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THE ROLE OF HISTONE H4 ACETYLATION IN DNA REPLICATION IN
SCHIZOSACCHAROMYCES POMBE

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ABSTRACT

The role of Histone H4 Acetylation in DNA Replication in

Schizosaccharomyces pombe.

The dynamic addition and removal of acetyl groups to lysine residues on histone proteins is one of the critical events that modulate DNA structure. This process, called “histone acetylation”, has been known to impact the regulation of transcription, but its role in DNA replication is not well understood. Here I report on my study of histone H4 acetylation in the fission yeast *Schizosaccharomyces pombe*. Using the two-hybrid method for detecting protein-protein interactions, it was demonstrated that Swi1, a component of the replication fork protection complex, interacts with a NuA4 histone acetyltransferase complex subunit, Vid21. Using quantitative Western blot I show that the level of bulk histone H4 acetylation is greatly reduced in the absence of Swi1 or Vid21. Furthermore, the cell-cycle dependent histone H4 acetylation was deregulated in *swi1* and *vid21* mutants. Importantly, mutations at acetylation sites in histone H4 rendered cells highly sensitive to camptothecin, a compound that induces replication fork breakage. These results suggest that histone H4 acetylation has critical roles in regulation of DNA replication and replication fork repair used to preserve genomic integrity.

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INTRODUCTION

Histones and DNA packaging

The completion of the sequencing of the human genome revealed that the human genome was only 1% coding regions (Venter, 2001) and contained less than 2 times as many genes as the nematode worm (Alberts, 2007).

Epigenetics, the study of phenotypic changes while the DNA sequence remains constant, has become an area of increasing interest to scientists to explain human's high level of complexity. An area of particular interest is the complex structure of DNA and packaging proteins which make up the chromosomes, known as chromatin. In particular, mechanisms of inheritance based on chromatin structure have proven to be vital in regulating the correct expression of genes in daughter cells (Smale, 2002). Disruption of the chromatin structure has been shown to cause faulty gene expression, suggesting that the ability to reestablish the chromatin structure during cell division is an important mechanism for preventing developmental disorders and various genetic diseases (Groth, 2009) .

The basic structural unit of chromatin is the nucleosome. The nucleosome is formed by 147 base pairs of DNA wrapped around an octamer of core histone proteins (Groth 2009). The histone octamer is made up of two H3-H4 dimers (also known as the histone tetramer) and two H2A-H2B dimers (Figure 1). The nucleosome structure limits the access of transcription, replication and repair machinery to the DNA, by wrapping the DNA around the histone proteins. These nucleosomal complexes sterically hinder the access of proteins to the DNA

(Simpson, 1993). To overcome this hurdle, the cell uses at least two major mechanisms: ATP dependent chromatin remodeling, and covalent modification of the histone proteins (Dion, 2005). ATP dependent chromatin remodeling involves a remodeling complex recognizing a segment of DNA, binding it, and altering the DNA-histone contacts in an ATP dependent manner (Imbalzano, 1994). Conversely, multiple covalent modifications can be made to the histone proteins (ex. methylation, acetylation, phosphorylation) which can disrupt chromatin interactions or recruit chromatin remodeling complexes (Kouzarides, 2007) Both of these mechanisms have been shown to be vital for transcription and the repair of damaged DNA (Dion, 2005). This thesis focuses on the covalent modification of the histone protein H4 and its role in maintaining genomic stability during DNA replication.

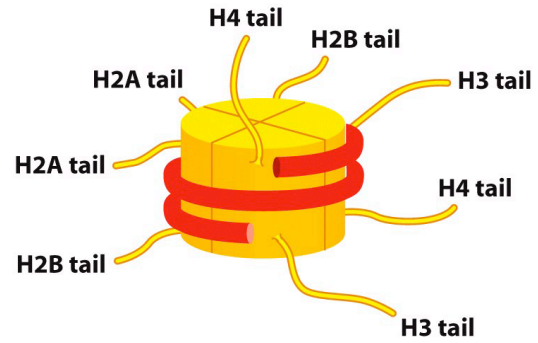


Figure 1: Core histone structure. Histone cores are made up of 4 dimers, two dimers of H3-H4 and 2 of H2A-H2B. The histone tails are the sites of posttranslational modification. (Alberts, 2007)

The histone code

The histone code hypothesis states that covalent modifications to histones and nucleosomal structure play a role in directing gene expression (Figure 2) (Dion, 2005). It is hypothesized that specific modifications to the histone proteins may create the binding sites recognized by DNA binding proteins (Dion, 2005). It is thought that these chromatin modifications play an important role in regulating transcription, repair and replication (Krebs 2007).

By modifying the histone proteins, cells can regulate DNA transactions by creating new binding sites, block protein-DNA interactions, or direct DNA structure by altering charge distributions along the DNA. These modifications can create binding sites for non-histone proteins such as bromodomain proteins that have been shown to bind to acetylated lysines on the histone tails (Figure 2) (Jenuwein, 2001). The Kaposi's sarcoma associated herpes virus uses the bromodomain protein Brd4 to bind to the host chromosomes during mitosis (You, 2006). Other modifications have been shown to inhibit protein binding by creating DNA conformations that hinder the binding of various proteins to the DNA (Jenuwein, 2001). For example, DNA methylation at histone H3 Lysine 9 has been shown to direct the formation of highly condensed and silenced heterochromatin by stimulating further DNA methylation (Bernhard, 2003).

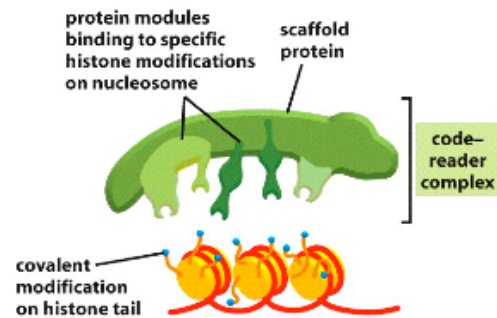


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Figure 2: Histone Code reader. To interpret the histone code, a catalytic subunit can be associated with several proteins that bind to specific modifications (Alberts, 2007)

Finally, some histone structures have been shown to change the conformation of DNA by altering the electrostatic charge of the DNA. For instance, it has been demonstrated that the histone proteins asymmetrically shield DNA phosphates, creating an uneven charge distribution which contributes to nucleosomal folding (Mirzabekov, 1979).

Recently, it has been shown that this code plays a role not only in transcription, but also in replication and repair (Verreault, 2008). For example, lysine 16 of histone H4 can be deacetylated in response to a double stranded DNA break (Jazayeri, 2004). In addition, acetylation of histone H3 at lysine 9 results in genes being replicated earlier in S phase, while genes with low levels of H3K9 acetylation are replicated later in S phase (Shukla, 2009).

Histone modifications

The histone code utilizes a series of covalent modifications, including methylation, phosphorylation, ubiquitination and acetylation to regulate various DNA transactions. Demonstrating the importance of these mechanisms, homologous histone methyltransferases have been identified in organisms ranging from yeast to humans. Originally these proteins main function was thought to be inducing long-term gene silencing through the establishment of heterochromatin. For example, the addition of methyl groups to histone H3 at lysine 9 has been shown to recruit HP1's (heterochromatin protein 1), which play an important role in gene silencing (Rea, 2000). For example, one of the heterochromatin proteins in fission yeast, Chp2, mediates gene silencing by

recruiting a complex to deacetylate H3K14 (Motamedi, 2008). Acetylated H3K14 is associated with active gene transcription so removing this signal leads to gene silencing. This is also an example of different types of histone modifications working together to bring about a desired pattern of gene expression.

