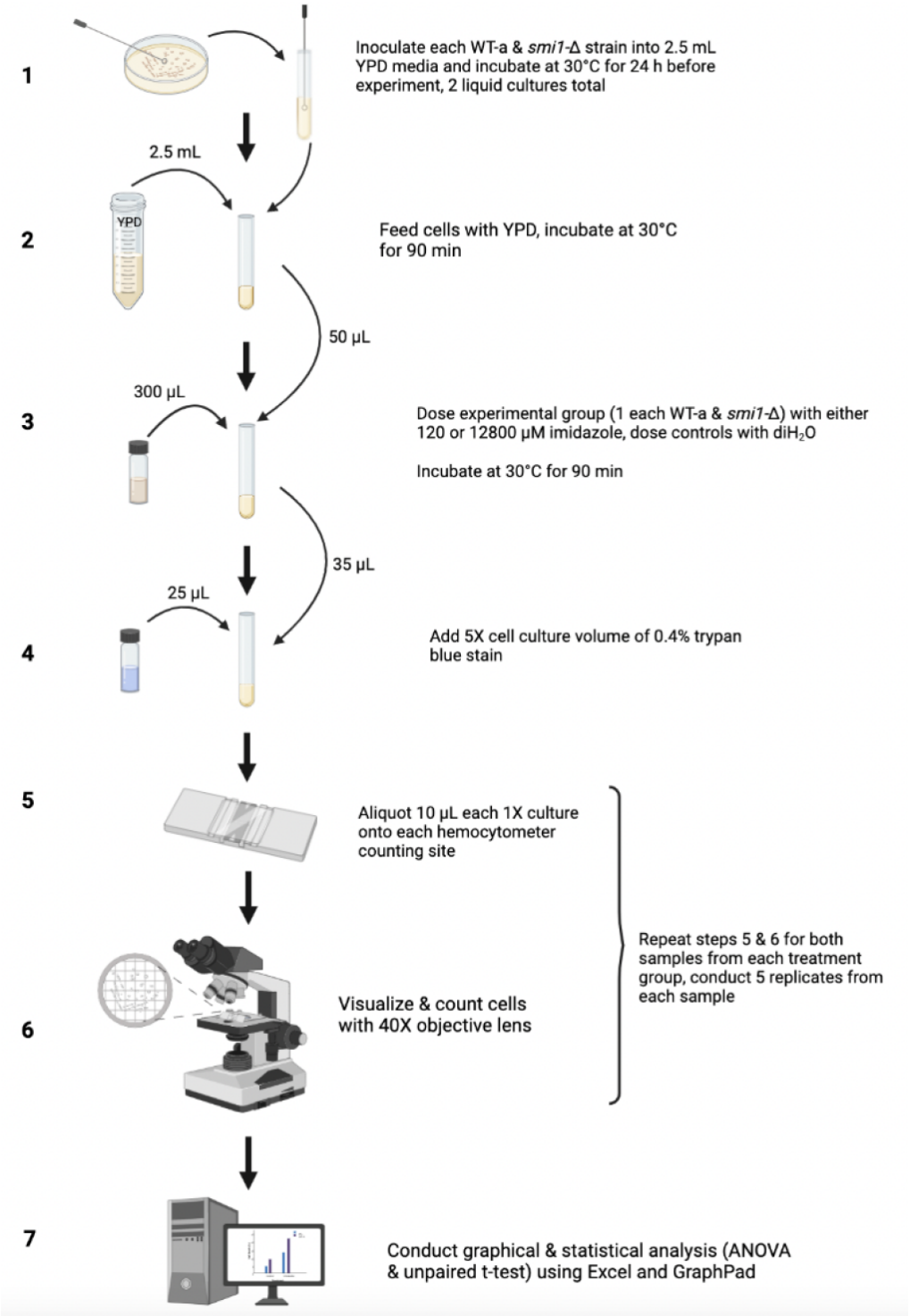
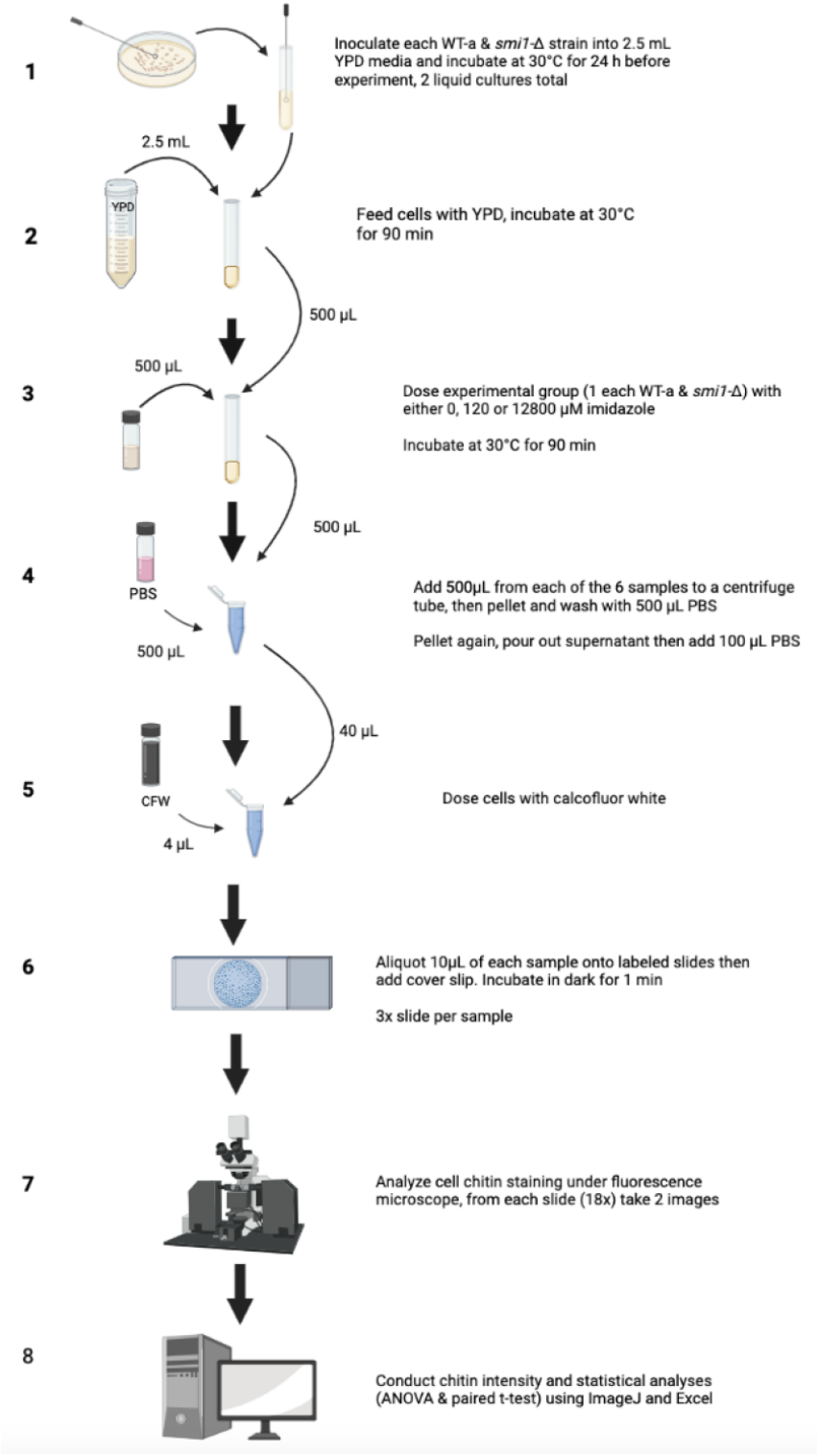
Appendix



Appendix A. Preliminary experiment to determine the appropriate imidazole dosage and incubation time using spectrophotometry in WT-a. Produced using BioRender.



Appendix B. Procedure of quantifying the cell death proportion in WT-a and *smi1-Δ* cells using trypan blue staining and hemocytometry. Produced using BioRender.



Appendix C. Procedure of quantifying the chitin fluorescence intensity in WT-a and *smi1-Δ* cells using CFW staining and fluorescence microscopy. Produced using BioRender.