Investigating the Effects of Oncohistones on mRNA Processing

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Abstract

This paper examines the effects of oncohistones on mRNA processing by specifically focusing on oncohistone-associated post-translational modifications (PTMs) and whether those modifications have interaction with 3'-end RNA processing units. PTMs lead to epigenetic modification of genes such as those associated with DNA synthesis, regulation of gene expression, or DNA repair (Khorasanizadeh, 2004; Qui et al., 2018; UniProt, 2019; Young, 2001). PTMs on histone H3 have been an area of recent research due to its activity within the nucleosome and the number of mutations - specifically oncogenic - associated with it (Mandel et al., 2008; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). To examine histones' function on epigenetic processes, previous studies using assays and *Drosophila melanogaster* found a strong connection to polyadenylation of the 3'-end of RNA (Li et al., 2016; Mandel et al., 2008; Mathieu & Bouché, 2014; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). This research sought to determine whether a genetic connection between the histone mutation and polyadenylation existed by utilizing *Saccharomyces cerevisiae*, budding yeast. High-copy suppressor screen transformations were performed to identify any potential genetic interactors between mutated histone H3 and 3'-end mRNA processing factors. Candidates that suppressed the growth defect of histone H3 mutants were sent for sequencing and their genes were identified. Finally, these genes were examined through literature searches to determine whether they were related to 3' end mRNA processing. Overall, preliminary results suggested a potential connection to mRNA processing, but results also introduced a new connection to mitochondrial function.

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Introduction

Histone Overview

Deoxyribonucleic acid (DNA) is stored in the nucleus as a condensed form called chromatin. Chromatin consists of subunits called nucleosomes, and these nucleosomes are the basic units of genetic structure, consisting of about 150 base pairs and four core histones, illustrated in Figure 1. The DNA wraps around each of the four histones, maintaining its structure, and they unfold at promoters such as TATA boxes to easily start transcription (Khorasanizadeh, 2004).

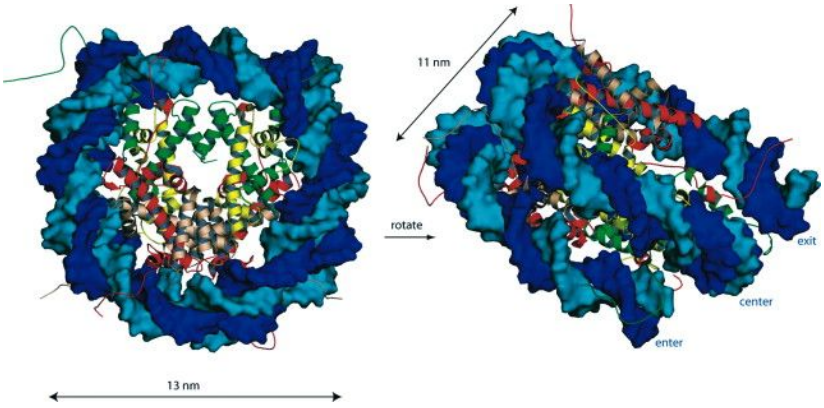


Figure 1: Nucleosome structure with the four core histones H2A, H2B, H3, and H4. Adapted from "The nucleosome: From genomic organization to genomic regulation" by S. Khorasanizadeh, *Cell*, *116*(2), 260. Copyright 2004 by Cell Press.

The four core histones are H2A, H2B, H3, and H4. The histones create weak hydrogen bonds, called heterodimers, with each other, through handshake agreements (Khorasanizadeh, 2004). Because of their structural function, these core histones are not diverse, and they have specific functions (Qiu et al., 2018). These proteins contain long, modifiable tails that impact

gene expression by altering the accessibility of DNA to transcription factors (Khorasanizadeh, 2004; Qui et al., 2018; Young, 2001).

Post-Translational Modifications (PTMs)

Each core histone consists of a globular domain and an N-terminal tail that can be modified (Alberts et al., 2015), and modification of the tails is a post-translational modification (PTM), as seen in Figure 2. These modifications have a significant impact on gene expression, dictating DNA repair sites, and marking parent and daughter DNA. There are several common modifications, such as methylation, acetylation, phosphorylation, etc., which serve as markers for proteins to help the nucleosome as needed as well as work with the DNA to regulate transcription of the genome. Another PTM is imprinting, or adding methyl groups to amino acids, which can pass on epigenetic information not stored in the DNA. While most of these modifications are reversible, methylation is not (Khorasanizadeh, 2004; Young, 2001).

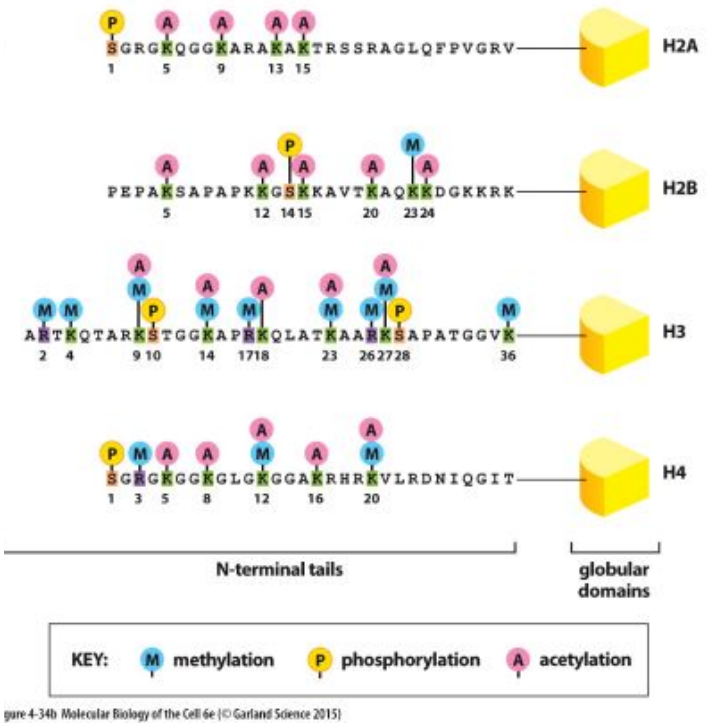


Figure 2: Core histone structure with some common post-translational modifications. Adapted from "Molecular biology of the cell" by Alberts et al., 2015, New York, NY: Garland Science.

Histone H3

One of the most intriguing histones studied is the core histone H3. Its tail goes through the nucleosome, making it easy to modify, and directly contacts the DNA. This proximity suggests a more important role in genetic processes (Khorasanizadeh, 2004). Due to its position, PTMs on H3 not only affect one part of the genome, but they can turn off multiple genes (Young, 2001). Like other histones, H3 has different variants with the most impactful being H3.3; it is the most prevalent form of H3 when the cell is not dividing, and it is located around transcribed genes (UniProt, 2019). It is also the most studied variant of H3 because of the many mutations associated with it (Qui et al., 2018).

Histone Mutations

Histone mutations have become of greater interest due to their associations with diseases, especially cancers. Multiple studies, including Maze et al. (2014; Figure 3) describe histone variations by detailing each mutation and what cancers are associated with it.

Table 2 | **Core histone variants in human disease**

Histone	Number of gene copies	Cell-cycle expression	Mutation and expression pattern	Tumorigenic consequences	Refs
H2A.X	1	RI	Reduced expression	Increased cancer progression in p53-knockout mice	60,61
H2A.Z	2	RI	Over-expression; oncogene	Numerous cancers	121–123
MacroH2A	2	Possibly RI	Reduced expression; tumour suppressor	Melanoma and other cancers	141
H3.1	10	RD	K27M in H3.1B	Adult and paediatric gliomas, including GBMs and DIPGs, respectively	83
H3.3	2	RD and RI	K27M, G34R and G34V in H3.3A	Adult and paediatric gliomas, including GBMs and DIPGs, respectively	82,83
			K36M in H3.3B	Chondroblastoma	101
			G34W and G34L in H3.3A	Giant cell tumours in bone	
CENP-A	1	RI	Over-expression; oncogene	Numerous cancers	115–119

CENP-A, histone H3-like centromeric protein A; DIPG, diffuse intrinsic pontine glioma; GBM, adult glioblastoma; ND, not determined; RD, replication dependent; RI, replication independent.

Figure 3: Histone variants in human diseases, the emphasis on H3.3 mutations. Adapted from "Every amino acid matters: essential contributions of histone variants to mammalian development and disease" by Maze et al, *Nature Reviews Genetics*, *508*, 259–271. Copyright 2014 Macmillian Publishers Limited.

In 3.8% of tumors, researchers found histone mutations, the same prevalence as the activation of certain oncogenes such as *BRCA2* and *NOTCH1* (Nacev et al., 2019). Histone H3 is especially important due to the number of cancers associated with it which are likely caused by the frequent mutations of PTM-affecting genes (Maze et al., 2014; Qui et al., 2018). This prompted researchers to take a closer look at these cancer-causing histone mutants such as H3.

The histone H3 mutations that this study focused on primarily involves the amino acids lysine and glycine. For example, K36M is a mutation at the 36th amino acid on the histone tail, that results in a lysine to methionine amino acid change. In H3K36, if the amino acid is acetylated, transcription proceeds as normal, but if methylated, the transcription is blocked and creates less H3K36me3 leading to less methylation of oncogenic (cancer-causing) genes (Nacev et al., 2019; Qui et al., 2018). The G34 mutation causes mutations in other regions, leading to abnormal telomere lengths which is a common feature in cancers (Qui et al., 2018). K36M leads to more methylation, activating Polycomb Repressor Complex-regulated genes, which can promote tumors in mice (Nacev et al., 2019). Examination of these mutations and their pathways (Qui et al., 2018) have provided a stronger link between histones and tumorigenesis.