Gene silencing by methylation was thought to be non-reversible until the discovery of lysine-specific demethylases (Chen, 2006). This discovery suggests that the dynamic addition of methyl groups may play a major role in regulating gene expression (Huang, 2007). Even more surprisingly, heterochromatin protein 1 in *Drosophila* has been shown to play a role in stimulating the activation of the *Hsp70* gene (Piacentini, 2003). Clearly, methylation is a dynamic and important process for modulating both short and long-term gene expression pathways.

In addition to methylation, nucleosomal phosphorylation has been shown to be an important signal. A well characterized phosphorylation site is histone H3 at serine 10 (Groth, 2001). This modification has been shown to induce the condensing of chromatin prior to replication in systems ranging from *T. Thermophila* to *C. Elegans* (Groth, 2001). The process of H3S10 phosphorylation has been shown to progress in an orderly and predictable pattern, beginning in G2, reaching a peak during metaphase and returning to normal levels by the end of telophase (Groth, 2001). Perhaps surprisingly, phosphorylation at this same site has also been linked to transcriptionally active DNA during interphase (Maile, 2004).

Histone acetylation is thought to play an important role in the regulation of both transcription and replication. Early on, histone acetylation was shown to be linked to active transcription (Allfrey, 1964). Hyperacetylation of histone H3 and H4 has been shown to correspond to active genes (Clark, 1993) and current research has shown that different transcription factors can be recruited by specific patterns of histone acetylation (Agalioti, 2002). In regards to replication, histone acetylation has been shown to correspond to accelerated timing of DNA replication (Aoki 1999), as well as with the formation of heterochromatin (Imai, 2000).

This study, however, will focus on the correlations between histone H4 acetylation and the DNA damage response and replication. Acetylation at H3 K56 provides an example of a connection between DNA replication and repair and histone acetylation. It was shown that H3K56Ac facilitates nucleosome assembly onto DNA *in vivo* through CAF-1 (Chromatin assembly factor 1) and Rtt106 (regulation of Ty1 transposition 106) (Li, 2008). As histones are incorporated into the newly synthesized DNA, the presence of H3K56Ac allows for more efficient recruitment of CAF-1 and Rtt106, allowing these histone chaperones to then assemble the nucleosomes (Li, 2008). In addition, it has been demonstrated, that strains lacking H3K56Ac are significantly more susceptible to DNA-damaging agents which act specifically during S-phase (Li, 2008)

Histone acetyltransferases

Histone acetyltransferases (HATs) are a class of enzymes which add acetyl groups to core histones, typically on lysine residues. These modifications affect both chromatin structure and gene activation (Lee, 2007). Histone acetyltransferases are usually found in protein complexes (Kimura, 1996) and are conserved from yeast to humans, demonstrating their importance to proper cell function (Lee, 2007).

HAT complexes display substantial diversity which corresponds to the variety of roles they perform. HAT complexes are able to bind specific regions of DNA by recognizing DNA structure and chromatin associated proteins through HAT subunits (Kusch, 2004). This allows HATs to carry out specific functions in both transcription and DNA repair. In addition, there are several other proteins with HAT-like function. These include the circadian rhythm protein CLOCK and the CERB binding protein p300/CBP (Kimura, 1996). Despite this great diversity of functions, HAT complexes can be divided into two groups, categorized by their catalytic subunit. The two main groups are the GNATS (Gcn5-N-acetyltransferases) and the MYST ('MOZ, Ybf2/Sas3, Sas2 and Tip60) HATs (Kimura, 1996).

The role of GNAT HATs in histone acetylation was first identified in *Tetrahymena*. The SAP130 subunit of the TFTC HAT complex was shown to have significant homology to DDB1, a protein that binds DNA that has experienced UV damage (Brand 2001). It was subsequently shown that UV-damage resulted in a global increase in histone H3 acetylation in both humans

and yeast (Yu, 2005). These results taken together suggest that SAP130 recruits the TBP-free TAFII complex (TFTC) complex to UV damaged DNA.

Recent work has focused on GNAT HATs interaction with the *MFA2 promoter* in yeast. This promoter controls the production of a-protein, a signal protein used by yeast to distinguish a type from alpha type cells during mating. In response to UV damage, the yeast chromosome is globally acetylated at histone H3 and H4 except at the *MFA2* promoter where only histone H3 is acetylated (Teng, 2002). It has been shown that a deletion of *GCN5* results in a decreased efficiency of nucleotide excision repair at the *MFA2* promoter (Teng, 2002). Interestingly, this effect is not seen genome wide (Teng, 2002). Adding further complexity is the fact that Gcn5 also plays a role in activating the *MFA2* promoter. It has been suggested that the function of Gcn5 may vary depending on its associated subunits (either repair or transcriptional activator), but this has yet to be shown (Teng, 2002). Most importantly, however, Gcn5 is an example of a HAT with a role in both transcription and DNA repair in response to UV damage.

The MYST family of HATs, in contrast, has been shown to play a significant role in double-strand break (DSB) repair. In yeast, the HAT complex NuA4 has been shown to bind to DSB and to be vital for DSB repair (Ikura, 2000). NuA4 mediated DSB repair offers an elegant example of histone modifications regulating protein function. In response to damage, histone H2A is phosphorylated at Ser129, recruiting the NuA4 HAT, which then acetylates the histones containing the damaged DNA (Utlely, 2005). Conversely,

phosphorylation at Ser1 of histone H4 inhibits the binding of NuA4 and leads to deacetylation (Utley, 2005). In humans, the NuA4 orthologue TIP60 complex has likewise been shown to play an important role in DSB repair, acting through the histone variant H2Av(Kusch, 2004). H2Av is phosphorylated in response to DNA damage and then recognized and acetylated by the TIP60 complex. A separate component of the TIP60 complex then replaces the acetylated H2Av with an unmodified H2Av (Kusch, 2004). Removing the phosphorylated H2Av allows the cell to signal that DNA repair has been completed. This study will focus on a novel system for the recruitment of NuA4 to the DNA during S-Phase of the cell cycle.

Preliminary results and Swi1

The Noguchi lab has shown that Vid21 (also known as Eaf1), a regulatory subunit of the NuA4 HAT, and Swi1 (unpublished data), a subunit of the replication fork protection complex (FPC), interact in *S. pombe*. A cDNA library of *S. pombe* was screened for physical interaction with Swi1 using the two-hybrid method. For this screen, Swi1 was fused to the DNA binding domain of the Gal4 transcription factor (Gal4DBD) and expressed in an *S. cerevisiae* host strain containing the HIS3 reporter gene for two-hybrid interaction. Cells expressing Gal4DBD-Swi1 were transformed with the cDNA library fused to the activating domain of the Gal4 transcription factor (Gal4AD). Cells which express the cDNA of a protein that associated with Swi1 would be able to grow on His- media, as the interactions of the two proteins would bring both the activating and DNA

binding domains of Gal4 to the promoter of the HIS3 reporter gene to promote transcription. Vid21 proved to be one such protein (Figure 3). The association of Vid21 and the replication fork protein Swi1 suggests that histone acetylation may play a role in DNA replication.

Swi1 has been shown to maintain DNA stability and integrity during replication. Swi1 forms a dimer with Swi3 and travels with the replication fork as a component of the replisome complex during normal DNA replication (Noguchi, 2004). It has also been shown that the Swi1-Swi3 replication fork protection complex is necessary for the efficient activation of cell cycle checkpoint stabilization of the fork in the event of fork stalling (Noguchi, 2004). The association of Vid21 with Swi1 suggests that histone acetylation plays a role in maintaining stability of the replication fork during replication.

