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Clairine Larsen

Denison University

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Author: Clairine Larsen

Denison University, Granville OH

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Advisor: Dr. Jeff Thompson, Biology Department

Secondary reader: Dr. Eric Liebl, Biology Department
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Role of Histone Modifications in Novel Epigenetic UV Hyper-resistance Phenotype in

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Clairine Larsen, Denison University Biology Department

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**Abstract**

Epigenetics is the phenomena of changes to gene expression without altering the physical DNA which can be inherited through generations of cells. Epigenetics enables cells to change gene expression rapidly in response to changes in the environment. This paper seeks to further our understanding of epigenetics through investigation of a novel epigenetic phenotype of UV hyper-resistance in the yeast species *Saccharomyces cerevisiae*. Yeast cells descendent from cells that received a primary exposure to mild UV radiation show an increase in survival and hyper-resistance to further UV exposures. Histone modifications are the only known mechanism of epigenetics found in yeast and we determined two histone modifications to be essential to development of this UV hyper-resistance phenotype. We found that a balance of acetylation and deacetylation at H3K56 and methylation at H3K4 serve as the histone modifications needed for the inheritance of UV hyper-resistance. This work is a key advancement in our understanding of epigenetics in response to environmental changes and in understanding the mechanism of this phenomenon on a single-cell level.
Introduction

Epigenetics is the phenomena of heritable changes to gene expression without altering the physical DNA. Each organism carries within its cells a unique genome which serves as a blueprint for cellular functions. However, while each cell holds an identical copy of DNA, difference in the expression of the genes contained within facilitates the function of an individual cell. To obtain this specialized control, a variety of molecules are involved in turning specific genes “on” or “off”. This control can be modified by temporal, locational, or environmental influence. Additionally, these particular expression states controlled by regulatory molecules are just as inheritable as the sequence of A, T, C, and Gs (Deans and Maggert, 2015). The discovery of this additional layer of control led to the creation of the field of epigenetics. Epigenetics is defined as changes to the genome that lie above, “epi”, the genetic code, “genetics”, and these changes are heritable. While there is limited consensus on what constitutes as epigenetic, the heritability aspect of the definition is essential (Deans and Maggert, 2015). The heritability of epigenetics constitutes a very interesting effect on the expression states in multiple generations of cells as, unlike changes made to the genetic sequence to modify expression states, epigenetic mechanisms allow for dynamic control that is ever changing. This enables cells to rapidly respond to environmental changes through changes in gene expression. Whether certain genes are expressed is dependent on the chromatin environment and the interaction of transcription factors with the DNA. Epigenetic changes to gene expression utilize both of these mechanisms.

An important control of gene expression is the interaction of transcriptional factors and the DNA. Changes in the ability of these factors to bind to DNA can affect
the expression of a target gene. DNA methylation in the genome can affect this interaction and is typically associated with repression or silencing of genes. DNA methylation is the addition of a methyl group to a cytosine with in a specific CpG sequence (Almouzni, 2016). This chemical modification to the DNA functions to either block the ability of transcription factors to bind to the DNA or recruit proteins associated with gene repression to the site (Moore, 2013). Both of which result in DNA methylation having a repressive effect on gene expression. DNA methylation is a well-established epigenetic mechanism as patterns of this modification are known to be highly conserved during replication. CpGs are symmetrically present on both strands of DNA and the methylation states are mirrored. During DNA synthesis a hemi-methylated state occurs and is recognized by DNA methyltransferase 1 which methylates the new strand of DNA, preserving the DNA methylation states found in the parental cell (Almouzni, 2016). The ability to change whether a site is methylated or not and the ability of cells to dutifully pass down DNA methylation states make DNA methylation a major mechanism of epigenetics.

In addition to influencing the ability of transcription factors binding to DNA, changes in the accessibility of regions of the genome is another mechanism of epigenetics and greatly influences gene expression. The ability of transcriptional machinery to access regions of the genome is dependent on the chromatin structure. Chromatin structure can be categorized as either heterochromatin or euchromatin (Quina et. al., 2006). Regions of heterochromatin are characterized by transcriptionally inactive genes. In these regions the DNA is densely packed contributing to the inability of genes located here to be transcribed. On the other hand, regions of euchromatin are open and associated with
higher levels of expression. Genes in regions of euchromatin are generally considered transcriptionally active. Cells contain both regions of heterochromatin and euchromatin within their genome and contribute to differences in gene expression. The relative chromatin state of a particular region of the genome can differ not only between individual cells but can change within a single cell’s life cycle (Quina et. al., 2006). This mobility of the chromatin structure enables cells to easily change their genomic expression profile in a cell cycle dependent manner, in the activation of cellular pathways, or in response to environmental changes.

The generation of these heterochromatin and euchromatin regions is achieved through the incorporation of histones proteins. Four core histones (H2A, H2B, H3, and H4) make up an octamer complex (two of each core histone) around which 147bp of DNA is wrapped, creating a nucleosome (Kouzarides, 2007). The organization of the genome around histones can change the accessibility of different genes and therefore modifying their expression states. Modifications to the histone protein itself governs the histone-DNA interaction and intern influences the accessibility of genes to be active. There are eight known histone modifications and over 60 different sites where these modifications can occur. Each modification and the particular placement of said modification can have different impacts on chromatin structure, lending to great diversity in control (Kouzarides, 2007).

The most well studied modifications are the small covalent modifications of phosphorylation, methylation, and acetylation. Phosphorylation events have been found to occur on serine, threonine and tyrosine amino residues and are associated with activation and inactivation of genes (Bannister, 2011). The addition of a phosphate group
induces a significant negative charge on the histone. Acetylation, meanwhile, is associated with lysine and the addition of this group neutralizes the charge of this amino acid loosening the DNA-histone interaction. Due to this, acetylation events have been associated with activation of genes. Methylation is more complex than the previous modifications mentioned, as this modification occurs on both arginine and lysine and includes different methylation states. Methylation on arginine can be mono- or di-methylated, with di-methylation being symmetrical or asymmetrical, while methylation on lysine can be mono-, di-, or tri-methylated (Bannister, 2011).

Modifications of histones through acetylation, methylation and phosphorylation changes the chromatin environment through histone-DNA, histone-histone, histone-other regulator protein interactions (O’Kane and Hyland, 2019). Further, many histone modifications have been associated with epigenetic phenomena and have been researched as the possible mechanism of heritability that is an essential element of epigenetics (Kouzarides). A well-studied example of histone modifications being a source of epigenetic inheritance is in telomeric silencing in the yeast species *Saccharomyces cerevisiae* (O’kane and Hyland, 2019). Recent studies have shown that gene silencing in the telomeric regions is very precise and is impacted by the removal of specific histone modifications. The removal of these specific modifications results in control of different proteins that create a heterochromatin environment. In the presence of these genetic markers, the heterochromatin environment sufficiently silences the telomeric region. While the model for inheritance of these sites is not well known, research has shown that this is an epigenetic phenotype as these modifications are dutifully passed down to daughter cells (O’kane and Hyland, 2019).
While the previous example is focused on an epigenetic phenotype that is constant in cells, the dynamic nature and temporariness of epigenetic elements makes them particularly well-suited for influencing a cell’s response to environmental changes (D’urso et al., 2016). A common cellular stimulus that triggers changes in gene expression is exposure to ultraviolet radiation (UV). Exposure to UV radiation can cause damage to the cell on the DNA level, commonly inducing double stranded breaks (DSB) and cyclobutane pyrimidine dimers (CPD) (Novarina et. al., 2011). These damages are repaired through the highly conserved DNA repair pathways of nucleotide excision repair (NER) and DNA damage check points. These repair mechanisms are essential to genomic stability and cells deficient of NER and unable to activate an essential G1 DNA damage check point result in many DNA lesions proceeding through the S phase (Novarina et. al., 2011). The involvement of histone modifications has been characterized in the normal cellular response to DNA damage and control of these repair pathways. During replication, there are high levels of acetylation present in S-phase associated with newly synthesized histones needed for organization of the new DNA strands (Gershon and Kupiec, 2021). However, these modifications are removed once the cell exits this phase. When DNA damage is present however, the removal of these modifications has been shown to be delayed, allowing for DNA repair pathways to proceed.