3’ RNA Processing and Histones

Many PTMs on H3 lead to alterations in transcription such as K36 leading to disruption in transcript elongation, DNA repair, and splicing out introns (Maze et al., 2014). A study by Meers et al. with H3K36R mutations suggests that H3K36 plays a larger role in the human genome than previously thought (2017). The enzymes that modify it make it difficult to study the function of H3K36, so researchers mutated fruit flies to create an unmodifiable version. The researchers discovered that the mutants failed to develop past the larval stage and that mutants had less mature transcripts of highly expressed genes, and the length of the polyadenylated tails were reduced (Meers et al., 2017). These discoveries suggest H3K36 has a post-transcriptional role, possibly with the complex involved in polyadenylation of the 3’-end of mRNA (Meers et al., 2017).

Yeast Modeling

Saccharomyces cerevisiae, or budding yeast, has become a popular model for eukaryotic processes due to its flexibility, low cost, amount of manipulatable DNA, quick growth, ability to switch mating types, well-defined genetic system and the ability to exist in haploid or diploid states (Duina, Miller, & Keeney, 2014; Hübel, 2009; Sherman, 1998). Budding yeast proteins are similar to human proteins, and human-native proteins can be altered to function in yeast (Hübel, 2009). Many non-coding genes in yeast are associated with RNA translation which makes it a useful model to explore translation in eukaryotes (Duina et al., 2014). Further, yeast genes are easily modifiable and can replicate genetic information in plasmids, giving researchers a greater level of control (Duina et al., 2014; Sherman, 1998). This simplicity and flexibility has allowed researchers to study telomeres and centromeres, and gain understanding of basic eukaryotic

functions as a whole (Duina et al., 2014; Hübel, 2009). However, some limitations to this model include the inability to encode drug targets in the genome, no similar genes to cancer-causing genes, and the limitations of extrapolation to more complex eukaryotic organisms (Duina et al., 2014; Hübel, 2009).

Additionally, yeast is a powerful tool to study human histones because most of the core histones in yeast are conserved meaning they are similar to human histones (Eriksson, Ganguli, Nagarajavel, & Clark, 2012; McBurney et al., 2016). In regards to this study, histone H3 is highly conserved between yeast and humans with a similarity of almost 90% (McBurney et al., 2016). In budding yeast, H3 is encoded by only two genes: *HHT1* and *HHT2*, and only one of them is needed to function (Eriksson et al., 2012). This adds an extra layer of control for researchers and for flexibility of mutant creation.

There are several methods of using yeast to study eukaryotic function. This study will primarily utilize a screen to overexpress and identify suppressors of growth defects caused by mutations in histone H3. Suppression of the growth phenotype will only occur when certain genes are overexpressed and, thus, identified in the screen (Sherman, 1998); for this study, one of the histone-encoding genes, *HHT1* (Eriksson et al., 2012), was knocked out of mutants, and any mutation phenotypes to examine suppression are specific to mutants in the *HHT2* gene. These techniques were utilized heavily in the study to determine potential suppressors on different drugs.

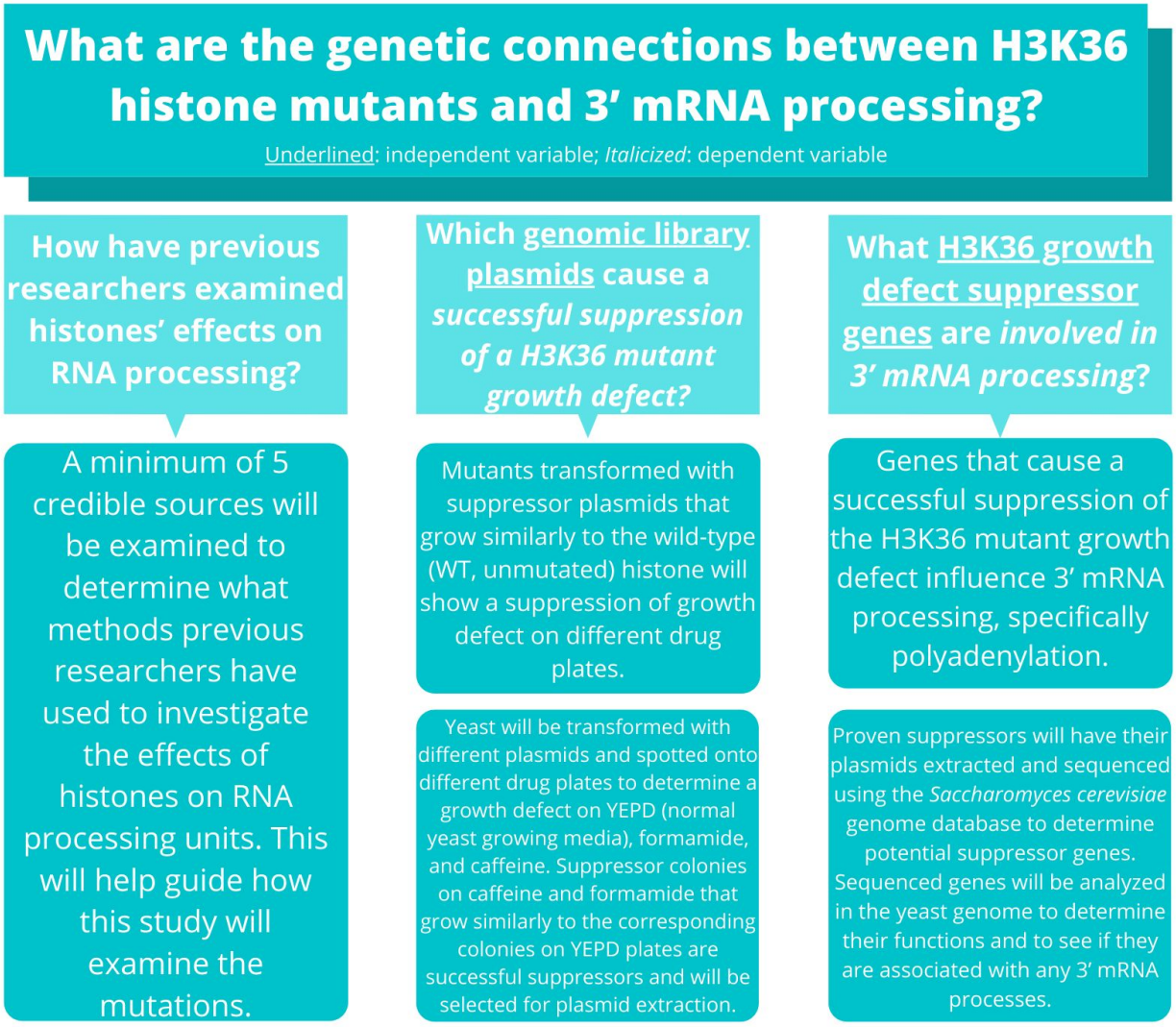
Rationale

This study investigated how RNA processing is affected by mutations on the H3 histone tail. Throughout elementary and middle school, I was encouraged by my mentor, Ms. Susan Peebles, to pursue my academic interests, especially in science. When she was diagnosed with liver cancer in seventh grade, this amplified my desire to pursue cancer research. This led to me taking several internships and advanced scientific courses to aid me in developing a cure. I developed a mental plan, but was unsure of its feasibility. However, after learning of my mentor's work with H3 oncohistones, I decided to use this opportunity to jumpstart my dream of developing a cure.

Histone H3's proximity to DNA, frequent mutations, position next to transcription genes, and ability to affect multiple genes (Khorasanizadeh, 2004; Qui et al., 2018; UniProt, 2019; Young, 2001) has increasingly intrigued researchers to study its function in more detail. Histone mutations occur with modifications on their tails, and they have become widely researched in the past few years after researchers discovered a significant percentage of tumors contained them (Qui et al., 2018). H3 mutations are the most studied and most common, and they are dominant negative. These mutations can cause issues such as affecting transcription, abnormal telomere lengths, and amplifying oncogene expression. The H3K36 mutation has a profound effect on transcriptional quality, and researchers concluded this mutation controls a large part of the genome and could have a post-translational role with the polyadenylation complex, a 3' mRNA processing element used to enhance the transcript (Mandel et al., 2008; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). While this literature establishes a link between histone mutations and RNA processing, there is no literature that definitively defines what this link is.

To further understand the link between RNA processing and H3 histone mutations, this study utilized yeast modeling to determine whether H3 histone mutations genetically interact with 3' mRNA processing elements. This research contributed to an enhanced understanding of histone mutations' role in eukaryotic cell growth, and it can be used as an additional consideration when examining cancer cell function in complex eukaryotes.

Research Map



Scope of Study

Delimitations/Limitations

- This study did not create histone H3 mutants.
- This study did not examine the effects of the H3K36 mutation by itself. It only investigated the effects of suppressors identified in the high copy suppressor screen on H3K36 mutant growth defects.
- The study utilized mutants made previously by the mentor.
- The study used plasmids from a premade genomic library for the high-copy suppressor screen.
- The study's researchers sent plasmids to a company for sequencing.

Assumptions

- This study assumed any growth defect suppression is due only to the plasmids or lack thereof, not because of any other genetic factors.
- This study assumed that all sequencing data received from McLab is accurate and adheres to established scientific standards.
- This study assumed that all the genomic information in the *Saccharomyces cerevisiae* database is updated and accurate.
- This study assumed that all H3K36 mutants created by the mentor previously were viable and unchanged.
- This study assumed the *Saccharomyces cerevisiae* database has functions for all genes listed.

Key Terms

Key Term	Definition
Nucleosome	Subunit of chromatin, contains genetic information, wrapped around four core histones with a linker histone connecting two units (Khorasanizadeh, 2004).
Post-translational modifications (PTMs)	Biochemical modification at amino acids on histone tails, impacts gene expression (Alberts et al., 2015; Khorasanizadeh, 2004; Qui et al., 2018; Young, 2001).
Histone H3	Core histone and one of the most important parts of nucleosomal structure, has several variants and modifications associated with cancer (Khorasanizadeh, 2004; Maze et al., 2014; Qui et. al, 2018; UniProt, 2019; Young, 2001).
NCBI BLAST search	The process of inputting a string of genetic sequence (nucleotides) and searching for a match in the *Saccharomyces cerevisiae* database (Engel et al., 2014).
High-copy suppressor screen (HCSS)	Transforming a genomic library of plasmids into yeast and checking for suppression of a growth defect (Ramer, Elledge, & Davis, 1992; “Yeast Genetics”, 2004).
Polyadenylation complex	Cellular elements used to polyadenylate mature mRNA transcripts, helps enhance the quality of the transcript, has a theoretical connection to histone modification (Mandel et al., 2008; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018).