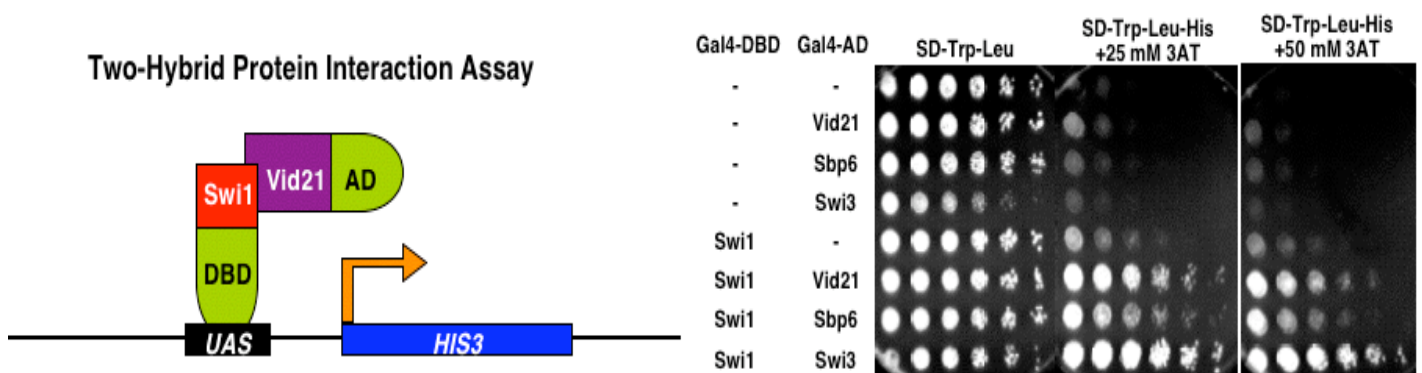


Figure 3: Swi1 and Vid21 interact physically *in vivo*. Swi1 was known to bind to both Swi3 and Sbp6. It is shown here that Vid21 binds Swi1 as well. The binding of Swi1 and Vid21 brings the activating domain and the DNA binding domain of the Gal4 transcription factor at the promoter of the HIS3 reporter gene, allowing transcription of the gene and growth of the cells on His⁻ media. Growth by cells containing Swi1 and Vid21 demonstrates Swi1 and Vid21 interact *in vivo*. Sbp6 and Swi3 are both known to bind Swi1 and served as positive controls (E. Noguchi, unpublished data).

***Schizosaccharomyces pombe* as a model organism**

The fission yeast *Schizosaccharomyces pombe* (Figure 4) was originally isolated from African millet beer. It is a unicellular eukaryote with 4979 genes and about 14Mb of DNA. The *S. pombe*

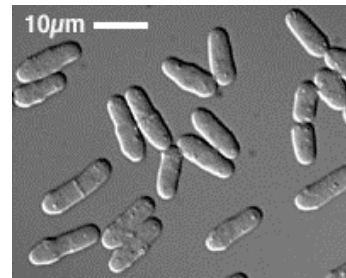


Figure 4: Asynchronous *S. Pombe* cells

genome, however, was shown to share many features with higher eukaryotes when it was first

published in 2002 (Wood, 2002). Specifically, the cell cycle and DNA repair genes of *S. pombe* have been shown to be very similar to those present in humans (Forsburg 2010). The powerful genetic approaches available in *S. pombe* have made it an excellent tool for elucidating mechanisms of various cellular processes (Ding, 2004).

In particular *S. pombe* has proven to be an outstanding model organism to study genome maintenance mechanisms. The wide variety of genetic manipulations available in *S. pombe* make it ideal for determining the functions of genes and proteins involved. The *S. pombe* genome is packaged into 3 chromosomes that exist in a haploid state making genetic manipulation and analysis significantly easier than in higher eukaryotes. Despite this fact, *S. pombe* chromosome structure is remarkably similar to higher eukaryotes (Fukuda, 2006). Indeed, there are approximately fifty *S. pombe* genes that are closely related to human genes associated with disease, half of these being cancer related (Wood, 2002). Therefore the information quickly obtained by yeast studies can be extrapolated to human systems.

Importance of the study

While the role of histone modifications in transcription has received considerable study, significantly less research has been conducted concerning the role of histone acetylation in DNA replication and repair. There is emerging evidence that these modifications play important roles in both these pathways (Ding, 2004, Li, 2008). For the

DNA to be replicated, the replication fork must pass through the nucleosome complexes formed by the histone proteins. For efficient replication, the DNA needs to be made accessible to the replication fork, then repackaged into newly synthesized histones (Lipford, 2001). Additionally, the chromatin structure must be reestablished (Verreault, 2008). Due to the ability of histone acetylation to alter chromatin structure, it seems likely that the replication fork proteins would play a role in recruiting and regulating the activity of HAT proteins.

Histone chaperones, have been shown to be capable of closely coordinating their activity with the DNA replication machinery. ASF1, a histone chaperone, has been shown to associate with MCM proteins, subunits of DNA

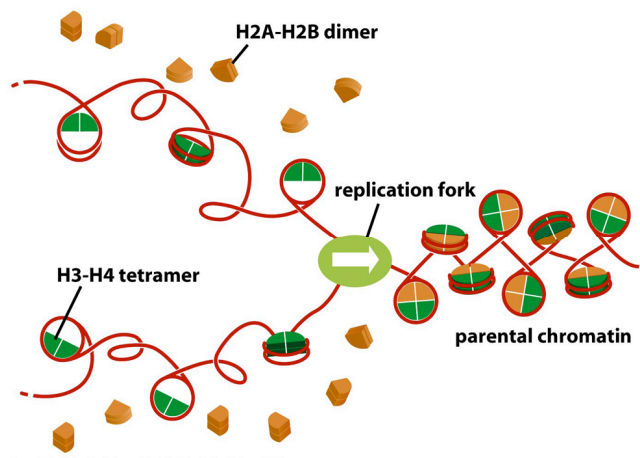


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Figure 5: Disruption of histones as replication fork moves through the DNA. After DNA replication, the DNA must be repackaged in newly synthesized histone proteins, and the histone code must be reestablished. Errors in repackaging the DNA could lead to aberrant gene expression or DNA damage. (Alberts, 2007)

helicase (Verreault, 2008). Additionally, CAF-1 has been shown to deposit histone H3-H4 behind the replication fork, through an interaction with PCNA, a protein often associated with DNA polymerases (Verreault, 2008). In this study, we show the association of Swi1 and Vid21 may be an additional pathway that is required to coordinate replication fork maintenance and histone regulation.

I describe a series of experiments aimed at understanding of the role of histone H4 acetylation in DNA replication and repair. Western blotting was used to show that levels of H4 acetylation are significantly decreased in asynchronous cells upon the depletion of Swi1 or Vid21. Likewise, the depletion of Swi1 or Vid21 lead to a significant change in the patterns of histone H4 acetylation across the cell cycle showing the link between replication fidelity proteins and histone acetylation. Finally, I show that mutants with a decreased ability to acetylate histone H4 are susceptible to DNA damage during S phase. All of these results together reveal a possible mechanism of DNA protection reliant upon HAT mediated histone acetylation during DNA replication.

MATERIALS AND METHODS

General techniques for *S. pombe*

S. pombe strains were grown in Yeast Extract + Supplements (YES) media (0.5% yeast extract, 3% glucose) (US Biological, Swampscott, MA) supplemented with the appropriate amino acids. The methods used for genetic and biochemical analyses of fission yeast have been previously described (2, Moreno, 1991). G418 sulphate, hygromycin B and nourseothricin (clonNAT) were used to select *S. pombe* transformants containing *kanMX6*, *hphMX6*, and *natMX6* genes, respectively. These drugs were used in solid YES medium in concentrations of 150 µg/ml G418, 100 µg/ml hygromycin B and 0.025 µg/ml clonNAT. For drug sensitivity assays, camptothecin, hydroxyurea and methyl methanesulfonate were used in solid YES medium at the indicated concentrations.

