A GENETIC SCREEN FOR HIGH-COPY SUPPRESSORS OF THE GROWTH DEFECT OF *SACCHAROMYCES CEREVISIAE SET1* NULL MUTANTS UNDER HISTIDINE STARVATION CONDITIONS

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ABSTRACT

A Genetic Screen for High-Copy Suppressors of the Growth Defect of *Saccharomyces cerevisiae* *set1* Null Mutants under Histidine Starvation Conditions

Previous research indicates that Set1 is the catalytically active protein in COMPASS, a protein methyltransferase complex associated with transcription in budding yeast cells. However, the mechanistic role that Set1 and COMPASS plays in the regulation of transcription remains poorly characterized. Current research in the Bryk lab indicates that mono-methylation of histone H3 on lysine 4 (K4) is required for 3-aminotriazole-induced transcription of the *HIS3* gene by RNA polymerase II (PolII). The research shows that yeast cells lacking a functional *SET1* gene (containing null alleles, either *set1Δ* or *set1-Y967A*) grow poorly on medium lacking histidine and containing 3-aminotriazole (3-AT). Overexpression screens are being performed to identify genes that suppress the growth defect of *set1* null mutants. Genes when over-expressed are expected to either bypass the need for Set1 or replace Set1 function through interaction with a non-functional Set1 complex. Studying genetic suppressors may uncover clues to the role of *SET1* in the Pol II transcription mechanism, providing new information on transcription, a ubiquitous vital process in prokaryotic and eukaryotic cells.

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NOMENCLATURE

H3K4	Histone 3 Lysine 4
3-AT	3-aminotriazole
URA	Uracil
TRP	Tryptophan
HIS	Histidine
LEU	Leucine
LEU - HIS	Leucine - Histidine

CHAPTER I

INTRODUCTION

The *SET1* gene encodes for the protein, Set1. Set1 is one of eight proteins that make up COMPASS, a large multiprotein complex (Shilatifard, 2012). Set1 is important for the formation and stability of COMPASS. Set1 and COMPASS play a role in both gene activation and repression (Margaritis et al., 2012). Set1/COMPASS regulates transcription through methylation of histone 3 lysine 4 (H3K4) (Takahashi & Shilatifard, 2010). H3K4 can be either mono-, di-, or tri-methylated by Set1/COMPASS. Histone methylation, such as H3K4 methylation, is associated with RNA polymerase II elongation (Tanny, 2014). H3K4 trimethylation occurs at the 5' end of genes while H3K4 mono- and di-methylation occur in the middle of the coding region (Rando, 2007). Set1 is also required for silencing of PolII transcription units in the ribosomal DNA locus of *S. cerevisiae* via histone 3 methylation (Bryk et al., 2002). The loss or reduction of the three types of H3K4 methylation is associated with a decrease in gene silencing at rDNA and telomeres (Mueller, Canze, & Bryk, 2006).

Preliminary data from the Bryk lab in collaboration with Dr. Shelley Pozzi showed that 3-aminotriazole, a herbicide that competitively inhibits the *S. cerevisiae HIS3* gene product, imidazoleglycerol-phosphate dehydratase (Kanazawa, Driscoll, & Struhl, 1988), prevents robust growth of yeast cells that lack functional Set1 histone methyltransferase. In the histidine anabolic pathway, imidazoleglycerol-phosphate dehydratase is the sixth enzyme in the pathway needed to make histidine, an amino acid required for cell survival (Sinha et al., 2004). In *set1* mutant cells, 3-aminotriazole causes histidine starvation. A protein that confers 3-aminotriazole

resistance is Atr1 (Kanazawa et al., 1988). Atr1 is a transmembrane efflux protein that helps to pump 3-aminotriazole out of the cell, reducing the intracellular concentration, allowing imidazoleglycerol-phosphate dehydratase to function properly. Previous research in the Bryk lab showed that *set1* null mutants, including *set1*Δ cells and *set1*-Y967A cells, exhibit growth defects when plated on medium lacking histidine with 3-aminotriazole. If a deletion allele of *ATR1* (*atr1*Δ) is introduced into these cells, the growth defects are exacerbated.

The goal of this project is to identify suppressor mutations that make *set1* null mutants resistant to histidine starvation in the presence of 3-aminotriazole. After transformation of a high-copy yeast gene library into *set1*Δ and *set1*-Y967A cells, screens for suppressors of the growth defect will be performed. The *set1*Δ cells do not contain the *SET1* gene and do not produced Set1 protein; therefore, COMPASS does not form. A bypass suppressor screen will be performed using the *set1*Δ cells. In bypass suppression, an alternative pathway to the Set1/COMPASS pathway would rescue the cell's growth defect (S. L. Forsburg, 2001). The *set1*-Y967A cells are mutants that form a defective Set1 protein that has no detectable methylation activity (Williamson et al., 2013). Preliminary evidence from the Bryk lab suggests that in these mutants an inactive COMPASS complex forms that is unable to mono-, di, or tri- methylate lysine 4 of histone H3. An interaction suppressor screen will be performed using the *set1*-Y967A cells. In interaction suppression, overexpression of a gene restores the interaction between effectors required for *HIS3* transcription to rescue cell growth on medium lacking histidine with 3-aminotriazole (S. L. Forsburg, 2001).

CHAPTER II

METHODS

Media Preparation

Yeast strains were cultured on various types of media, depending on the selection required. Plates used include i) SC – Uracil (URA), ii) SC – Tryptophan (TRP), iii) SC – Histidine (HIS), and iv) SC – Leucine -Histidine (-LEU-HIS). All plates were made using the same recipe: 2 g of SC dropout mix, 1.45 g of Yeast Nitrogen base, 5 g of ammonium sulfate, and 20 g of bactoagar per 1 Liter of media. All components were dissolved and then autoclaved. Following autoclaving and stirring for 30 minutes, 50 mL of 40% glucose was added to a final concentration of 2%. The SC-HIS and the SC-LEU-HIS plates also had 3-AT added to them. Concentrations of 3-AT used include 2.5 mM, 5 mM, 10 mM, 15 mM, and 20 mM. For plates containing 3-AT, a 1M stock of 3-AT was used.

Broth used included $YPAD_{TU}$ and $2xYPAD_{TU}$. The $YPAD_{TU}$ broth was made as follows: 10 g of Yeast Extract, 20 g of peptone, and 40 mg of adenine hemisulfate per 1 Liter of broth. All components were dissolved and then autoclaved. Following autoclaving and stirring for 30 minutes, 50 mL of 40% glucose was added to a final concentration of 2%, as well as 10 mL of 20 mM uracil, and 10 mL of 40 mM tryptophan were added. The $2xYPAD_{TU}$ broth was made as follows: 20 g of Yeast Extract, 40 g of peptone, 80 mg of adenine hemisulfate, and 40 g of dextrose per 1 Liter of broth. All components were dissolved and then autoclaved. Following autoclaving and stirring for 30 minutes, 20 mL of 20 mM uracil, and 20 mL of 40 mM tryptophan were added.

Bacterial strains were cultured on LB + Kanamycin plates (10 g tryptone, 5 g Yeast Extract, 10 g NaCl, 15 g of agar per L). All components were dissolved in water and autoclaved. Following autoclaving and stirring while cooling for 30 min, 0.05 g of Kanamycin was added. Bacterial strains were also cultured on SOC broth (20 g tryptone, 5 g Yeast Extract, 0.5 g NaCl per L). All components were allowed to dissolve in water and 10 mL of 250 mM KCl was added. The pH was adjusted to 7.0 using 5 N NaOH and autoclaved. After allowing the broth to cool, 5 mL of 2 M $MgCl_2$ and 20 mL of 1 M glucose (final concentration of 20 mM) were added.

Table 1. Genotypes of Yeast Strains used in this study.