The variety and interchangeability of histone modifications make them an attractive mechanism for inducing changes to the gene expression without changing the genomic sequence. However, the question remains, how are these distinct genetic markers and transcriptional states inherited? During replication the genome undergoes major chromosomal reorganization, and the process of semi-conservative replication
generates genome wide disruption of histones (Reverón-Gómez et. al., 2018). If a histone’s position in the chromosome is disrupted during every replication event, there is a possibility that either the histone position or modifications changed during replication, which could affect the ability of epigenetic inheritance. Reverón-Gómez et. al. explored the process of histone position and modifications being accurately inherited in *S. cerevisiae*. They showed that during replication, post-replication histones were reflective of the position of the pre-replication histones on both copies of the newly synthesized strand. In addition, they overserved the presence of H3K4me3, H3K36me3, H3K79me3, and H3K27me3 modification states, which are markers of both active and repressed chromatin states, before and after replication. They found all of these states to be conserved in histones following replication. While the exact mechanisms of how these modifications are maintained or restored if removed during disruption remains unclear, it is thought that the parental histones in the region act as a template for redeposited histones (Reverón-Gómez et. al., 2017).

Interestingly, they found that similarly to the semi-conservative nature of DNA replication, the final histone population of the replicated cells included both parental and de novo histones. Both the presence of parental histones and de novo histones have the opportunity to influence the epigenetic markers inherited by the daughter cells. The modification states present on the parental histones prior to replication have shown to be conserved and copied onto newly synthesized and incorporated histones. This in turn preserves the transcriptional environment in the new cell (Reverón-Gómez et. al., 2017). However, the newly synthesized histones provide the flexibility for new modifications to be added and new epigenetic states to be incorporated into the genome. While there is
supportive research on the histone modified environment being conserved through normal replication events, not much is known about the mechanism of epigenetic changes that occur from environmental stressors, like UV exposure. While researchers have long suspected that histone modifications in response to UV exposure have a lasting effect on cells and potentially confer evolutionary advantages, it has yet to be proved (Xue and Acar, 2018).

However, previous research completed in the Thompson Lab has established the presence of a novel epigenetic phenomenon of UV hyper-resistance in the yeast species *Saccharomyces cerevisiae*. When *S. cerevisiae* is pre-exposed to a non-lethal dose of UV radiation, subsequent generations of yeast cells showed a hyper-resistance to further exposure and a higher survival rate compared to those without pre-exposure (figure 1). While these daughter and granddaughter cells had never experienced a UV exposure event, those decedent for parental cells that were exposed, had acquired a phenotype that equipped them to better handle UV exposure. This trait is particularly evident with cells exposed to a secondary UV dosage of 200 J/m². At this dosage, subsequent generations of the cells that had been pre-exposed showed about a 200-fold increase in survival compared to their unexposed counterpart (figure 1).

This provided a very interesting phenotype to explore as the UV hyper-resistant cells themselves had never experienced this environmental stressor, yet they were better equipped for survival. The seemingly heritability nature of this phenotype generated the question of if this was an epigenetic phenomenon. Previous work has established that UV hyper-resistance is in-fact an epigenetic mechanism and not a product of mutations made by the exposure to UV damage. One piece of evidence for this was that the phenotype is
shown to diminish over time if no secondary exposure event occurs, showing it is not a permanent change in the genome (Reardon, unpublished). As UV exposure is known to cause DNA damage and triggers DNA repair pathways, it is possible that UV hyper-resistance is due to increased DNA repair response. However, previous work has established that there was no difference in DNA repair mechanisms in cell descendent from exposed cells. Instead of having enhanced DNA repair, these cells were seeing inherently less DNA damage, suggesting a different mechanism of protection (Schmidberger and Thompson, unpublished). Further supporting this hypothesis, preliminary results of transcriptome analysis revealed about 500 genes were upregulated in response to UV exposure. However, virtually none of these effected genes were involved in DNA repair but rather involved in cell wall synthesis. Suggesting this DNA protection comes from changes in cell size or cell wall composition (Thompson, unpublished). This and other work done in the lab has provided unequivocal evidence that the UV hyper-resistance phenotype is an epigenetic memory in response to UV. However, this begs the question, what epigenetic mechanism is involved in the development of this particular phenotype?

In past years, yeast have been dubbed as the model organism for epigenetic studies and most of our knowledge on epigenetic mechanisms comes from its role in yeast. Histone modifications are the only known epigenetic mechanism present in yeast and this simplicity allows for clearer understanding of epigenetic traits.(O’kane and Hyland, 2019). Due to this, we hypothesize that histone modifications are the epigenetic mechanism in UV hyper-resistance. This paper seeks to determine the underlying histone modifications that function as epigenetic markers in the development of UV
hyper-resistance. Further, we are working to identify the genes that may be influenced by these modifications to gain a complete picture of the phenotype associated with UV hyper-resistance. In order to understand this epigenetic phenomenon, we examined numerous histone modifications to identify ones that were important in the acquisition of UV hyper-resistance. The results of this work have the potential to significantly impact our understanding of how epigenetic phenotypes develop and are inherited in response to environmental stressors, specifically UV exposure. Further, characterizing the epigenetic mechanism of UV hyper-resistance yields important insights into the broader mechanism of epigenetics in eukaryotes.
Figure 1. Fractional survival of cells after secondary exposure events, with 50 J/m$^2$ pre-exposure and 4 hour incubation period. Cells received either no primary exposure (-UV) or a 50 J/m$^2$ primary exposure (+UV), were incubated for 4 hours, and then received a secondary exposure (0, 50, 100, 150, or 200 J/m$^2$). After 6 days of incubation, colonies were counted and fractional survival of each group was calculated. Pre-exposed cells displayed significantly increased survival at 50 100, 150, and 200 J/m$^2$ (ANOVA with Tukey-HSD, p=0.0122, p=0.0001, p=0.0003, p=0.0020, respectively). Fold differences between +UV and -UV groups were 1.3 for the 50 J/m$^2$ secondary exposure, 3.9 for 100 J/m$^2$, 25 for 150 J/m$^2$, and 190 for 200 J/m$^2$. Some data provided by Amanda Walsh. Experiments, figure, and figure legend was performed and completed by Rachel Reardon unpublished data.

Methods

Strains

Saccharomyces cerevisiae strain BY4741 was used as the wildtype strain (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Knockout strains were isogenic to BY4741 and all were obtained from the Saccharomyces Genome Project. These knockout strains were
generated using PCR-mediated gene deletion and genes were replaced with the antibiotic resistance gene KanMX. Hst3/hst4 knockout mutant (MT15) was obtained from the lab of Dr. Brian Kennedy. Set1 knockout mutant (SDBY1210) was obtained from the lab of Dr. Scott Briggs.

Cell survival

96 well plate UV Double-Exposure Assay

Using a sterile 96 well plate wild type and mutant strains were inoculated in a single well of the first column in 100 µl of YEPD broth. Cultures were incubated at 30°C on Microjive shaker at 1000 rpm overnight. Cultures were then serially diluted in the preceding columns of 96 2. 1 µl of the original culture was transferred into 100 µl of YEPD broth then serially diluted 10-fold out to 1 X 10^-5. Cultures were then incubated as previously for about 18 hours/mid-log phase. A singular dilution of each strain was selected based on visualization of the cell pellet and cells were washed and resuspended in sterile water. Half of selected cultures were transferred to a new 96 well plate and exposed to 50 J/m2 of UV radiation (254 nm, Philips 30 W G30T8 UV lamp) (+UV condition). The remaining half of the original unexposed culture was then transferred to an additional 96 well plate (-UV condition). 10X YEPD and sterile water was added to both +UV and -UV plates to dilute the cell culture by half. Plates were covered to avoid further light exposure and incubated at 30°C on Microjive shaker at 1000 rpm for 4 hours.

After incubation cells were washed and resuspended in 100 µl or 35 µl of sterile water for -UV and +UV respectively to compensate for cell growth differences. Cultures
were then serially diluted out to $1 \times 10^{-5}$ in the preceding columns of the 96 well plate and was done for both the -UV and +UV conditions. Each +UV culture was spot plated at each dilution onto a YEPD agar plate in triplicate, this was repeated with the -UV cultures. A single +UV and -UV plate was then exposed to a second UV dosage of 0, 150, or 200 J/m$^2$ (+UV/0, +UV/150, +UV/200, -UV/0, -UV/150, -UV/200). Plates were incubated at 30° and covered to limit light exposure for ~4 days.

Plates were then qualitatively analyzed through comparison of the mutant strain’s response to the secondary dosage of UV with or without the primary exposure event. Each strain was evaluated at least in duplicate.

