Investigating High Copy Suppressors of hat1∆ and rad52∆ Mutations in Fission Yeast

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INVESTIGATING HIGH COPY SUPPRESSORS OF *HAT1*Δ AND *RAD52*Δ MUTATIONS IN FISSION YEAST

a thesis

by

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Table of Contents

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Investigating High Copy Suppressors of *hat1*Δ and *rad52*Δ Mutations in Fission Yeast

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The histone acetyltransferase Hat1 is an enzyme that specifically acetylates newly synthesized histone H4 at positions K5 and K12 (or their homologous positions) in all eukaryotes. In *Schizosaccharomyces pombe*, the deletion of *hat1* presents a mutant phenotype. The telomeres in a *hat1Δ* strain become permissive for transcription, as analyzed by a telomeric *ura4* marker gene. In this study, we evaluate the efficacy of high copy suppression of this *hat1* deletion. Due to high-frequency recombination events in the telomere, it became necessary to create a *hat1-rad52* double deletion strain that also contains a telomeric *ura4* reporter. High copy suppressor screens for recovery of telomeric silencing yielded several promising transformants. Multiple rounds of testing were performed to assess the recovery of transcriptional repression at the telomere. It was found that despite the anti-recombination effect of deleting *rad52*, the *ura4* reporter was still lost from the telomere through recombination. Additional observation of the *hat1*Δ *rad52Δ ura4-tel* strain revealed a significant synthetic slow-growth phenotype. The double mutant displays a greatly decreased growth rate compared to *hat1*Δ, as well as increased cellular length. Further study showed unique phenotypes on various media, and gene expression studies showed unique patterns of regulation in this double mutant when compared to both a wild-type and its single mutant counterparts (*hat1*Δ, rad52Δ). In summary, the telomeric *ura4* marker in a *hat1*Δ strain of *S. pombe* is not stable and is lost by recombination at a high frequency. This has led to the discovery of a double mutant (*hat1*Δ *rad52*Δ) that displays a severe synthetically sick phenotype.

Introduction

Chromatin

Chromatin is the combination of DNA and proteins that are located in the nucleus of the eukaryotic cell. Chromatin plays an essential role in genomic regulation, mitosis, meiosis, and replication. One of its many functions is the condensation of DNA. To achieve proper packaging of DNA, there must be several tiers of chromatin structure. Chromatin fibers have been known as “beads on a string” for 40 years, and these beads are the first order of chromatin structure; they are called nucleosomes. Nucleosomes are the fundamental repeating unit of chromatin, and they are composed of approximately 166 base pairs, or two turns, of DNA arranged around an octamer of proteins called histones. The octamer is composed of four core histones: H2A, H2B, H3, and H4. Each of these histones has a protein fold, or histone fold, to enable a “handshake arrangement” that allows the formation of H2A-H2B and H3-H4 heterodimers (3, 17). H3-H4 dimers then form a tetramer, about which two H2A-H2B dimers arrange (1). There is also a linker histone, H1, which helps organize the histone octamer into higher order structures. The next order of structure is the 30nm fiber, which can be folded into the most condensed chromatin, metaphase chromosomes.

Chromatin Assembly Pathways

The assembly of chromatin is dependent on many proteins, and there are several pathways by which this assembly occurs. One of these pathways is mediated by

chromatin assembly factor 1 (CAF-1). CAF-1 associates H3 and H4 in the chromatin assembly complex (CAC) to assemble nucleosomes preferentially on replicating DNA (1). CAF-1 has been shown to associate specifically in a complex with H3 variant H3.1 (5). There is another chaperone, HIRA, that deposits the histone variant H3.3 independently of replication (5). Nucleoplasmin, a protein found only in eggs and oocytes, both disassembles and remodels sperm chromatin after fertilization of the egg (6,7). Nucleosome assembly protein Nap1 is another chromatin assembly factor. It is known to associate with H2A/H2B and act as a histone chaperone *in vitro* (6). Nap1 has also been shown in *Drosophila melanogaster* to associate with heterochromatin protein HP2 as well as NURF, a chromatin remodeling complex, which could implicate it in transcriptional repression (6,8). Anti-silencing function 1 (ASf1) is a part of the replication-coupling assembly factor complex RCAF, along with histones H3 and H4 (6). It has been found in both CAF-1 and HIRA complexes, and thus associates with both histone H3.1 and H3.3 (6). In *S. cerevisiae*, overexpressing Asf1 disrupts telomeric and mating type silencing and in the fission yeast *Schizosaccharomyces pombe*, loss of Asf1 is lethal (19).

Histones and Posttranslational Modifications

Each of the four core histones mentioned above possesses a disordered N-terminal tail that extends away from the nucleosome, which allows the tails to interact with both DNA and other proteins (2,17). These N-termini are highly

conserved among organisms. Only one conservative amino acid substitution distinguishes the H3 and H4 N-termini in yeast and humans (31). The deletion of either the H3 or H4 amino-terminal tails in *S. cerevisiae* results in a viable mutant, but the deletion of both these tails creates a synthetic lethal (4). This suggests that the functions of the H3 and H4 tails are essential, but redundant for viability. Due to the protrusion of these tails away from the nucleosome, they are subject to posttranslational modifications, which affect their interactions with DNA and proteins.

The most commonly studied modifications of the N-terminal tails include acetylation and methylation on the lysine residues of the tails. Posttranslational modifications of N-terminal histone tails are responsible for changing chromatin stability, increasing DNA accessibility, and for providing opportunities for more interactions with other proteins. These epigenetic markers have been called the "histone code," and are important for both transcriptional activation and repression (43). Site-specific methylation of histone H3 at lysine 9 results in an affinity for Heterochromatin Protein 1, linking this mark to silencing activity as well as heterochromatin assembly (43, 44). However, the phosphorylation of serine 10 on the H3 tail prevents K9 methylation, and is associated with other acetylated positions and transcriptional activation. H3 phosphorylation has also been implicated in mitotic chromosome condensation, indicating that a specific covalent modification doesn't itself determine transcriptional state, but that the

position is important (45). In general, histone modifications include phosphorylation of serine and threonine, methylation of lysine and arginine, and acetylation and ubiquitination of lysine residues. These modifications can be either transient or long-term.

Histone Acetylation

It has been shown in more than one study that histone acetyltransferases are necessary for proper transcription, and thus the acetylation of histones has traditionally been associated with increased transcriptional activity (23-26). When the lysine residues are acetylated, their positive charge is neutralized. As a result, their affinity for the negatively-charged DNA is decreased, which allows proteins that regulate transcription to better access the DNA (27-29).

Histone acetylation facilitates the communication between DNA and histones by regulating chromatin higher order structure, but acetylation has other roles in the cell. Acetylated lysines can also act as molecular tags and are recognized by bromodomains (25). Bromodomains are structures found in chromatin remodeling complexes, as well as in other complexes that modify histones (30). Therefore, acetylated lysines on histones may act as transcriptional activators and recruit transcriptional machinery (25).

Acetylation is the most widely studied posttranslational modification of histones. When nucleosomes are assembled during S phase, newly synthesized histone H4 is acetylated at K5 and K12 before being deposited onto DNA (9-13). This acetylation pattern is highly conserved, and has been found in humans, *Tetrahymena*, and *Drosophila* (14). Deacetylation of this new H4 is necessary for the proper maturation of chromatin (6). Hat1 is the enzyme responsible for the acetylation of new H4 (2, 15, 16).