Primers, and *S. pombe* strains

Primers used in this study are listed in Table 1. The *S. pombe* strains used in this study were constructed using standard techniques (Alfa, 1993) and their genotypes are listed in Table 2. Histone H4 mutants (*hhf2-K8 to R* and *hhf2-K16 to R*) were generated by Kunkel site-directed mutagenesis (Kunkel, 1985) and gene replacement method as described in the next section.

Table 1. Primers used in this study

Primer	Primer No.	Sequence	Use
H4.2 K5	1029	ctttaccacctcttccaccgaccagac	Mutagenesis
H4.2 K8	1030	cctttccaatcctctaccacctttcc	Mutagenesis
H4.2 K12	1031	cttagcaccacccttccaatcctttac	Mutagenesis
H4.2 K16	1032	cttagcagtgccgcttagcaccaccctttc	Mutagenesis
H4.2 K20	1033	ggtatcacgaaggatcctacgatggc	Mutagenesis
H4.2 K5 K8 K12	1029	caccacccttccaatcctctaccaccttccacgacc	Sequence
H4.2-seq1	982	aattcaaatcataggaat	Sequence
Hhf2-5'	1008	caaggaaaaccaaccattttcgtgc	Amplify
Hhf2-3'	1009	cgcctaactacagcacttacacag	Amplify

Table 2. *S. Pombe* (Y) and *E. coli* (CJ) strains

Strain	Genotype	Source
Y1	<i>h-</i>	Laboratory stock
Y2	<i>h+</i>	Laboratory stock
Y211	<i>h- swi1::kanMX6</i>	Noguchi et al, MCB 2003
Y256	<i>h+ swi3::kanMX6</i>	Noguchi et al, MCB 2003
Y2444	<i>h+ h3.2/h4.2Δ::kanMX6 his3 D1</i>	Toru Nakamura
Y2594	<i>h- kanMX6-nmt81-3FLAG-vid21</i>	Mikael Garabedian
Y2778	<i>h- mst1-L344s-5FLAG:kanMX6 his3 D1</i>	Eishi Noguchi
Y2719	<i>h- h3.1/h4.1-K5,8,12,16R-hphMX6 his3 D1</i>	Tanu Singh
Y2721	<i>h+ h3.2/h4.2-K5,8,12,16-natMX6 his3 D1</i>	Tanu Singh
Y2800	<i>h+ h3.2/h4.2-K5,8,12,16-natMX6 rad22-YFP his3 D1</i>	Tanu Singh
Y6	<i>h- cdc25-22</i>	Laboratory stock
Y2797	<i>h+ swi1::hphMX6 cdc25-22</i>	This Study
Y2609	<i>h- kanMX6:nmt81-3FLAG-vid21 cdc25-22</i>	This Study
Y655	<i>h- cdc25-22 swi3-3FLAG:KanMX6</i>	Chiaki Noguchi
CJ239	<i>DH5α</i>	Laboratory stock

Table 3. Antibodies used in this study

Antibody	Type and Company	Catalogue Number
Anti-H4	Polyclonal-Abcam, Cambridge, MA	ab10158
Anti-Ac H4	Polyclonal-Upstate, Lake Placid, NY	06-866
Anti-Rabbit	Polyclonal-eBioscience,	18-8816

Extraction and crude purification of histones

To evaluate the level of histone acetylation present in cells, a crude extraction of histones was performed followed by Western blot analysis. *S. pombe* cells were grown overnight in 50mL of YES media to a final OD of ~1.0. The cells from 50 ml media were collected by centrifugation at 1500 G for 3 minutes (4° C) , then washed with 20 ml twice and 1 ml of water once and collected by centrifugation at 1500 G for 3 minutes (4° C). The samples were resuspended in 0.4 mL NIB buffer (0.25 M sucrose, 60 mM KCL, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 15 mM PIPES pH 6.8, 0.8% Triton X-100) supplemented with protease inhibitors {0.1mM *p*-4-amidoinophenyl-methane sulfonyl fluoride hydrochloride monohydrate (*p*-APMSF) and Complete EDTA-free protease inhibitor cocktail at manufacturer's recommended concentration.(Roche Diagnostics, Basel, Switzerland 04719999001)} and lysed with a Fastprep cell disrupter {four times, 20 seconds at 6x (Q-biogene Irvine CA FP120)}. The insoluble fraction containing histones were collected by centrifugation at 16,100 G for 10 minutes (4° C) in 1.5 ml screw top centrifuge tubes, and histones were extracted from the insoluble fraction by nutating with 500 µl of 0.5 N HCl overnight (4° C) in 1.5 ml centrifuge tube. The extract containing histone was clarified by centrifugation at 16,100 G for 15 minutes at 4° C, and histones were precipitated in the presence of tri-chloro acetic acid (TCA) before being washed sequentially in acetone (-20°C) then twice in acetone containing 0.5N HCl (-20°C), each time being collected by centrifugation at 16,100 G for 10 minutes (4° C). The histone-containing pellet was allowed to air

dry on the bench and then resuspended in 50 μ l of 8M urea. The protein concentrations were determined using Bio-Rad protein assay dye reagent concentrate (BioRad, Hercules, CA 500-0002) and then equalized with 8M urea before adding SDS-PAGE loading buffer.

Quantitative western blot analysis of histone

To allow comparison of histone acetylation between various strains, protein levels in crude histone extracts were adjusted before Western blotting. Histones were separated in 15% polyacrylamide gels using Bio-Rad Mini-PROTEAN 3 Cell system and transferred to PVDF membrane using Bio-Rad Semi-Dry protein transfer system. Histone H4 was detected with anti-histone H4 antibodies or anti-acetyl-histone H4 (K5, K8, K12, K16) antibodies. The levels of histone H4 acetylation relative to total histone H4 level were quantified using EZQuant-Gel software (EZQuant, Israel). Acetylation levels in different mutants were evaluated by setting the acetylation level from wild-type cells to 100%.

***S. pombe* synchronization**

Cells containing the *cdc25-22* mutation and the mutation of interest were grown overnight at 25°C in 500mL YES to an O.D.₆₀₀~0.4. The cells were then incubated at 35.5°C for 4 hours to arrest the cells at the G2/M transition. Cells were then released at 25°C and allowed to grow for 3 hours with a 50mL sample taken every 30 minutes. To determine cell cycle progression, 200 cells were counted under a microscope and scored as positive or negative for septum formation (Figure 6). The samples were washed with 50 ml and 1 ml of water and frozen at -80°C for storage before being thawed and the histones purified via the histone preparation method described above.