**Double exposure mutant survival assays**

Cultures of wildtype and mutant strain were grown overnight to mid-log phase in YEPD broth then washed and resuspended in water. Half of the cell suspension was exposed to 50J/m$^2$ UV at 254 nm using a Philips 30W G30T8 UV lamp (+UV) or left unexposed (-UV). This served as the primary exposure event. All cultures were incubated at 30° for 4 hours in 10 YEPD broth. Cell cultures were washed and resuspended based on culture densities in water. Cell suspension was then serially diluted and plated onto YEPD agar in duplicate at a dilution projected to obtain ~200 colonies per plate after secondary exposure. +UV and -UV primary exposure groups for each strain were exposed to a secondary UV dosage of 0, 50, 100, 150, or 200J/m$^2$ at at 254 nm using a Philips 30W G30T8 UV lamp. Plates were immediately incubated in the dark at 30°C for 5-6 days and colony numbers were counted. For each primary and secondary dosage
condition average percent survival relative to the unexposed control (0J/m2) was calculated. Average of replicate plates for each exposure set was taken. Each strain was tested in at least triplicate (however, the hpa3 mutant was only run once). Mean of replicates were taken with error bars representing standard deviation. When applicable T-tests were used to analyze significance at individual secondary exposure dosages. (Procedure adapted from Bostelman et. al. (2007)).

**Western Blot analysis**

**Exposure**

Cultures were grown to mid-log phase in YEPD broth and optical density was used to ensure cultures were in the appropriate phase. Cells were then washed and resuspended in sterile water. Half of the original culture was transferred into 10X YEPD broth (-UV). Half of the -UV culture was then added to cold ethanol to serve as a baseline control (-UV/0hr). After 5 minutes on ice this culture was washed, and supernatant was decanted before pellet was stored at -80°. The remaining half of the original culture was exposed to 50 J/m² at 254 nm using a Philips 30W G30T8 UV lamp (+UV) before combined into YEPD broth. The +UV and -UV flasks were incubated at 30° for 4 hours (+UV/+4hr, -UV/+4hr). After incubation cultures were added to ice cold ethanol before being washed and supernatant was decanted.

**Histone prep**
-UV/0hr, -UV/+4hr, +UV/+4hr cell pellets were resuspended in 2M NaOH with 10% β-mercaptoethanol. Aliquots of 300 µl for each condition were incubated on ice for 5 minutes before being twice washed with high extraction salt buffer (40 mM HEPES-NaOH pH = 7.5) and resuspend in 2X SDS buffer. Samples were heated at 95°C for 10 minutes and supernatant was transferred into new microcentrifuge tubes before storage at -20°C.

**Western blot and analysis**

Western blots were run with all UV conditions (-UV/0hr, -UV/+4, +UV/+4) of the particular strain. Appropriate amounts of samples were loaded onto Any kD Mini-PROTEAN TGX gels (Bio-Rad) and transferred to PBDF membranes with a Trans-Blot Turbo Transfer System (BioRad). Membranes were blocked by rocking in 5% BSA in TBS-tween 0.05 and 0.02% NaN₃ solution for 60 minutes at room temperature. Membranes were then incubated in the primary antibody concentration indicated in a solution of 5% BSA in TBS-tween 0.05 solution overnight at 4°C (table 1). The following day membranes were washed in TBS-tween 0.05 and incubated in the secondary antibody for 60 minutes at room temperature. The secondary antibody is the goat anti-rabbit IgG-HRP antibody at a 1:3000 dilution in 3% milk in TBS-tween 0.05. After incubation membranes were wash sequentially in TBS-Tween at 0.3, 0.1, and 0.05%. Blots were then prepared with ECL reagent before being imaged with a FluorChem HD2 Chemiluminescent Workstation for 30 min exposure.
Densitometries of modification levels were measured using the computer software system ImageJ. A general αH3 antibody was used as a standard to correct for loading amounts. All band intensities were normalized to the H3 antibody densitometries. Then levels were normalized to the +0hour specific antibody levels. Each histone prep set was analyzed through at least two western blots and with at least three different histone prep sets. Fold differences were averaged within each histone prep set then averaged across different histone preps. Fold differences for single strain (WT) experiments were statistically analyzed using a t-test comparing to a theoretical mean of 1 meaning there was no fold difference. Mutant strain experiments were analyzed using a t-test comparing the +4/-4fold difference for the mutant strain to the fold difference of the WT. (Procedure was adapted from Rossodivita et. al. (2014)).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company/cat#</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>αH3</td>
<td>Primary (general)</td>
<td>Abcam/1791</td>
</tr>
<tr>
<td>αH3K56ac</td>
<td>Primary (specific)</td>
<td>Millipore/07-677-I</td>
</tr>
<tr>
<td>αH3K4me1</td>
<td>Primary (specific)</td>
<td>Activemotif/39297</td>
</tr>
<tr>
<td>αH3K4me2</td>
<td>Primary (specific)</td>
<td>Millipore/07-030</td>
</tr>
<tr>
<td>αH3K4me3</td>
<td>Primary (specific)</td>
<td>Activemotif/39159</td>
</tr>
<tr>
<td>αRabbit IgG-HRP</td>
<td>Secondary</td>
<td>Millipore/12-348</td>
</tr>
</tbody>
</table>

Table 1. Antibody information used in the western blot analysis.

Results

*Histone modifications involved in UV hyper-resistance*
We have previously seen that following a pre-exposure event, subsequent generations of cells develop an epigenetic phenotype of UV hyper-resistance. Histone modifications have been characterized as the only known epigenetic mechanism in yeast and served as a logical starting place for determining the mechanism of the UV hyper-resistance phenotype. In order to screen for histone modifications that could contribute to this phenotype, we used *S. cerevisiae* strains knocked out for specific histone modification enzyme genes. Using a qualitative UV exposure assay we are able to rapidly evaluate if any of the knockout strains had an effect on the cells ability to acquire the UV hyper-resistance phenotype. Cells either received a primary exposure event or were left unexposed and then incubated for 4 hours to allow for any DNA damage repair pathways to function and for at least two rounds of division. Dilutions of each strain were all spot plated onto agar plates that received no secondary exposure or two distinct dosages. Following incubation, relative growth of colonies at the dilutions under different exposure conditions was qualitatively compared to the wildtype yeast strain run in parallel.

As this is a novel qualitative assay, we first established that we could observe the expected phenotype in wildtype cells. The wildtype strain was used as a control and did show the expected acquisition of the UV hyper-resistance phenotype and growth behavior. In each exposure set, the wildtype strain grew similarly with no secondary UV dosage at every dilution with or without a pre-exposure event (figure 2). However, wildtype cells that received a pre-exposure event grew at higher dilutions of the culture after receiving a secondary UV exposure (both at 150 and 200 J/m²) than those that did not have the primary exposure event. In each trial the UV exposed primary exposure
wildtype cells grew 10 to 100-fold higher compared to the non-pre-exposed cells following secondary exposures (figure 2). This fold difference in growth was seen at both the 150 J/m² and 200 J/m² exposure events. This increased survival in the wildtype is exemplary of the UV hyper-resistance phenotype and is comparable to prior observations and past quantitative survival assays (figure 1).

Mutant growth was compared to the wildtype growth to understand if the loss of a particular histone modification enzyme influenced the presence of the UV hyper-resistance phenotype. If a particular histone modification is involved in the establishment of this phenotype, we would expect there to be no difference in the growth of the mutant strain after a secondary exposure with or without a primary exposure event. If the histone modification is not involved, we expect the growth to be similar to the wildtype growth patterns.

**Figure 2. Example of wildtype S. cerevisiae growth after exposure.** Serial dilutions of cells were plated decreasing in concentration from left (undiluted) to right (1X10⁶). During the first exposure cells were either exposed to 50 J/m² (+UV) or unexposed to UV radiation (-UV). After a 4-hour incubation cells were then received a secondary exposure of 0 J/m², 150 J/m², or 200 J/m². There was no visible growth differences between the two primary UV conditions with no secondary exposure, this served as a control. First exposure +UV cells had a visibly higher survival rates of at least 10-fold increase with both a 150 J/m² and 200 J/m² secondary exposure event.
Twenty-eight histone modifications were analyzed through knockouts of genes encoding for various modifier enzymes with most histone modifications having little to no impact on the presence of the UV hyper-resistant phenotype compared to the wildtype (supplementary figure 1). However, four histone modification enzymes showed a moderate influence on the UV hyper-resistance phenotype.

The first of these was the *hpa3* single knockout mutant. A loss of *HPA3* induced an increase in UV hyper-resistance. *HPA3* encodes for an acetyltransferase of H4 (Sampath et. al., 2013). *Hpa3* null mutants had similar growth compared to wildtype cells at +UV 150 J/m² and +UV 200 J/m² secondary exposures with no primary exposure. In addition, there was no significant difference in growth in the cells with a primary exposure and no secondary exposure between *hpa3* null mutants and wildtype strains. However, we see that with a primary exposure event following a secondary exposure event of 150 or 200 J/m² the *hpa3* knockout mutants experienced 10-fold increase in survival compared to wildtype (Figure 3).
Figure 3. Growth of *hpa3* mutant after exposure events compared to wildtype growth. Serial dilutions of cells were plated decreasing in concentration from left (undiluted) to right (1X10⁶). During the first exposure cells were either exposed to 50 J/m² (+UV) or unexposed to UV radiation (-UV). After a 4-hour incubation cells were then received a secondary exposure of 0 J/m², 150 J/m², or 200 J/m². Qualitative visual analysis was used to compare growth of *hpa3* mutants to wildtype growth under difference primary and secondary UV exposure conditions. *Hpa3* mutants (highlighted by the red box) displayed an increase UV hyper-resistance of ~10-fold at +UV pre-exposure followed by either a 150 J/m² or 200 J/m².

