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A Genetic Screen for Second Site Suppressor Mutations of Extended Gene Silencing in *Saccharomyces cerevisiae*

by

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Undergraduate honors thesis under the direction of

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& Agricultural and Mechanical College
Baton Rouge, Louisiana

Introduction

Living cells contain an abundance of genetic information in the form of DNA; however, all the genetic information within a cell cannot be expressed at once in a functioning eukaryotic organism. Gene expression in eukaryotic cells is regulated at various stages including DNA replication, transcription of DNA to mRNA, translation of mRNA into proteins, and post-translational modifications of proteins to control their activity. To save energy and resources within a living cell, the most crucial step in the regulation of genetic information is the initiation of transcription. Different activating and repressing transcription factors are activated at specific chromosomal regions to either initiate or inhibit transcription of DNA into mRNA. Disruptions of cellular processes that lead to altered gene expression patterns can cause abnormal cell proliferation and cancer cell growth. Because of the importance of understanding these cellular processes, especially in humans, a model organism is needed to efficiently study gene regulation in the lab. The most commonly used organism for studying gene regulation and chromosome structure is the budding yeast, *Saccharomyces cerevisiae*. Yeast are useful model organisms because they are small enough to be cultured quickly and easily, their DNA is relatively simple to manipulate in the lab, the yeast genome was the first genome completely sequenced from a eukaryote, and they can exist with either diploid or haploid genomes (Engel *et al.*, 2014). These small single-celled eukaryotes package and regulate their DNA in the same way as other larger eukaryotes, including humans, allowing them to be a powerful tool for studying transcriptional regulation, nuclear organization, chromatin structure and function.

In all eukaryotes, DNA is packaged as chromatin, a complex made up of DNA, nucleosomes, and non-histone chromosomal proteins. Histones are small, positively charged proteins that make up about half of the protein within the nucleus, and they are highly

evolutionarily conserved in eukaryotes. Each nucleosome is a disc-shaped structure made up of 146 base pairs of DNA wrapped 1.7 times around a histone octamer made up of 2 subunits each of histones H2A, H2B, H3, and H4. Additionally, each nucleosome is separated by about 20 base pairs of DNA in *S. cerevisiae*. Another important feature of nucleosomes is the amino terminal tails of histone proteins which extend out from the nucleosome core as shown in Figure 1. These tails are the sites of important enzymatic reactions and modifications that can affect the level of gene expression in the region.

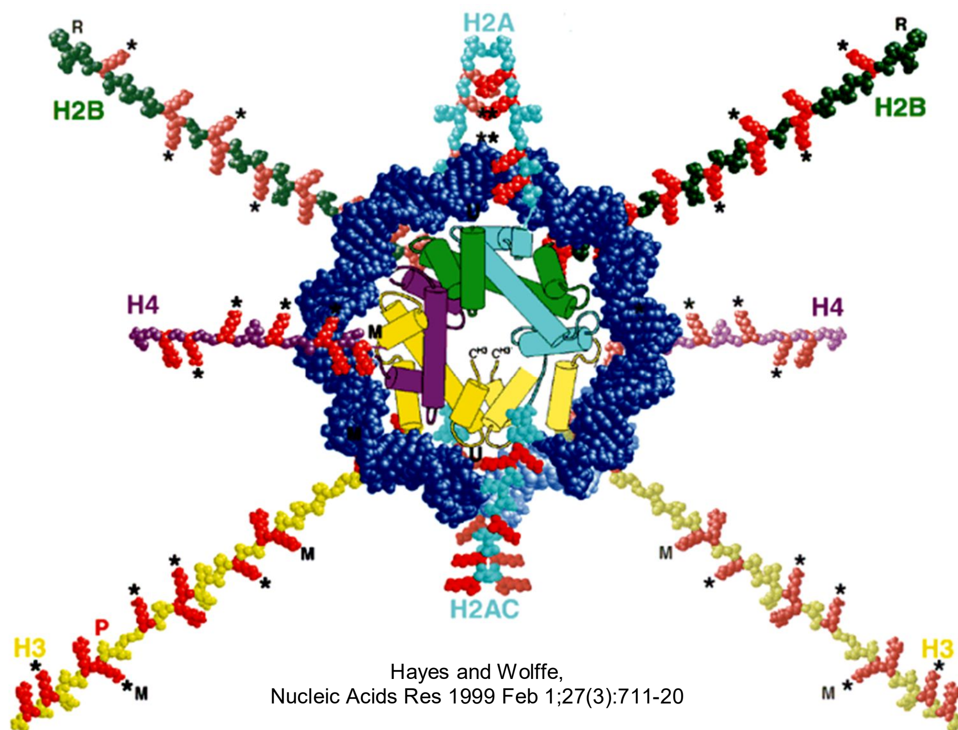

Figure 1. Nucleosome depiction highlighting amino acid residues on histone tails where important amino acid modifications take place.

One such modification that is critical to chromatin structure and function is the acetylation of amino groups on histone lysine residues. Acetylation of lysine alters the structure and chemistry of the amino acid residue, making it less hydrophilic and changing it from positively charged to uncharged. Modifying the proteins that make up histones in this way marks

chromatin regions as binding sites for proteins that can activate or repress gene expression. Acetylated histones are commonly associated with actively transcribing regions of the genome known as euchromatin. On the other hand, regions of the genome associated with deacetylated nucleosomes that are more tightly compacted and therefore resistant to DNA binding proteins are known as heterochromatin.