Hat1

Hat1 is part of the family of enzymes called histone acetyltransferases (HATs), of which there are two types: A and B. Type A histone acetyltransferases are localized to the nucleus and can modify histones already incorporated into chromatin (46, 47). Type A HATs are likely involved in gene activation (55). Type B HATs, however, are found in the cytoplasm as well as the nucleus, but acetylate free histones before incorporation into nucleosomes on newly replicated DNA (16, 46, 56-61). Hat1 is a Type B histone acetyltransferase.

The structure of Hat1 is composed of both α-helices and ß-pleated sheets, which forms a curvature. This allows the N and C terminal residues of HAT1 to lie away from each other, connected by an extended loop (20). The acetyl CoA molecule that Hat1 binds is situated at the C-terminal domain of the protein, and it makes contact on the concave surface of Hat1 (21). Interestingly, it has been found that

the C-terminal 54 residues are not necessary for catalytic function of the enzyme (22). It is believed that Hat1 itself does not undergo dramatic structural changes after binding the substrate, which leads to a proposed structure: when binding the H4 tail, association with Hat1 lysine-12 is thought to be positioned adjacent to the acetyl group of acetyl-CoA (21).

Hat1Δ Mutants

The deletion of the *HAT1* gene in *S. cerevisiae* does not result in a visible phenotype under normal growth conditions (47-49). However, when this deletion is combined with mutations of certain acetylable lysines of H3, *S. cerevisiae* exhibits an increased sensitivity to MMS, a DNA-damaging agent (50). Previous studies in the Annunziato laboratory examined the evolutionary conservation of this phenomenon by deleting *hat1* in *S. pombe*. While this deletion did not affect cell growth or doubling time, *hat1*Δ cells were sensitive to MMS without concomitant H3 mutations (18). This indicates that in fission yeast, Hat1 has a nonredundant function in DNA repair as compared to *S. cerevisiae*.

Despite the typical growth observed in *S. cerevisiae hat1*Δ cells, this deletion has been shown to cause defects in DNA damage repair, as well as a reduction in telomeric silencing (37, 51). This derepression at the telomeres is dependent on concurrent mutations in the H3 N-terminal domain (38, 51). In *S. pombe*, however, the deletion of *hat1* is sufficient to cause a loss in telomeric silencing

(37). No abrogation of silencing was seen at the mating type locus or in centromeric regions (37). There was a significant increase in acetylation of subtelomeric chromatin in the H4 N-terminal domain, which is consistent with a loss of transcriptional silencing. Treatment of *hat1*Δ *S. pombe* with the deacetylase inhibitor trichostatin A also caused a loss of telomeric silencing, further supporting that increased acetylation is responsible for the silencing loss (37).

Telomeres

Telomeres are regions located at both ends of chromosomes that contain repeated nucleotide sequences. These structures help to ensure that the chromosomes are completely replicated during DNA replication. Telomeres also protect the ends of chromosomes from degradation, as important information would be lost during each replication cycle without them. Additionally, telomeric structure prevents chromosomal ends from fusing with other chromosomes. Telomeres are maintained by an enzyme called telomerase, but adult cells lack telomerase. As a result, telomeres in adult cells become progressively shorter over the lifespan of a cell (32).

Silencing of telomeric genes in *S. cerevisiae* is likely due to a different chromatin structural domain that starts in the telomeric region (33). This is supported by the fact that there are mutations in histones H3 and H4 that cause derepression at

the telomeres (33). While H4 is normally acetylated in actively transcribed regions of the genome, it is in a comparatively hypoacetylated state in the telomeric regions (33). Gene repression also decreases with greater distance from the telomere (34).

The silencing of heterochromatin in yeast is due to many factors. Normally, both mating type loci and telomeres are silenced. In *S. cerevisiae*, mutations in the amino termini of histones H3 and H4 cause derepression of the telomeres (35). Additionally, chromatin assembly factor CAF-1, when deleted, also reduces telomeric silencing in *S. cerevisiae* (36). Deletion of ASF1 or HIR1 also affects telomeric silencing (36). It was later found that Hat1p participates in telomeric silencing, and the deletion of *hat1* leads to telomeric derepression (37, 38).

Schizosaccharomyces pombe

The choice of organism in any investigation is a crucial one, and the fission yeast *S. pombe* is an ideal genetic model organism. *S. pombe* contains only 3 chromosomes, as opposed to 16 in the budding yeast *S. cerevisiae*. Additionally, *S. pombe* shares some genes with humans that *S. cerevisiae* does not (39).

Yeast colony size is easily monitored, and it is an important trait to note. This characteristic makes growth vs. non-growth very clear, and it allows the use of

auxotrophic markers as a genetic tool. The diploid sexual cycle of this yeast, though not unique to *S. pombe*, is useful for both complementation and recombination tests (40). The haploid life cycle is also very important, and haploidy permits scientists to study and utilize recessive mutations. Null mutations, causing a loss of function, allow the study of the normal role a gene plays in the cell (40). Fission yeast also accepts plasmids well, which allows transformation using cDNA libraries and subsequent genetic analysis.

Suppression

The study of suppression mechanisms is an important tool for genetic analysis. It has the ability to characterize interactions between genes that may not have been identified with other genetic experimental procedures. Typically, a mutation in a gene makes the pathway involved susceptible to study, and allows suppressor screens to reveal other components of that pathway (41). A common method for this type of investigation is the use of a plasmid library. A library that overexpresses wild-type genes is transformed into the mutant strain. From that transformation, suppressors are selected based on phenotype.

To classify a gene as reversing a particular mutation would place it in the general category of “suppressors.” There are several different kinds of suppressors, which are extensively described in Gregory Prelich’s 1999 review (41). An intragenic suppressor would be a second mutation in the same gene as the

original mutation. The phenotype of this second mutation would ameliorate that of the primary mutation, and would restore the phenotype to wild-type, at least partially. Suppressor screens are usually performed to identify new genes or proteins involved with that of the original mutation, so intragenic suppressors are often not the goal of such screens. Informational suppressors restore function by affecting translation—the flow of information from RNA to protein. The other four suppressors characterized by Prelich are self-describing: they alter the activity or amount of the mutant protein, the pathway of the mutant protein, or the activity of a pathway other than that in which the mutant protein is involved (41). It is important to note that suppressors frequently do not have a direct effect on the mutant, but can regulate other participants in a pathway to compensate.

In this investigation, we were not seeking a particular type of suppressor. Using a cDNA library, we were looking to identify any suppressor of the *hat1*Δ mutation. During the course of the investigation, the project transformed into the characterization of a new mutation, which causes synthetic sickness with *hat1*Δ.

The deletion of *rad52* (formerly *rad22*) causes a severe slow growth phenotype, as well as dramatically elongated cells. This gene has been proven to be very important in double stranded break repair (42, 52). In line with that function, it has also been shown that *rad52* is important for homologous recombination (52, 53).

It has been shown that deleting *rad52* in *S. pombe* results in increased doubling time as well as cell elongation (52, 54).

The following investigation is twofold. First, we searched for high copy suppressors of the *hat1*Δ mutation in *S. pombe*. This led to the second phase, where the properties and gene expression of the *hat1*Δ-*rad52*Δ synthetically sick strains were studied.

Materials and Methods

Transformation of S. pombe

LiOAc
100mM LiOAc
10mM Tris HCl
1mM EDTA

40% PEG in LiOAc
100mM LiOAc
10mM Tris HCl
1mM EDTA
40% PEG

S. pombe strains were cultured in YES liquid overnight to 10^7 cells/ml. Subcultures were made in EMM complete to target for 10^7 cells/ml overnight. Cells were washed in sterile water and 1X LiOAc/TE, and then brought to 2 x 10^9 cells/ml in 1X LiOAc. 2 x 10^8 cells were added to boiled carrier DNA (10mg/ml) and transforming DNA (1:5 ratio). Cells were incubated 10 minutes at room temperature, and 40% PEG/LiOAc/TE was added. After gentle mixing, cells were incubated 4 hours at 30°C. DMSO was added and cells were heat shocked 5 minutes at 42°C and then spread onto EMM-Leu media.