Another histone modification gene that was of interest in the presence of the UV hyper-resistance phenotype is *HPA2*. *HPA2* has been shown to be related to *HPA3* and both code for an acetylation enzyme (Sampath et. al., 2013). *HPA2* acetylates at H3K14, H4K5, and H4K12, while *HPA3* acetylates at H4K8. Contrary to the effect of a loss of *hpa3*, the loss of the *hpa2* gene decreased the overall growth of cells that received a primary exposure at every condition of a secondary exposure event (0, 150, and 200 J/m²). However, we see a slight increase in cell survival in *hpa2* knockout cells compared
to wildtype with no pre-exposure event at every secondary exposure event (0, 150, and 200 J/m²) (figure 4).

A loss of the NAT4 gene induced UV hyper-resistance in cells without a pre-exposure event. NAT4 codes for the acetylation enzyme for H4 and H2A (Song et. al., 2003). In yeast cells deficient for NAT4 and without pre-exposure, there was an increase in survival following a secondary UV exposure event of about a 10-fold increase. However, cells receiving a pre-exposure event have the same increase in survival over the wildtype strain as seen in the non-pre-exposed (figure 4). There was no further increase in the UV hyper-resistance after a +UV primary exposure in the nat4 knock out strain.

The final histone modification gene of interest was SPT10. SPT10 is an important regulator of histone gene transcription and is essential for acetylation, particularly at H3K56 (Mei et. al., 2017). In cells with a loss of SPT10 there was a loss of the UV hyper-resistance phenotype. When spt10 knockout cells did not receive any exposure events (no primary or secondary) there is a noticeable slight decrease of growth in the higher dilutions (10⁻³ to 10⁻⁵) (Figure 4). This is consistent with known growth defects in spt10 null strains (Saccharomyces Genome Database). When cells received only a secondary exposure spt10 mutants had a slight decrease in survival at both exposure events. However, this decrease in growth is consistent with the decrease seen in cells that did not receive a primary or secondary UV exposure. When cells receive a primary exposure, we see a loss of the ability to acquire the UV hyper-resistance phenotype fully. This is seen in the slight decrease in growth at both the 150 and 200 J/m² secondary exposure events. This decrease in growth warrants further investigation.
To further assess the possible role of *HPA3, HPA2, NAT4*, and *SPT10* on the development of UV hyper-resistance we used a quantitative survival assay. This double exposure survival assay allowed for more precise determination of which histone modifications are involved in the UV hyper-resistance phenotype and to what extent. Mutant and wildtype cells were either exposed to a primary UV exposure event or unexposed prior to a 4-hour incubation. All cells were then plated on agar plates and exposed to a secondary UV dose of either 0, 50, 150, or 200 J/m² followed by an
incubation period. Fractional cell survival of each condition was calculated for an individual exposure set. Survival was averaged for each cell condition across multiple exposure sets (with the exception of the hpa3 knockout, which included only one trial).

Analysis of hpa2, hpa3, and nat4 knockout mutants revealed no significant impact on the ability of the cells to acquire the UV hyper-resistance phenotype (figure 4, 5, 6). In each case wildtype cells displayed the UV hyper-resistance phenotype. At each secondary exposure condition wildtype cells that received a primary exposure event had an increased fractional survival compared to cells that did not receive a primary exposure event. At the 200 J/m$^2$ secondary exposure condition there is about a 200-fold increase in fractional survival of cells that were pre-exposed. For the hpa3, hpa2, and nat4 knockout strains there was no statistical difference (due to small trial size we were unable to runs statistical analysis on hpa3) in fractional survival of the pre-exposed mutant and pre-exposed wildtype cells or between the unexposed mutant and unexposed wildtype cells at a secondary UV dosage of 200 J/m$^2$ (figure 5,6,7). If the histone modification was involved in the phenotype, we would expect a loss of the gene involved in establishment of the modification to result in the complete loss of the increase in the fractional survival after a primary exposure. While these findings are contradictory to the qualitative assay, it is important to note that the subtle differences observed in the qualitative assay are less reliable than the results presented here and that the trends seen here are in all likelihood biologically significant.
Figure 5. Fractional survival of hpa3 knockout mutant strain after various exposure events compared to the wildtype strain. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV). Cells were incubated for 4 hours prior to receiving a secondary exposure event at a particular dosage (0, 50, 100, 150, or 200 J/m²). Cells were incubated for 6 days. Colonies were counted and fractional survival was calculated for each group. Statistical tests were not able to be performed due to a small number of replicates. However, there is a very clear trend with the mutant strain behaving similarly to the wildtype.
Figure 6. Fractional survival of hpa2 knockout mutant strain after various exposure events compared to the wildtype strain. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV). Cells were incubated for 4 hours prior to receiving a secondary exposure event at a particular dosage (0, 50, 100, 150, or 200 J/m²). Cells were incubated for 6 days. Colonies were counted and fractional survival was calculated for each group. At 200 J/m² there was no statistical difference between the wildtype +UV and mutant +UV or between wildtype -UV and mutant –UV. There is a statistically significant difference between both +UV conditions and both -UV conditions (p = 0.0001). There were no statistical differences at any other secondary dosage. Analysis was done through ANOVA tests at the individual secondary UV dosages followed by Tukey-Kramer HSD post-hoc test when applicable.
Figure 7. Fractional survival of *nat4* knockout mutant strain after various exposure events compared to the wildtype strain. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV). Cells were incubated for 4 hours prior to receiving a secondary exposure event at a particular dosage (0, 50, 100, 150, or 200 J/m²). Cells were incubated for 6 days. Colonies were counted and fractional survival was calculated for each group. At 200 J/m² there was no statistical difference between the wildtype +UV and mutant +UV or between wildtype -UV and mutant –UV. There is a statistically significant difference between both +UV conditions and both -UV conditions (p = 0.0008). At 150 J/m² there is no statistical difference between wildtype and mutant +UV but these two conditions were statistically different from wildtype and mutant -UV. There was statistical difference between the wildtype and mutant -UV conditions (p = 0.0001). At 100 J/m² there was no statistical difference between the wildtype +UV and mutant +UV or between wildtype -UV and mutant –UV. There is a statistically significant difference between both +UV conditions and both -UV conditions (p < 0.0001). Analysis was done through ANOVA tests at the individual secondary UV dosages followed by Tukey-Kramer HSD post-hoc test when applicable.