Statement of Focus and Sub Problems

Histone H3 has become increasingly relevant due to its proximity to DNA, ability to affect multiple genes, and frequency of mutation (Khorasanizadeh, 2004; Qui et al., 2018; UniProt, 2019; Young, 2001). In addition, these H3 mutations appear frequently in cancers (Maze et al., 2014; Nacev et al., 2019; Qui et al., 2018; UniProt, 2019) and can lead to disruption of cellular function. One of these mutations, those on H3K36 are associated with abnormal transcription and a potential role in polyadenylation (Maze et al., 2014; Meers et al., 2017). In current literature, many researchers have posited a link between mRNA processing and H3 histone modification (Li et al., 2016; Mandel et al., 2008; Mathieu & Bouché, 2014; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). However, there is no defined genetic link between the two processes. Therefore, the following focus question was addressed through this study to fill a gap in literature:

- What are the genetic connections between H3K36 histone mutants and 3' mRNA processing?

To answer the focus question, this study investigated the following sub-problems:

- Basic Sub Problem: How have previous researchers examined histones' effects on RNA processing?
- Applied Sub Problem: Which plasmids cause a *successful suppression of a H3K36 mutant growth defect*?
- Applied Sub Problem: What H3K36 growth defect suppressor genes are *involved in 3' mRNA processing*?

Literature Review

Introduction

This study aimed to find a link between histone mutations and 3'-end RNA processing. The study took a sub-problem based approach by determining candidate suppressors of histone H3 mutant growth phenotypes and categorizing them according to function. First, researchers performed a transformation of yeast containing a genomic library of unique plasmids and growing master plates. These plates were "copied" via replica plating onto media containing drugs (caffeine, formamide, hydroxyurea, and phleomycin) to look for initial growth defects. Colonies that exhibit growth defects were resuspended, and a spotting assay was performed. After reconfirming suppression of the initial growth phenotype, the plasmids were extracted and sequenced to look for 3'-end RNA processing elements.

To aid the study, literature was analyzed to determine previous methods of examining histones and their effects on 3'-end RNA processing units as well as methods of determining abnormalities in yeast histones. Studies have indicated that histone H3 has an important, post-translational role in the genome (Khorasanizadeh, 2004; Qui et al., 2018; UniProt, 2019; Young, 2001), and examinations of cancer cells have revealed the abundance of H3 histone mutations (Qui et al., 2018) leading to increased interest. They have also linked a histone H3 mutation, H3K36R, to transcriptional genes and potentially to the polyadenylation complex, a 3' processing function to create a better transcript (Mandel et al., 2008; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). Despite the speculation, no literature has attempted to create a definitive link between H3 mutations and the polyadenylation complex which is what this study aimed to establish through histone yeast modeling.

Histones and their Effects on RNA Processing

Since this study focused on the effect of histone modifications on 3'-end RNA processing machinery, previous studies were examined to determine what questions to investigate. One piece of literature that catalyzed the examination of histones' connections to RNA processing is a 1987 study about histone removal. Yeast cells were plated onto different sugars after removal of the core histone H2B. When examining the cells, the researchers found them in a mitotic state, but the removal of the histone nearly halted division (Han, Chang, Kim, & Grunstein, 1987). They also discovered that any cells without H2B would not grow, even on different sugars, suggesting permanent damage to the yeast chromosomes (Han et al., 1987). Without the histone, normal DNA replication occured, but there was a decrease in histone mRNA (Han et al., 1987). These findings implied that nucleosomal histones have a stronger role in genetic processes than previously examined.

Histone Defects and pre-mRNA Splicing

Subsequent literature has associated histone defects with pre-mRNA splicing. RNA processing factors are linked to either RNA Polymerase II (RNAPII) or histones, establishing a general connection between the two (Böhm & Farrants, 2011). RNA polymerase copies DNA sequences into pre-mRNA. A defect in the amount of DNA-dependent, or canonical, histones led to an increase in the DNA-independent histone H3.3 (Jimeno-González et al., 2015), a histone heavily associated with tumorigenesis (Maze et al., 2014). However, histone removal caused RNAPII delays and thus delayed pre-mRNA splicing, leading to less non-coding regions being spliced out (Böhm & Farrants, 2011; Jimeno-González et al., 2015). Retaining the non-coding regions leads to replication defects, uncontrolled growth in non-mitotic cell phases, and genetic

instability (Jimeno-González et al., 2015). Researchers also noticed that coding regions, or exons, in the mature mRNA have more histones with elongated PTMs on their tails (Böhm & Farrants, 2011; Jimeno-González et al., 2015), specifically H3K36me3 (Böhm & Farrants, 2011) which is associated with RNA processing (Meers et al., 2017).

Histone Defects and Effects on Other RNA Processing Elements

These PTMs have also been associated with other aspects of RNA processing such as capping and polyadenylation (Li et al., 2016; Mathieu & Bouché, 2014; Meers et al., 2017). Chromatin helps recruit units associated with RNA processing, such as spliceosomes to take out non-coding units, methylation complexes to modify histone tails (Li et al., 2016; Mathieu & Bouché, 2014), selecting polyadenylation sites on mRNA transcripts (Mathieu & Bouché, 2014) and works in part with the 5' capping machinery to improve the quality of the transcripts (Li et al., 2016). The capping complex and chromatin modifications work co-transcriptionally to regulate gene expression, and interruption of these processes could negatively impact splicing and methylation (Li et al., 2016), potentially leading to activation of oncogenes and Polycomb complexes leading to tumorigenesis (Nacev et al., 2019; Qui et al., 2018). These methylated histone tails create better transcripts (Mathieu & Bouché, 2014) which makes them an important aspect of gene expression. Since literature has indicated a strong connection between histones affecting pre-mRNA processing complexes, this study could add to the existing knowledge by positing a link between the two.

Detecting Abnormalities in Yeast Histones

Previous researchers have examined several histone residues such as those at H3K36, H3K27, H3G34, and H3L61 (Jha & Strahl, 2014; Johnson et al., 2015; Mandel et al., 2008;

Maze et al., 2014; Meers et al., 2017; Nacev et al., 2019; Qui et al., 2018). This study utilized the H3K36R/M mutants previously developed in the lab since they are commonly associated with tumorigenesis (Maze et al., 2014). These residues have been examined in three main methods: plating on different media and examining growth defects, using Western blots to examine protein levels, and assessing spotting assays for dominant-negative expression (Duina and Winston, 2004; Jha & Strahl, 2014; Johnson et al., 2015; Mei et al., 2019).

Plating with Different Media.

Studies have utilized plating on media with different drugs or sugars to examine growth defects in yeast. Plating on these drugs can indicate the viability of a mutant (Duina & Winston, 2004; Jha & Strahl, 2014; Johnson et al., 2015), can help determine a connection between chromatin and transcription (Johnson et al., 2015), and act as a method for detecting drug sensitivity (Figure 4; Duina & Winston, 2004; Jha & Strahl, 2014).

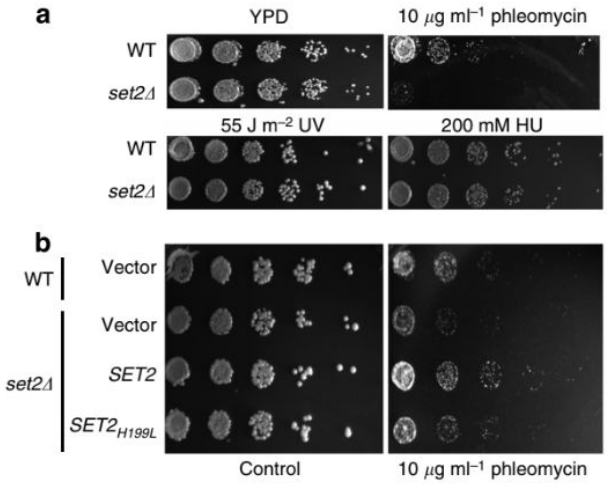


Figure 4: Example of a sensitivity to phleomycin for the *set2Δ* mutant in part **a**, and the rescue of the growth defect in part **b** with the addition of normal *SET2*. Adapted from "An RNA polymerase II-coupled function for histone H3K36 methylation in checkpoint activation and

DSB repair" by Jha & Strahl, 2014, *Nature Communications*, *5*(1). Copyright 2014 Macmillan Publishers Limited.

Some common drugs/sugars used for detecting inhibition of cellular functions are phleomycin (Jha & Strahl, 2014), formamide, caffeine, hydroxyurea (Duina & Winston, 2004; Johnson et al., 2015), dextrose, and galactose (Jha & Strahl, 2014). The growth defect on these plates can help researchers determine the issue within the cell since each drug is associated with a certain cellular pathway. Mutants can also be tested for sensitivity by utilizing different temperatures while growing cultures (Duina & Winston, 2004; Johnson et al., 2015). Growth defects can also be examined on plates lacking an amino acid to test viability and chromatin interactions (Duina & Winston, 2004; Johnson et al., 2015). By utilizing these known growth defects in conjunction with a high copy suppressor screen to find a rescue of the growth defect ("Yeast genetics", 2004), this study identified suppressors of the H3K36 mutations.

Summary and Implications

While researchers have previously proposed a connection between processing and histone removal (Li et al., 2016; Mathieu & Bouché, 2014), not many studies have provided a connection between histone H3 and 3'-end mRNA processing (Mathieu & Bouché, 2014; Meers et al., 2014). In addition, many yeast genetics studies have utilized different methods of inducing histone H3 mutations such as enzyme modification (Jha & Strahl, 2014), modification of an amino acid (Johnson et al., 2015), or modification with plasmids (Duina & Winston, 2004; Jha & Strahl, 2014). Even though these mutants have been studied for growth defects, changes in protein levels, and whether they behave in a dominant negative fashion (Duina & Winston, 2004; Jha & Strahl, 2014; Johnson et al., 2015; Mei et al., 2019), not many have utilized a high copy

suppressor screen to find factors, specifically those involved in 3'-end mRNA processing, that genetically interact with modified residues on histone H3 ("Yeast Genetics", 2004). Based on previous research which led to the discovery of a link between histone modifications and RNA processing, this study aimed to contribute to literature by using a high copy suppressor screen and yeast modeling to propose a link between histone mutations and 3' mRNA processing.