MBY3044	*MAT**a** leuΔ1 ura3-52 trp1Δ63 set1Δ::KANMX4 lys2-128δ*
MBY3080	*MAT**a** leuΔ1 ura3-52::pRS406 trp1Δ63 set1Δ::KANMX4 lys2-128δ atr1Δ::TRP1*
MBY3081	*MAT**a** leuΔ1 ura3-52::pRS406-set1-Y967A trp1Δ63 set1Δ::KANMX4 lys2-128δ atr1Δ::TRP1*
MBY3107	*MAT**a** leuΔ1 ura3-52::pRS406 trp1Δ63 set1Δ::KANMX4 lys2-128δ [pRS425 LEU2-2μ]*
MBY3108	*MAT**a** leuΔ1 ura3-52::pRS406 trp1Δ63 set1Δ::KANMX4 lys2-128δ atr1Δ::TRP1 [pRS425 LEU2 2μ]*
MBY3109	*MAT**a** leuΔ1 ura3-52::pRS406-set1-Y967A trp1Δ63 set1Δ::KANMX4 lys2-128δ [pRS425 LEU2 2μ]*
MBY3110	*MAT**a** leuΔ1 ura3-52::pRS406-set1-Y967A trp1Δ63 set1Δ::KANMX4 lys2-128δ atr1Δ::TRP1 #25 [pRS425 LEU2 2μ]*
MBY3111	*MAT alpha leu2Δ1 lys2-128δ ura3-52::pRS406-SET1 trp1Δ63 set1Δ::TRP1 [pRS425 LEU2 2 micron] isolate #1*
MBY3113	*MAT**a** leuΔ1 ura3-52::pRS406-SET1 trp1Δ63 set1Δ::KANMX4 atr1Δ::TRP1 lys2-128δ [pRS425 LEU2 2 μ] isolate #1*

Yeast Strain Construction

Table 1 lists strains that were created and used for this study. Strains were created from MBY3044, a *set1Δ* strain. The *atr1::TRP1* fragment was created from the plasmid template pRS404 using PCR. The reaction required 0.5 µL of 200 µM oligonucleotides OM1193 (5'-AAAGAGAGGCAATTAGAGAATCTCAAACAGGTAATAATACTGTGCGGTATTTCACACCG-3') and OM1194 (5'-GGTGTATTTCTATCTATTTACCTTAATAACCGCTTTCCAGATTGTACTGAGAGTGCAC-3'). In addition, 5 µL of 10x PCR buffer, 2.5 µL of $MgCl_2$, 1.0 µL of 10 mM dNTPs, 0.5 µL of Taq DNA polymerase, and 39 µL of sterile milliQ H_2O (reverse osmosis purified water, mqH_2O) for each sample. The PCR machine ran 30 cycles. Plasmids, either empty vector (pRS406) or *set1-Y967A* (pRS406-*set1-Y967A*), were linearized with *StuI* and transformed into yeast cells for integration into the *ura3-52* locus.

Both the *atr1Δ::TRP1* fragment and the *Stu*I-linearized plasmids were integrated into the genome in one step, using the lithium acetate transformation method. MBY3044 cells were added to 3 mL of $YPAD_{TU}$ and placed in a rotating wheel in a 30°C incubator. The following day, 1.2 mL of culture was inoculated into 60 mL of $YPAD_{TU}$ and allowed to shake at 225 rpm in a 30°C incubator for four hours. After four hours, the early log-phase culture (1-2 $x10^7$ cells/mL) was transferred to a 50 mL conical tube and pelleted in a centrifuge at 2,000 rpm for 3 min at 4°C. The media was decanted and cells were washed by resuspension in 10 mL of sterile mqH_2O. The cells were pelleted in the centrifuge at 2000 rpm for 3 min at 4°C. The water was decanted and the cells were resuspended in the residual water. A volume of 500 µL of 0.1M LiAc/1xTE solution was added and the cells were transferred to a microfuge tube. The tube was place in a microcentrifuge and spun for 2 minutes at 5,000 rpm and the solution was removed using a

pipette. The pellet was gently resuspended in 230 µL of 0.1M LiAc/1xTE solution, to raise the volume to a total volume of 300 µL. Boiled carrier DNA (12.9 µL of 11.7 µg/µL solution) was added to the cell solution. Cell and carrier DNA solution was aliquoted into three microfuge tubes (100 µL per aliquot). For the empty vector (pRS406) and *set1::Y967A* transformation, aliquots include one no-DNA control, one for the *Stu*I-linearized empty vector (pRS406), and one for *Stu*I-linearized pRS406-*set1-Y967A* vector. For the *atr1Δ::TRP1* transformation, aliquots were made to include one no-DNA control and two for the *atr1Δ::TRP1* fragment. To the tubes that receive transforming DNA, 10 µg of the appropriate linearized vector was added and mixed. All tubes were incubated for 30 min at 30°C to allow for integration. Following incubation, 700 µL 0.1 M LiAc/1x TE/40% PEG 3350 was added to each tube. Following another incubation for 30 min at 30°C, cells were heat shocked for 15 minutes in a 42°C water bath. Sterile mqH_2O (500µL) was added to each tube and gently mixed. Following a 10 sec 13K rpm spin in the microcentrifuge, the liquid was removed using a pipette. Cells were resuspended in 1 mL of sterile mqH_2O and pelleted for 10 sec at 13K rpm. All but 100 µL of the water was removed and the cells were resuspended. For the *atr1::TRP1* transformation, cells were plated on SC-TRP agar. For the empty vector (pRS406) and *set1-Y967A* transformation, cells were plated on SC-URA agar. All plates were incubated at 30°C for 2-3 days.

Genomic DNA was extracted following the protocol described by Hoffman and Winston (Hoffman & Winston, 1987). Using this genomic DNA, the empty vector (pRS406) and *set1-Y967A* transformants were verified by *Bam*HI linearization and Southern blot analysis, as previously described by Southern (Southern, 2006). The *atr1::TRP1* transformants were verified by PCR amplification. The two oligonucleotides used were OM1195 (5'-

CGTCTGAAGAATGAGACG-3') and OM1196 (5'- CTGGTTACAGTTCAAGGC-3'). Following amplification, the product was run on a 1% agarose/1x TEA gel (1.5 g agarose per 150 mL of 1x TEA). In order to visualize the DNA, 15 µL of ethidium bromide (10 mg/mL) was added to the molten gel. Each lane was loaded with 20 µL of sample and ran at 85 V for 1.5 hrs.

Library Transformation of Yeast

Cells [*set1*Δ (MBY3080 with pRS406) or *set1-Y967A* (MBY3081 with pRS406-set1-Y967A)] were transformed using a high-copy *Saccharomyces cerevisiae* genomic DNA plasmid library carrying a *LEU2*-selectable marker (Jones et al., 2008). For the bypass suppressor screen, MBY3080 was transformed with the library. For the interaction suppressor screen, MBY3081 was transformed with the library. The transformation procedure was performed using a high-efficiency transformation protocol, with a few modifications (Gietz, 2014). Cells were added to 3 mL of $YPAD_{TU}$ and placed in a rotating wheel in a 30°C incubator. The following day, 1.2 mL of the overnight culture was inoculated into 50 mL of $2xYPAD_{TU}$ shaking at 225 rpm at 30°C four hours. After four hours, the culture was transferred to a 50 mL conical tube and cells were pelleted at 2,000 rpm for 5 min at 4°C. The media was decanted and cells were washed with 25 mL of sterile mqH_2O. The pellet was resuspended by vortexing and the cells were pelleted in the centrifuge at 2,000 rpm for 3 min at 4°C. The water was decanted. The pellet was resuspended in 1 mL of sterile mqH_2O and transferred to a microfuge tube. The tube was spun for 10 sec at full speed in a microcentrifuge and the water was decanted. Sterile mqH_2O was added to the pellet to increase the total volume to 0.5 mL. Following resuspension, 100 µL of cell solution were aliquoted to 4 microfuge tubes, one for a no-DNA control and three tubes for library DNA. After pelleting the cells and decanting the water, 360 µL of transformation mix was added to