Heterochromatic regions of the genome are called silenced chromatin in *S. cerevisiae* because the ordered, compact structure of regularly spaced, hypoacetylated nucleosomes blocks transcription machinery from gaining access to promoters necessary for transcription to occur. Because of epigenetic effects, which allow histone modifications to persist on the chromatin through replication for ten or more generations, heterochromatin regions can be inherited. In *S. cerevisiae*, several regions of the genome consist of inherited heterochromatin: the telomeres at the ends of chromosomes, rRNA-encoding DNA, and the silent mating loci (Rusche, 2003). This project focuses on silencing in the area around the silent mating locus *HMR*a.

The two yeast silent mating loci, *HML*α and *HMR*a, are silenced regions that flank the yeast mating type locus *MAT*. These silent mating loci help to determine whether haploid yeast are mating type a or α, and they are involved with mating type switching. Each silent mating locus is flanked by two silencers, which bind proteins that lead to transcription repression. The two silencers are called E and I. E, which is essential for silencing, includes binding sites for the ORC (origin recognition complex), and the DNA binding proteins Rap1p and Abf1p. I, which is important for silencing, includes the binding sites for Abf1p and ORC (Figure 2). Mutation in any one of these three proteins or their binding sites can lead to loss of cryptic loci silencing.

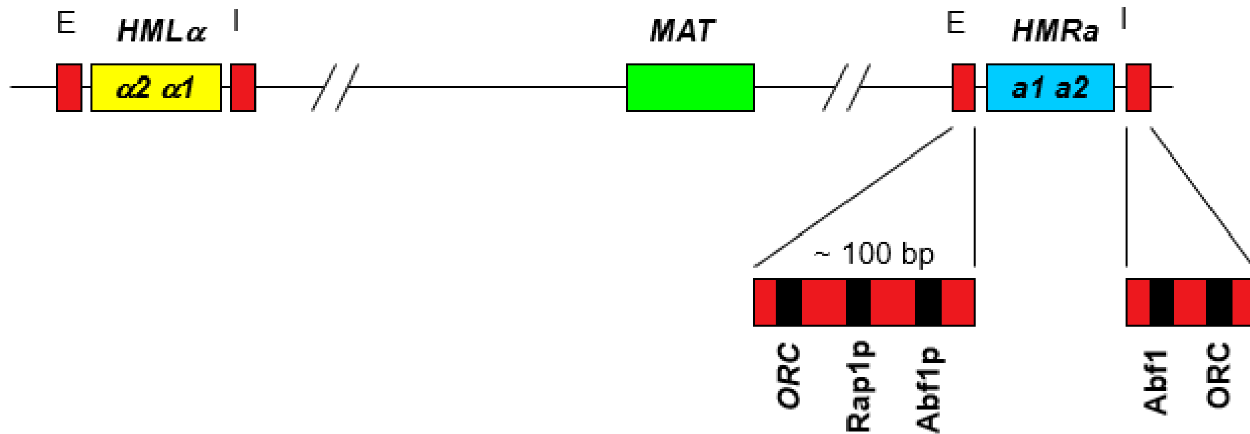

Figure 2. Yeast mating type loci (MAT) and silent mating loci flanked by silencers E and I.

The formation of heterochromatin at the *S. cerevisae* telomeres, rDNA, and silent mating loci requires Silent Information Regulators, referred to as SIR proteins (Cockell *et al.*, 1995. Oakes *et al.*, 2006). At the silent mating loci, a group of proteins assemble at the silencer, recruiting Sir proteins Sir2p, Sir3p, and Sir4p to spread silenced chromatin. Sir2p is an NAD^+ dependent histone deacetylase (HDAC) that removes acetyl groups from histones. Sir3p and Sir4p have higher binding affinity to deacetylated histones; therefore, they bind deacetylated histone tails, preventing transcription by physically blocking access of RNA polymerase II and transcription factors to the promoters (Rusche, 2003). The formation of silenced chromatin is shown in Figure 3.