This study utilized pre-made mutants, but to fill a gap in literature, it included a high-copy suppressor screen ("Yeast Genetics", 2004) in conjunction with drug plating on caffeine and formamide for growth defects since these are reliable methods that have been used heavily in previous studies (Duina & Winston, 2004; Jha & Strahl, 2014; Johnson et al., 2015). By combining these techniques, the study generated a list of plasmid suppressors that impact 3' end mRNA processing.

Methodology

The methodology for this study is detailed below. The methods detail the process of transforming yeast mutants, screening for suppressors, and analyzing sequencing results. This process of gathering suppressors allowed the study to create a list of suppressor genes that may be involved with 3'-end mRNA processing elements.

Yeast Transformation

Mutated histone H3 yeast colonies were grown in test tubes in 10mL of nutrient-rich (YEPD) media. After growth, these cells were spun in a centrifuge at 3000 rotations per minute (RPM) for 3 minutes until a pellet is formed. The pellets were resuspended in 1 mL of water and transferred to a microfuge tube, and the tubes were spun at 13000 RPM for one minute. The water was removed and the pellets washed with 1 mL of TE buffer and lithium acetate (LiOAc). The TE/LiOAC was removed and the pellet resuspended in 300 µL of TE/LiOAc mixture. Once the pellet was resuspended and aliquoted 100 µL into tubes, 1 µL of plasmid DNA was added to the tubes.

Salmon sperm (ss) carrier DNA was heated at 100°C and snap-chilled for 5 minutes, and 10 µL was added to the yeast/TE/LiOAc mixture. After the addition of ssDNA, 600 µL of a PEG/LiOAc/TE solution was added to the yeast tubes and then vortexed for about 10 seconds. The tubes were then incubated at 30°C with agitation for 30 minutes. After agitation, tubes were mixed with 70 µL of DMSO and inverted to mix. Those tubes were heat shocked at 42°C for 15 minutes, then spun down at 13000 RPM for a minute and washed with water, then spun again. The water was removed from the tube and resuspended in 100 µL. The yeast was plated on uracil drop-out plates- to select for colonies containing the high-copy suppressor plasmid- using glass

beads, and incubated for growth. This screening process was repeated until the same suppressors appeared repeatedly and with another plasmid library to verify the results of the screen.

Determining Suppressors

Scanning for Preliminary Suppressors

After the plates were incubated for growth, preliminary scanning for suppressors began. The yeast colonies were patched onto dropout uracil plates by picking colonies off of the growth plates and patched onto a gridded plate. These plates acted as master plates for colonies used in suppressor detection. The plates were incubated until fully grown colonies appeared. Once the patched colonies grew, the plates were replica-plated onto yeast growth media (YEPD), caffeine, and formamide (Duina & Winston, 2004; Johnson et al., 2015). A replica-plating block was sterilized with ethanol, and a piece of sterilized velvet was placed on the block, secured with its ring. The master plate was imprinted onto the velvet. Once the cells were imprinted, each drug plate was pressed onto the imprinted cells until on the plate. These plates were left to incubate to determine preliminary suppressors. The plates grew at 30°C until the plates showed clear colonies, up to five days. Growth defects were examined on the formamide and caffeine plates in comparison to the YEPD plates which contain all essential nutrients for growth and, according to previous lab data, acted as a normal growth comparison for mutants compared to wild types. The colonies on caffeine and formamide that grew similarly to their YEPD counterparts were marked as preliminary candidates and selected for spotting assays.

Assay for Potential Suppressors

After selecting colonies to further screen for suppressors, a spotting assay was performed. The preliminary suppressors were picked off of the master plate and resuspended in - Ura media.

To perform the spotting assay, a 1:10 serial dilution was performed with each suppressor culture and positive & negative controls, and then samples were spotted using a multi-channel pipette onto YEPD, caffeine, and formamide plates (Duina & Winston, 2004; Johnson et al., 2015). The plates were allowed to dry and then incubated at 30°C until all plates had grown. Once the plates showed growth, they were examined to reconfirm suppressors. Similarly to replica plating, the suppressor rows on caffeine and formamide were compared to the empty control plasmid rows and wild type rows to determine successful suppression. Those colonies were labeled as final suppressors and selected for plasmid extraction.

Plasmid Extraction

Isolation

Next, the candidate suppressor plasmids were isolated from yeast for sequencing. The candidate suppressor colonies were resuspended in -Ura media and incubated overnight. The cultures were aliquoted into screw cap tubes, centrifuged, and the supernatant dumped, leaving the pellet. After only the pellet is left, 100 µL of STET buffer was added to the pellet and vortexed briefly. A cap full of small glass beads was added to the tubes, and then the cultures were vortexed for four minutes, rested, then vortexed again for four minutes at 4°C. Once finished, another 100 µL of STET buffer were added and then vortexed again briefly. The tubes were placed into a 100°C heat block for 3 minutes. The tubes were then cooled on ice for about two minutes; then, a blue tip was used to transfer the liquid into an eppendorf tube. The tubes were centrifuged at 4°C for 10 minutes. The supernatant was removed, put into a fresh tube with 500 µL of chilled 7.5 M ammonium acetate, and mixed. The tubes were incubated at -20°C overnight.

After incubation, the tubes were centrifuged for 10 minutes at 4°C, creating a small, faint pellet. One-hundred μL of the supernatant was added into a fresh eppendorf tube containing ice cold 100% ethanol. The tubes were mixed and left to stand for five minutes at room temperature and then centrifuged for ten minutes. The supernatant was discarded and the pellet washed with 200 μL of 70% ethanol. The tubes were centrifuged for five minutes; after, the supernatant was removed and the pellet tubes dried on paper towels.

Once dry, the pellets were resuspended in 20 μL of TE buffer. To ensure that the most pure plasmid is extracted, the plasmids were dialyzed for ten minutes. After dialysis, the plasmids were transferred into clean eppendorf tubes to prepare for bacterial transformation to amplify the plasmids for later use.

Bacterial Transformation

Along with the dialyzed plasmids, a 1 μL positive control was added in a separate tube, and 50 μL of *E. coli* bacteria was added with the plasmids. The bacteria was incubated on ice for 30 minutes, heat shocked for 50 seconds at 42°C, and incubated on ice again for two minutes. After incubation, 800 μL LB media was added and mixed in by tilting. The tubes were incubated at 37°C while shaking for an hour. The cells were spun down for three minutes at 8000 RPM, 900 μL LB was removed, and the pellet was resuspended in the remaining 100 μL of LB. The resuspended cells were spread on an antibiotic plate and incubated overnight. Once grown, single colonies were picked and resuspended in LB and antibiotics and then incubated overnight. This process amplified the plasmids in case they were needed again.

Sequencing

Preparing Plasmids

The plasmid extraction used a QIAGEN extraction kit. After the cultures grew, 5 mL of the bacteria was centrifuged at 8000 RPM for three minutes at room temperature. The media was dumped out and the pellet was resuspended in 250 μL of P1 buffer then transferred into a microcentrifuge tube. After resuspension, 250 μL of P2 buffer was added and mixed together by inverting it until the solution becomes clear. Once the solution is mixed, 350 μL of N3 buffer was added in and mixed by inverting it. The tubes were centrifuged for 10 minutes at 13000 RPM, and the supernatant was poured into the QIAprep spin column. The columns were spun for one minute and the flow-through discarded, then washed with 750 μL of PE buffer. The columns were spun again for a minute and the flow-through discarded again, and centrifuged again for a minute to remove any more flow-through. The QIAprep spin column was placed in a clean eppendorf tube and 50 μL of EB buffer added to it. The columns stood for a minute, and then were centrifuged for a minute. The QIAprep columns were discarded and the plasmids stored at -20°C.

After plasmids were extracted, a restriction enzyme digest gel was performed using the enzyme EcoRI and EcoRV to compare the similarity of suppressor genes between plasmids. While the mixture incubated, a 1% ethidium bromide gel was prepared. The gel ran for 30 minutes at 130 V and was then imaged to examine the bands. By examining the bands, it showed whether the genes on the final candidate suppressor plasmids had similar genes.

Analyzing Sequences

Sequencing results were analyzed using SnapGene software (from Insightful Science; available at snapgene.com) to select a string of plasmid bases. The base sequences were inputted into the *Saccharomyces cerevisiae* database and a NCBI BLAST search was performed to map the plasmid sequence to the corresponding gene region. Those genes were catalogued by suppressor and their functions analyzed to see if any were associated with 3'-end RNA processing.

Validity and Reliability

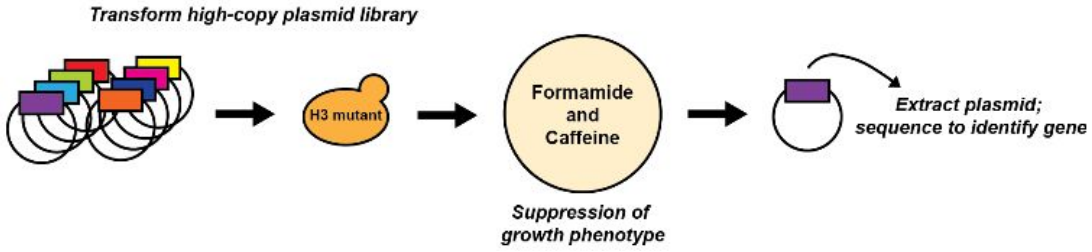
Previous researchers have utilized caffeine and formamide to detect growth suppression (Duina & Winston, 2004; Johnson et al., 2015), and the high-copy suppressor screen plasmid library has been used by different researchers (Ramer, Elledge, & Davis, 1992; "Yeast Genetics", 2004). In addition, all instruments, protocols, and databases such as centrifuges, assays and restriction enzyme digests, and the yeast genome database are valid tools for this type of study (Engel et al., 2014). Because these techniques have been used in literature before, this established the validity and reliability of the study.