Library Screening

Cells plated onto EMM-Leu were grown 5 days at 30°C. Cells were then replica plated onto 5FOA medium and back on to EMM-Leu. These plates were replica plated back to 5FOA five times, with 3 days of growth each. Well-grown colonies were streaked on EMM-Leu medium and grown 4 days. Colonies were then

patched onto YES and grown 1 day. They were then replica plated every 2 days for one week. Each candidate was streaked for single colonies, grown 3 days, replica plated on 5FOA, EMM-Leu, and YES media. Appropriate candidates were chosen to streak on YES, grown 3 days, and were then replica plated again onto 5FOA, EMM-Leu, and YES.

Serial Dilutions

Each strain was diluted from $2.5x10^7$ cells/ml in ten-fold adjustments to a 100,000-fold dilution on both YES and 5-FOA medium.

PCR of Transformants

Master mix per reaction:
10µl sterile H_20
12.5µl 2x Buffer G or J
0.5µl pLEV3-Forward
0.5µl pLEV3-Reverse

TAE Buffer
40mM Tris Buffer
20mM Acetic acid
1mM EDTA

A pinhead of freshly grown cells was added to each tube of master mix. The PCR program was run as follows: 10 min at 98°C, 30 sec at 95°C, 30 sec at 59°C. As the machine was cooling to 58°, 1.25U of FailSafe Taq polymerase was added to each tube. 40 cycles were run, with a 5 minute annealing period.

Samples were separated on a 1% agarose gel in TAE buffer for one hour at 100V. The agarose gel was incubated in ethidium bromide at room temperature for 7 minutes.

PCR Purification

PCR products were purified according to the purification protocol (QIAquick®). DNA was eluted for 5 minutes with water.

Medium Preparation

YES medium
5g/L Yeast extract
30g/L Glucose
0.225g/L adenine, histidine, lysine, leucine, uracil

5FOA medium
80g/L Glucose
0.4g/L 5-FOA
1.45g/L Yeast nitrogen base
5.0g/L Ammonium sulfate
2.0g/L Synthetic complete lacking uracil
0.05g/L Uracil

EMM Complete
12.3g/L EMM
30g/L Glucose
0.075g/L Each adenine, histidine, lysine, uracil
0.15g/L Leucine

Cell Imaging

DIC images were taken using a Zeiss Axioplan 2 and Image J software. Cell measurements were taken using Fiji and analyzed in Microsoft Excel.

Silencing Assay

3mL cultures were seeded at $1.25x10^6$ cells/mL with 10μM splitomicin or DMSO (vehicle). Cultures were grown for 3 doublings in splitomicin; vehicle-treated cultures were diluted if overgrown. Cells were resuspended at $2x10^6$ cells/mL and 5 5-fold serial dilutions were made. 5μL of each dilution were spotted on EMM, EMM-ura, 5-FOA (1g/L), and YES plates in the absence of splitomicin. Plates were incubated 2-3 days at 30˚C.

Gene Expression Profiling

Microarray analysis was performed as described previously (66). Strains *hat1*Δ*, rad52*Δ*, hat1*Δ*–rad52*Δ*, and* 975 were grown to an OD_{595} of 0.4. Gene ontology (GO) analysis was performed using the Princeton Gene Ontology Term Mapper tool. GO terms were obtained from PomBase (www.pombase.org).

Results

Early High Copy Suppressor Studies

As stated, previous data in our lab showed that deletion of *hat1* in *S. pombe* results in derepression of the telomeres (37). The strain used in that study, KTP36, also contains a *ura4* marker in the telomeric region. The goal of the present study is to find high copy suppressors of that deletion, in order to better understand the pathway(s) involved with Hat1 and telomeric silencing. There are multiple possibilities for the mechanism that Hat1 is involved in, two of which are described in Figure 1.

The assay used to screen for suppressors of transcription of the *ura4* marker gene involves use of the 5FOA system. Cells with functioning *ura* genes create a toxin when using the compound 5-fluoroorotic acid (5FOA) during the uracil biosynthetic pathway. However, if the *ura* gene is silenced, no poison is produced and the cells are able to grow on medium containing the compound 5FOA.

To begin, the *hat1*Δ (referred to as KTP36, see Table 1) strain was transformed with two DNA libraries with the intent that the DNA inserted would suppress the *hat1* deletion phenotype. Plating on EMM-Leu was used to screen for positive transformants. Subsequent replica plating on 5FOA medium permitted the identification of colonies whose *ura4* markers were repressed. This first round of testing showed promising results (Figure 2). There was an important discrepancy

between the number of colonies that grew on EMM-Leu, and a smaller number of colonies that continued to grow on 5FOA. This indicated that there were a significant number of positive transformants, a fraction of which had the *ura4* marker silenced. Further tests, PCR, and sequencing (data not shown) revealed, however, that the *ura4* marker was recombining and had been lost, thereby giving false positive results. Note that a silenced *ura4* marker and a nonexistent *ura4* gene would exhibit the same growth on 5FOA medium.

Strain Construction

The recombinatorial loss of *ura4* required a solution, and the creation of a new strain was the most viable option for this problem. Rad52 is a protein homolog of the budding yeast's RAD52, and it is involved in recombination and binding DNA double-stranded breaks (42). The deletion of *rad52* causes a dramatic decrease in recombination events in *S. pombe* (62). Therefore, we hypothesized that the introduction of this mutation would decrease loss of the *ura4* marker and allow the study of high copy suppression. A *rad52* deletion, *ura4* mutant strain (courtesy of Tim Humphrey via Matthew Whitby) was crossed with KTP36 to yield CHP1619, a strain that contains *hat1*Δ*, rad52*Δ*,* and a telomeric *ura4* marker (Table 1). There was significant difficulty in creating this strain, due the ability of the KTP36 strain to switch mating type. The *rad52*Δ strain was very sickly, and therefore KTP36 was more likely to switch type and mate with itself.

This strain CHP1619 (*hat1*Δ-*rad52*Δ) was tested on complete medium as well as 5FOA medium to observe its growth characteristics (Figure 3). It was found that CHP1619 reverts to 5FOA resistance less frequently than KTP36, indicating that it can maintain the *ura4* marker. This set the stage for further experimentation with this strain. Interestingly, a significant slow growth phenotype was observed for CHP1619 on complete medium, which will be discussed later.

Transformation and Candidate Testing

A transformation was performed again with two libraries, this time with CHP1619. The same criteria as previous experiments were used to screen for candidates: growth on EMM-Leu as well as 5FOA. These strains were replica-plated several times to 5FOA medium to weed out background growth. This is very important because there was a significant number of transformants in the first round of experiments, which was seen again with the new strain. Multiple rounds of 5FOA growth would allow only the colonies with true 5FOA resistance to remain alive.

Colonies that maintained strong growth on 5FOA were chosen, and another round of replica plating experiments was performed. This time, however, the medium was rich and complete (YES), to allow for plasmid loss. This was an extremely important step because only colonies that contain plasmid should be

able to grow on EMM-Leu and 5FOA. Once the plasmid is gone, these conditions would no longer be permissive.