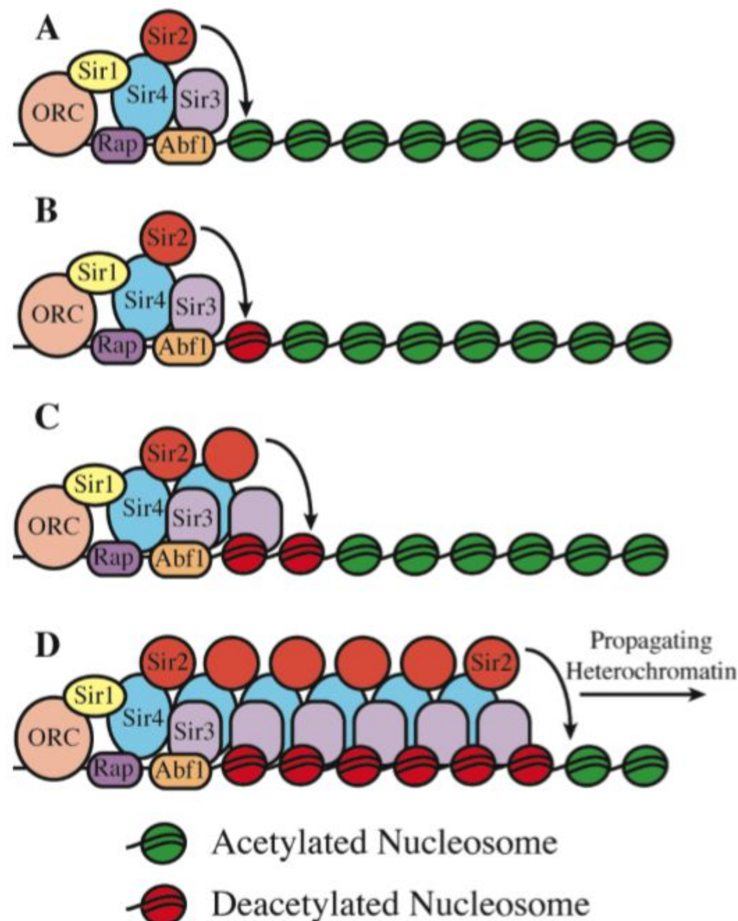

Figure 3. Establishment and propagation of heterochromatin at *HMR*α in *S. cerevisiae*. A) Depicts interactions between the proteins bound at the silencer and the Sir proteins that initate the Sir protein complex. B) Sir2p deacetylates lysine 16 on the tails of Histone 3 of the closest nucleosome, creating a high affinity binding site for Sir3p and Sir4p. C) Sir3p binding recruits another Sir2p/Sir4p complex to acetylate the neighboring nucleosome. D) This process of deacetylation and recruitment continues to spread the heterochromatic structure (Donze, 2003).

This silenced chromatin structure can propagate until it reaches a boundary element. Boundary elements on the DNA block the spread of silencing by either creating a nucleosome free region to prevent the deacetylation of nucleosomes, binding other proteins to physically block heterochromatin spreading, or recruiting histone acetyltransferases (HATs) to re-acetylate the histone tails (Figure 4).

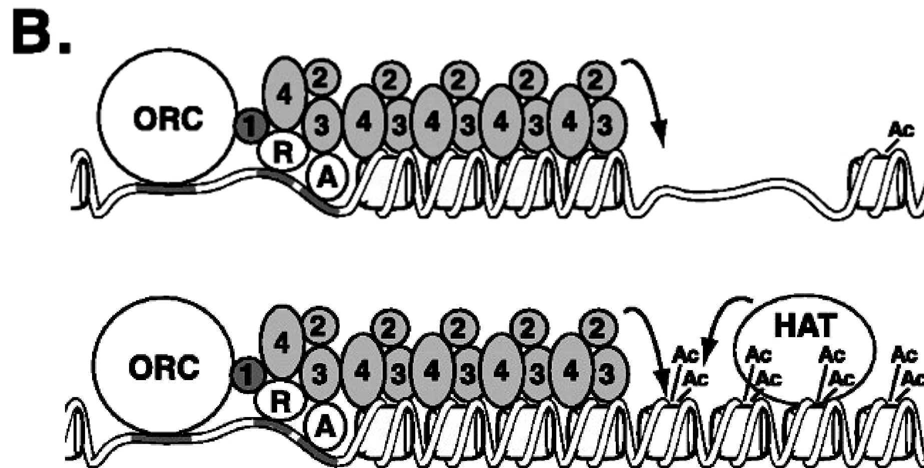

Figure 4. The halting of heterochromatin spreading by reaching a nucleosome free region (top) and the counteracting of deacetylation by HAT histone acetylation (bottom). (Rusche, 2003)

Because altered gene expression patters can lead to cancer and gene expression is controlled by the spread of heterochromatin, inhibiting histone deacetylases is currently being tested as a cancer treatment (Walkinshaw and Yang, 2008). The specific class of histone deacetylase being targeted is the *RPD3*/Hda1 family of HDACs; however, the global effects of HDAC-mediated silencing and its inhibition by candidate drugs are not fully understood. The *RPD3* histone deacetylase is interesting because it behaves differently at silenced regions of the genome such as *HMR* than it does at other areas of the chromosome. *RPD3* as an HDAC generally functions to increase gene silencing, preventing incorrect transcription initiation by deacetylating nucleosomes behind an elongating RNA polymerase II and directly inhibiting transcription at some promoters. Because histone deacetylases promote heterochromatin formation, it would be expected that deletion of a histone deacetylase would decrease heterochromatin formation; however, at certain regions of the chromosome, deletion of *RPD3* increases heterochromatin formation past the normal boundaries. In *S. cerevisiae, rpd3* deletion displays this contradictory activity at rDNA loci, telomeres, and the silent mating loci (Oakes *et*

al., 2006. Sun and Hampsey, 1999). For this reason, we have undertaken this experiment to screen for second site mutations that affect the increased gene silencing due to *RPD3* deletion.