However, unlike the previous mutants, the *spt10* null strain showed a significant decrease in the fractional survival of mutants exposed to a primary exposure event
compared to the wildtype cells at all secondary dosages (figure 8). This supports the trends seen in the qualitative assay. In the wildtype strain, as expected, there is a statistically significant increase in fractional survival between cells that received a primary exposure compared to those that did not, exemplifying the UV hyper-resistant phenotype ($p < 0.05$). However, this pattern was not seen in the $spt10$ null mutants. There is a significant decrease in the fold difference between pre-exposed and non-pre-exposed cells in the mutant compared to the fold difference seen in wildtype cells. At 200 J/m$^2$ the wildtype cells have a 547.00-fold increase while mutant cells have a 42.70-fold increase. This fold decrease is seen at all other secondary UV dosages. At both 100 J/m$^2$ and 150 J/m$^2$ secondary exposure events the fractional survival of pre-exposed wildtype cells was higher than both the unexposed wildtype cells and both mutant conditions ($p = 0.0002$ and $p = 0.0016$ respectively). However, there was no statically significant difference between the knockout mutant that did receive primary exposure compared to both the wildtype and mutant cells that did not receive UV exposure ($p > 0.05$). At the 200 J/m$^2$ we still see that the pre-exposed wildtype cells have a higher survival rate than all other groups, however at this secondary exposure, pre-exposed mutant cells had significantly higher fractional survival than the mutant and wildtype unexposed cells. This dramatic loss of fractional cell survival in $spt10$ mutant cells with a pre-exposure event suggests an essential role for $SPT10$ in the UV hyper-resistance phenotype. Since $SPT10$ is involved in H3K56 acetylation, this modification is a potential epigenetic marker required for UV hyper-resistance.
Figure 8. Fractional survival of spt10 knockout mutant strain after various exposure events compared to the wildtype strain. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV). Cells were incubated for 4 hours prior to receiving a secondary exposure event at a particular dosage (0, 50, 100, 150, or 200 J/m²). Cells were incubated for 6 days. Colonies were counted and fractional survival was calculated for each group. Spt10 mutant +UV showed a significant decrease in survival compared to the wildtype +UV at secondary exposure dosages of 100 J/m² and above (100 J/m² \( p = 0.0049 \), 150 J/m² \( p = 0.0252 \), 200 J/m² \( p = 0.0136 \)) (due to low replicates statistical analysis at 150 J/m² should be reconsidered). The fold differences for mutant +UV and -UV were significantly lower than the fold difference for wildtype. The fold differences between wildtype +UV and -UV were 2.18 for 50 J/m², 10.05 for 100 J/m², 80.41 for 150 J/m², and 547.00 for 200 J/m². There was no difference in survival between mutant +UV and -UV for 50 J/m² or 100 J/m² (1.23 and 1.74-fold difference). The fold difference for 150 J/m² was 5.00 and 42.7 for 200 J/m². At 100 J/m² and 150 J/m² wildtype and mutant -UV and mutant +UV survival were statistically significantly lower than wildtype +UV. At 200 J/m² however, wildtype and mutant -UV were statistically different from mutant +UV and wildtype +UV was statistically significant from all other groups. Analysis was done through ANOVA tests at the individual secondary UV dosages followed by Tukey-Kramer HSD post-hock test when applicable.
As the spt10 mutant data suggests an essential role of H3K56ac we further investigated the role of this modification site as a potential epigenetic marker in UV hyper-resistance using the acetyltransferase of this site, RTT109. RTT109 encodes for an acetylation enzyme for H3K56 (Gershon et. al., 2021). Research from our lab completed by previous lab member Rachel Reardon analyzed the effect of the rtt109 knock out mutant on the UV hyper-resistance phenotype using the double exposure survival assay (figure 9). A loss of rtt109 induces UV hyper-resistance in cells without a primary exposure event. However, there is no further increase in the cell survival following a pre-exposure event as we would expect to see in the development of the UV hyper-resistant phenotype. Mutant cells that did not receive a primary exposure event had a significant increase in fractional survival compared to wildtype cells at all secondary exposure dosages and was significant at dosages of 100 J/m² and higher. Following a pre-exposure event in mutant cells there was a slight increase in survival compared to the unexposed cells, but this fold increase is substantially lower than the survival fold increase we see in wildtype cells between cells that received and did not receive a primary exposure. In-fact mutant cells had a lower survival compared to wildtype cells after a primary exposure event at each secondary UV dosage indicating mutant cells were not able to develop the hyper-resistance phenotype despite having an increased baseline survival without primary exposure. This further supports the finding that H3K56ac is essential for UV hyper-resistance.
Figure 9. Fractional survival of wildtype and rtt109 knockout cells after secondary exposure events, with 50 J/m² pre-exposure and 4-hour incubation period. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV), were incubated for 4 hours, and then received a secondary exposure (0, 50, 100, 150, or 200 J/m²). After 6 days of incubation, colonies were counted, and fractional survival of each group was calculated. Mutant cells in the -UV group displayed significant increases in survival compared to WT cells at 100 and 200 J/m² (p=0.0261, p<0.0001, respectively). Fold differences between the mutant and WT -UV cells were 0.94 for the 50 J/m² secondary exposure, 1.48 for 100 J/m², 2.93 for 150 J/m², and 44 for 200 J/m². Mutant cells in the +UV group displayed significant decreases in survival relative to WT cells at 50 J/m² (p=0.0118). Fold differences between the mutant and WT +UV cells were 0.74 for the 50 J/m² secondary exposure, 0.72 for 100 J/m², 0.74 for 150 J/m², and 0.43 for 200 J/m². P-values were calculated using ANOVA followed by Tukey HSD for pairwise analysis. Experiments, figure, and figure legend was performed and completed by Rachel Reardon unpublished data.
Additionally, we wanted to explore the complement of *RTT109* by examining the effect of the double knock out mutant, *hst3/hst4*, which encodes for the deacetylase for H3K56 (Gershon et. al., 2021). With a loss of the deacetylase enzymes for H3K56 cells are unable to induce UV hyper-resistance as effectively as wildtype cells (figure 10). There is no statistical difference in the fractional survival between mutant and wildtype cells without a pre-exposure event. Mutant cells that received a primary UV exposure event had a significant decrease in survival compared to wildtype cells that received primary exposure at all secondary UV dosages. The effect of a loss of *HST3* and *HST4* indicates that deacetylation is important for the ability of cells to fully develop UV hyper-resistance.
Figure 10. Fractional survival of wildtype and hst3/hst4 knockout cells after secondary exposure events, with 50 J/m² pre-exposure and 4 hour incubation period. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV), were incubated for 4 hours, and then received a secondary exposure (0, 50, 100, 150, or 200 J/m²). After 6 days of incubation, colonies were counted and fractional survival of each group was calculated. Mutant cells in the -UV group did not show statistically significantly changed survival compared to the wildtype at dosages greater than 50 J/m² (p<0.0001). Fold differences between the mutant and WT -UV cells were 0.211 for the 50 J/m² secondary exposure, 0.626 for 100 J/m², 0.581 for 150 J/m², and 2.75 for 200 J/m². Mutant cells in the +UV group showed statistically significantly decreased survival compared to the non-pre-exposed knockout cells at 150 J/m² (p=0.0094) and 200 J (p=0.0007). Fold differences between the mutant and WT +UV cells were 0.251 for the 50 J/m² secondary exposure, 0.203 for 100 J/m², 0.169 for 150 J/m², and 0.211 for 200 J/m². P-values were calculated using ANOVA followed by Tukey HSD for pairwise analysis. Experiments, figure, and figure legend was performed and completed by Rachel Reardon unpublished data.
The phenotypic impacts of knocking out *SPT10, RTT109, and HST3/HST4*, suggests that H3K56 acetylation is important for the development of UV hyper-resistance. However, we see that development of this phenotype requires a balance of acetylation and deacetylation events at H3K56 across the genome.

*Analysis of global acetylation and deacetylation of H3K56*

In order to further understand the role of H3K56ac we used western blot analysis to analyze the global levels of this modification in cells following UV exposure (this work was completed in collaboration with previous lab member Rachel Reardon). Cells were either exposed to UV or left unexposed. A portion of unexposed cells and exposed cells were incubated for 4 hours, based on the approximate time of the presence of the phenotype. The remaining unexposed cells were no incubated and served as a baseline control (0 hour). Protein isolation was performed on all samples simultaneous followed by western blot analysis for the desired histone modification. Samples were probed with a general αH3 antibody and used to normalize relative densitometries of modification levels. Samples were additionally probed with αH3K4ac specific antibodies and densitometries were taken. Relative levels of the modification were normalized to the wildtype 0 hour incubation and fold difference was calculated (exposed/unexposed).

Following UV exposure and a 4 hour incubation period, H3K56 acetylation levels increased in cells relative to both baseline levels and levels in unexposed cells after 4 hour incubation (figure 11). In cells that did not receive UV exposure, acetylation levels did not change with a 4 hour incubation period (-UV mean ~1). However, cells that received a UV exposure had increased levels of H3K56ac after a 4 hour incubation relative to the
baseline levels at 0hours. UV exposed cells additionally had statistically significant increased levels of H3K56ac compared to unexposed cells with a 4hour incubation (p = 0.0467). There was a 2.76-fold increase in H3K56ac levels between exposed and unexposed cells after 4hours (p = 0.01).

Figure 11. Relative H3K46ac levels in -UV and +UV primary exposure event with a 4hour incubation normalized to -UV +0hour incubation. Relative modification levels represent the average of runs (ranging from one to three) of at least three distinct protein prep sets with standard error bars. T-test analysis was used for comparison of the -UV and +UV. There was a statistically significant increase in H3K56ac levels between the +UV and -UV condition (-UV mean = 0.96±0.19, +UV mean = 2.50±0.63, p = 0.0467). There is a statistically significant 2.76-fold increase between +UV and -UV H3K56ac levels (p = 0.01, compared to a fold change of 1). Data supplemented with results from R. Reardon.
While the \textit{rtt109} and \textit{hst3/hst4} knock out data suggests an importance in deacetylation this is contrary to what we hypothesized given the role of \textit{SPT10}. This begs the question that the UV hyper-resistance phenotype may be dependent on both acetylation and deacetylation events at H3K56.