Plasmid loss experiments for whole colonies showed dramatic results (Figure 4). While strains were later streaked to allow for less widespread plasmid loss, this image shows how growth is lost entirely on EMM-Leu when the plasmid is lost. The plasmid contains a functioning *leu* gene, which has been mutated in the strains in these experiments.

Unexpected Growth

When promising candidates were allowed to lose plasmid, the growth on EMM-Leu vs. YES yielded promising results. The growth on EMM-Leu was variable, indicating that plasmid had been lost from some single colonies and they could therefore no longer grow without a leucine supplement (Figure 5). However, as is visible in the right panel of Figure 5, the growth on 5FOA is robust. This indicates that the plasmid is not conferring growth on 5FOA medium.

There were other candidates that did not show varying growth on medium lacking leucine, even after culturing on YES to allow for plasmid loss (Figure 6). They also showed uniform growth on 5FOA medium. This indicates that the plasmid was integrated into the strain's genome, which would allow the strain to retain all

genes necessary for growth on restrictive media after extended growth on YES. The 5FOA growth also indicates loss of the *ura4* reporter.

To test this hypothesis, multiple PCR experiments were performed with varying buffers to try and obtain a product. There was a weak product at approximately 2kb (Figure 7). Sequencing for this product was unsuccessful, and further PCR experiments were not able to replicate the earlier results. It does not appear that there was an integration event based on these data. Thus, the phenomenon that caused the uniform growth remains unclear.

While studying CHP1619 and constructing strains, it was observed that cells were dying frequently on MEA, a medium used to promote mating. It contains maltose as a carbon source, but a death phenotype has not been observed for most strains when doing mating experiments. To better observe this phenomenon, a compound called Phloxine B was incorporated into MEA medium. Living cells can efflux the compound, but dead cells are stained dark red (63). To investigate this phenotype, another series of transformations was performed in search of colonies that both grow up more quickly than CHP1619 and do not stain as darkly on MEA+Phloxine B. After screening thousands of colonies, PCR was performed using primers for the DNA library and several candidates were sequenced (Figure 8). The samples each appeared to have a product at the same length of about 1.3kb. Sequencing revealed that only the

Ura3 gene was identified, which coincides with the product size. While this validated that the screen is functional, it did not move the project forward.

To further the studies of the double mutant, a spot test was performed on several strains to look for a slow growth phenotype, without the *Ura4* deficiency (Figure 9). It was observed that an *ade6*- strain stained much darker on MEA+Phloxine B, but red cells are characteristic of this mutant. While the double mutant may stain slightly darker than either single mutant (*hat1*Δ or r*ad52*Δ), the slow growth phenotype is distinctive. Repeating the aforementioned procedure, transformants were screened to look for recovery of growth as well as lighter staining on MEA+Phloxine B. After PCR and sequencing, three genes were identified: Yop1, Grx3, and a glyoxylate reductase gene, which is involved in gluconeogenesis. Yop1 is an ER membrane protein and Grx3 is monothiol glutoredoxin, and it contributes to the removal of superoxide radicals. The plasmids from these candidates were isolated and used to retransform the strain. This re-transformation yielded colonies that grew more quickly and stained less darkly on MEA+Phloxine B than the double mutant. Further experiments showed some candidates did not have phenotypes conferred by the plasmid, and these genes did not show differential regulation in gene expression studies.

CHP1619 Phenotype

As previously mentioned, the *hat1*Δ-*rad52*Δ strain has a dramatically lengthened doubling time, compared to either *hat1*Δ or *rad52*Δ. Additionally, the cells have a significant elongation phenotype (Figure 10). Using the student's t-test, the average cell length of the double mutant was shown to be significantly different than both *hat1*Δ and *rad52*Δ, with p values of 0.01 and 0.04, respectively (Table 2). The standard deviations of both *rad52* mutants are very high, indicating that cell length at septation varies greatly. Notably, the difference between average cell lengths of the single mutants was not statistically significant, indicating that the combination of mutations drives the phenotypic change.

Splitomicin

It has previously been established that hyperacetylation is sufficient to abolish telomeric silencing by treating with the HDAC inhibitor trichostatin A (TSA) (37). We sought to expand upon these results with the use of a different HDAC inhibitor. It has been shown that Sir2 is a histone deacetylase that is required to maintain telomeric silencing (64). Splitomicin is an HDAC inhibitor that targets Sir2 (65). Treating with splitomicin, however, does not appear to have the same effect as TSA (Figure 11).

Microarray Analysis

Given the transcriptional derepression at the telomeres, looking at global gene expression was essential. A microarray was performed to look at the gene expression of three strains compared to a wild-type strain: a *hat1*Δ, *rad52*Δ, and the double mutant, *hat1*Δ-*rad52*Δ. A broad look at gene expression showed varying degrees of upregulation in each strain. The *hat1*Δ strain had the greatest number of probes showing upregulation over the wild-type of twofold or greater, followed by *rad52*Δ, and *hat1*Δ-*rad52*Δ had the fewest (Figure 12). These gene expression levels are consistent with the growth rates of each of these strains.

The probes that showed twofold or greater upregulation in each of the three strains were analyzed and separated into different genes to avoid multiple probes to a single gene. These were separated by strain, and then compared across strains. While the relative number of genes remained consistent with the number of probes, the proportions of unique genes were different. Over 80% of *rad52*Δ upregulated genes are shared with the other two mutants, compared to only 68% of *hat1*Δ-*rad52*Δ and 63% of *hat1*Δ (Figure 13).

Using Princeton's Gene Ontology Term Mapper, the origins of the upregulation in each strain were observed. The double mutant showed a significant upregulation in ribosome biogenesis and cytoplasmic translation, a trend that was not observed to the same degree for the other two strains. In the double mutant,

ribosome biogenesis and cytoplasmic translation each account for about 25% of the upregulated genes (Figure 14). One specific area of probes that localize to chromosome 3 showed significant upregulation, with up to an 18.5-fold increase in expression of rDNA over the wild-type (Figure 15).

Tables and Figures

Table 1. *S. pombe* strains

Strain	Genotype	Source
975	h^+, wild-type	67
FY1872	h^{90} *ade6-210 leu1-32 ura4-DS/E otrRSph1::ade6 TEL2L-ura4*	68
KTP36	h^{90} *hat1*Δ::*kan ade6-210 leu1-32 ura4DS-E otr1 Rsph1::ade6 TEL2L-ura4 12C*	37
CHP1614	h^+ *ura4::fbp1-lacZ leu1-32 rad52*Δ::*ura4*$^+$ *hat1*Δ::*kan*	This study
CHP1619	$h^?$ *ura4-DS/E leu1-32 ade6-210 rad52*Δ::*ura4*$^-$ *hat1*Δ::*kan TEL2L-ura4*$^+$	This study
CHP1677	h^+ *ura4-DS/E leu1-32 rad52*Δ::*ura4*$^+$	This study

Table 2. Cell lengths of mutant strains

Strain	Average Cell length (μM)	Standard Deviation
*hat1*Δ	15.49	0.85
*rad52*Δ	15.98	3.07
*hat1*Δ-*rad52*Δ	18.12	5.53