One assay that has provided an effective screen to identify genes involved in silencing by utilizing *S. cerevisiae* is the colony color assay (Sussel, 1993). In this assay, a copy of the *ADE2* gene, which catalyzes a step in the biosynthesis of Adenine nucleotides, is placed downstream of the normal tRNA boundary in a yeast strain that is defective for the normal *ADE2* gene. This *ADE2* reporter gene is used to detect whether silencing at the *HMR* locus passes the tRNA boundary. During normal silencing, the Sir proteins spread until they reach the tRNA boundary element, the *ADE2* gene is transcribed downstream by RNA polymerase, and yeast colonies grow white on media with low adenine concentrations because they can synthesize more adenine on their own. If silencing spreads past the tRNA element, as it does with *rpd3Δ* mutants, *ADE2* is silenced, a red pigment caused by the interrupted adenine biosynthesis pathway accumulates, and the colonies appear pink to red when grown on media with sub-optimal adenine concentrations. This phenomenon is shown in Figure 5. In addition to purely white or purely pink colonies observed, this assay has revealed that some previously identified mutations cause genetically identical cells to switch between repressed and derepressed states of transcription resulted in variegated colonies, or colonies that display both red and white cells in the same colony (Sussel, 1993).

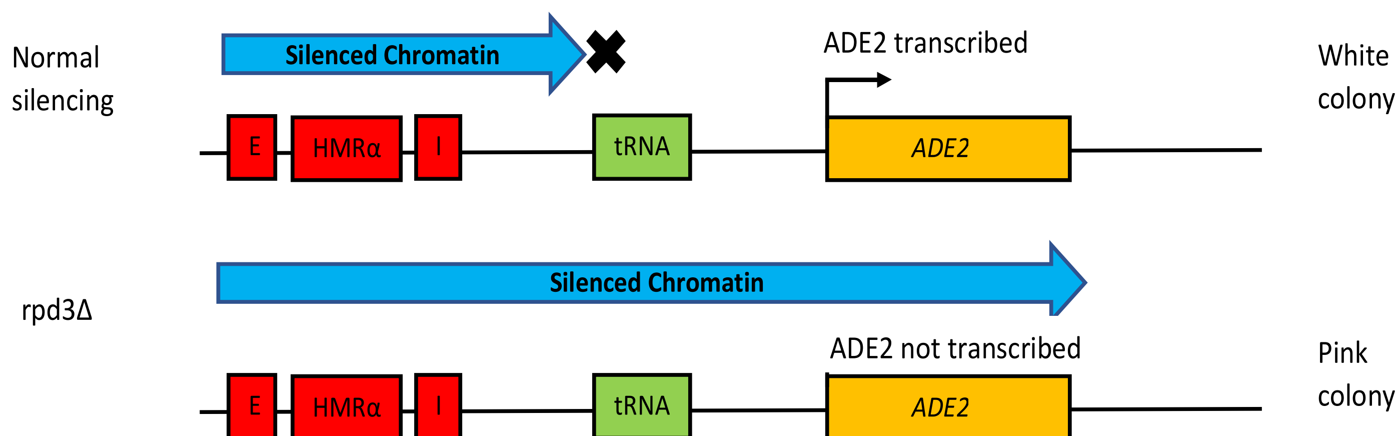

Figure 5. Schematic of *HMR* silencing in the colony color assay. Under normal conditions, Sir proteins spread to the tRNA boundary, *ADE2* is transcribed, and colonies appear white. In *RPD3* deletion mutants, silencing spreads past the boundary element, *ADE2* is not transcribed, and colonies appear pink. *rpd3*Δ colonies with second site mutations that reverse the increased silencing appear like the top figure, leading to a white colony phenotype.

In this experiment, we used the colony color assay with *RPD3* deletion yeast (referred to as *rpd3*Δ) to determine secondary mutations that would reverse the increased silencing effect, producing white colonies. To create the second site mutations, we used a plasmid library containing transposon insertions at various points in the yeast chromosome. A plasmid is an extra-chromosomal piece of DNA that replicates independently of the cell's chromosome, and it is helpful to use as a vector to maintain additional DNA within a cell. Transposons are small DNA elements that can move from one location to another (Weaver, 2016). When transposons insert within a gene, they can effectively "knockout" or decrease the effect of this gene by interrupting the normal sequence. Using these transposon insertions which integrate into random sites of the chromosome, secondary site mutants can be found using the colony color assay to identify white mutants that lessen the *rpd3*Δ-mediated spread of silencing at the *HMR* locus. The use of transposon insertions is also beneficial for this experiment because they can be easily found using PCR techniques because their sequence is known. Polymerase chain reaction (PCR) amplifies a region of DNA by using forward and reverse primers that flank the region along with

repeated cycles of DNA polymerase action (Weaver, 2016). Because the DNA sequence of the transposon is known, primers can be created that will amplify from one primer within the transposon to a secondary known site, created by adding a string of known nucleotides to the end of a piece of DNA close to the transposon insertion. This amplified sequence contains both a fragment of the transposon and a piece of the gene that the transposon inserted into. Sequencing the DNA of this amplified fragment can reveal the gene interruption responsible for the reversal of increased silencing.

Although many genes were found in this screen, two genes were particularly of interest for further study, *NGG1* and *CRC1*. According to the NCBI BLAST database, *NGG1* is a subunit of histone acetyltransferase (HAT) complexes which, as mentioned previously, are involved in chromatin modification. This mutation is interesting because its deletion showed less repression than the original *rpd3*Δ parent strain. Because HAT complexes generally function to increase gene transcription, a deletion of part of this complex would be assumed to show more transcription repression, not less. The other gene of interest, *CRC1*, encodes a carnitine transporter in the mitochondrial inner membrane. This transporter is required for carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria during fatty acid beta-oxidation. Because it transports acetyl-CoA, which is also needed for the acetylation of lysine residues in silencing, it could be taking acetyl CoA away from areas where histone modification are occurring, resulting in reduced global histone deacetylation. Additionally, our analysis of RNA-seq data from the Tsukiyama lab indicated that *CRC1* expression is upregulated nearly four-fold in *rpd3* mutants (Tsukiyama, 2015). Both *NGG1* and *CRC1* were further investigated in this study by experiments designed to confirm the results found in the colony color assay.