Results

This study utilized a previously constructed H3K36 mutant altered at the *HHT2* locus. A high-copy suppressor screen (Figure 1) was performed by transforming a genomic plasmid library into the H3K36R/M mutant yeast strains. The mutants were identified and confirmed by replica plating and spotting assays, respectively, onto caffeine and formamide. To determine successful growth defect suppression, mutants with suppressors were compared to a wild-type (WT) control with an unmutated *HHT2* locus and a mutant control containing an empty plasmid. The colonies that grew the most similarly to the WT control were categorized as suppressors and were coded according to their suppression strength. After finding successful candidates, the plasmids were extracted and sent for sequencing. Those genes were analyzed according to their function using literature dives to see if any were connected to 3'-end processing.

Figure 1.

General Processing of High-Copy Suppressor Screen Transformation



Each spotting assay result is in Appendix 1. While all assays were conducted on both caffeine and formamide, this study disregarded the formamide results due to inconsistencies in

the colony formation and their unusual appearance. All suppressors were categorized by strengths: weak, medium, and strong, as shown in Table 1.

Table 1.

Suppressor ranking with examples.

Category	Definition	Exemplar
Strong	Grows almost exactly like the WT control on caffeine	 WT (top row) and S11 (bottom row)
Medium	Grows similarly to the WT control on caffeine	 WT and S41
Weak	Grows on caffeine, but not similarly to the WT control	 WT and S29

Table 1. This table shows how this study categorized the strengths of its suppressors, what made a suppressor a specific strength, and a visual aid from data.

Out of all the suppressors listed in Table 1, the set consisted of 34.6% strong suppressors, 28.8% medium suppression, and 36.6% weak suppressors in HCSS plasmids (Appendix 2). These suppressors, when sequenced (Appendix 3), yielded the following genes in Table 2 with corresponding suppression levels. It should be noted that any genes listed in the table were found

to be completely intact within the suppressor plasmid during sequencing. While some partial genes were uncovered during sequencing, they were not investigated further because they most likely would encode partial proteins. These partial proteins are not physiologically relevant since they are not normally produced by yeast cells (L. Lemon, personal communication, April 9, 2020).

Table 2.

List of suppressors and associated genes.

Suppressor(s)	Gene(s)
2	PAN5, SSZ1
3	SGV1
12	YHR21C-D, YHR214C-E, YAR069C, YAR070C
15	FHN1, PHB1, PEX4
16, 17, 18, 19, 26, 27	TRP1, YDR008C, ARS1
21	YEL020C-B, TIM9, RPR1
22	YJRCΔ15, SUF23
31	YEL020C-B, TIM9, RPR1, YEL Ctau1
42, 44	YL154W-B, TAR1, ITS21

Note. Green indicates an overall strong suppression, and yellow indicates an overall medium suppression.

Table 2. This table provides a list of suppressor plasmids with their corresponding sequenced genes. These genes are color-coded to represent their overall level of suppression over the suppressor(s).

When examining the functions of the genes in Table 2, there are only four that relate to mRNA processing: TRP1, ARS1, RPR1, and SGV1 (Bodmer-Glavas, 2001; Christie, Croft, & Carroll, 2011; Coughlin, Pleiss, Walker, Whitworth, & Engelke, 2008; Gallegos & Rose, 2019; Snyder, Sapolsky, & Davis, 1988; Table 3; Wlotzka, Kudla, & Tollervey, 2011). The preliminary results suggest that there are some factors that could be associated with mRNA processing, such as introns, acting as a transcription factor, and modification of poly(A) tails (Bodmer-Glavas, 2001; Christie, Croft, & Carroll, 2011; Coughlin, Pleiss, Walker, Whitworth, & Engelke, 2008; Gallegos & Rose, 2019; Snyder, Sapolsky, & Davis, 1988; Table 3; Wlotzka, Kudla, & Tollervey, 2011). However, more screens will need to be performed to confirm the individual genes as suppressors and to search for any additional factors. An interesting trend emerged with the remaining suppressor genes. Many of the genes found were associated with the mitochondria, such as PHB1, TIM9, and TAR1 (Alliance of Genome Resources, 2020a; Coelho, 2002; Merkwith & Langer, 2009; Table 3; UniProt, 2020b; Wasilewski, Chojnacka, & Chacinska, 2017). With the abundance of mitochondrial genes in Table 3, this indicates that along with modifying mRNA transcripts, the H3K36 histone mutation may be involved in deregulation of metabolism (Hanahan & Weinberg, 2011).

Table 3.

List of suppressor genes and function.

Gene	Function
TRP1	Catalyzes synthesis of trp, acts as auxotrophic marker, could increase mRNA accumulation, affects introns and gene expression (Christie, Croft, & Carroll, 2011; Gallegos & Rose, 2019)
ARS1	Chromosomal replicator sequence, interacts with the galactose (GAL) promoter, transcription factor that can compensate for loss of a factor (Bodmer-Glavas, 2001; Snyder, Sapolsky, & Davis, 1988)
FHN1	Works with yeast membrane, homolog to NCE102 and can replace its function of regulating membrane proteins with overexpression (Loibl et al., 2010)
PHB1	Part of prohibitin complex which helps stabilize new proteins, affects mitochondrial structure and cell proliferation (Merkwith & Langer, 2009); human ortholog is linked to breast, cervical, and prostate cancers (Alliance of Genome Resources, 2020a)
PEX4	Makes an enzyme involved in ubiquitin-protein transferase action, interacts with peroxisomes (UniProt, 2020a)
SUF23	Makes glycine-tRNA, regulates translation during cellular stress (Alliance of Genome Resources, 2020b; *Saccharomyces* Genome Database, 2020)
TIM9	Important protein that works in the intermembrane space of mitochondria, part of a complex that imports proteins, can lead to less protein in the mitochondria and selectively translate mRNA that works with stress response (UniProt, 2020b; Wasilewski, Chojnacka, & Chacinska, 2017)
RPR1	Transcribed by RNA polymerase III and interacts with the TRAMP complex which modifies poly(A) tails (Coughlin, Pleiss, Walker, Whitworth, & Engelke, 2008; Wlotzka, Kudla, & Tollervey, 2011)
TAR1	Regulates metabolism, in mitochondria, interacts with RPO41 (mitochondrial RNA polymerase) and COQ5p (ubiquinone biosynthesis in mitochondria), overexpression maintains stability of mtDNA (Coelho, 2002)

Note. All mRNA processing related genes are highlighted in maroon, and mitochondrial genes are in orange. Genes listed in dark green are cancer-related.

Table 3. This table shows the suppressor genes and their functions. Each gene is highlighted in colors to show the functions.

Conclusions

This study investigated the genetic relationship between histone H3 modifications and 3' end processing using a high-copy suppressor screen, a technique not previously utilized to investigate this experimental question. Although studies have primarily focused on intron splicing and occasionally capping or poly(A) tail formation, none have found a defined genetic link (Böhm & Farrants, 2011; Jimeno-González et al., 2015; Li et al., 2016; Mathieu & Bouché, 2014; Meers et al., 2017). The study succeeded in finding plasmids that suppressed the H3K36 mutant growth defect, but it does not provide a majority of 3'-end processing genes (Table 3). Further assays will be needed to find more 3'-end elements, confirm suppressors, and examine additional factors. Despite not having a majority of 3'-end mRNA processing elements, the genes associated with mRNA processing were intriguing. The four genes' functions (Table 2, Table 3) included recruiting cellular machinery, accumulation of mRNA, splicing, and a few were associated with polyadenylation machinery. Out of these genes, PHB1 was the only gene that was related to cancers (Alliance of Genome Resources, 2020a). These preliminary results indicate that the H3K36 histone modification may be affecting 3'-end processing, but further studies will be needed to confirm the current genes and discovering new genes.

Another interesting finding during the study was that many suppressor genes were associated with the mitochondria (Table 3), indicating that the H3K36 histone modification may also be involved in regulation of metabolism and/or cellular respiration by forceful activation of alternative energy pathways to stimulate cell growth (Hanahan & Weinberg, 2011). This is plausible due to this activation being a characteristic of cancers (Hanahan & Weinberg, 2011). For example, one suppressor was part of the GAL4 gene (Appendix 3) which is a transcription

factor that activates galactose genes, possibly activated as an alternative energy source to suppress growth defects (Hanahan & Weinberg, 2011; Hopper, Broach, & Rowe, 1978; Muratani, Kung, Shokat, & Tansey, 2005). However, to accurately determine the role of the H3K36 histone mutation and create an accurate model, more assays will need to be performed with the study's current mutant, and future studies will have to consider mitochondrial pathways as a potential cause of growth defect suppression. By considering modification of metabolism as a cause of growth defect suppression, it could aid in further understanding the role of the mitochondria in cancers and demystifying the role of histone modifications on gene expression. However, because suppression levels were determined only using caffeine, similar results may not appear on different media such as formamide or hydroxyurea because caffeine activates stress response pathways. The stress response pathway of the yeast cells may be why the suppressors found in this study were mostly mitochondrial. Still, this study could further the investigation of histone modifications and cancer, and would be greatly beneficial as an additional step towards finding a cure.