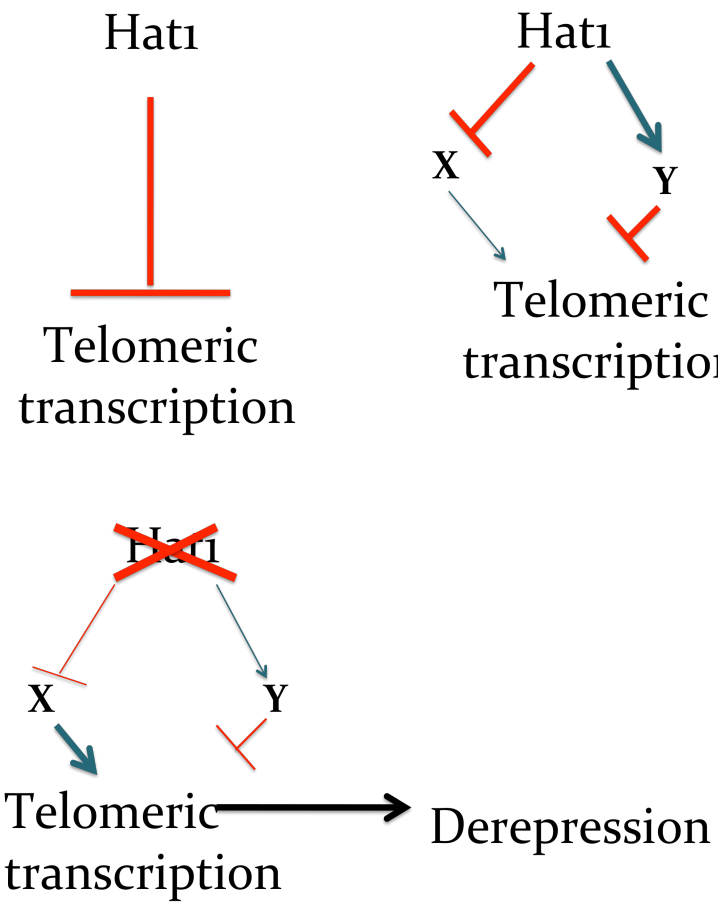

Figure 1. A schematic of possible Hat1 mechanisms. This is a depiction of possible mechanisms of Hat1's inhibition of telomeric transcription, and the resulting derepression upon its deletion.

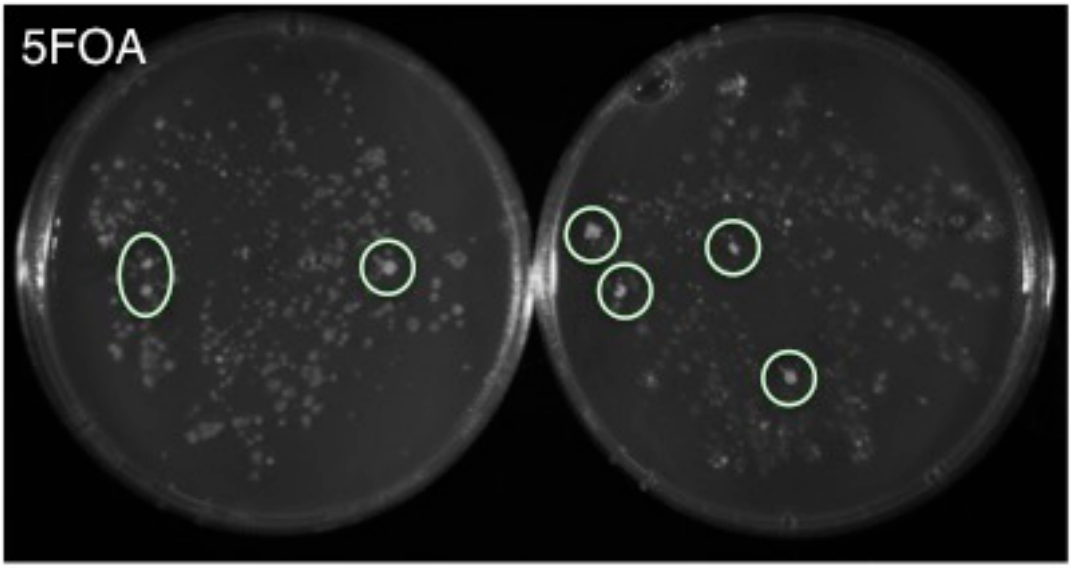

Figure 2. 5FOA-resistant transformants. KTP36 (*ura4*-tel, *hat1Δ*) cells were transformed onto medium lacking leucine and then replicated onto medium containing 5-FOA. Circled colonies are those that stand out against background growth.

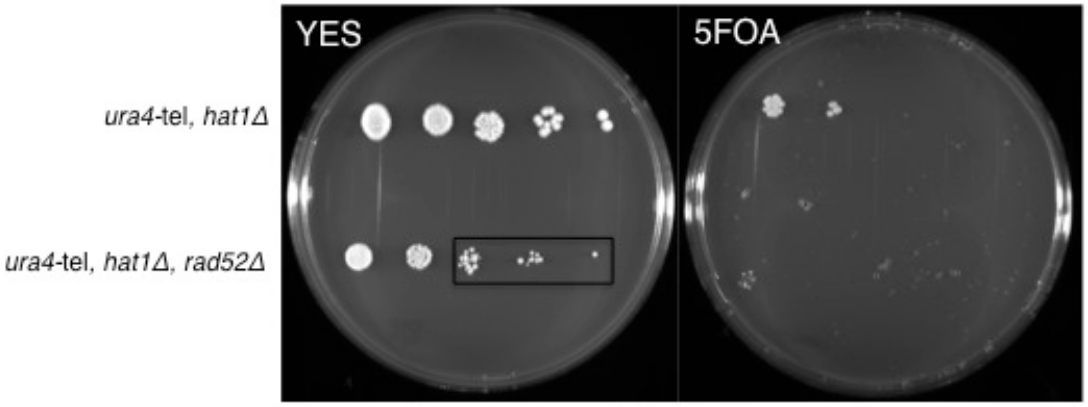

Figure 3. Growth characteristics of KTP36 and CHP1619. The left panel shows growth rates on complete medium. The right panel shows rate of reversion to 5FOA resistance. Box indicates slow growth phenotype.

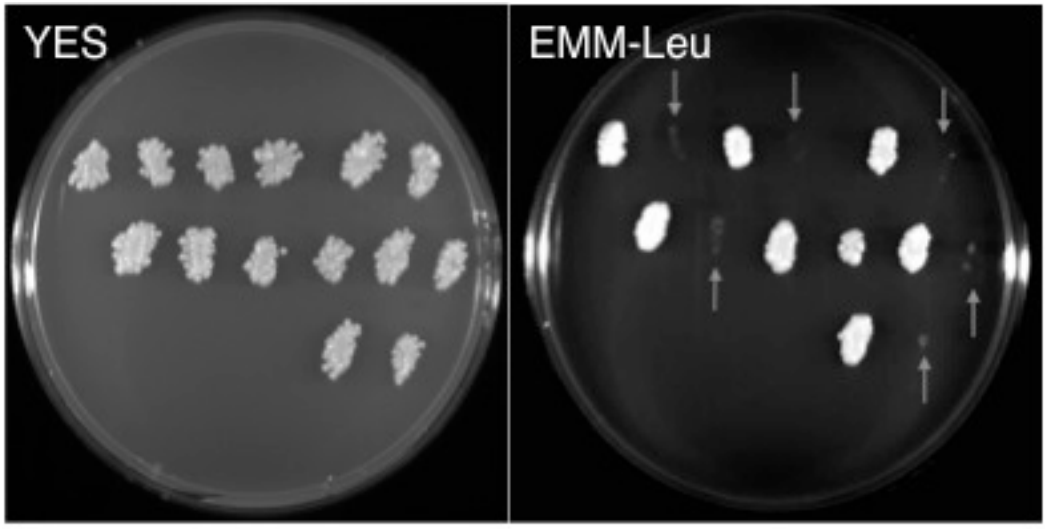

Figure 4. Growth is prevented by plasmid loss. CHP1619 transformants were patched onto complete medium (left) and then replicated to medium lacking leucine (right). Arrows indicate complete loss of plasmid.