Methods and Results

All protocols are referenced from the Donze Lab Protocol Manual

To determine which gene deletions could reverse increased gene silencing caused by rdp3Δ at the *HMR* locus, we first inserted transposons into the yeast genome using a plasmid library. These transposon library pools containing the *LEU2* gene were already prepared in the Donze lab as plasmid DNA (library obtained from Nithya Jambunathan). The entire transposon library includes plasmids with transposon insertions flanked to each *S. cerevisiae* gene. Several of the transposon *LEU2* library pools were cut with the restriction enzyme Not1, and 6ul of these linear fragments were transformed into the yeast strain DDY 3133, which contains *rdp3*Δ and an *ADE2* reporter gene, to perform the colony color assay. This transformation was plated on YMD -leu 15% ade plates. The lack of leucine in the media is used to select for yeast that have undergone successful recombination of the transposon insertion, as the transposon contains the *LEU2* gene, into the yeast genome. 15% adenine is used to test the resulting recombinants for silencing. Strains with increased silencing caused by *rpd3*Δ grow pink because the silencing passes the tRNA boundary element, silences the *ADE2* gene, and limitation of adenine in the cell causes the buildup of a red pigment. White transformant strains indicate the reversal of the increased silencing caused by *rpd3*Δ because the *ADE2* gene is transcribed. Therefore, the white colonies from this transformation were picked out and streaked for isolation onto another YMD -leu 15% ade plate to confirm the white color. Over the course of several transformations and verifications, 32 independent white colonies were isolated. Colony of interest #11 appeared to be variegated, meaning that each plate had a combination of pink and white colonies, including some colonies that were both white and pink, even after re-streaking a single pink colony and a single white colony onto YMD -leu 15% ade. The white colonies that were later found by

sequencing to have transposon insertions in genes of interest (shown later in Table 1) are shown in Figure 6.