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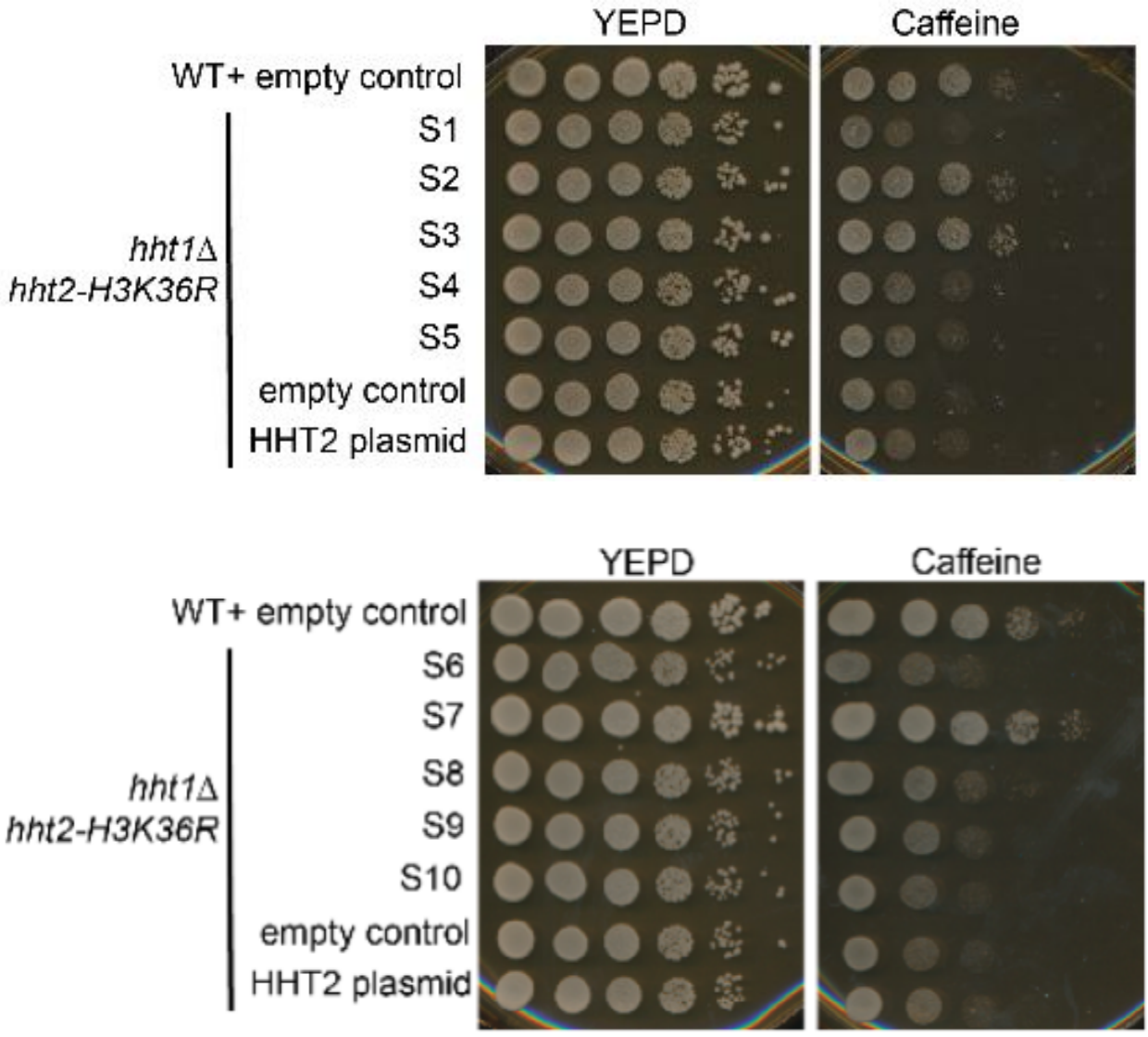
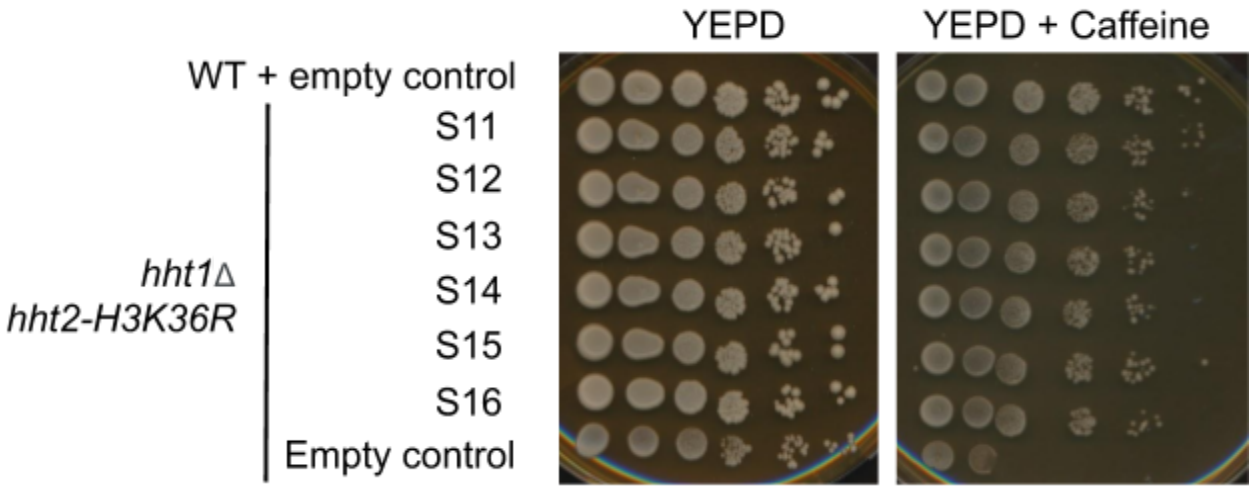
Appendices