each tube. A transformation mixture with 240 µL of 50% PEG 3500, 36 µL of 1 M lithium acetate, 7.6 µL of boiled carrier DNA (13.24 µg/µL), and 68 µL of sterile mqH_2O was added to each tube. To the tubes with cells for transformation with the library DNA, 2 µL of the genomic DNA plasmid library was added. After mixing, tubes were incubated for 30 min in a 30°C incubator and then 30 min on a roller in a 30°C incubator. Following incubation, 36 µL of 10% DMSO was added to each tube and after gentle mixing, the cells were heat shocked in a 42°C water bath for 15 minutes. The cells were pelleted by centrifugation, the transformation mix was decanted, and 1 mL of sterile mqH_2O was added to each tube. For the no DNA control sample, 100 µL of solution was spread onto a SC-Leu- His plate. For the library transformation samples, 100 µL of cell solution was spread onto a single SC-Leu-His agar plate and the remaining solution was spread equally (100 µL per plate) onto nine SC- Leu- His + 10 mM 3AT agar plates. Plates were placed in a 30°C incubator for five days. After five days, colonies on the SC-Leu-His + 10 mM 3AT were single-colony purified on SC- Leu agar plates and incubated for two days in a 30°C incubator. After single-colony purification, one colony was selected and patched onto a SC- Leu plate and incubated at 30°C. The plates with the patches were stored at 4°C for use for the growth assays described below.

Growth Assays

Growth assays were performed in order to i) determine the correct concentration of 3-AT to induce growth defects using MBY3101, MBY3108, MBY3111, and MBY3113 cells and ii) to examine the ability of transformants to suppress the growth defects of the *set1* null mutants (*set1Δ* and *set1-Y967A*). In order to do this, five-fold serial dilutions were made and spotted on agar plates as described below for each growth assay. To verify the suppression phenotype of

high-copy library suppressor candidates, cells from each candidate were used to inoculate separate tubes containing 3 mL of $YPAD_{TU}$ and left to grow overnight on a rotating wheel in a 30°C incubator. The following day, in a 96-well plate, 160 µL of sterile mqH_2O was added to each well. For each suppressor candidate, 40 µL of culture from the tube was added to the first well. After gently mixing, 40 µL of solution was then transferred into the second well. This was continued until there were six or eight dilutions for each culture. For the test to determine the correct concentration of 3-AT to induce growth defects using MBY3101, MBY3108, MBY3111, and MBY3113 cells, 5 µL of cell dilutions 3-8 were then spotted onto SC- His, SC- His + 2.5 mM 3AT, SC- His + 5 mM 3AT, SC- His + 10 mM 3AT, and SC- His + 15 mM 3AT. For the growth assays to verify the suppression phenotype of high-copy library suppressor candidates, 5 µL of dilutions 1-6 were spotted into SC- Leu- His, SC- Leu- His + 10 mM 3AT, and SC- Leu- His + 20mM 3AT. The plates were incubated for 2-5 days in a 30°C incubator. For the 3-AT sensitivity assay, images were taken after 2 days and for the growth assays, images were taken after 5 days.

Plasmid Isolation

Plasmids were isolated from bypass and interaction suppressor candidates using the QIAprep Spin Miniprep Kit (Qiagen). Cells from each suppressor candidate were added to 10 mL of SC-LEU + 2% glucose and placed in a rotating wheel in a 30°C incubator. The following day, the cells were pelleted in a centrifuge at 2,000 rpm for 5 min at 4°C. Media was decanted and 1 mL of sterile mqH_2O was added to the tubes. After resuspending cells, the cells were transferred to a microfuge tube. The cells were then pelleted in a centrifuge at 13K rpm for 30 sec. Water was decanted and the pellet was resuspended in 250 µL of Buffer P1 containing 0.1 mg/mL of RNase

A. One scoop of acid washed beads was added and the tubes were vortexed for 5 min. After allowing the beads to settle, the supernatant was transferred to a fresh microfuge tube and 250 µL of lysis buffer P2 was added. Tubes were incubated for 5 min at 22°C (room temperature). Following incubation, 350 µL of neutralization buffer N3 was added, solution was mixed and the tubes were spun in a microcentrifuge for 10 min at 13K rpm. The lysate was transferred to a QIAprep Spin Column in a collection tube and the column was spun in the centrifuge for 1 min at 13K rpm. The flow-through in the collection tube was discarded. The column was washed with 750 µL of Buffer PE and spun for 1 min. The flow-through was discarded and the column was spun again for 1 min. The QIAprep Spin Column was transferred to a fresh microfuge tube and 50 µL of Buffer EB was added. After allowing the column to sit for one minute, the column was spun for 1 minute to transfer the isolated plasmid from the column to the microfuge tube. The plasmid DNA preparations were stored at -20°C.

Plasmid Amplification

In order to amplify the plasmids, before transforming the isolated plasmids back into yeast, the individual plasmids were transformed into *Escherichia coli* using electroporation following the instructions from the manufacturer. Electrocompetent *E. coli* cells ($DH_5\alpha$) were transformed with 1 µL of the isolated plasmid. Sterile 2 mm electroporation cuvettes chilled on ice and the white cuvette chamber slide was stored at -20 °C until ready for use. Chilling helps prevent too much heat transfer to the cells during electroporation. The cells and DNA solution is transferred to the bottom of the sterile electroporation cuvette, ensuring that there are no air bubbles introduced. For a 2 mm cuvette, the Bio-Rad Gene Pulser is set to 25 µF, 200 O, and 2.5 kV. The cuvette is loaded into the chamber slide and slid into the chamber until it touched the

electrodes. Once the sample is pulsed, 1 mL of SOC broth is immediately added to the cuvette. The solution is transferred to a test tube and the cultures are incubated for 1 hr on a rotating wheel at 37°C. After incubation, the cells were pelleted and about 500 µL of media was decanted. The pellet was resuspended in the remaining media. The cells were plated onto two LB + Kanamycin plates (250 µL per plate). The plates were incubated at 37°C for two days. After single-colony purification, one colony was selected and patched onto a LB + Kanamycin plate and incubated at 37°C.

Plasmids were isolated from *E. coli* in order to be transformed back into the parent yeast strains. Isolation was performed using the QIAprep Spin Miniprep Kit (Qiagen). A single *E. coli* colony was added to 5 mL of LB + Kanamycin (50 µg/mL) and placed in a rotating wheel in a 37°C incubator. The following day, the cells were transferred to a microfuge tube and pelleted. The cells were resuspended in 250 µL of buffer P1 and 250 µL of buffer P2 was added. After inverting, 350 µL of buffer N3 was added and the tube was quickly inverted. Tubes were centrifuged for 10 min at 13K rpm and the supernatant was transferred to QIAprep Spin Column in a collection tube and the column was spun in the centrifuge for 1 min at 13K rpm. The flow-through in the collection tube was discarded. The column was washed with 500 µL of Buffer PB and spun for 1 min. The flow-through was discarded. The column was washed with 750 µL of Buffer PE and spun for 1 min. The flow-through was discarded and the column was spun again for 1 min. The QIAprep Spin Column was transferred to a fresh microfuge tube and 100 µL of Buffer EB was added. After allowing the column to sit for one minute, the column was spun for 1 minute to transfer the isolated plasmid from the column to the microfuge tube. The plasmid DNA preparations were stored at -20°C.