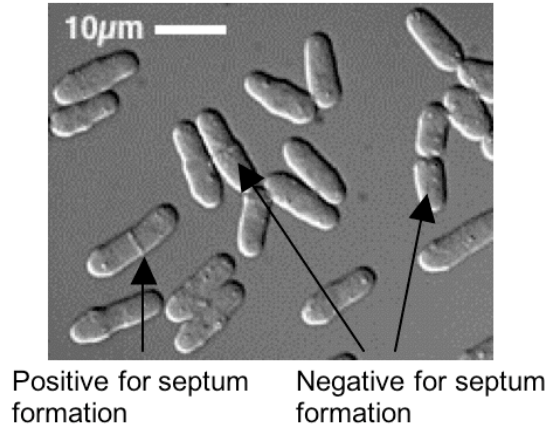


Figure 6: Positive and negative examples of septum formation. Cells were counted as positive for septum formation only if the septum was distinct and the cells had not begun to divide, as seen by “pinching” at the border between the daughter cells

Serial dilution growth assays

Serial dilution growth assays, also known as “spot assays” provide an effective way to qualitatively visualize the effects of DNA damaging agents on cell viability. Cells were grown in YES overnight to an O.D ~ 1.0. Cell concentration was determined by counting cells using a hemocytometer. Cells were then equalized to 2.0×10^7 cells per ml in YES media. 250µl of the cell suspension was added to the first well of a 96 well plate. 200µl of milliQ water

was added to the second to sixth wells of the 96-well plate. 50 µl of cell suspension was serially transferred to the next well creating a 5-fold serial dilution of cells. Using a 96-well plate, serial dilutions of 8 strains were achieved using an 8-channel pipetter. A sterilized 48-pin-replicator was used to transfer the cells from the 96-well plate to YES media, and YES media containing DNA damaging agents. The cells were allowed to absorb into the YES-agar medium and then grown at the appropriate temperatures to a level of comparable growth. Alpha Innotech software was used to document results.

Site-directed mutagenesis

The Kunkel method (Kunkel, 1985) was used to mutagenize histone H4 genes. *S. pombe* contains three genes coding for Histone H4: *hhf1*, *hhf2*, *hhf3*. Each histone H4 gene was cloned into a pBluescript-based vector containing the nourseothricin (*natMX6*), hygromycin (*hphMX6*) or kanamycin (*kanMX6*) resistant gene. These vectors were obtained from Dr. Toru Nakamura at University of Illinois Chicago. *E. coli* strain CJ236 were transformed with pBluescript-hhf-drugMX6 and then infected by R408 helper phage (Promega, Madison, WI P2291). Phage particles were then collected, and single stranded DNA (ssDNA) was extracted. Oligonucleotide primers containing the desired mutations were phosphorylated and annealed to the ssDNA. T4 DNA polymerase and T4 DNA ligase were then used to extend the primers and replicate the plasmids containing the desired mutations. The plasmids were transformed into CJ236 *E. coli* cells. The plasmids were amplified and sequenced to confirm the correct

mutagenesis. The plasmids were used to transform *S. pombe* cells containing corresponding deletion of histone H4 genes, allowing the replacement of the deleted genes with the mutated histone H4 genes. Since drug resistant marker genes in mutant plasmids were different from the marker used to delete corresponding histone H4 genes, we were able to confirm gene replacement by monitoring switches of drug marker genes. Correct gene replacement was further confirmed by sequencing histone H4 genes isolated from the resulting *S. pombe* strains.

RESULTS

Swi1 (FPC) and Vid21 (NuA4) are required for proper histone H4 acetylation

First, the impact of selected proteins on histone acetylation levels was investigated. In this study, the investigated proteins were 1) Bdf1 and Bdf2: bromodomain proteins implicated in maintaining histone acetylation 2) Vid21 and Mst1: regulatory and catalytic subunits, respectively, of the NuA4 HAT complex 3) Swi1: a component of the replication fork protection complex (FPC). Deletion strains of all these proteins were created except for Mst1 and Vid21. It was impossible to simply delete these genes, as these genes are essential for viability. Instead, a temperature sensitive *mst1* (*mst1-L344S*) mutant was created while *vid21* was put under the control of the *nmt81* promoter (*nmt81-vid21*), allowing production of Vid21 to be turned off with the application of thiamine to the media. It was hypothesized that a deletion or knockdown of any of these proteins except for Swi1 would lead to a decrease in histone H4 acetylation.

S. pombe cells, with the described mutations were grown and a crude histone extraction was performed. The histone-containing extracts were then analyzed via quantitative western blot analysis. Two samples of the same cell extract were run on a western blot. One membrane was probed with anti-acetylated histone H4 antibodies, while the other was stained with anti-histone H4 antibodies. A ratio of total histone H4: acetylated histone H4 was then obtained using the EZQuant quantifying software. A significant and reproducible reduction of histone H4 acetylation was observed in the *swi1* Δ , *bdf1* Δ and *mst1*-

L344S strains as well as *nmt-vid21* with trace thiamine in the media (Figure 7), error bars created based on three repeated experiments). The *bdf2Δ* strain demonstrated an inconsistent decrease in histone H4 acetylation, while *nmt-vid21* with excess thiamine demonstrated a drastic reduction in histone H4 acetylation that was not repeated due to time constraints (Figure 7). I hypothesize that the *nmt-vid21* mutation was partially activated with trace thiamine and only fully activated with the addition of excess thiamine. These results confirm the hypothesis that FPC, NuA4 and bromodomain proteins play an important role in promoting histone H4 acetylation

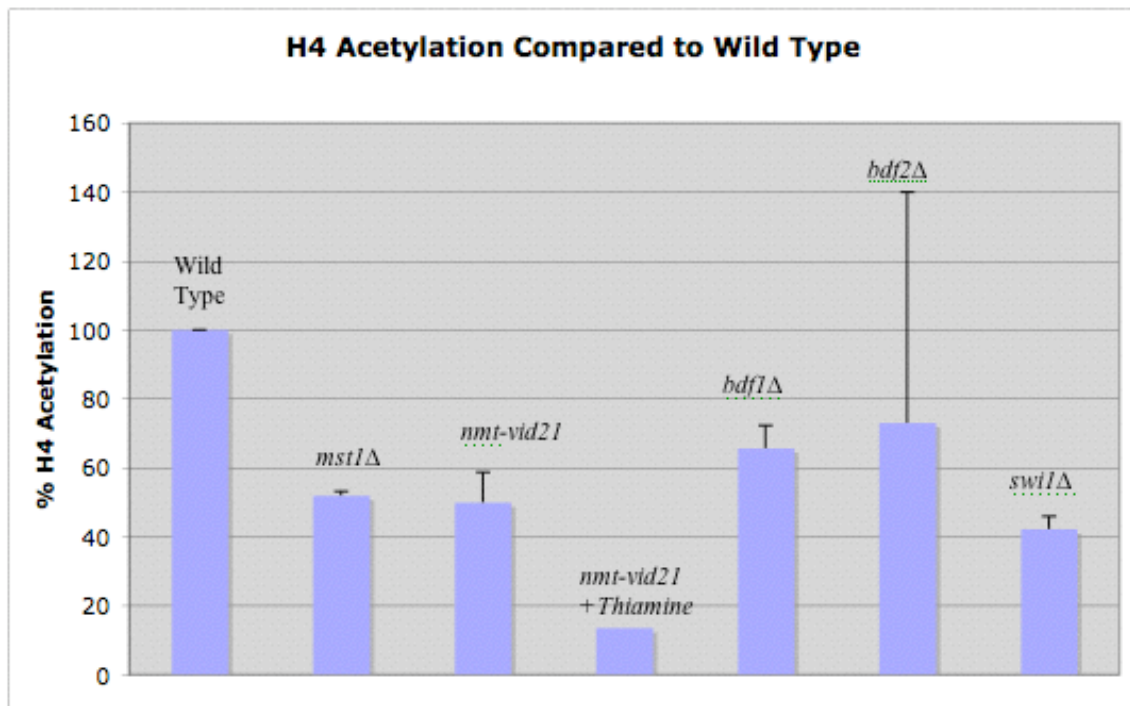


Figure 7: Effect of various mutations on histone H4 acetylation. All numbers are reported as a ratio of acetylated H4 to total H4. The percentages were then equalized by setting the wild type value to be equal to 100%. A significant and reduction of histone H4 acetylation was seen in each strain. Mst1, Vid21, Bdf1 and Bdf2 have all been previously implicated in either establishing or maintaining histone H4 acetylation. The role of Swi1 in maintaining histone acetylation suggests a connection between DNA replication and histone H4 acetylation.