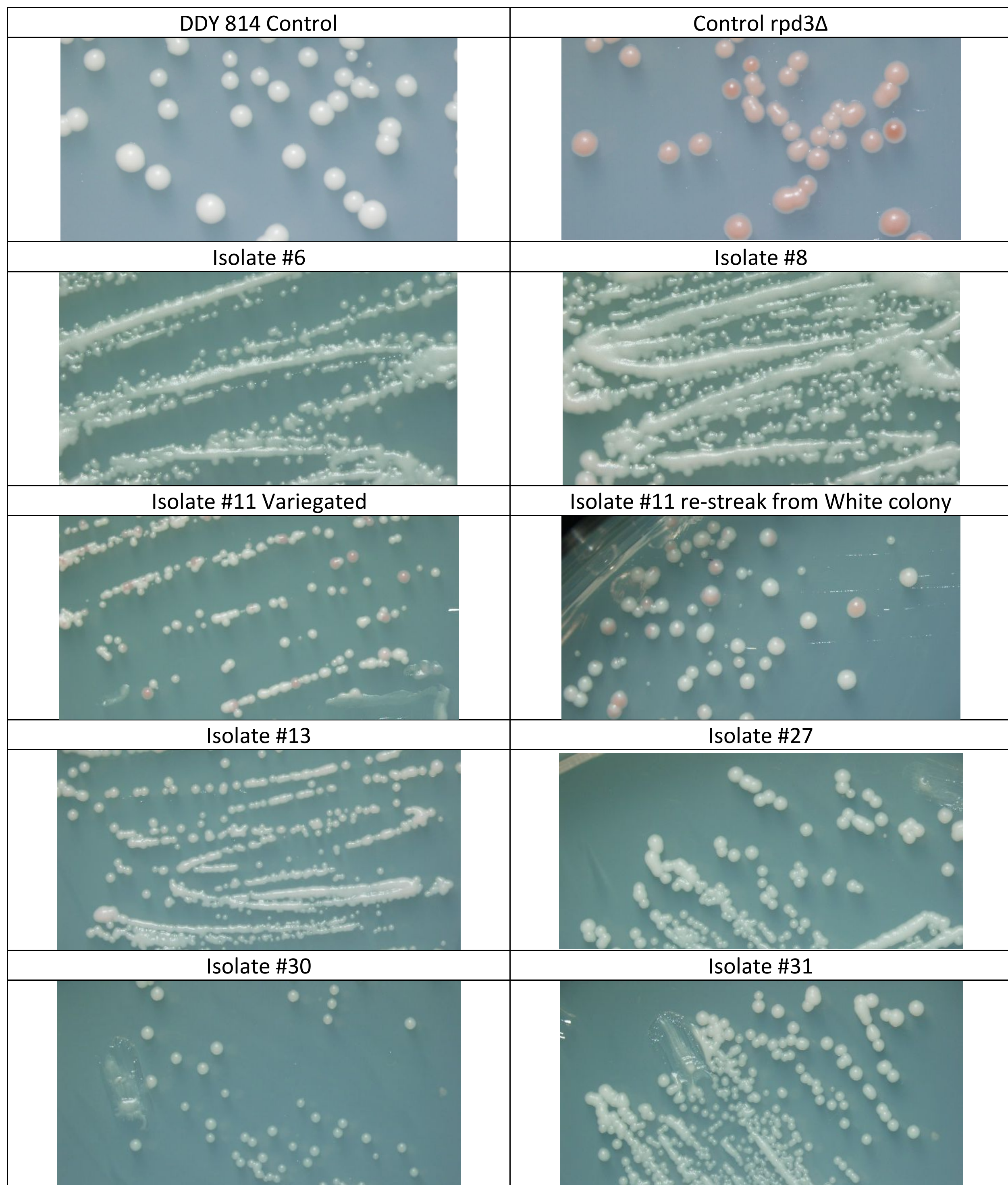

Figure 6. White colonies from color colony assay re-streaked onto YMD -leu 15% ade. Note the variegation of Isolate #11 which persists after re-streaking a white colony from the original streak.

After the white color was confirmed, we determined which gene the transposon inserted into using DNA extraction, restriction digest, anchor bubble ligation, PCR, and sequencing. A Winston Prep protocol was used to extract and purify the DNA from the white colony cells. The purified DNA was cut using either AluI, DraI, or RsaI. Each of these restriction enzymes has a 4 base pair cut site, meaning that it will cut at more sites in the genome than restriction enzymes with a longer cut site. Because it has many cut sites, these enzymes will cut the DNA within the transposon at a known defined site, and many times outside of the transposon sequence. Three enzymes were used to give a higher probability that one would cut not too close or not too far from the transposon cut site, ideally 100 to 300 base pairs. Next, anchor bubble adaptors were ligated to the end of the cut fragment, shown in Figure 7. A kinase was used to add a phosphate group to the oligonucleotide DDO 46. This phosphate allows oligonucleotide DDO 47 to ligate to the cut site outside of the transposon and create a double stranded bubble adaptor.

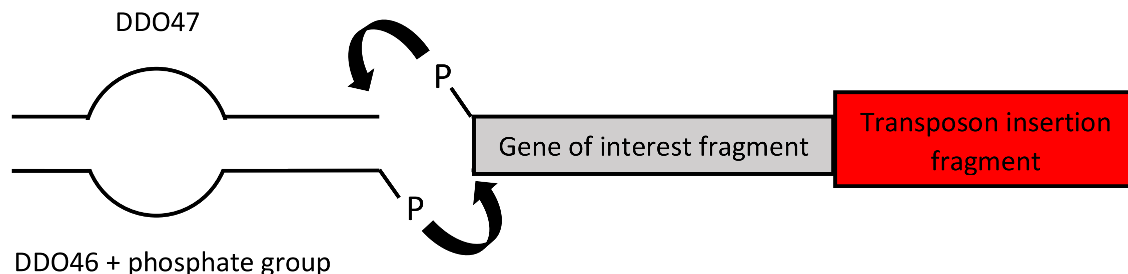

Figure 7. Ligation of anchor bubble adaptor to locate the gene that the transposon inserted into to cause the white colony phenotype.

A PCR reaction was used with the forward primer DDO45, which amplified from one part of the anchor bubble, and the reverse primer DDO48, which recognizes and amplifies within the transposon in the opposite direction, shown in Figure 8.

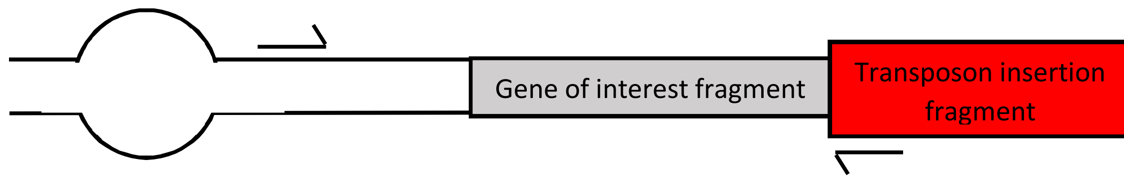

Figure 8. Primers used to amplify fragment for DNA sequencing of gene of interest.

The DNA from this PCR was separated by molecular weight in an agarose gel electrophoresis and the main band was excised and gel purified to remove the low molecular weight primers that ran further down the gel. If these primers were not removed, they would interfere with the sequencing reaction. The purified DNA fragments were then sequenced by Sanger DNA sequencing using Big Dye terminators**.** The DNA sequence was compared to the *S. cerevisiae* BLAST database to determine which gene the transposon inserted into. Some pertinent genes found in this screen are shown in Table 1 along with a brief description of the gene.