The isolated plasmids were then transformed back into parent yeast strains, MBY3080 and MBY3081. A medium-sized colony of yeast cells were added to a microfuge tube in addition to 7.5 µL of boiled carrier DNA (12.9 µL OF 11.7 µg/µL), 10 µL of plasmid DNA, and 500 µL of PLATE solution (4 mL 50% PEG 3500, 500 µL 1M lithium acetate, 50 µL 1M Tris HCl pH 7.5, 10 µL 0.5M EDTA pH 8.0, 440 µL sterile mqH_2O). Cells were incubated overnight at 22^oC (room temperature). The following day, the cells were heat shocked at 42^oC for 15 min. Cells were pelleted for 10 s at 13K rpm. The solution was decanted and cells were resuspended in 200 µL of sterile mqH_2O. Cells were plated on SC-LEU agar and incubated at 30^oC for 2 days. Colonies were single colony purified and patched. Plates were stored at 4^oC and cells used to recheck the suppression phenotype using the growth assay procedure as described above.

Sequencing of Suppressor Plasmids

The plasmids were sequenced by MCLAB using primers provided by the company (KS and M13 reverse). After the plasmid is sequenced, BLAST search of the yeast nuclear genome was used to determine the genes present on the suppressing plasmid. The function of each gene on plasmid 78 was investigated using the Saccharomyces genome database and literature search.

CHAPTER III

RESULTS AND DISCUSSION

Strain Creation

Either an empty vector (pRS406,(Sikorski & Hieter, 1989)) or pRS406-*set1-Y967A* (Williamson et al., 2013) was integrated into the *ura3-52* locus via homologous recombination, as illustrated in Figure 1. The *ura3-52* locus is located on Chromosome *V* and is involved in the synthesis of the nucleotide uracil. The Ty element is a transposable element in yeast that is approximately 5,700 bp long sequence (Rose & Winston, 1984). In the case of *URA3*, the Ty element is inserted in the coding region, causing a non-reverting mutation. The *ura3-52* allele is not functional; however, the plasmid being integrated contains a functional *URA3* gene. By plating on selective SC-URA agar plates, the cells that have the plasmid integrated into the genome will grow, however, cells that did not integrate the plasmid into the genome will not grow.

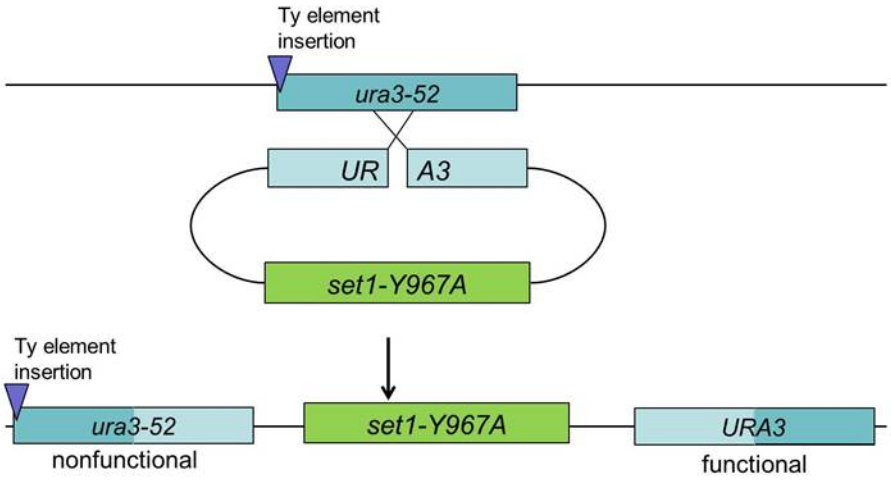

Figure 1: Integration of *set1* alleles into the *ura3-52* locus. The *set1-Y967A* allele was introduced into yeast strains using a one-step integration coupled with the lithium-acetate transformation method. Plasmids containing *set1-Y967A* allele or no *set1* allele were linearized with *Stu*I and transformed into yeast cells. Transformed cells were selected on SC-URA agar plates.

After transformation, Ura$^+$ colonies were single-colony purified and examined to determine if a single plasmid integrated into *ura3-52*, as opposed to integration of more than one plasmid in tandem array (a multiple integration event). Yeast strains with multiple integration events are not desired because they may overexpress *set1-Y967A*, which would add an unknown variable to the suppressor screen. Southern blotting of *Bam*HI-digested genomic DNA was performed. Figure 2 shows the sites where *Bam*HI cuts the genome and integrated plasmid, and the expected sizes of the fragments.

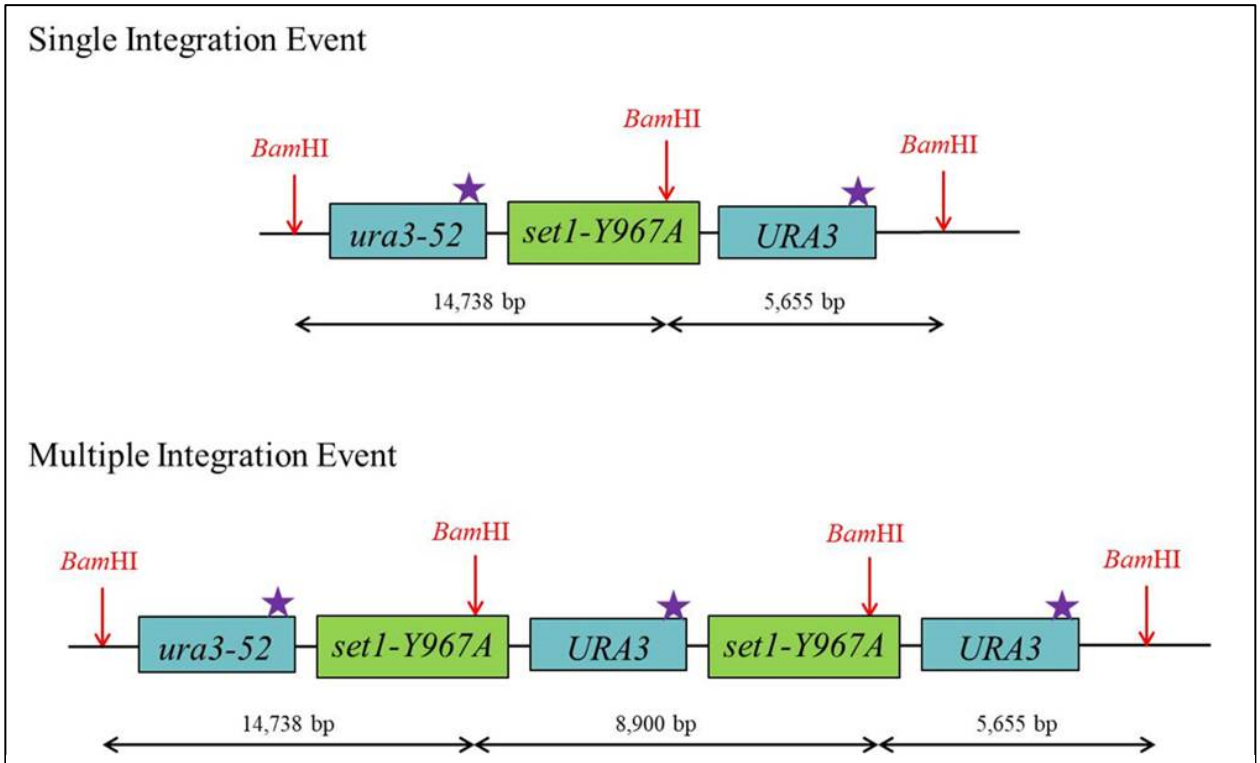

Figure 2: Sizes of expected *Bam*HI fragments in pRS406 and pRS406-*set1-Y967A* transformants. Each *Bam*HI site is marked with a red arrow and the corresponding fragment size is indicated below. The purple stars represent where the radiolabeled *URA3* probe hybridizes during Southern blotting.