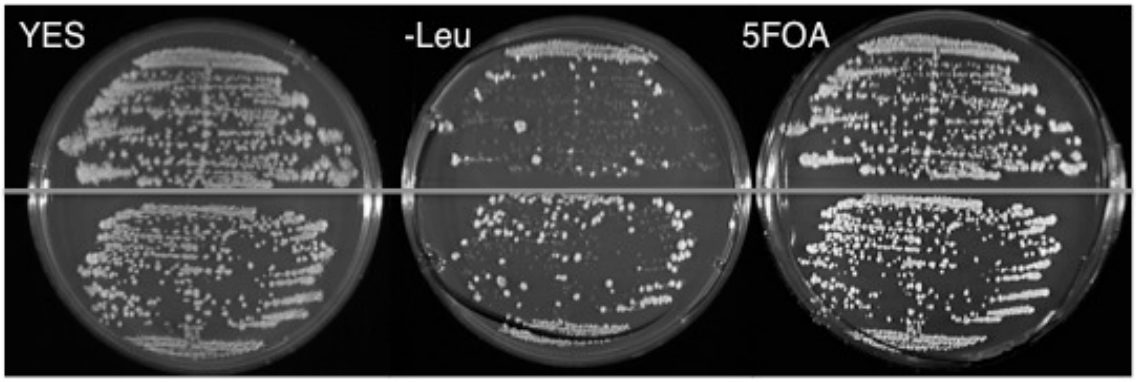

Figure 5. Transformants exhibit plasmid loss and 5-FOA resistance. CHP1619 transformants show some plasmid loss with varied growth on medium lacking leucine (center) compared to complete medium (left). Growth is uniform on 5FOA. Gray line separates two individual transformants.

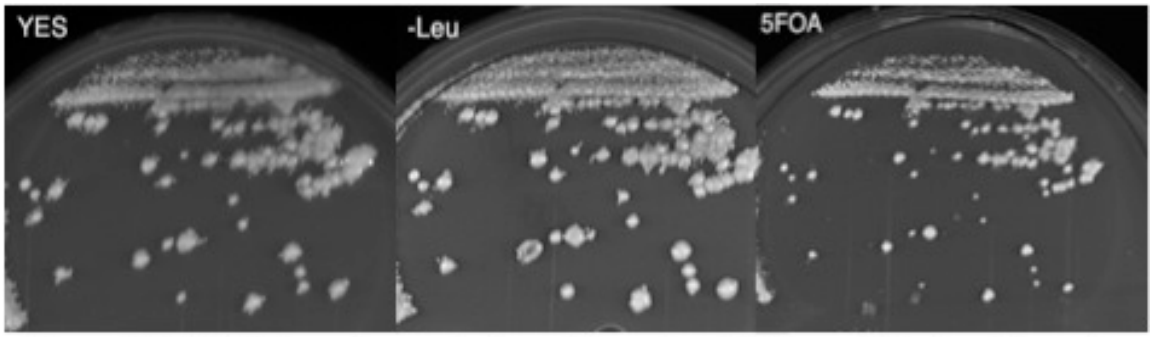

Figure 6. CHP1619 transformants do not lose plasmid. A candidate transformant that had been cultured multiple days on YES was streaked onto the three plates above. It shows uniform growth across YES, EMM-Leu, and 5FOA medium.

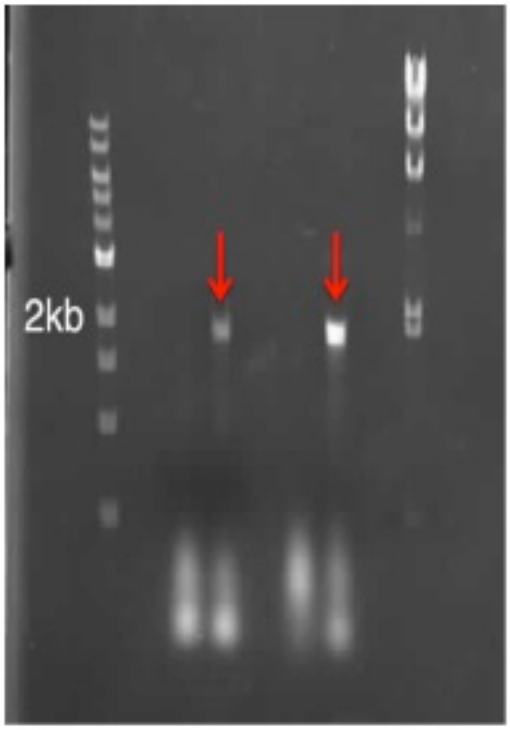

Figure 7. Agarose gel of PCR product for plasmid integration. PCR was performed on CHP1619 candidates that showed uniform growth on varying media. The detected product (arrows) can be seen at 2kb. Standard latter is shown at left and right.

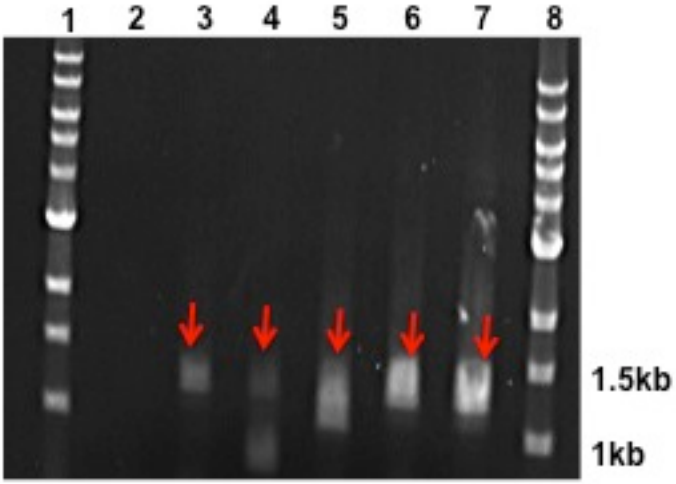

Figure 8. PCR products from transformants on MEA+Phloxine B. PCR reactions contained pLEV3-forward and reverse primers (lanes 3-7). Products were separated on a 1% agarose gel. Molecular weight standards can be seen in lanes 1 and 8.

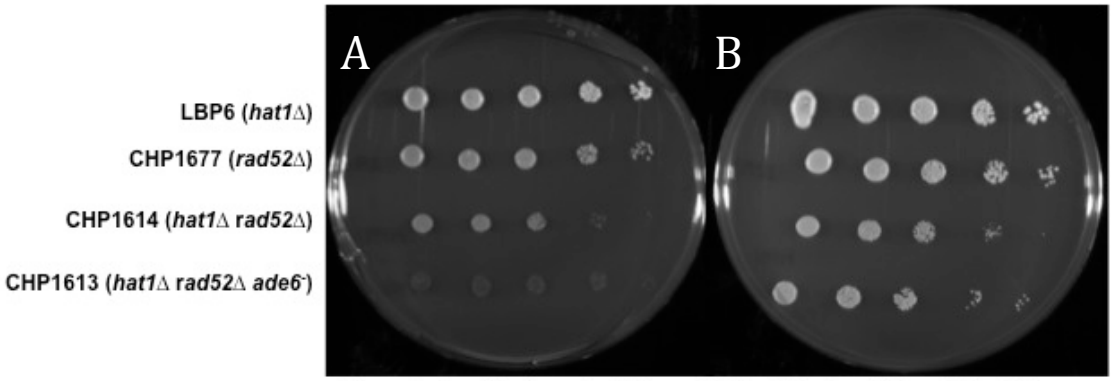

Figure 9. Growth characteristics on MEA+ Phloxine B. Ten-fold serial dilutions are shown on MEA+Phloxine B (A) and YES (B) media. Plates were incubated 3 days at 30˚C. Gray cells in A indicate those that stain red with Phloxine B.