Gene	**Isolate #**	**Brief Description**
ADE3	6	Catalyzes a step in the *de novo* purine nucleotide biosynthetic pathway; red pigment accumulates in mutant cells deprived of adenine
ADE4	30	Catalyzes first step of the *de novo* purine nucleotide biosynthetic pathway
SIR4	31	Sir protein involved in assembly of silent chromatin domains along with *Sir2p* and *Sir3p* at telomeres and silent mating-type loci
BRE2	8	Subunit of COMPASS (Set1C) complex; COMPASS methylates Lys4 of histone H3 and functions in silencing at telomeres
NGG1	11	Subunit of chromatin modifying histone acetyltransferase complexes (with Gcn5p), showing less repression when a mutation in HAT should show more repression
SET1	13	Involved with *BRE2*, histone methyltransferase, subunit of COMPASS complex, modulates histone acetylation levels in promoter proximal regions to ensure efficient Nrd1p-dependent termination; required in transcriptional silencing near telomeres and at silent mating type loci
CRC1	27	Mitochondrial inner membrane carnitine transporter; required for carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria during fatty acid beta-oxidation
YNL033W	22	Protein of unknown function, but it is upregulated 1.5X in *RPD3* mutants
NUP1	M2	FG-nucleoporin component of central core of the nuclear pore complex; contributes directly to nucleocytoplasmic transport and maintenance of the nuclear pore complex permeability barrier

Table 1. Genes implicated in phenotype switch of Colony Color Assay which are preliminarily assumed to reverse the increased silencing caused by *rpd3*Δ. Gene descriptions from NCBI BLAST database for *S. cerevisiae*.

The results of the *ADE3, ADE4*, and *SIR4* serve as controls for the color colony assay, confirming that the assay was run correctly. Interruption of the *ADE3* or *ADE4* genes prevents accumulation of the red pigment made later in the adenine nucleotide synthesis pathway. Inactivation of *SIR3* or *SIR4* results in complete derepression of the *HMR* locus, expressing the

ADE2 gene and producing white colonies (Sussel, 1993). *BRE2* is of interest because it has been found in previous experiments, and *SET1* codes for a histone methyltransferase whose function requires the *BRE2* gene product (Kleinschmidt, 2011). We decided to further investigate the genes *NGG1* and *CRC1* because they have not been found in other screens performed in this lab, and their descriptions in the NCBI database appear to show potential connections to chromatin modification.

To further investigate *NGG1*, we conducted two matings of haploid yeast strains to determine if the change in phenotype from pink to white was caused by the *LEU2* insertion or a random mutation. The sequence of *NGG1* was isolated from transformant #11 which displayed a variegated colony morphology, meaning that even when a white colony was streaked onto YMD -leu 15% ade, it gave rise to both pink and white colonies. All transformants are mating type a (MATα), so to create mating crosses with the transformants, a MATa strain, DDY 814, was used. DDY 814 also contains a wild type LYS2 gene, an *ADE2* reporter gene at the *HMR* locus, and functioning *RPD3* (Appendix Table 2). To test whether the change in phenotype was caused by *LEU2* or random mutation, a white #11 transformant colony was crossed with DDY 814. This mating and subsequent sporulation into four haploid spores confirmed that the *LEU2* phenotype always co-segregated with the white phenotype, meaning that the change in phenotype from pink to variegated was due to the *LEU2*-marked transposon insertion rather than a random mutation. Another control cross was performed with DDY2093 and DDY2461 (Appendix Table 2). DDY2093 contains a transposon with a *LEU2* marker that disrupts *RPD3*, and DDY2461 has a deletion of *GCN5*. Ngg1 and Gcn5 are both part of the SAGA complex involved in transcription activation, and Gcn5 is responsible for the histone acetyltransferase (HAT) activity. Because *RPD3* deletion has been shown to increase silencing at *HMR* and because the HAT Gcn5p

generally increases transcription, we assumed that disrupting *GCN5* in addition to *RPD3* would disrupt SAGA more, increasing gene silencing. However, while the *rpd3* deletion alone grew pink, the *rpd3* and *gcn5* delete mating cross grew white, meaning that the double deletion decreased gene silencing at the *HMR* locus. Though these results are conclusive that *NGG1* variegated colony morphology is caused by the transposon-mediated *LEU2* insertion, the results that disrupting the SAGA complex reversed the increased silencing is the opposite of what would be expected.

To further investigate *CRC1*, we first performed a deletion of *CRC1* to observe the phenotype displayed with a complete loss of function of *CRC1*p. The primers DDO 2113 and DDO 2114 were used to create a knockout *crc1Δ*::*LEU2* fragment using the plasmid pRS405 (pDD672) as the *LEU2* template. This knockout DNA was transformed into DDY 814, containing wild type *RPD3* and *HMR-ADE2*, and DDY 5604, containing *RPD3* delete (full genotype in Table 2). The transformation was plated onto YMD -leu to select for transformants containing the *CRC1* knockout. Though the transformation was performed multiple times, all colonies were extremely slow growing. This phenotype suggests that the complete deletion of *CRC1* and *RPD3* together is in some way deleterious to growth.

Due to the slow-growing phenotype observed in the *CRC1* knockout and based on the observation by Tsukiyama that *CRC1* is upregulated 4X in *RPD3* deletion mutants, we decided to test whether the overexpression of *CRC1* would reverse the increased silencing phenotype in *RPD3* mutants. The overexpression experiment was performed at two levels by cloning *CRC1* into both a low copy number plasmid (pDD637) to slightly overexpress *CRC1* and a high copy number plasmid (pDD639) to greatly overexpress *CRC1* (Sikorski et al., 1989. Christianson et al., 1992). First, a restriction digests of both vector plasmids were performed using NotI and SalI