For a single integration event, two bands that hybridize with the *URA3* probe are expected on the Southern blot, one at 14,738 bp and the second at 5,655 bp. If there are multiple copies of the

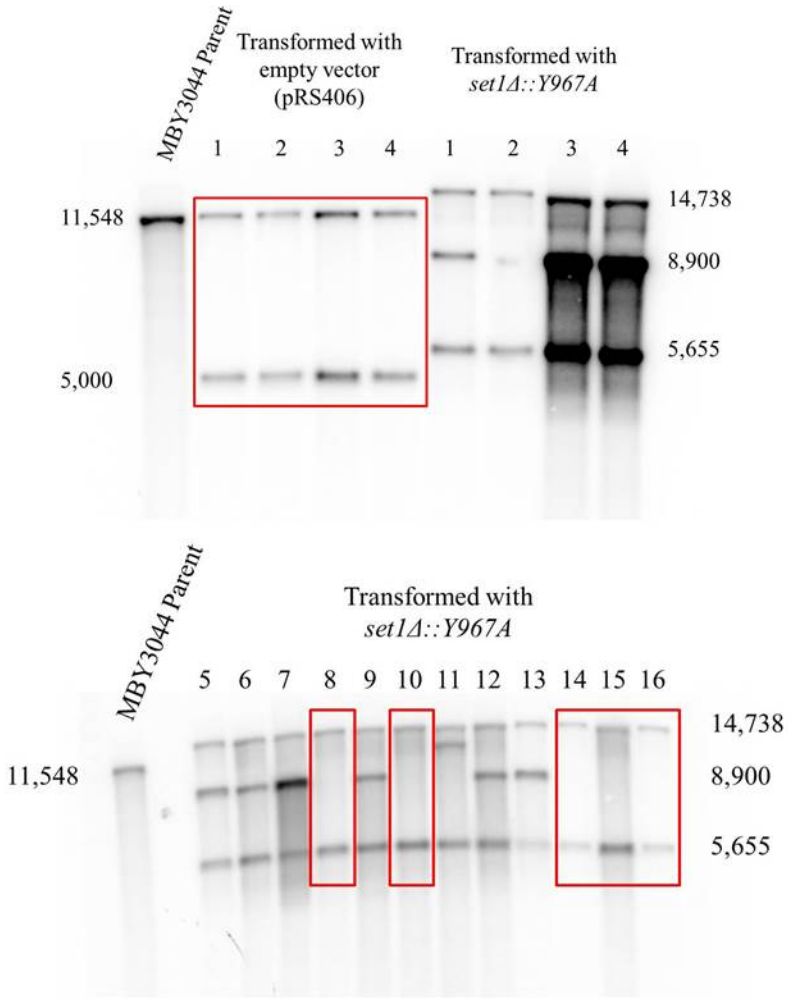

Figure 3: Verification of integration events by Southern blotting. Southern blots showed that a single copy of the transformed plasmid carrying a functional copy of the *URA3* gene and either no insert (empty vector, pRS406) or the *set1-Y967A* allele had integrated into the *ura3-52* locus. Numbers on sides of blot images indicate fragment length in base pairs.

plasmid integrated at *ura3-52*, a third band would be detected at 8,900 bp. When integrating the empty vector (pRS406), two bands that hybridize with the *URA3* probe are expected on the Southern blot, one at 11,548 bp and the second at 4,550 bp. Figure 3 shows the Southern blots performed to identify yeast strains with a single copy of either the empty plasmid or the *set1-Y967A* gene integrated into the *URA3-52* locus. The red boxes indicate genomic DNA from yeast strains with successful single-copy plasmid integration events. For the integration of the empty vector (pRS406), candidates 1-4 contained one copy of the empty vector, as seen by the

two bands at 11,548 bp and 4,550 bp. For the pRS406-*set1-Y967A* transformants, most of the candidates (1-7, 9, 12, 13) had multiple copies of the plasmid integrated into the *ura3-52* locus as shown by the presence of a third *Bam*HI fragment of 8,900 bp. Candidate 11 contained a new fragment that was unexpected. This is likely due to a misincorporation of the plasmid, resulting in a larger fragment when cut. Candidates 8, 10, 14, 15, and 16 had single copy of the plasmid pRS406-*set1Y967A* integrated into the *ura3-52* locus, because the digest of genomic DNA gave rise to only two *Bam*HI fragments, one at 14,738 bp and another at 5,655 bp, that were detected by the *URA3* probe.

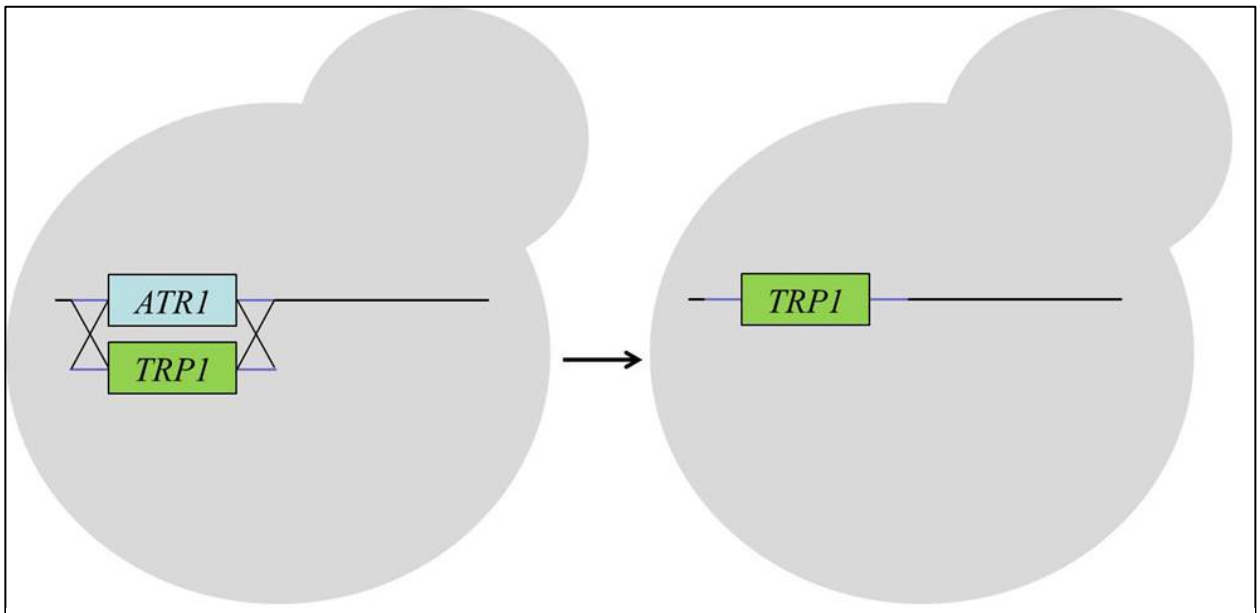

Figure 4: Creation of *atr1Δ::TRP1* strains using the One–Step Gene Disruption Method. An *atr1Δ::TRP1* fragment amplified by PCR was transformed into yeast to replace the *ATR1* gene by homologous recombination.

With the proper yeast strains constructed, preliminary transformation experiments were performed to determine the appropriate conditions for transformation of the high-copy yeast genomic DNA library and screening of transformants that suppress the *SET1* growth defects. During the initial suppressor screen transformation experiments, the *set1Δ* growth-defect

phenotype was not exhibited by cells that were transformed with the high-copy genomic DNA library when transformants were plated on agar plates containing 3-AT, regardless of concentration. This observation hinted that the large number of cells being plated on the selective plates was limiting the ability to detect the growth defect. To overcome this problem, the *ATR1* gene that encodes a multi-drug efflux pump was deleted (Kanazawa et al., 1988). The Atr1 multi-drug efflux pump pumps 3-AT out of yeast cells; therefore, removing the *ATR1* gene is expected to make the cells more sensitive to 3-AT. *ATR1* was deleted using gene replacement of the *ATR1* coding sequence with the *TRP1* gene via homologous recombination (Figure 4).