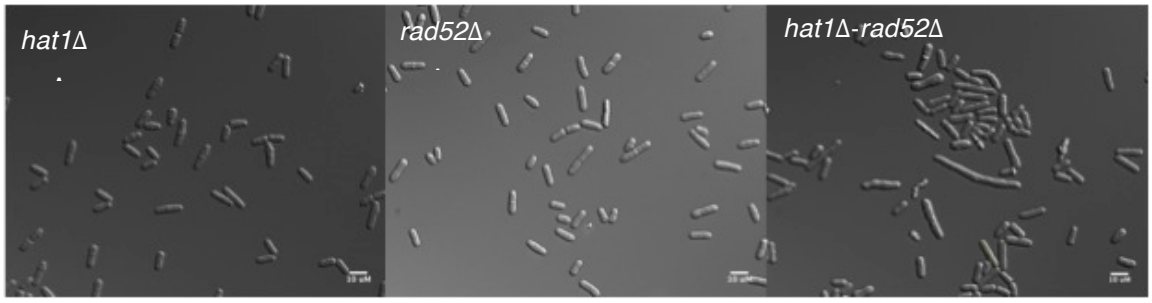

Figure 10. Average cell length of double mutant is larger than either single mutant. DIC images show cell length differences between three strains (*hat1*Δ, *rad52*Δ, *hat1*Δ-*rad52*Δ) grown in YES. Scale bar represents 10μM.

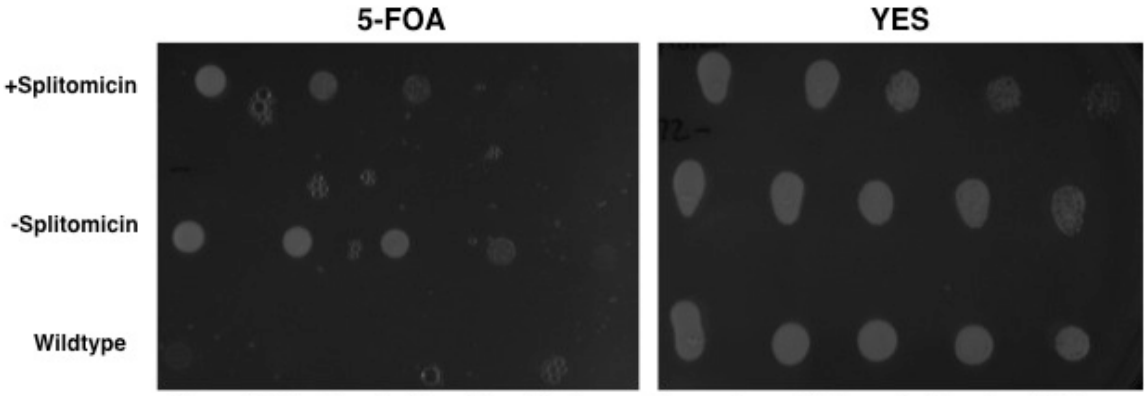

Figure 11. Treatment with splitomicin does not cause loss of telomeric silencing. Wild-type (975) and telomeric marker strain FY1872 were cultured in medium with and without splitomicin for three generations at 30°C. Cells were grown three days in the absence of splitomicin on 5-FOA or YES medium. Fivefold serial dilutions are shown.

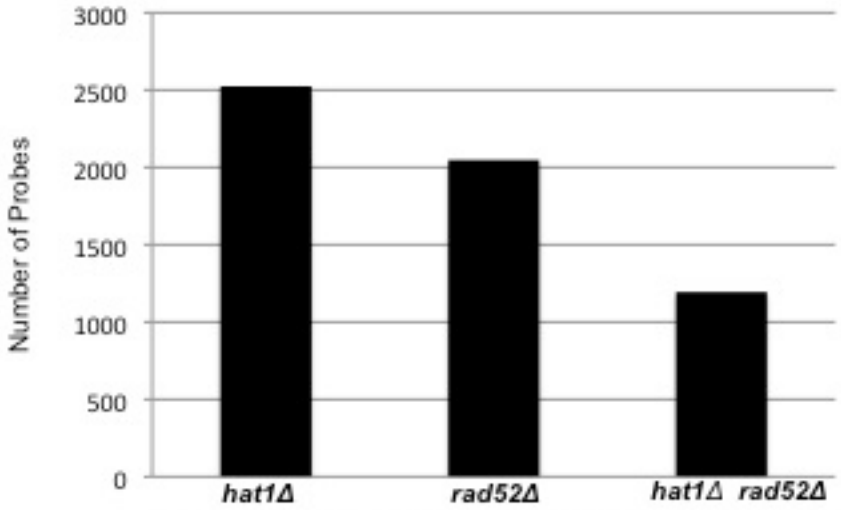

Figure 12. The number of probes upregulated in each strain varies. Graph represents the number of probes in each strain (hat1*Δ*, *rad52Δ*, *and hat1Δ rad52Δ)* with a twofold or greater increase in expression when compared to a wild type strain.

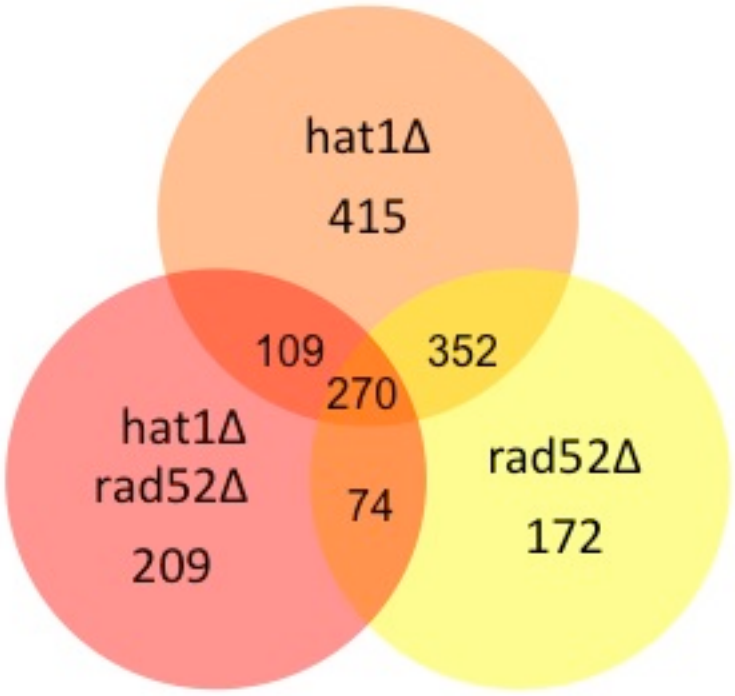

Figure 13. Venn diagram represents the number of unique genes in each strain with a twofold or greater increase in expression compared to the wild-type. The number of genes overlapping between mutants is also shown.