Vid21 and Swi1 are required for proper timing of histone acetylation across the cell cycle

To investigate histone levels across the cell cycle, a time course experiment was performed. Strains *swi1* Δ , *swi3* Δ and *nmt81-vid21* cells were crossed with *cdc25-22* cells. The *cdc25-22* mutation is a temperature sensitive mutation causing the cells to become stalled at the G₂/M transition when grown at the restrictive temperature of 35.5°C. Cells were grown in culture, raised to the restrictive temperature for 4 hours to synchronize the cells at the G₂/M transition. The cells were then grown at the non-restrictive temperature for 3 hours with samples taken every 30 minutes and histone ratios were determined by western blot and EZQuant analysis. A septation index, or percentage of cells which possessed a septum, was likewise obtained for each time-point to monitor cell cycle progression.

cdc25-22 cells served as the wild type control. These cells demonstrated maximum levels of histone H4 acetylation during G₂/M and minimum levels during S phase (Figure 8a). *cdc25-22 nmt81-vid21* with excess thiamine *cdc25-22* Δ *swi1* cells, showed maximum levels of histone acetylation during M/G₁ and low levels of histone acetylation during S phase (Figure 8b and 8c). Interestingly, *cdc25-22* Δ *swi3* showed a phenotype similar to *cdc25-22* (Figure 8d). These results support the hypothesis that Vid21 and Swi1 play a role in regulating histone H4 acetylation.

The results of the qualitative western blot experiments are exciting, but the procedure of histone extraction needs to be improved before further work can be

accomplished. Due to an unknown technical difficulty, our histone purification method occasionally yielded insufficient levels of histone H4 for quantification. This made it extremely difficult to obtain reproducible time course results. An ideal purification method would yield comparable levels of protein each time the purification was carried out. The histone purification process was a long, multi-stepped process which took 2 days to complete. Perhaps a simpler process, with less room for variation between samples, would allow for more consistent results.

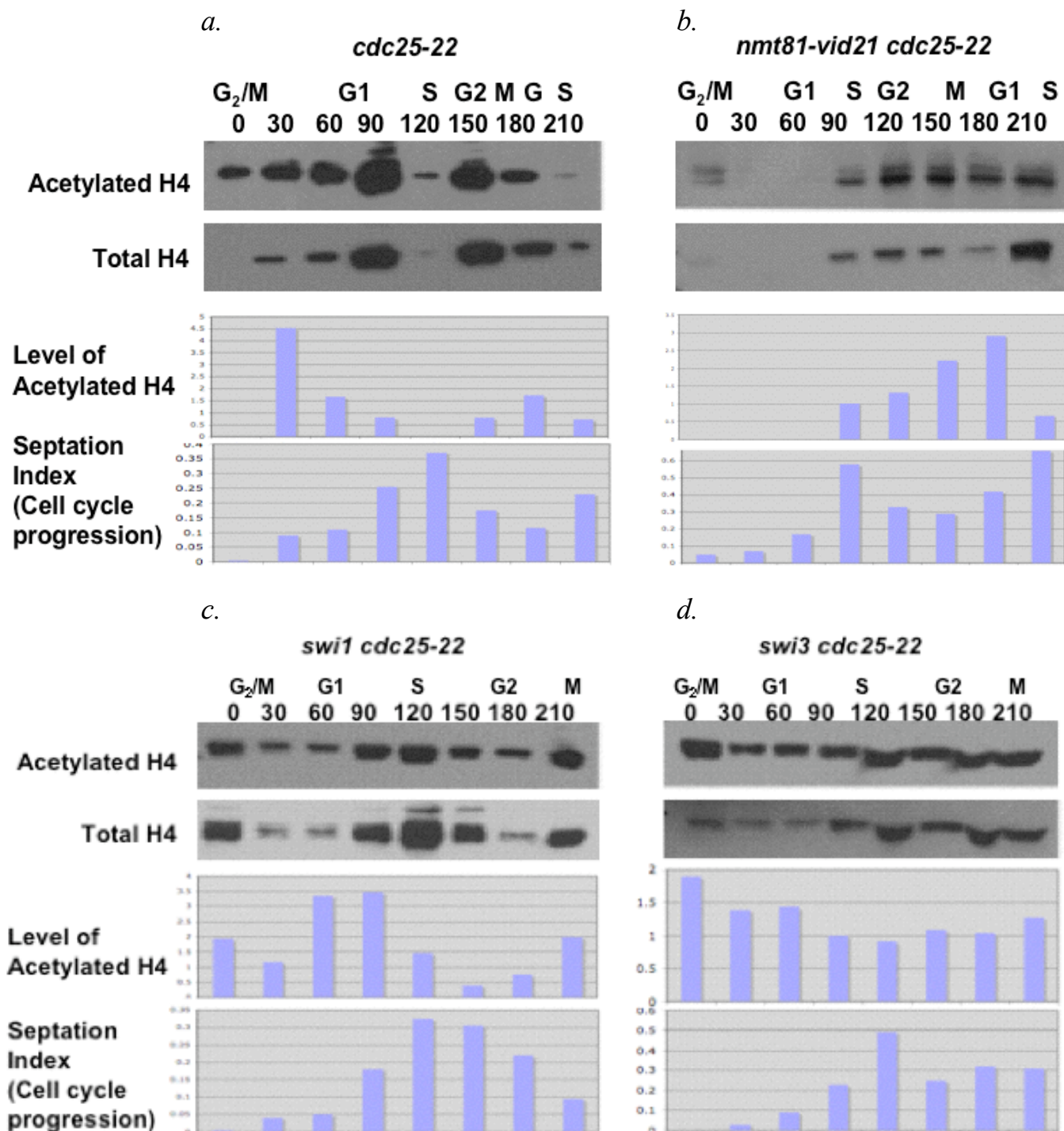


Figure 8: Swi1 and Vid21 both play a role in maintaining correct timing of histone H4 acetylation across the cell cycle. Cells were stalled at the G₂/M transition and then released. Cell cycle progression was monitored by counting 200 cells and noting the percentage of cells in S phase, demonstrated by the presence of a septum. Histone H4 acetylation levels were reported as a ratio of acetylated H4 to total histone H4. Control cells experienced the lowest level of histone H4 acetylation during S phase. *nmt81-vid21* cells showed an opposite pattern of histone acetylation with their highest levels of histone H4 acetylation during S phase. The Δ *swi1* strain showed of histone H4 an intermediate phenotype with the lowest level of H4 acetylation occurring between G₂ and S phase. Surprisingly, Swi3, which is known to form a dimer with Swi1, appears to play no role in the timing of histone H4 acetylation.

Histone H4 acetylation is involved in genomic integrity during S-phase

A serial dilution assay growth was used to determine the importance of histone H4 acetylation to genomic stability during S phase. *S. pombe* has three genes that encode histone H4, each possessing 4 lysines (at positions 5, 8, 12 and 16) that are commonly acetylated. Three strains were created where one of the three genes had all of these lysines (at positions 5, 8, 12, and 16) changed to arginines while other two histone H4 genes were wild-type (*hhf1-KR*, *hhf2-KR* and *hhf3-KR*) (Tanu Singh, unpublished results). These strains' susceptibility to DNA damaging agents was evaluated along with *nmt81-vid21* and *mst1-L344S* mutants. For this purpose, we used hydroxyurea (HU), a compound that depletes dNTP leading an arrest of the replication fork. We have also used camptothecin that induces DNA double-stranded breaks at replication forks. A five-fold serial dilution of each strain was replica plated to YES with or without genotoxic agents and grown for 2 to 5 days to compare their growth rates.

mst1-L344S and *nmt81-vid21* cells showed hypersensitivity to camptothecin (Figure 9). The *hhf2-KR* mutants also displayed significant camptothecin sensitivity while *hhf1-KR* and *hhf3-KR* mutant cells showed mild sensitivity to camptothecin (Figure 9). Since camptothecin induces double stranded DNA breaks specifically during S-phase, this supports the hypothesis that histone acetylation plays a role in genomic integrity during replication.