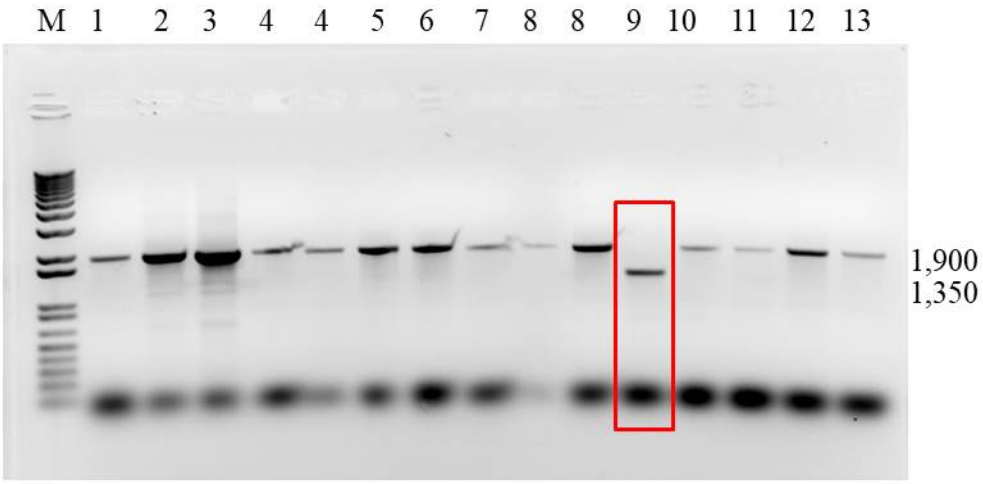

Figure 5: Verification of *TRP1* replacement of the *ATR1* gene by PCR and agarose gel electrophoresis. PCR amplification experiments showed that the *TRP1* gene had replaced the *ATR1* gene in yeast strain 9. Numbers on sides of blot images indicate fragment length in base pairs.

Following transformation, cells that grew on SC-TRP agar were examined for replacement of the *ATR1* gene with *TRP1* using PCR. If the *TRP1* gene was successfully integrated at *ATR1*, then a band at about 1,350 bp was expected. Verification of the deletion of *ATR1* is important because the *atr1Δ::TRP1* fragment could also integrate into the *trp1Δ63* locus via homologous integration. In this case, *ATR1* would not have been deleted. If the integration was not

successful, a single band at about 1,900 bp was expected. Figure 5 shows the image of the agarose gel used to verify replacement of *ATR1*. The red box indicates that yeast strain number 9 has a band at 1,350 bp and therefore *TRP1* replaced the *ATR1* locus. The new genotype is written: *atr1Δ::TRP1*. Other candidates (1-8, 10-13) had a band at 1,900 bp indicating that *TRP1* did not replace the *ATR1* locus. Candidate 9 was used in preliminary transformation experiments with the high-copy genomic DNA library, and it was determined that the *atr1Δ::TRP1* strain allowed the *set1* growth-defect phenotype to be observed when transformants were plated on low concentrations of 3-AT (5-20 mM).

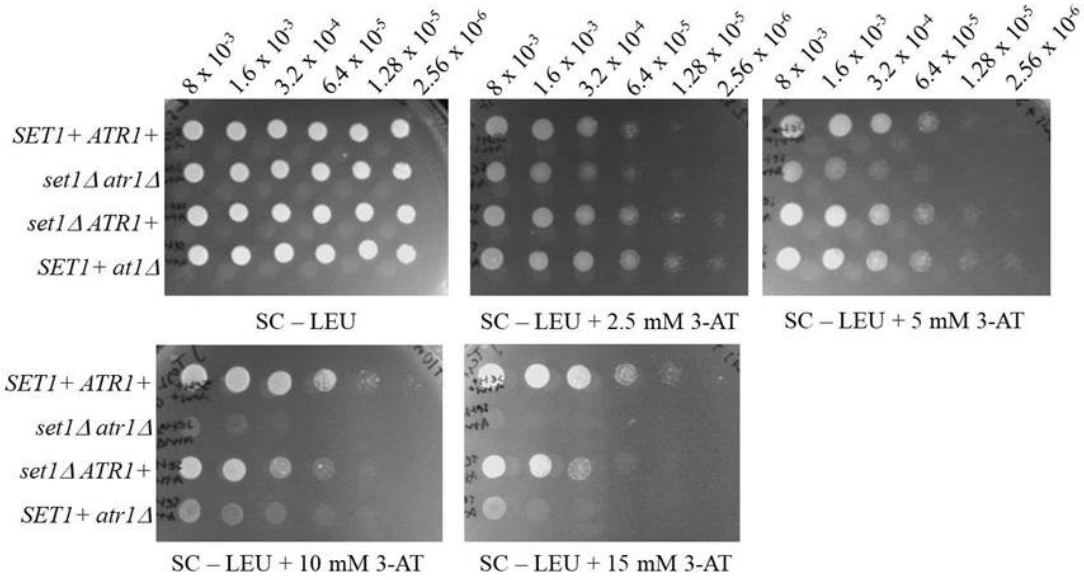

Figure 6: Results from the test to determine optimal concentration of 3-AT. The plates that are SC-Leu-His+10mM 3AT were selected due to the change in growth between each strain.

Determination of 3-AT concentration to use in yeast cell plate growth assays

To determine the best concentration of 3-AT for detecting the *set1* null mutant growth phenotype and for plating of yeast cells transformed with the high-copy genomic DNA library, cell growth was analyzed on a series of plates with different concentrations of 3-AT. Figure 6 contains

images of the plates used for the growth experiment to determine the optimal concentration of 3-AT for detecting a *set1* null mutant growth phenotype. On SC-Leu His plates, as the cells are diluted, fewer cells are plated so less growth is expected. The addition of 10 mM 3AT to the plates caused a gradient of growth that was evident among all four stains.

Bypass Suppressor Assay

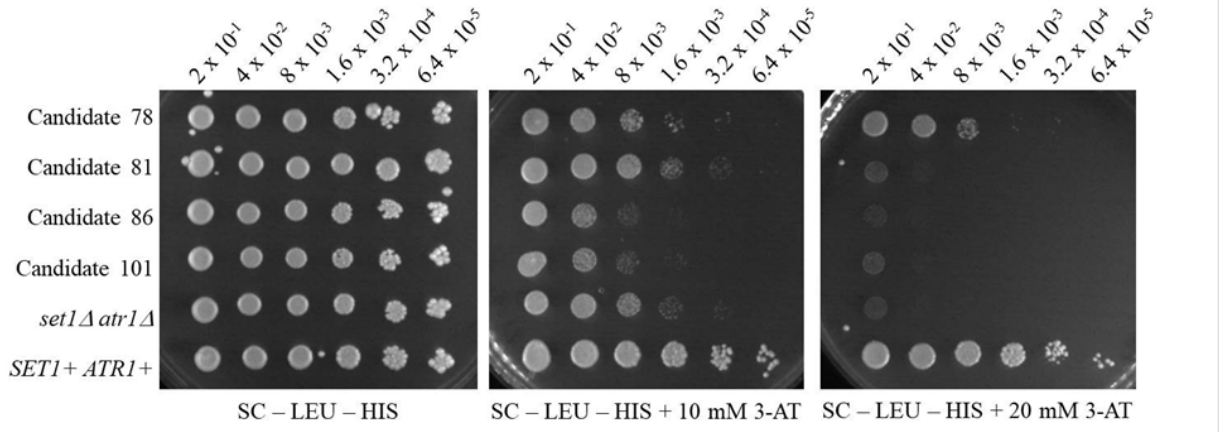

Figure 7: Retesting the suppression phenotype of Candidates 78, 81, 86, and 101. From this growth assay, it was determined that transformants 78 and 81 were possible suppressors of the *set1* null mutant growth defect, due to growth that was better than that of the parent stain (MBY 3080).

After transformation of the high-copy genomic DNA library into MBY3080, four colonies grew on the SC-Leu-His+ 10 mM 3AT. These suppressor candidates were colony-purified and retested for suppression of the *set1Δ* growth defect (Figure 7). Two candidates, numbers 78 and 81, were identified as possible bypass suppressors. Candidate 78 was pursued because its growth on SC-Leu-His+ 20mM 3AT plates was better than the parent strain MBY3080. Candidate 81 was selected as a possible candidate due to its robust growth on SC-Leu-His+ 10 mM 3AT. Its

growth was better than the growth of its parent, MBY3080; better growth than the parent indicates that candidate 81 might contain a possible suppressor.