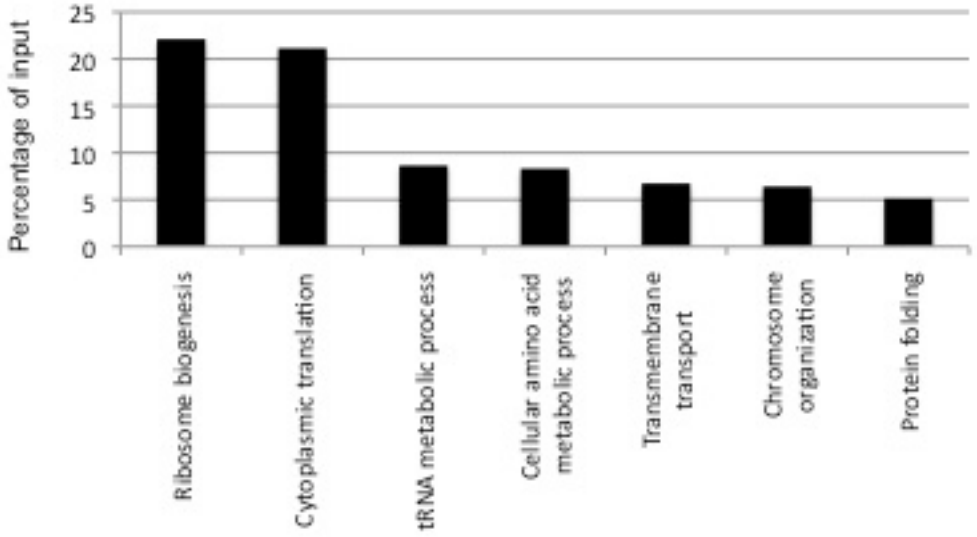

Figure 14. **Ontological enrichment in the double mutant.** Using Princeton's Gene Ontology Term Mapper, hat1Δ -rad52Δ genes with a greater than twofold increase in expression over the wild-type were analyzed. All terms shown represent greater than 5% of input genes.

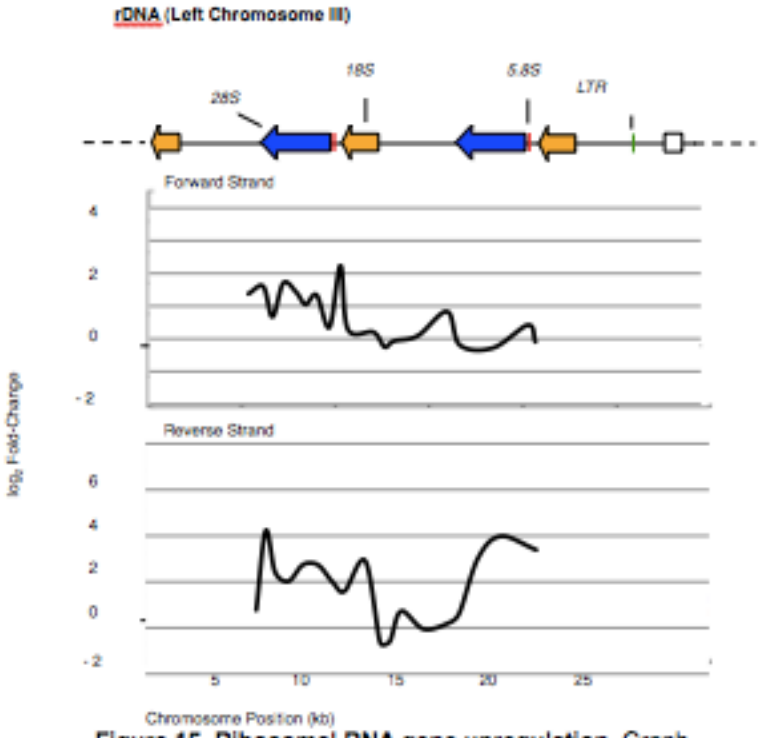

Figure 15. Ribosomal RNA gene upregulation. Graph represents fold change in expression of several probes localized to ribosomal RNA genes on chromosome 3 when compared to the wild-type. Expression changes are plotted based on position, with closest to the centromere on the left and closest to the telomere on the right.

Discussion

Here, two strains of *S. pombe* were examined for high copy suppressors of a *hat1* deletion through the use of a *ura4* telomeric marker. After transformation, we chose to study colonies that grew on medium lacking leucine, which would show presence of the plasmid, and then select those that grew on 5FOA from that group. These cells would indicate regained telomeric silencing, since strains expressing *ura4* genes produce a toxin using 5FOA as a substrate. However, the earliest experiments showed loss of the *ura4* marker at a high frequency, which warranted the creation of a second strain (CHP1619) to reduce recombinatorial activity. Despite multiple rounds of experimentation, it was found that the *ura4* marker is simply not well maintained in the telomeric position, as judged by growth on 5FOA medium.

While the goal of this project was to identify high copy suppressors of the *hat1* deletion, the discovery of frequent *ura4* recombination is a unique phenotype in and of itself. The cause of this loss is uncertain, but it could be that there are recombination events that occur independently of the *rad52* gene.

During the course of this project, a second interesting phenotype was identified: CHP1619, the strain with a *hat1-rad52* double deletion, exhibits slow growth. It appears that the deletions have caused synthetic sickness. Not only do these cells grow much more slowly than KTP36, but the cells are shaped differently.

They show a significant elongation in comparison to the KTP36 cells.
Due to this unforeseen development, the focus was shifted to this synthetically sick phenotype. These experiments did not rely on the *ura4* telomeric marker. While transformations and PCR yielded some colonies that grow more quickly and genes that were possibly responsible, the death and slow growth phenotypes of this strain yielded more questions than answers. Two distinct further courses of study were taken to try and understand both the abrogation of telomeric silencing and the cause of this phenotypic change.

It was previously reported that the use of the histone deacetylase (HDAC) inhibitor TSA can cause loss of telomeric silencing, due to increased acetylation at the telomere (37). In an effort to expand on this result, the HDAC inhibitor splitomicin was tested as well. However, the same effect was not observed and it is a possibility that another deacetylase can compensate for the loss of Sir2p function at the telomere. However, it may also be that splitomicin was not inhibiting Sir2p under our experimental conditions. Further studies of this compound should include adding it to the solid media to test the effects of prolonged exposure.

Microarray analysis of gene expression yielded significant results. First, it revealed a greater global increase in gene expression for the fastest-growing mutant, *hat1*Δ, compared to the other two mutants. Additionally, the gene

expression profiling confirmed telomeric derepression in the *hat1*Δ strain: telomeric probes showed an increase in gene expression, however the same upregulation was not seen in the *rad52*Δ strain.

Ontological analysis of expression data for the double mutant yielded interesting results. Ribosome biogenesis and cytoplasmic translation were significantly upregulated, however this strain grows much more slowly than either of the other two strains. This trend was not observed to such a heightened degree in either of the other two mutants. The double deletion appeared to have eliminated some functional redundancy in keeping ribosome biogenesis at an appropriate level.

At first observation, the upregulation of ribosomal RNA genes was counterintuitive. Upon closer examination, however, it was found that the antisense transcripts are even more upregulated than the sense transcripts. The largest spikes in upregulation of antisense (and sense) probes correspond to the 28S portion of the ribosome, based on position. These increases could effectively downregulate ribosomal RNA and ribosome biogenesis. The lack of ribosome production would likely lead to a severe decrease in protein production. If that were the case, then it may explain, in part, the ontological results as well as the slow growth phenotype of the double mutant strain. These findings should be explored further in additional gene expression studies.

The study of the pathway by which *hat1*Δ strains abrogate telomeric silencing transformed into a study of much wider scope investigating multiple strains and their characteristics. While many important observations have been made regarding phenotypes of the *hat1*Δ, *rad52*Δ, and *hat1*Δ-*rad52*Δ strains, there is still much to learn. These results have, however, identified a novel synthetically sick phenotype and further studies should aim at elucidating the cause.

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