to make 5.5kb and 6.0kb fragments. Then the *CRC1* DNA fragment and appropriate plasmid vector were transformed into *E. coli* and grown on LB +ampicillin plates to select for cells with transformed vector plasmids containing *CRC1*. Then, a miniprep was performed to isolate and purify the *CRC1* + vector plasmid DNA from the *E. coli.* Additionally, a restriction digest with NotI and SalI was performed on a portion of the miniprep DNA to check for both the 5.5 or 6.0 kb vector plasmid fragment and the *CRC1* fragment. Sequencing of the plasmids was performed with primers DDO 183, a T3 sequence that base pairs upstream to the plasmid sequence just outside of the cloning site, and DDO 184, a downstream primer. Both the T3 fragment that elongates in one direction and the T7 fragment that elongates in the other direction were sequenced, and the sequences overlapped for each plasmid tested. In addition to the sequencing, the plasmids were transformed into DDY 814, the *RPD3* wild type, to see if overexpression of *CRC1* alone leads to increased silencing without *RPD3* deletion. Five transformations were performed: one with the *CRC1* low copy number vector, one with the *CRC1* high copy number vector, one empty low copy number vector without *CRC1* as a control, one high copy number vector without *CRC1* as a control, and one no DNA control. All transformed colonies for both the low and high copy number vector containing *CRC1* remained white, showing that overexpression of *CRC1* does not lead to increased silencing on its own.

Conclusions and Further Study

Overall, the colony color assay appeared to be useful, as genes of interest were identified from the white colonies that showed reversal of increased silencing in *RPD3* deletion mutants. Because only several pools of the transposon plasmid library were used in this experiment, the possibility remains that all 20+ pools of DNA need to be used to cover the entire library of transposon insertions to find all the genes involved in reversal of the *RPD3* deletion phenotype. There is also the possibility that two transposons inserted into one yeast strain causing reversal of the phenotype, and the PCR primers only amplified one of the transposon insertions. In this case, the gene causing the reversal is still at large, but it could be found by repeated trials of the colony color assay.

Since the knockout of *CRC1* was slow growing and the overexpression of *CRC1* did not cause silencing on its own, the original mutation that increased the reversal of silencing in the *rpd3*Δ mutant could be caused by an increase of *CRC1* and a change in expression level of some other unknown protein. One future study that could be used to further test the *CRC1* gene is to test an insertion with a marker gene flanked to the site of the transposon. This experiment is similar to the knockout performed previously, but with the insertion of an oligonucleotide instead of a deletion. Because the insertion will allow the beginning of the gene to be expressed, this experiment could determine if the originally observed phenotype was due to a partial protein with partial protein activity or a partial protein with dominant negative activity.

Though the results of *NGG1* deletion were the opposite of what was expected and the results of *CRC1* were generally inconclusive, the methods used to further study these genes can still be used in studies of other genes of interest later. Additionally, the colony color assay

performed using *rpd3*Δ strains can still serve as a useful method to determine genes of interest that can relieve the increased silencing caused by deletion of *RPD3*. It is important to understand the global genomic effects of histone deacetylase inhibition, especially because this approach is beginning to be considered for treatment of cancer. Because cancer itself can be caused by improper genetic regulation, it is of the utmost importance to understand all genetic effects of changing gene regulation, especially when disease treatments for human patients are involved. In this way, a "basic" science study, such as these molecular genetics experiments using a single-celled eukaryote model, can have far-reaching applications for human health and understanding the mechanisms that underlie the most basic units of inheritance.

Appendix

S. cerevisiae strains	Genotype
DDY814	MATa ade2 his3 leu2 LYS2 trp1 ura3 HMR-ADE2
DDY3133	MATα ade2 his3 leu2 lys2 trp1 ura3 VII-L-URA3-TEL ppr1Δ::TRP1 HMR-ADE2 rpd3Δ::KanMX
DDY2093	MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2 Tn:LEU2:rpd3
DDY2461	MATa ade2 his3 leu2 LYS2 trp1 ura3 gcn5::TRP1 HMR-ADE2

Table 2. List of yeast strains used in this study and their genotypes.

Plasmid	Description
pDD637	*S. cerevisiae* ARS CEN LEU2 vector, Sikorski et al. (1989)
pDD639	*S. cerevisiae* 2 micron LEU2 vector, Christianson et al. (1992)
pDD672	*S. cerevisiae* LEU2 vector, Sikorski et al. (1989)

Table 3. List of plasmids used in this study.

Oligonucleotides	Sequences
DDO45	CGCCAGGGTTTTCCCAGTCACGAC
DDO46	GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGA GAAGGGAGAG
DDO47	GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTG TCCTCTCCT
DDO48	CGAATCGTAACCGTTCGTACGAGAATCGCT
DDO183	AATTAACCCTCACTAAAGGG
DDO184	GTAATACGACTCACTATAGGGC
DDO2113	AGGTTTTCCTTGGATTCTACGTATTGTACGACTTTCTTATCCTCC ACAAACGTCATCGTGTCAGTGCAGATTGTACTGAGAGTGC
DDO2114	ACAAAAAGTTCCCAAAAGGAATGCCAGAAAAAAATGGGAGGCA GGTCCACCTAACAAAAATTTAACTCCTTACGCATCTGTGCGG
DDO2117	GGGTAGCAGCGTGCGGCCGCGCGATCAAGGGTGTGCTAAG
DDO2118	TCGGTTGATTATGTCGACATCAGCCCACGTTGTCTTTG

Table 4. List of oligonucleotide primers used in this study.

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