Interestingly, *nmt81-vid21* and histone H4 mutants showed no detectable reduction in viability compared to wild type when challenged with hydroxyurea, while *mst1-L344S* cells displayed significant sensitivity to hydroxyurea (Figure 9).

Hydroxyurea stalls replication forks during S phase by depleting the dNTP, suggesting that Mst1 may have a role in protecting stalled replication forks or activating the necessary repair pathways.

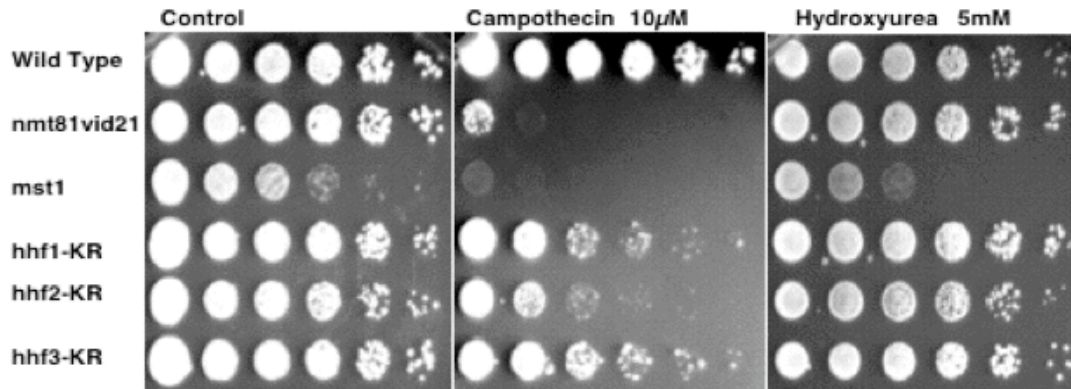


Figure 9: Proper histone H4 acetylation is necessary for DNA damage response during S phase. Camptothecin and hydroxyurea act specifically during S phase, by creating double stranded breaks and depleting the dNTP pool respectively. *nmt81-vid21* and *mst1* strains had been shown to be deficient in histone H4 acetylation. Likewise, the remaining three strains had all the lysine residues changed to arginines, leaving two unaltered H4 genes in each of these mutants. Each of these mutants showed sensitivity to camptothecin, while only *mst1* mutant cells were sensitive to hydroxyurea. These results suggest a role of histone H4 acetylation in damage response, but not to stalled replication forks during S-phase.

Creation of *hhf1*Δ *hhf3*Δ *hhf2*-KR mutants

Unlike the case in *S. cerevisiae*, a mutant with the four acetyltable lysines changed to arginines (K5/8/12/16R) in all three histone H4 genes proved to inviable (Tanu Singh and Eishi Noguchi, unpublished). We have also found that introducing K 5/8/12/16R mutations to *hhf2* in cells lacking *hhf1* and *hhf3* is lethal (Tanu Singh and Eishi Noguchi, unpublished). Therefore, we have decided to introduce single lysine mutation in the *hhf2* gene in *hhf1*Δ *hhf3*Δ background.

First, *E. coli* CJ239 cells were transformed with pBluescript-*hhf2*-natMX6 and infected by helper phage R408 to isolate a single stranded DNA version of the plasmid. Kunkel mutagenesis

A.	H4 Lysine 16 Original Sequence: GGGTGGTGCT AAG CGCCAT Lysine	Mutated Sequence: GGGTGGTGCT AGG CGCCAT Arginine
B.	H4 Lysine 20 Original Sequence: GCCATCGT AAG ATCCTTCGT Lysine	Mutated Sequence: GCCATCGT AGG ATCCTTCGT Arginine

Figure 10: Sequence confirmation of mutations. Sequencing of the mutated plasmids revealed that lysine to arginine mutations were successfully introduced into plasmids at lysines 16 and 20 (A and B respectively).

was performed on the ssDNA to introduce mutations at acetylatable lysine in *hhf2*. The intent was to create single lysine to arginine mutations at lysine 5,8,12,16 and 20 (K5R, K8R, K12R and K16R) as well as a double mutant (K5/8R) and a triple mutant (K5/8/12R). Point mutations were successfully introduced at K16 and K20 in pBluescript-*hhf2*-natMX6 (Figure 10).

Since the three histone H4 genes share significant homology at the nucleotide level, it was a possible problem that the mutated *hhf2* gene would replace the *hhf1* or *hhf3* gene through homologous recombination and not the *hhf2* gene when transformed into the wild-type cells. It is also possible cells incorporate the mutated *hhf2* gene cassettes at different genome locus when *hhf1*Δ *hhf3*Δ cells are used. To avoid this, the point mutated *hhf2* gene cassettes (for example: *hhf2*-K5R-natMX6) were transformed into a *hhf2*Δ::*kanMX6* strain (Figure 11). The *hhf2*-K5R-natMX6 (in the plasmid) and *hhf2*Δ::*kanMX6* (in the genome) cassettes share homology at their flanking regions, facilitating the correct gene replacement, which can be determined by a switch of drug resistance from *kanMX6* to *natMX6* (Figure 11). The resulting strain can then be

crossed with a *hhf1* Δ *hhf3* Δ (*hhf1* Δ ::*hphMX6* *hhf3* Δ ::*kanMX6*) strain to obtain the desired mutant. The resulting *S. pombe* strains should be examined by DNA sequencing to verify that the point mutations were maintained in the yeast.