Appendix 1: Spotting Assay Results

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YEPD + Caffeine
WT + empty control
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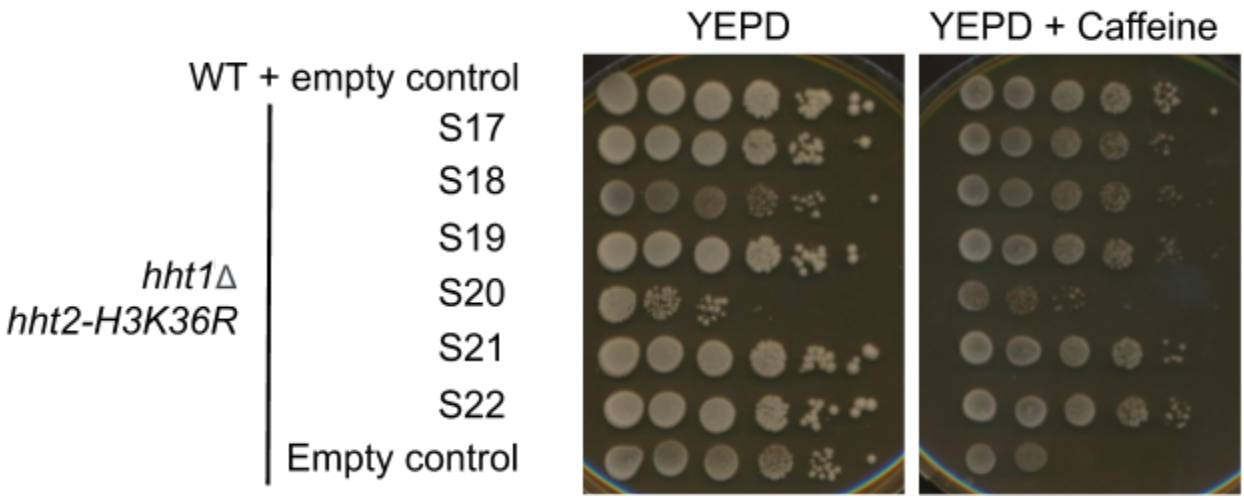
hht2-H3K36R
S11
S12
S13
S14
S15
S16
Empty control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YEPD + Caffeine
WT + empty control
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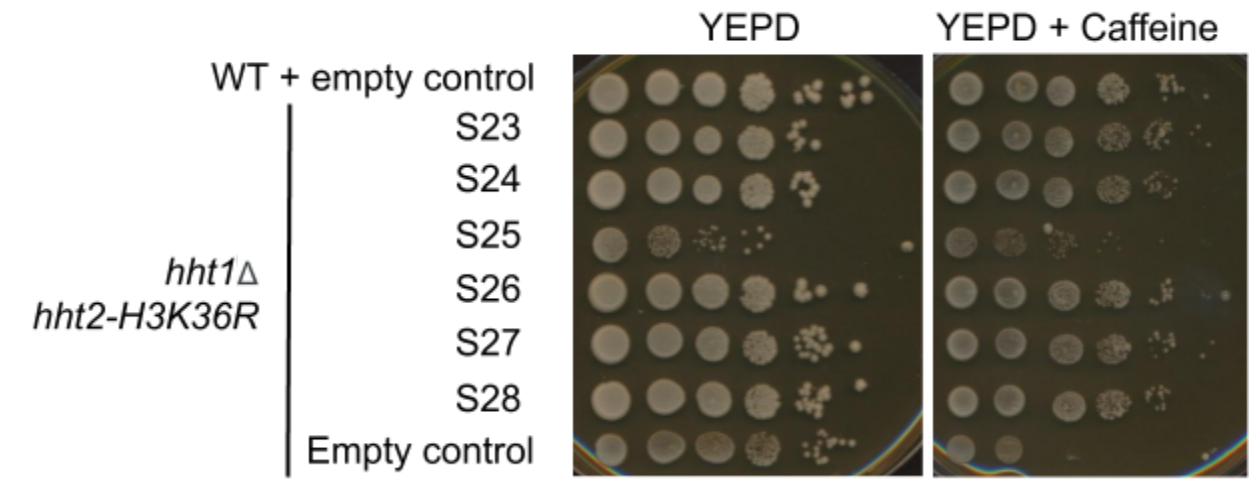
hht2-H3K36R
S17
S18
S19
S20
S21
S22
Empty control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YEPD + Caffeine
WT + empty control
hht1Δ
hht2-H3K36R
S23
S24
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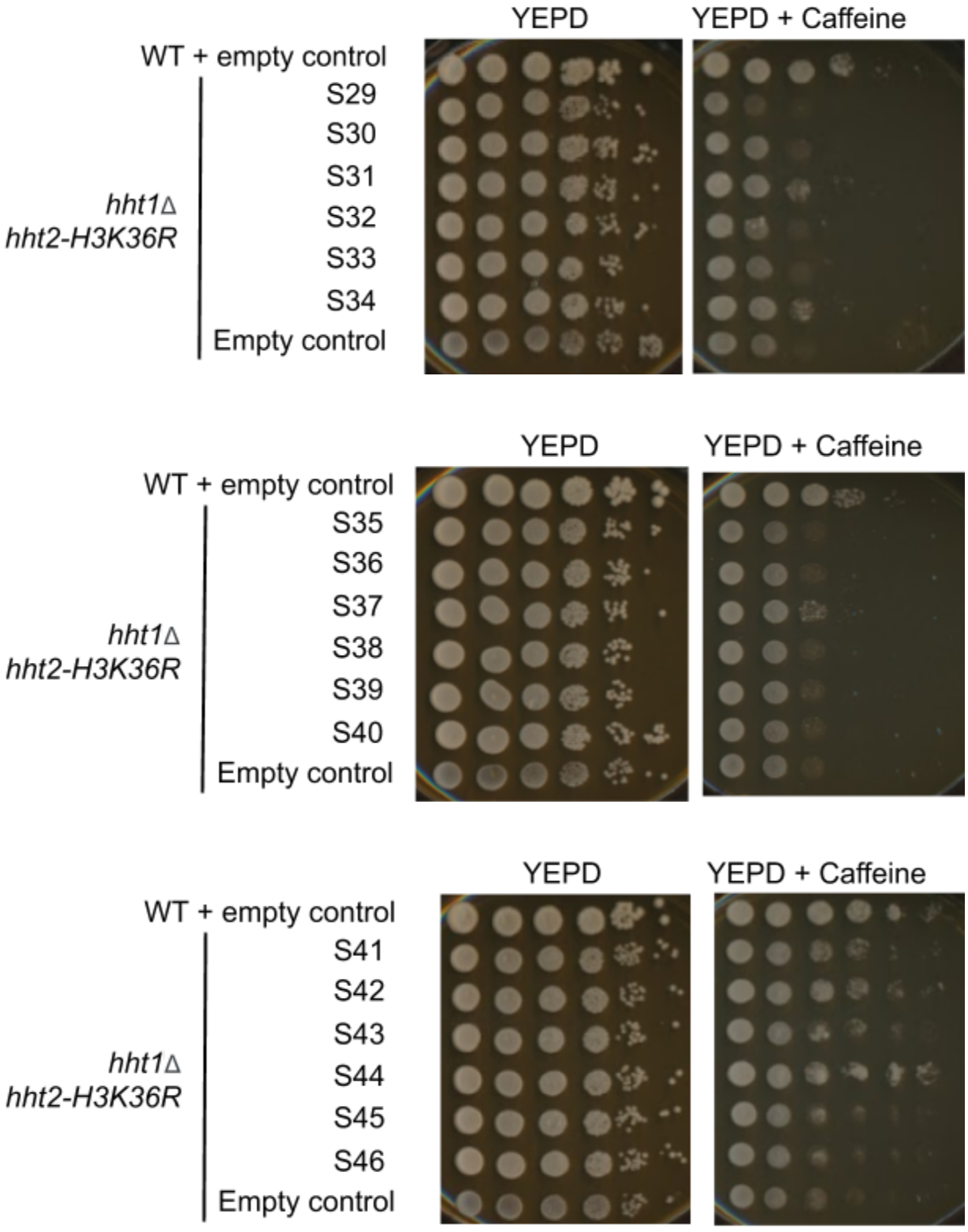
S26
S27
S28
Empty control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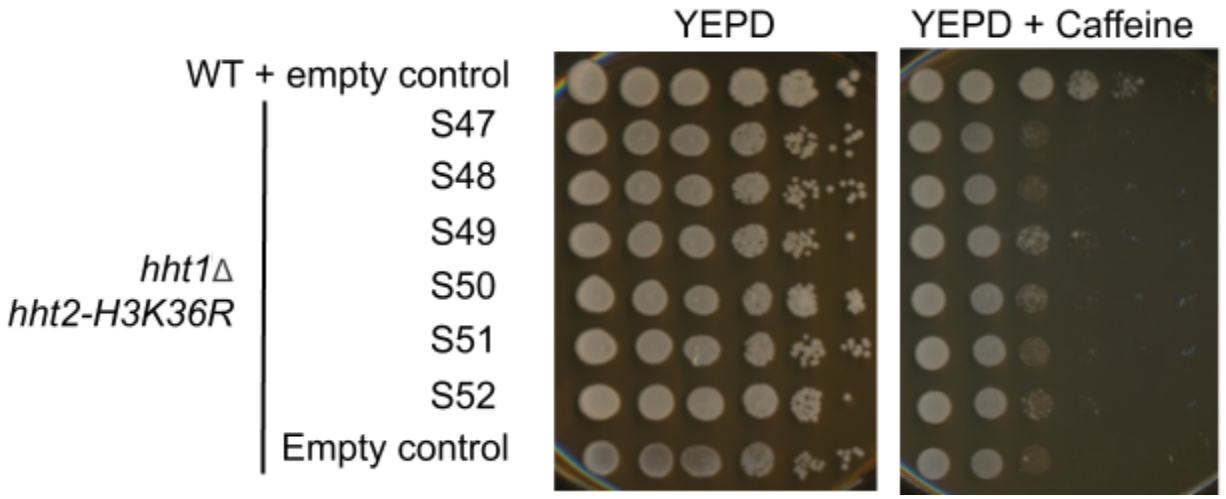
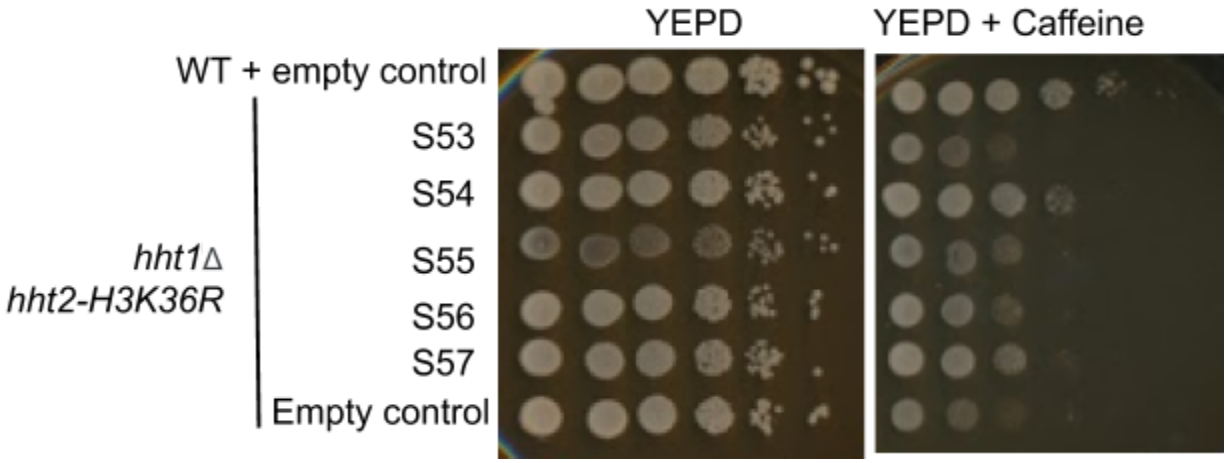
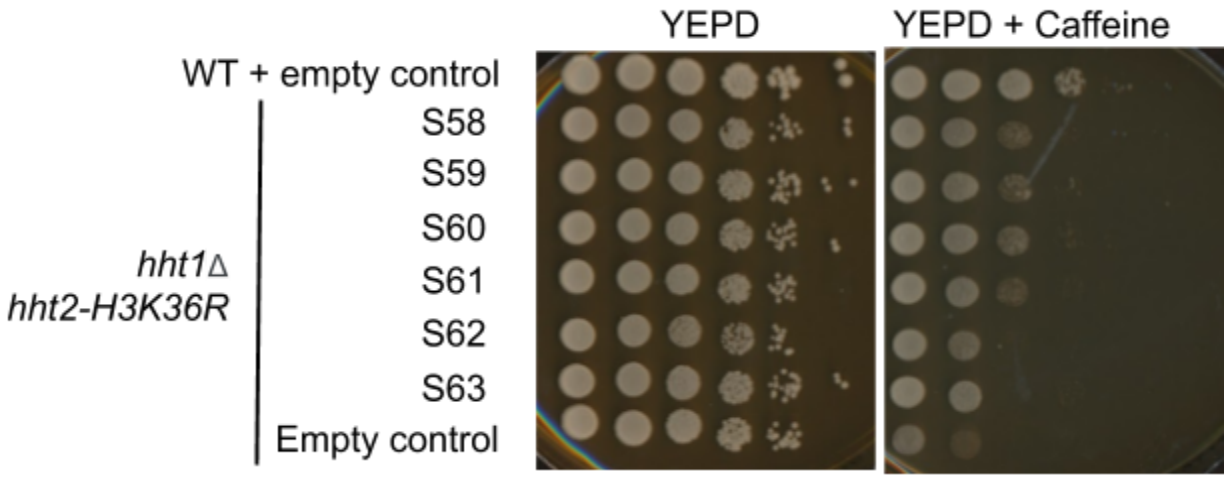
YEPD + Caffeine
WT + empty control
hht1Δ hht2-H3K36R
S29
S30
S31
S32
S33
S34
Empty control
YEPD
YEPD + Caffeine
WT + empty control
hht1Δ hht2-H3K36R
S35
S36
S37
S38
S39
S40
Empty control
YEPD
YEPD + Caffeine
WT + empty control
hht1Δ hht2-H3K36R
S41
S42
S43
S44
S45
S46
Empty control


YEPD
YEPD + Caffeine
WT + empty control
hht1Δ
hht2-H3K36R
S47
S48
S49
S50
S51
S52
Empty control


YEPD
YEPD + Caffeine
WT + empty control
hht1Δ
hht2-H3K36R
S53
S54
S55
S56
S57
Empty control


YEPD
YEPD + Caffeine
WT + empty control
hht1Δ
hht2-H3K36R
S58
S59
S60
S61
S62
S63
Empty control