Western blot analysis of wildtype cells displayed an increase in H3K56ac in response to UV exposure as we expected from our genetic data. However, the UV hyper-resistance phenotype seems to be dependent on both acetylation and deacetylation events. In order to confirm the deacetylation occurring in the cell in response to UV we used western blot analysis of H3K56ac levels in the \textit{hst3/hst4} knock out mutant compared to wildtype cells. By examining the H3K56ac levels in an acetylase knockout mutant we would be able to see if there are targeted sites of demethylation. If targeted demethylation events are occurring after pre-exposure to UV, we would expect acetylation levels to be higher in the mutant strain that is unable to deacetylate then the wildtype cells after UV exposure following a 4-hour incubation. \textit{Rtt109} knock out mutant strain was also used as a control to confirm that there were no additional acetylation enzymes that would acetylase H3K56 in place of \textit{RTT109} (data not shown). Western blot was performed as stated previously with all three strains exposed and blotted simultaneously. Each strain was probed with the general $\alpha$H3 antibody, used to normalize relative densitometries of modification levels, and the specific $\alpha$H3K56ac antibody. Densitometries were taken and relative levels of the modification were normalized to the wildtype 0hour incubation and fold difference was calculated.

When comparing the relative levels of H3K56ac in the wildtype and \textit{hst3/hst4} knockout strain cells we find no statistical significance between any of the UV and
incubation conditions (figure 12). However, we do see our expected trend in the wildtype data as +UV cells trend toward an increase in H3K56ac levels compared to both -UV groups. In the +UV group, there was no statistically significant difference between the levels of H3K56 acetylation between the mutant and wildtype strain. The mean relative H3k56ac increase in the wildtype strain was 2.94 and the mean H3K56ac increase in the mutant strain was barely higher at 3.09. This finding is supported by analysis of the fold changes comparing +UV cells to -UV cells. There was no statistically significant difference between the fold increase seen in wildtype strain compared to the mutant strain (figure 13). This data was suggesting that either the anticipated deacetylation events are either not occurring or that the western blot analysis is not sensitive enough to pick up small changes in the genome. It is also important to note that these assays were only run on two exposure sets, limiting the ability for statistical analysis.
Figure 12. Relative H3K46ac levels in wildtype and hst3/hst4 mutant strains for -UV +0-, -UV +4-, and +UV +4-hour incubation and primary exposure events normalized to -UV +0-hour incubation. Relative modification levels represent the average of two runs of two distinct protein prep sets with standard error bars. No statistical difference between any conditions was found. ANOVA analysis was used for comparison of the -UV and +UV (p = 0.5542). No Tukey-HSD test was used.
Figure 13. Levels of H3K56ac present in +UV/-UV cells in wildtype strain compared to hst3/hst4 knockout mutant strain. Fold differences levels represent the average of two runs of two distinct protein prep sets with standard error bars. T-test analysis was used to compare fold increases between wildtype and hst3/hst4 mutants. There was no statistically significant difference between the fold difference in +UV/-UV cells for wildtype and hst3/hst4 null strains (p = 0.5327).

H3K4 methylation may play a role in UV hyper-resistance

An additional histone modification gene that was previously found to be important in the UV hyper-resistance phenotype was JHD2 (Rachel Reardon, unpublished data). JHD2 encodes for a demethylase enzyme and targets modification of the H3K4 methylation site (Lee et. al., 2018). Jhd2 null strain cells show a significant decrease in the survival of both cells that received a primary exposure and those that did not compared to the wildtype fractional survival at all secondary UV dosages (figure 14).
Cells that are unable to demethylate at H3K4 have an inherent UV-sensitivity and are not able to establish the UV hyper-resistance following a pre-exposure event, indicating that \textit{JHD2} and demethylation of H3K4 is essential in establishment of UV hyper-resistance. The complementary methylase for H3K4 was also analyzed using a \textit{set1} knockout strain (work done by Rachel Reardon). \textit{SET1} encodes for the methyltransferase of H3K4 (Lee et. al., 2018). Cells that were unable to methylate H3K4 were still able to establish the UV hyper-resistance phenotype, however it was at a slightly lower magnitude as the wildtype strain (supplementary figure 2). Together these results support that H3K4me is important in the establishment of the UV hyper-resistance phenotype, specifically deacetylation (Reardon unpublished data).
Figure 14. Fractional survival of wildtype and *jhd2* knockout cells after secondary exposure events, with 50 J/m² pre-exposure and 4 hour incubation period. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV), were incubated for 4 hours, and then received a secondary exposure (0, 50, 100, 150, or 200 J/m²). After 6 days of incubation, colonies were counted and fractional survival of each group was calculated. Mutant cells in the -UV group showed statistically significant decreases in survival compared to the wildtype at and above 100 J/m² (p<0.0001). Fold differences between the mutant and WT -UV cells were 0.120 for the 50 J/m² secondary exposure, 0.0072 for 100 J/m², 0.0121 for 150 J/m², and 0.139 for 200 J/m². Mutant cells in the +UV group showed statistically significantly decreased survival compared to the non-pre-exposed knockout cells at and above 100 J/m² (p<0.0001). Fold differences between the mutant and WT +UV cells were 0.132 for the 50 J/m² secondary exposure, 0.0164 for 100 J/m², 0.00529 for 150 J/m², and 0.00738 for 200 J/m². P-values were calculated using ANOVA followed by Tukey HSD for pairwise analysis. Experiments, figure, and figure legend was performed and completed by Rachel Reardon unpublished data.
To further characterize this modification’s role in establishing the UV hyper-resistance phenotype we used western blot analysis to look at global levels of H3K4me. It is important to note that H3K4 can be methylated in three different methylation states, mono-, di-, and tri- methylation. All three modification states were analyzed to determine if there were specific responses of each methylation state in response to UV. Based on the previous findings from the genetic data, we would expect to see a decrease in one or more of the methylation levels in cells that receive a pre-exposure as demethylation was found to be important in establishing the phenotype.

Western blot analysis was done as above. Samples were probed with a general αH3 antibody and used to normalize relative densitometries of modification levels. Samples were additionally probed with αH3K4me1, αH3K4me2, or αH3K4me3 specific antibodies. Western blot analysis of H3K4me3 modification levels were completed previously in the lab (Reardon, unpublished data). Again, densitometry results are shown either as relative levels of the modification normalized to the wildtype 0 modification levels and the fold difference between the +UV and -UV cells (work was done in collaboration with previous lab member Rachel Reardon).

Contrary to what we expected to see, H3K4me1 and H3K4me2 levels did not change with exposure to UV and H3K4 tri-methylation levels increased in response to UV radiation. H3K4 mono- and di-methylation levels with or without UV exposure after 4 hour incubation did not change relative to the baseline levels of these modifications in cells (figure 15). Additionally, there was no difference in the levels of these modification between cells unexposed and cells exposed to UV (p = 0.3439, p = 0.6124 respectively).

Supporting the analysis of the relative modification levels there is no statistically
significant change in the levels of H3K4me1 (fold difference of 1.26) or H3K4me2 (fold difference of 1.00, or no fold change) between exposed and unexposed cells (p = 0.3329 and p = 0.99 respectively) (figure 17). However, H3K4me3 increased in the cell. When H3K4me3 levels are normalized to unexposed baseline cells (0 hour incubation), UV exposed cells had increased levels of this modification (mean of ~2) compared to -UV cells (mean of ~1, no difference) (p = 0.0414) (figure 16). This was a significant 2.76-fold increase in H3K4me3 levels between UV exposed and UV unexposed cells (p = 0.018, against a mean of 1) (figure 16). While this data suggests that H3K4me1 and H3K4me2 do not play a role in the UV hyper-resistant phenotype, the increase in H3K4me3 levels after UV exposure suggests this particular methylation state may be involved in the phenotype. However, the increase in H3K4me3 levels is contradictory to what we expected based on the genetic data, which had suggested the removal of methylation at H3K4 was important for establishing the phenotype.
Figure 15. Relative H3K4 methylation levels in -UV and +UV primary exposure event normalized to -UV +0-hour incubation for each methylation state. Relative modification levels represent the average of runs (ranging from one to three) of at least three distinct protein prep sets with standard error bars. T-test analysis was used for comparison of the -UV and +UV for each histone modification individually. There was a significant increase in H3K4me3 levels in +UV compared to -UV cells. There was no significant difference between the +UV and -UV condition for either H3K4me1 or H3K4me2. H3K4me1: -UV mean = 0.8±0.06, +UV mean = 1.14±0.28, p = 0.3439. H3K4me2: -UV mean = 0.74±0.10, +UV mean = 0.68±0.04, p = 0.6124. H3K4me3: -UV mean = 0.98±0.19, +UV mean = 2.01±0.38, p = 0.0414. H3K4me3 data was collected by previous lab member Rachel Reardon. H3K4me1 and H3K4me2 data was collected by Clairine Larsen.
Figure 16. Fold difference of each H3K4 methylation state in +UV/-UV cells. Fold differences represent average of runs (ranging from one to four) of at least three distinct protein prep sets with standard error bars. Means tests comparing to a fold difference of 1 (no change in levels) was used. There is no statistically significant increase or decrease in the levels of H3K4me1 or H3K4me2 following a pre-exposure event (mean = 1.26±0.20 p = 0.3329 and mean = 1.00±0.24 p = 0.99 respectively). However, there was a statistically significant increase in the fold difference for H3K4me3 modification following a pre-exposure event (mean = 2.16±0.30 p = 0.0188). H3K4me3 data was collected by previous lab member Rachel Reardon. H3K4me1 and H3K4me2 data was collected by Clairine Larsen.