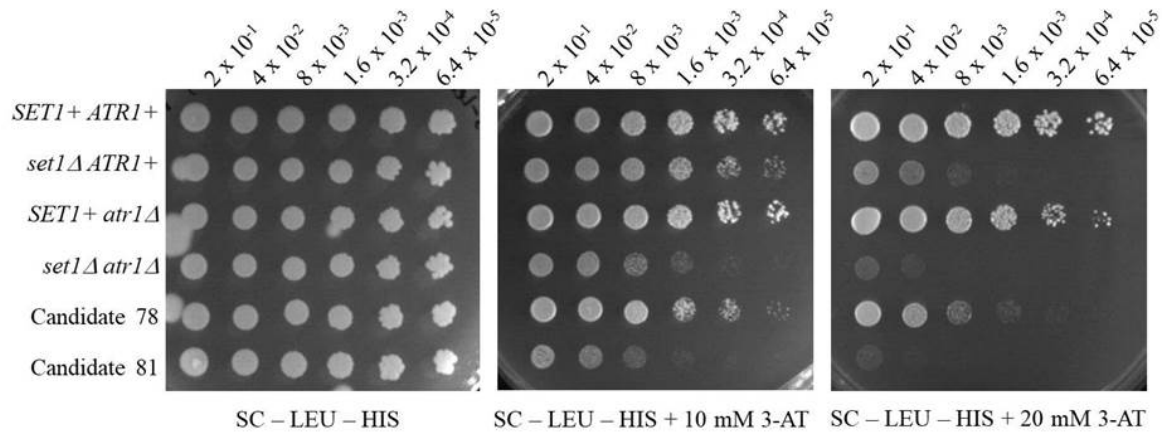

Figure 8: Verification of suppression of the *set1* slow growth phenotype on medium containing 3-AT by suppressor candidates 78 and 81. Following retransformation into MBY3080, candidate 78 recapitulated the growth defect suppression seen in the original assay but candidate 81 did not.

Because both candidates demonstrated suppression of the growth-defect phenotype, the plasmids from candidates 78 and 81 were isolated in order to verify the suppression phenotype. After transforming each plasmid back into yeast strain MBY3080 (Figure 8), candidate 81 failed suppress the *set1Δ* growth defect. The original growth defect suppression, seen in the first growth assay, is most likely a result of another mutation in the yeast cell that was not linked to the plasmid. However, candidate 78 displayed better growth on both the SC- Leu-His + 10 mM 3-AT and the SC-Leu-His + 20 mM 3-AT plates. This confirmed that the plasmid from candidate 78 contained a gene that acted as bypass suppressor of the *set1Δ* growth-defect phenotype.

Once candidate 78 was confirmed to be a suppressor, the insert in the plasmid was sequenced. The insert is a 10-kilobase fragment from Chromosome *V* (position 80864 - 90889). This fragment contains six genes, three on each strand (Figure 9). Another student in the Bryk Lab, Lyndsey Price, is isolating the individual genes on plasmid 78 and retransforming these smaller plasmids with one or two genes back into yeast strain MBY3080 in order to identify which of the genes on plasmid 78 is responsible for suppression of the *set1* null growth defect.

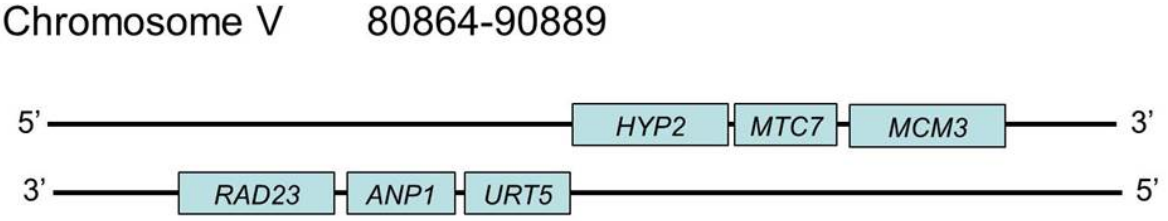

Figure 9: Genes found on Candidate 78. Using the yeast genome database, six genes were identified on the insert from Chromosome *V*.

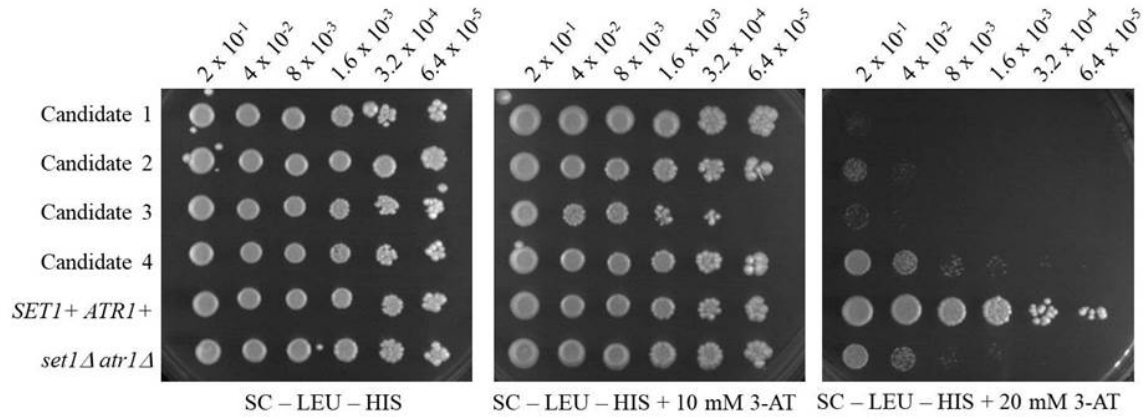

Figure 10: Original Growth Assay with Candidate 1-4. From this assay, it was determined that only Candidate 4 was a true bypass suppressor of the growth defect, due to growth that was better than that of the parent stain (MBY 3080).

In addition to Candidate 78, four possible candidates were found growing on SC – LEU - HIS + 10mM 3-AT, following another transformation with the library. The four candidates were

retested for the suppression phenotype (Figure 10). Of the four candidates, only Candidate 4 was identified as another possible interaction suppressor. Candidate 4 was the only candidate whose growth exceeded that of the parent strain MBY 3080, especially on SC – LEU – HIS + 20mM 3-AT. The other three candidates failed to express the growth-defect phenotype, as their growth was worse than the *set1Δ* and *atr1Δ* parent strain.