Appendix 2: Strength of Suppressor Plasmids

Suppressor	Type
S11	S
S12	S
S13	S
S14	S
S15	S
S16	S
S17	S
S18	S
S19	S
S20	W
S21	S
S22	S
S23	S
S24	S
S25	W
S26	S
S27	S
S28	S
S29	W
S30	W
S31	M

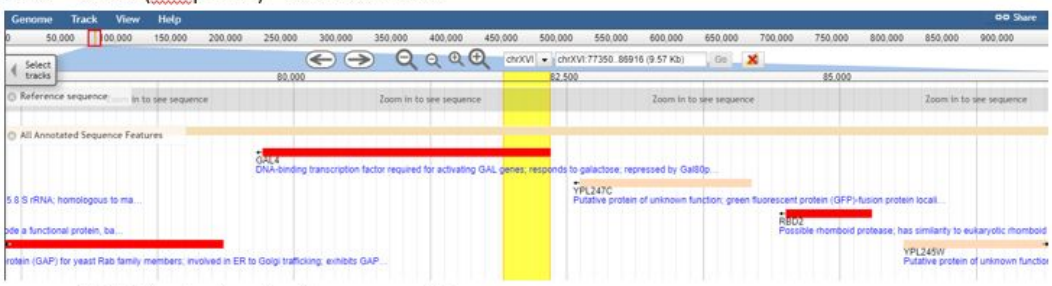
S32	W
S33	W
S34	M
S35	W
S36	W
S37	M
S38	W
S39	W
S40	W
S41	M
S42	S
S43	M
S44	S
S45	M
S46	M
S47	W
S48	W
S49	M
S50	W
S51	W
S52	W
S53	W
S54	M

S55	W
S56	M
S57	M
S58	M
S59	M
S60	M
S61	M
S62	W
S63	W

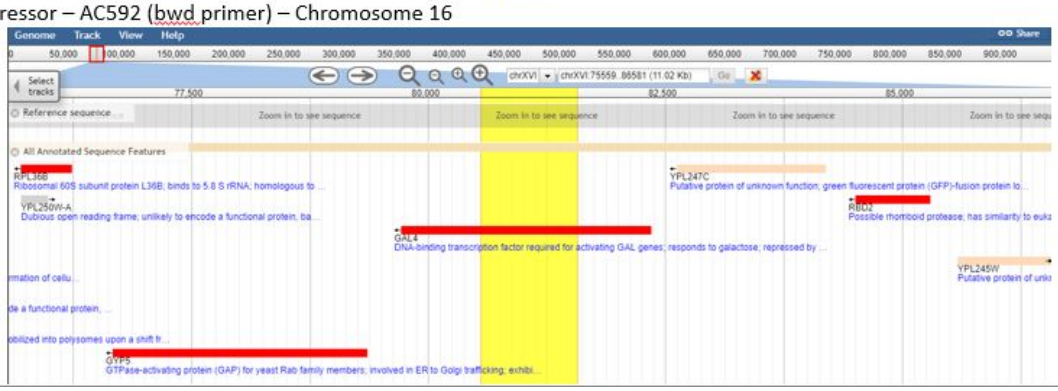
Appendix 3: Sequencing Results

S14: Incomplete GAL4

S14 suppressor – AC591 (fwd primer) – Chromosome 16

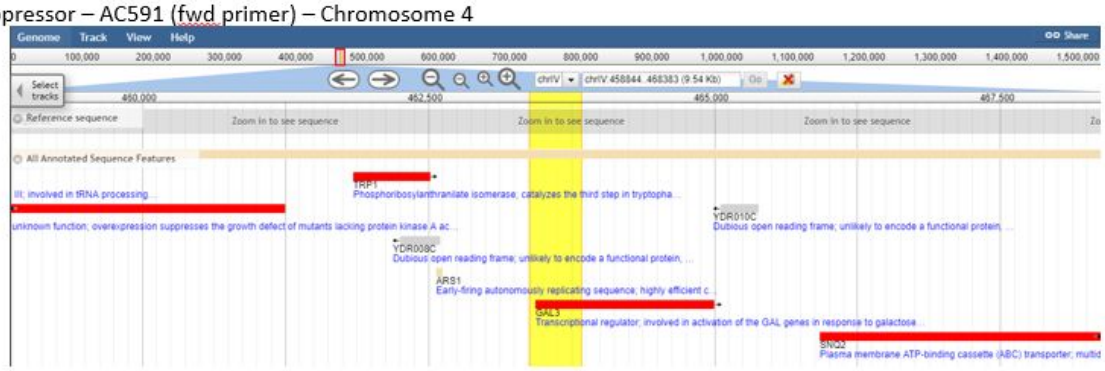


S14 suppressor – AC592 (bwd primer) – Chromosome 16

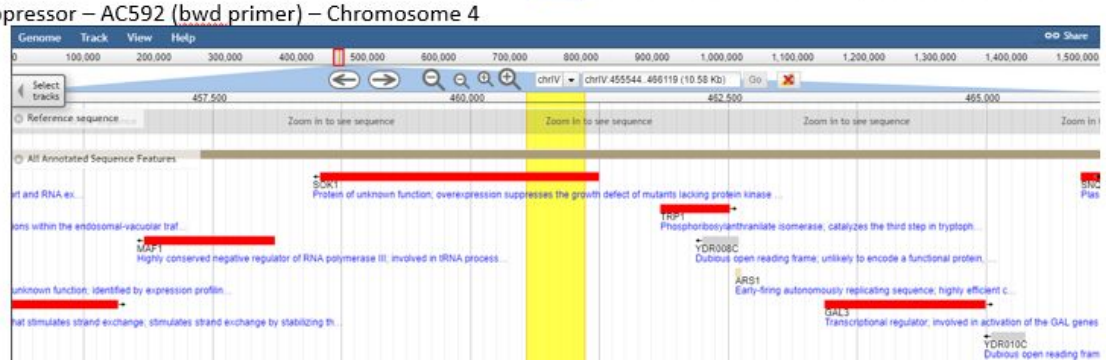


S16: TRP1 and ARS1

S16 suppressor – AC591 (fwd primer) – Chromosome 4

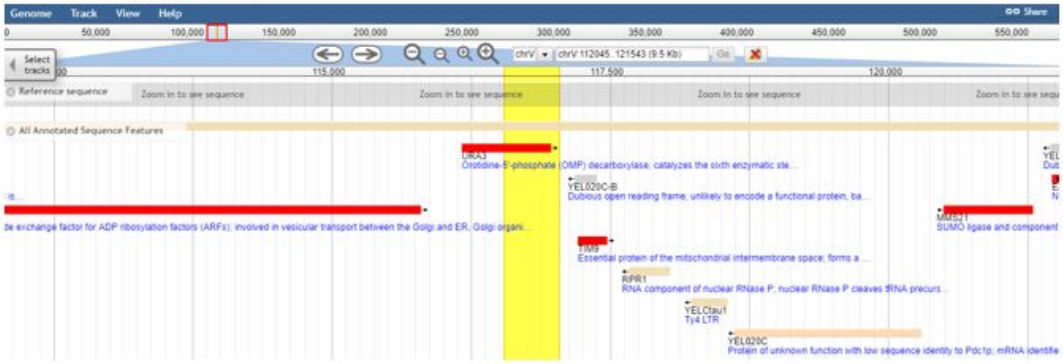


S16 suppressor – AC592 (bwd primer) – Chromosome 4

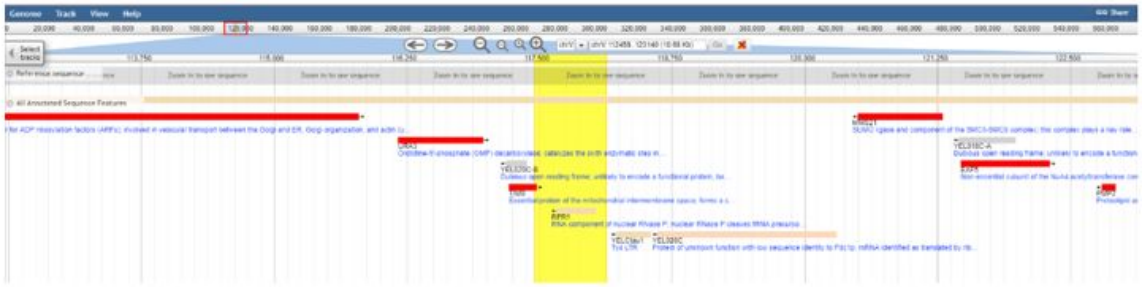


S21: TIM9 and RPR1

S21 suppressor – AC591 (fwd primer) – Chromosome 5

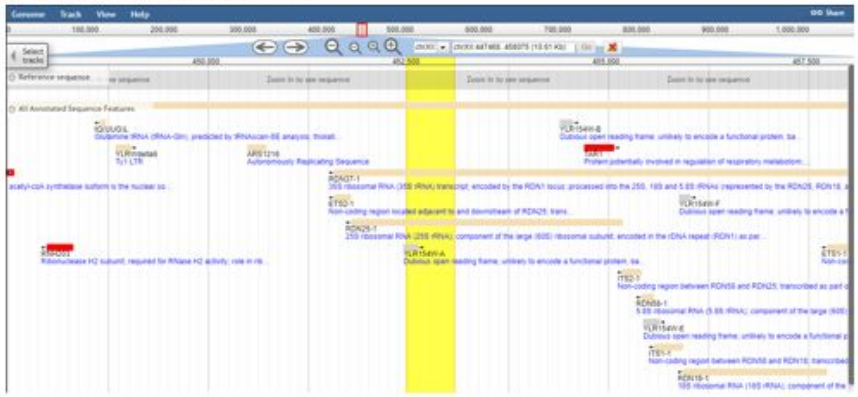


S21 suppressor – AC592 (bwd primer) – Chromosome 5



S42: TAR1

S42 suppressor – AC591 (fwd primer) – Chromosome 12



S42 suppressor – AC592 (bwd primer) – Chromosome 12