Discussion

UV hyper-resistance is a novel epigenetic phenotype seen in the yeast species *Saccharomyces cerevisiae*. Daughter cells of UV exposed cells have increased survival rates following exposure to UV radiation compared to their non-exposed counterparts. Through epigenetic changes to the parental cell’s genome, these daughter cells inherit a
modified chromatin environment that changes their gene expression to better equip them for survival against UV radiation. Histone modifications have been classified as a major heritable epigenetic mechanism. Through our analysis of histone modifications present in yeast cells we were able to identify two modifications that play a central role in the UV hyper-resistance phenomena. Both acetylation at histone 3 lysine 56 and methylation at histone 3 lysine 4 play central roles in the development of UV hyper-resistance in daughter cells. After a UV exposure event parental cells induce changes in the state of H3K56ac and H3K4me, this particular environment is then past to daughter cells and induces UV hyper-resistance and higher survival after an exposure event.

The first modification of importance is H3K56ac. This modification plays essential roles in cell survival and in the transmission of chromatin states during replication (Gershon, 2021). H3K56ac is unique compared to other acetylation sites, as lysine 56 is positioned at the “entry – exit” point of the nucleosome (Gershon, 2021). While many histone modifications occur further out on the free tail of the histone, this modification is uniquely situated to induce a greater impact on gene expression through directly affecting DNA-histone interaction. \textit{SPT10}, \textit{RTT109}, \textit{HST3}, and \textit{HST4} are genes involved in the acetylation state of H3K56. \textit{SPT10} and \textit{RTT109} are involved in acetylating this site, while \textit{HST3} and \textit{HST4} are redundant deacetylases of H3K56. We were able to show that a balance of acetylation and deacetylation events at H3K56 is necessary for daughter cells to induce hyper-resistance to UV radiation. Cells that lacked the ability to acetylate at H3K56 were unable to acquire UV hyper-resistance. However, at the same time, cells that were unable to deacetylate H3K56 were not able to induce UV hyper-resistance as well as wildtype cells. Given our findings, we hypothesize a model of
the role of H3K56ac in the development of UV hyper-resistance that includes both events of deacetylation and acetylation at this site to be important (figure 17). Following a UV exposure event, we know that there are increased levels of acetylation genome wide associated with DNA repair response pathways. However, we then believe targeted Spt10 mediated acetylation is occurring at certain regions of the genome. Occurring in parallel other H3K56 sites are being deacetylated by Hst3 and Hst4. The addition of H3K56ac at particular regions and the removal of this modification at others generates the overall histone modified environment that is inherited by daughter cells and induces UV hyper-resistance.
Figure 17. Model of H3K56 acetylation events that induce UV hyper-resistance in daughter cells by SPT10 and HST3/HST4. The yeast genome is represented by the black line with the ovals being nucleosomes. Acetylation at H3K56 is represented by the presence of triangles and an X represents deacetylation events. Yellow arrows represent modifications to H3K56 that are essential to the development of the UV hyper-resistant phenotype.

In cells with a loss of SPT10 there was a complete loss of the UV hyper-resistance phenotype. SPT10 is involved indirectly in increasing acetylation levels at H3K56, suggesting the importance of H3K56ac as a genetic marker for UV hyper-resistance. While SPT10 appears to contain a histone acyltransferase (HAT) domain and early research indicated SPT10 as a punitive acetyltransferase, there is no evidence suggesting
that *SPT10* has a direct acetylation role at H3K56 (Eriksson et. al., 2005). However, there is strong evidence suggesting *SPT10* regulates H3K56ac through the indirect recruitment of HATs and is necessary for H3K56ac, but not on a global level. Eriksson et. al. determined that Spt10 binds highly specifically to the upstream activator sequence (UAS) on the DNA near the promoter of all four histone genes. It has been shown that Spt10 is essential in H3K56 acetylation at these sites, further suggesting that Spt10 may mediate H3K56ac at these sites through recruitment of the acetyltransferase of H3K56, Rtt109. Recruitment of acetylation at these sites is thought to activate the expression of histone genes, in turn increasing the levels of nucleosomes in the cell. This leads to a complex role of Spt10 in regulating global gene expression. Through chip seq analysis it has been shown that Spt10 has a generally repressive effect on gene expression, this is due to the indirect effect Spt10 has on these genes. While Spt10 is an activator, it functions to activate histone genes which are considered repressors, leading to decreased expression in genes throughout the genome (Eriksson et. al., 2005).

Considering the effects of the loss of *SPT10* on cells ability to inherit UV hyper-resistance, this data suggests that increased H3K56 acetylation at histone gene promoters, functions as a genetic marker for UV hyper-resistance. Due to *SPT10*’s role as a global regulator, we hypothesize that the genes effected downstream of this increase in acetylation may be involved in the phenotypic changes in the cell that result in UV hyper-resistance. Additionally, this upregulation of histone levels in the cell may function to protect the DNA for damage. As previously discussed, the UV hyper-resistance phenotype is associated with less DNA damage occurring and cells were able to recover from DNA damage more efficiently. Work done by Brambilla et. al. showed a direct
relationship between the density of nucleosomes and the amount of DSB accrued through UV exposure. They found that in regions that remained nucleosome heavy for long periods of time had significantly decreased levels of DNA damage from UV exposure (Brambilla et. al., 2020). It is possible that the outcome of this epigenetic marker, increase in histone production in the cell, serves to protect daughter cells from DNA damage by increasing the nucleosome density present in the genome prior to UV exposure.

This finding of H3K56ac being important in the development of this phenotype is further supported as cells that are deficient for the direct acetyltransferase of this site were unable to induce UV hyper-resistance. Rtt109 acts globally through the genome to acetylate all H3K56 sites. Interestingly, rtt109 mutants that did not receive a primary exposure event were able to survive at higher rates after a secondary exposure than wildtype cells under the same conditions. Rtt109 is involved in acetylating H3K56 genome wide and the loss of RTT109 is, in all likelihood, affecting many functions of the cells. It is possible that the effect of a loss of RTT109 cells is triggering an additional pathway that induces higher survival rates to UV exposure. As no UV hyper-resistance was seen in rtt109 mutants following a primary exposure event, the increased survival is likely due to a separate effect of a loss of RTT109 and is not indicative of modifications related to our phenotype of interest. It is far more likely that the acetylation events that influence the development of hyper-resistance are associated with events mediated by Spt10 as previously discussed.

Further suggesting that H3K56ac to be important in UV hyper-resistance, we found significant global increase of H3K56 acetylation in cells following a UV exposure.
While some of this increase in H3K56ac may be important in the establishment of the UV hyper-resistance phenotype, previous research has shown following UV damage there is a general increase in H3K56ac. This is due to the activation of pathways, such as the DNA damage repair pathway and chromatin remodeling, that are characterized by high levels of H3K56 acetylation. H3K56 acetylation is critical in repair of double stranded break (DSB) which are commonly induced by UV radiation (Novarina et. al., 2011). In the event of DSBs, chromatin mobility increases genome wide in order to facilitate DNA repair through homologous recombination (Hauer and Gasser, 2017). This increased chromatin mobility is characterized by genome wide histone degradation which leads to the cell needing to synthesize new histones (Gershon and Kupiec, 2021). De novo histones are flagged with H3K56ac and increased levels of newly synthesized histones in turn increases the levels of H3K56ac in the cell following exposure to DNA damaging agents.

Consistent with the previous discussion of the specific Spt10 mediated Rtt109 acetylation events, it is possible that only a fraction of this significant increase in H3K56ac levels is associated with the UV hyper-resistance phenotype. Preliminary efforts were made to evaluate H3K56ac levels in the spt10 mutants in order to distinguish acetylation events directly associated with acquisition of the phenotype (data not shown). However, due to growth defects and other technical issues this work is incomplete (Saccharomyces Genome Database).