Interaction Suppressor Assay

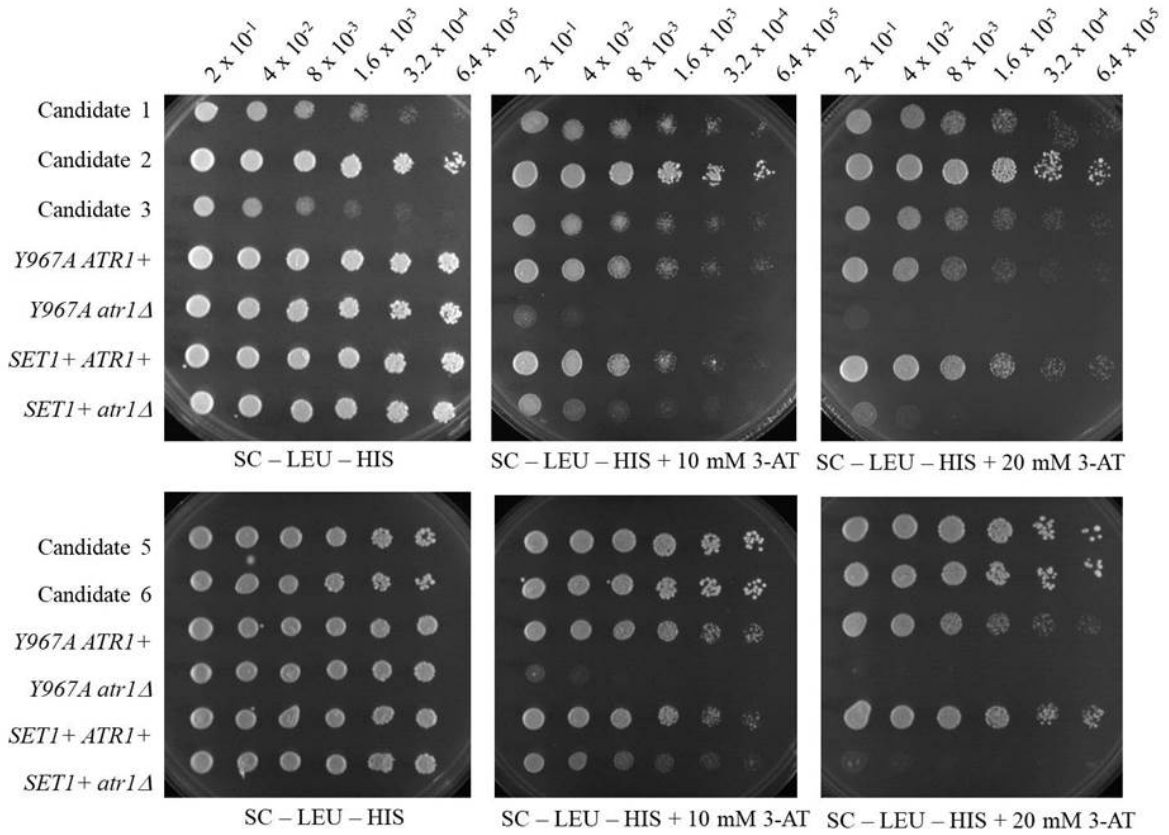

Figure 11: Original Growth Assay with Candidates 1, 2, 3, 5, and 6. From this assay, it was determined that candidates 2, 5, and 6 were interaction suppressors of the *set1Δ* growth defect, due to growth that was better than the *SET1+* and *ATR1+* control strain.

Two transformations of MBY3081 with the high-copy genomic DNA plasmid library yielded six colonies that grew well on SC-LEU-HIS+ 10 mM 3-AT. One of those colonies did not grow when single-colony purified onto SC-LEU-HIS, so that colony was not studied further. Of the remaining five colonies, candidates 2, 5, and 6 suppressed the *set1-Y967A* growth defect (Figure 11). Candidates 2, 5, and 6 outgrew the control strains, including the wild type *SET1*$^{+}$ and *ATR1*$^{+}$ strain MBY3111. Candidates 1 and 3 were not studied further because their growth, while better than the *set1-Y967A* and *atr1Δ* parent strain, was not sufficient enough to warrant further study. In addition, candidates 1 and 3 did not grow well when plated on SC-LEU-HIS, indicating that the parent cells contained a possible mutation that caused grow-defects when not plated on 3-AT.

CHAPTER IV

CONCLUSION

Of the genes on the insert from candidate 78, two of the genes encode for proteins with unknown functions. These two genes are *UTR5* and *MTC7*. Because the function of these genes is unknown, they are not suspected gene candidates that conferred the suppression of the growth-defect phenotype.

RAD23 encodes for a protein required for nucleotide excision repair, especially of UV damaged DNA (Prakash & Prakash, 2000). Rad23 associated with other proteins to form the Rad4-Rad23 complex (NEF2) that binds to damaged DNA. Rad23 helps to recruit NEF1 and NEF3, complexes that perform the nucleotide excision. *RAD23* is not expected to be a suppressor because the DNA damage repair process does not interact with histone methylation. Repair of DNA damage does allow for RNA Pol II to continue transcription; however, this continuation is due to repair of DNA lesions that cause the RNA Pol II to stall.

HYP2 encodes for a protein that is involved in translational elongation and termination, specifically as the elongation factor eIF5A. Its most prevalent role in translational elongation is in restarting translation after the ribosome stalls, especially at polyproline motifs, by stabilizing the peptidyl-tRNA (Schuller, Wu, Dever, Buskirk, & Green, 2017). During termination, eIF5A helps during the release of the polypeptide caused by the release factor, eRF1. *HYP2* is not expected to be the growth-defect suppressor because translation occurs after transcription. The

set1 null mutants struggle during transcription, so less stalling of ribosomes during translation is not expected to suppressor the growth defect of *set1* null mutants.

ANP1 encodes for a protein involved in the α-1,6 mannosylation of proteins. The addition of multiple mannose sugars is characteristic of proteins involved in secretion (Jungmann & Munro, 1998). *ANP1* itself is considered an amino nitrophenol propandiol resistance gene; the encoded protein is located in the endoplasmic reticulum (Chapman & Munro, 1994). Strains that are *anp1Δ* are sensitive to a product of chloramphenicol degradation, amino nitrophenol propandiol. *ANP1* is a possible candidate suppressor because of its involvement in the secretory pathway. During preliminary research, *ATR1*, a multi-drug efflux pump was found to also confer suppression of the 3-AT induced growth defect by pumping 3-AT out of the cell (Kanazawa et al., 1988). It is possible that the overexpression of *ANP1* helps remove 3-AT from the cell, thereby allowing the His3 protein to function at a level that is conducive with viability.

MCM3 encodes for open protein that is part of the hexameric complex (MCM2-7). This complex functions as a helicase that unwinds DNA during replication (Bochman & Schwacha, 2008). Mcm3 is a peripheral protein that does not possess the helicase function (Susan L. Forsburg, 2004). However, it is involved in recruitment of other Mcm proteins to the MCM2-7 complex. In addition to DNA replication, Mcm3 is has also been associated with transcription, as a MCM3/5 sub-complex, in response to gamma interferon (DaFonseca, Shu, & Zhang, 2001). The MCM3/5 sub-complex interacts with Stat1, a transcriptional factor involved in activation. Mcm5 is necessary for this interaction, as Stat1 binds to Mcm5. As levels of Mcm5 increase, so does the levels of transcriptional activation (Susan L. Forsburg, 2004). Because Mcm3 also

interacts with Stat1, overexpression of *MCM3* could increase transcriptional activation. Mcm3, as well as the other Mcm proteins, have been associated with RNA polymerase II during elongation when cells are exposed to gamma interferon (Snyder, He, & Zhang, 2005). Mcm3 specifically was found to be present in the middle of the *IRF-1* locus as well at the 3' UTR of the gene. The *IRF-1* locus is an interferon regulatory factor. Due to this association of Mcm3 during transcriptional activation and elongation, it is possible that overexpression of *MCM3* helps to suppress the growth defect by allowing elongation to occur, without H3K4 methylation.

Moving forward, each of the genes on the insert need to be studied individually to determine which gene confers the suppression phenotype. In addition, the plasmid from the one suppressor candidate from MBY3080 bypass suppressor screen and the plasmids from the three suppressor candidates from the MBY3081 interaction suppressor screen are being retransformed into MBY 3080 and MBY3081, respectively, and the ability to suppress the *set1* null growth defect on medium containing 3-AT is being verified. High-copy plasmid from the candidates that suppress the *set1* null growth defect after the re-transformation will be sequenced. Following sequencing, the genes on those inserts will be studied individually, similarly to how the genes on suppressor plasmid 78 are being studied. By identifying and characterizing bypass suppressors, alternative pathways that allow transcription in the absence of normal H3K4 methylation will be uncovered. The results are expected to reveal what role H3K4 methylation plays in transcription. By studying interaction suppressors, the role of the interacting protein can help illuminate the role of H3K4 methylation in transcription. These suppressors will help to further knowledge about transcription, a process that is conserved across all species of life, yet remains not fully understood.

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