While H3K56 acetylation is essential in cells ability to acquire UV hyper-resistance, the story is complicated further by a need for targeted deacetylation at H3K56. H3K56 deacetylation is mediated by redundant histone deacetylases (HDACs)
*Hst3* and *Hst4*. Cells unable to deacetylate H3K56 showed a reduced ability to develop UV hyper-resistance compared to wildtype cells. This suggest that in addition to acetylation at H3K56, deacetylation at other H3K56 sites are also markers that are needed for daughter cells to inherit this epigenetic phenotype. We hypothesize that these deacetylation events are more likely targeted to specific regions of the genome as the global levels of H3K56ac were shown to increase following UV exposure. In an attempt to see these targeted demethylation events in the cell we used western blot analysis of the deacetylation mutants. If our hypothesis is correct, we expected that cells unable to make these precise deacetylations of H3K56 would have higher levels of this modification after UV exposure compared to wildtype cells. An increase in H3K56ac in these mutants would represent the specific genetic markers that are inherited by daughter cells and confer UV hyper-resistance.

However, we were unable to see any difference in the H3K56 acetylation levels after UV exposure between wildtype and mutant cells. This analysis was unable to confirm the role of deacetylation of H3K56 raised by the genetic data. A possible explanation for this comes with the pitfall of western blot analysis. Western blot analysis assesses global levels of the modification and is not sensitive enough to pick up changes that occur on a smaller scale in the genome. As mentioned above in the discussion of *RTT109*, following DNA damage events there is an increase in acetylation levels genome wide with the activation of repair pathways. These events may be masking the targeted deacetylation events that are occurring in the cell following UV exposure, making western blot analysis an ineffective tool for detecting these essential modifications.
Additionally, acetylation and deacetylation of H3K56 are normally cell cycle dependent. Deacetylation is important in maintaining genomic stability whereas H3K56 acetylation is associated with high levels of genomic mobility. In normal replication there is an increase in H3K56ac levels during S phase due to the increased synthesis of new histones on which H3K56ac is deposited (Kouzarides, 2007). This modification is then removed in G phase in order to stabilize the chromatin region and diminish possible detrimental effects of increased genomic mobility (Kouzarides, 2007 and Gershon et. al., 2021). In the presence of a DNA damaging event, such as UV exposure, these deacetylation events are restricted and do not occur till later in order to allow for genomic mobility associated with DNA repair (Gershon et. al., 2021). Our analysis was done with asynchronized cells and showed levels of H3K56ac as an average of what is occurring the cell population. As cells may have been at different stages in the cell cycle this could contribute to the masking effect of the deacetylation events. Future work should be done with synchronized cells in order to show the balance of acetylation and deacetylation events in the cells and partition events that are genetic markers of UV hyper-resistance from those that are results of normal pathways.

In addition to H3K56 acetylation being a punitive genetic marker for UV hyper-resistance, genetic data shows H3K4 methylation to also be involved in the development of the phenotype. With a loss of the ability to demethylate H3K4 we saw a decrease in cell survival of both cells not exposed to UV and those that were and no ability of cells to induce UV hyper-resistance. Methylation appears in three distinct states, mono-, di-, and tri- methylation, however JHD2 is the sole demethylase for H3K4, interacting with each state. However, each methylation state plays a distinct role in
modification of the genome. We attempted to further understand the precise role of H3K4me by examining the global levels of each methylation state in cells after UV exposure. While our genetic data suggests the importance of demethylation at H3K4, following UV exposure there was no change in the levels of either H3K4me1 or H3K4me2. Even more interestingly following UV exposure there is an increase in H3K4me3 levels in the cell.

While these findings are inconsistent with the genetic data a possible explanation for this is the short coming of western blot analysis. As discussed above, western blot shows global levels of a modification in the cell and may not have been a sensitive enough tool to examine a small number of demethylation events that may be occurring following UV exposure. H3K4 mon-, di-, and tri-methylation are all distributed among transcriptional start sites, with H3K4me1 associated with enhancers while H3K4me2 and H3K4me3 are associated with transcriptionally active genes (Liu et al.). H3K4me has been shown to be highly involved in epigenetic memory of environmental stress (Fabrizio, 2019). One of the most studied examples of H3K4me states and transcriptional memory is of the response to inositol deprivation in yeast. In response to this particular stressor, H3K4 is first di- and tri-methylated at the INO1 promoter followed by demethylation to H3K4me2 states. This H3K4 di-methylation has been shown to be the genetic marker for transcriptional memory in response to inositol deprivation stress (D’urso et al., 2016). While there is no current work on the role of H3K4me2 memory states in response to UV, this state’s role in the epigenetic response to other environmental stressors suggests the need for further investigation into H3K4 particularly di-methylation.
The identification of the novel epigenetic UV hyper-resistance phenotype in *S. cerevisiae* is an important discovery in furthering our understanding of epigenetic mechanisms. We have been able to identify the importance of H3K56ac and H3K4me histone modifications in the establishment of this phenotype. Further we have characterized that a balance of locus specific acetylation and deacetylation events of H3K56 and deacetylation at H3K4 are genetic markers inherited by cells that induce UV hyper-resistance. While the precise locations of these markers in the genome are still unknown, the specificity of *Spt10* offers possible locations for acetylation markers. Future investigation of this phenotype, logically, would be identifying the specific genomic locations of these markers and subsequently the genes involved in conferring UV hyper-resistance. While determining the genes targeted by the deacetylation and demethylation events may require global analysis tools, like chip seq, genes determined to be affected by *Spt10* could serve as a logical starting place (Eriksson et. al., 2005). However, it is important to note that while genes seen to be targets of *Spt10* seem promising, these analyses were performed under normal cellular conditions and it is possible that during UV exposure there may be new targets of *Spt10* not previously identified. This idea additionally applies to *Rtt109* and *Hst3/Hst4*. However, preliminary work has suggested that changes in cell wall composition and cell size may be the mechanism by which the cells are protecting themselves from future damage (Thompson and Walsh, unpublished). We may be interested in connections between our modifications of H3K56ac and H3K4me and regulation of genes that are involved in synthesis of the cell wall or cell growth.
Another pathway for further investigation, would be into the role of methylation. This modification is complex in the various states and our western blot analysis was ineffective in providing a visualization of the demethylation events suggested necessary by the genetic data. In addition to the inability of western blot to detect small scale changes, it is possible that our analysis is limited by the time point in which we observed these modifications. For both H3K56ac and H3K4me, the time point when these modifications occur may contribute to our inability to visualize these changes in the cell. Further investigation into which methylation states are markers for this phenotype is needed.

Overall, this work has the ability to provide important insights to the growing knowledge around the field of epigenetics. While our understanding of this phenomenon has increased substantially there are still many gaps in our understanding of the impact of epigenetics. Research in epigenetics provides critical information about another layer of control of genetic expression. Particularly this work provides insight into how the environment can make non-permeant changes to gene expression and how these responses can be utilized to modify future generation’s susceptibility. As we have seen with UV hyper-resistance epigenetic markers, epigenetics has the ability to change many aspects of the cell and can target numerous genes at once, generating the possibility of phenotypic changes on a massive scale (Burrgren, 2016). Epigenetics provides cells with a novel way to rapidly respond to environmental changes and a clear understanding of how these mechanisms work on the single cell level is an essential step in understanding the phenomenon of transcriptional memories.
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**Supplemental**
S1. Growth of various yeast strain after exposure events compared to wildtype growth. Each row has an individual mutant strain. Serial dilutions of cells were plated decreasing in concentration from left (undiluted) to right (1 X 10^6). * indicates strains that show a possible effect but showed uncertainties between trials.
S2. Fractional survival of wildtype and Set1 knockout cells after secondary exposure events, with 50 J/m² pre-exposure and 4 hour incubation period. Wildtype and mutant strain cells received either no primary exposure (-UV) or a 50 J/m² primary exposure (+UV), were incubated for 4 hours, and then received a secondary exposure (0, 50, 100, 150, or 200 J/m²). After 6 days of incubation, colonies were counted and fractional survival of each group was calculated. Mutant cells in the -UV group did not show any significant difference relative to the WT cells at any secondary exposure dosage. Fold differences between the mutant and WT -UV cells were 0.815 for the 50 J/m² secondary exposure, 2.33 for 100 J/m², 1.34 for 150 J/m², and 1.43 for 200 J/m². Mutant cells in the +UV group did not show significant differences in survival relative to the WT cells at any secondary exposure dosage. Fold differences between the mutant and WT +UV cells were 0.836 for the 50 J/m² secondary exposure, 0.0586 for 100 J/m², 0.0385 for 150 J/m², and 0.0245 for 200 J/m². P-values were calculated using ANOVA followed by Tukey HSD for pairwise analysis. Experiments, figure, and figure legend was performed and completed by Rachel Reardon unpublished data.