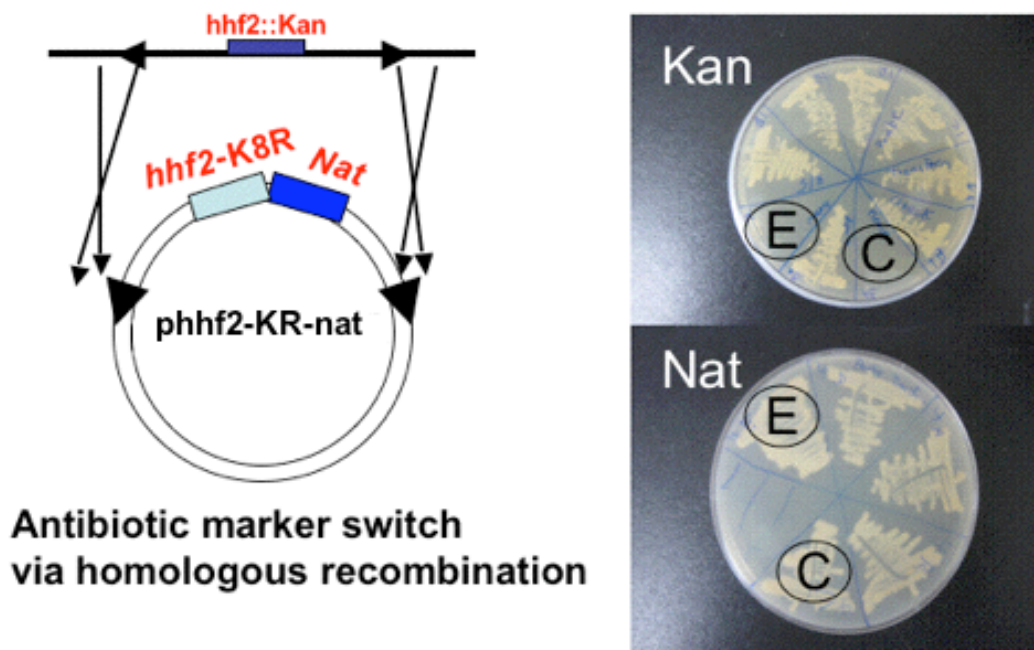


Figure 11: Marker switch in *S. pombe*, demonstrating homologous recombination. The plasmid with the altered *hhf2* gene and a nourseothricin (Nat) resistance gene was inserted into *S. pombe* cells where *hhf2f* had been deleted with a kanamycin (Kan) resistance gene. If the mutated gene was inserted correctly, the *S. pombe* cells should have lost the kanamycin resistance gene and gained a nourseothricin resistance gene, which could be visualized easily using antibiotic plates. It can be seen that strains E and C are susceptible to kanamycin and resistant to nourseothricin, demonstrating that the gene was correctly inserted into the genome.

DISCUSSION

Quantitative measurements of histone H4 acetylation levels

Histone acetylation is a vital process without which cells are unable to grow. We have found that mutating all four acetylation sites at histone H4 tail is lethal to *S. pombe* (Noguchi, unpublished). To analyze the effects of a knocking down Vid21 and Swi1, it was necessary to measure the levels of histone acetylation in our mutants. We found an acid extraction method, which uses hydrochloric acid to extract basic proteins, allowed us to obtain relatively reproducible results. Although this method was useful to purify histones from asynchronous culture, it was still difficult to reproducibly obtain histone proteins from synchronized culture. The cause of this technical difficulty is currently unknown, and we need to improve our protocols to obtain accurate results.

While the histone purification protocol proved inconsistent, an effective method of quantifying the western blot results was achieved. To measure the levels of histone acetylation, we utilized EZquant software. This software allows us to obtain numerical values of signal intensity from Western Blotting data. By normalizing histone H4 acetylation signals to total histone H4 protein levels, this software allowed us to quantitatively evaluate the level of histone H4 acetylation in our mutants. Therefore, once we have established protocols that reproducibly purify histones, we would be able to precisely evaluate the level of histone acetylation

Swi1 and Vid21 are required for proper histone acetylation

Using quantitative Western blotting protocol, we have shown that histone H4 acetylation levels are significantly reduced when Swi1 or Vid21 are depleted. We have also shown that Swi1 interacts with Vid21. A simple model to explain these observations is that Swi1 modulates histone acetylation by physically interacting with the NuA4 complex regulatory subunit Vid21 and thus recruiting the NuA4 complex. It has been shown that Swi1 forms the replication fork protection complex (FPC) and travels with the replication fork as a part of the replisome (Noguchi, 2004). As the replication fork passes through the chromosome, it is necessary for the newly synthesized DNA to be repackaged in newly synthesized nucleosomes. The NuA4 HAT may serve to reestablish the “histone code” following replication. Therefore, we speculate that Swi1 recruits the NuA4 complex to maintain the level of histone H4 acetylation on the newly synthesized DNA after the replication fork has passed by.

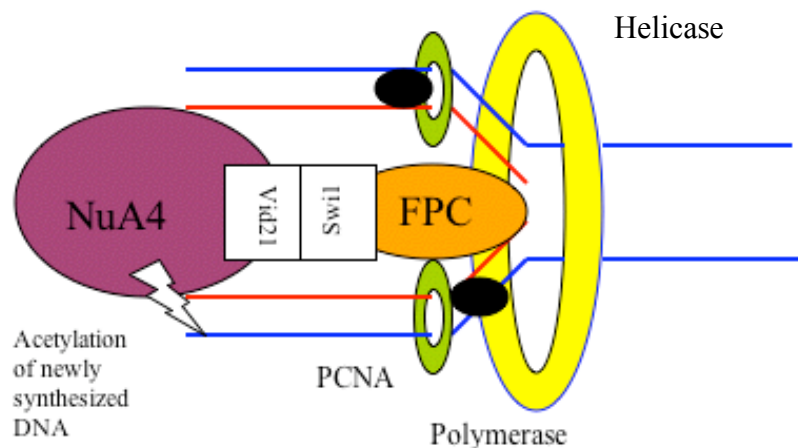


Figure 12: Model of interaction between Swi1 and Vid21 and the recruitment of the NuA4 histone acetyltransferase complex. It is thought that as the Fork protection complex moves with the replication fork, Swi1 recruits the NuA4 HAT through interaction with Vid21. NuA4 may serve to reestablish the histone code on the newly synthesized DNA, or as a mechanism to signal DNA damage.

This model seems likely but more confirmatory experiments need to be done to offer further evidence and insight into this mechanism. If this model is correct, it should be possible to co-immunoprecipitate purified NuA4 complex or purified Vid21 with purified Swi1 *in vitro*. During these experiments, it seemed as if Swi1 and NuA4 mutants had significantly lower total levels of histone proteins as well as proportionally less histone acetylation. This could be tested using the same histone preparation protocol. Total histone H4 levels could be compared to tubulin levels to determine relative levels of histone H4 protein in the mutant strains and then compared to relative levels of histone H4 in wild-type cells.

Vid21 and Swi1 may be required for proper timing of histone acetylation across the cell cycle

Although we need to repeat our experiments, our cell cycle analysis of histone H4 acetylation levels demonstrated that histone acetylation might be a dynamic process with fluctuation in level dependent on cell cycle progression. In wild-type cells, our preliminary data shows that the histone H4 acetylation level increases before S-phases, but it decreases once cells enter into S-phase. This cell cycle-dependent regulation of histone H4 acetylation seems to be deregulated in the absence of Swi1 or Vid21. A loss of proper control of histone H4 acetylation could cause aberrant reestablishment of the histone code, which in turn could lead to genetic instability and incorrect transcriptional control of genes, two processes implicated in a variety of genetic disease.

The improper regulation of histone acetylation should lead to DNA damage during S-phase. This damage could be visualized using fluorescently labeled Rad22, a protein which binds single stranded DNA at the site of damage. NuA4 mutants and Swi1 mutants with Rad22-YFP could be visualized with fluorescence microscopy. The deletion mutants should have significantly more Rad22 foci during S phase than wild type cells. Cell cycle progression could again be monitored using the septation index. The number of Rad22 foci in cells with a septum could be compared to cells without a septum.

Histone H4 acetylation is involved in genomic integrity during S-phase

In this study, we have shown significant sensitivity of NuA4 mutants (*nmt81-vid21* and *mst1-L344S*) and histone H4 mutants to camptothecin. Interestingly, these mutant, except for *mst1-L334S*, were not sensitive to hydroxyurea. Camptothecin acts by interfering with the action of topoisomerase and causing double stranded breaks at the replication fork. As expected from the role of NuA4, we have found that inactivation of Vid21 or Mst1 causes inefficient histone H4 acetylation. Intriguingly, our study has revealed that Swi1, a component of the replication fork protection complex, also serve to facilitate proper acetylation of histone H4. It seems likely that without proper acetylation, many of the repair mechanisms of damaged DNA may be rendered less effective. It has been shown that some DNA repair mechanisms are dependent upon specific histone acetylation patterns to be formed at the site of damage. Without proper acetylation, it may be more difficult for the cell to initiate an

effective damage response. It is also possible that without proper histone acetylation the DNA is inefficiently packaged and therefore more susceptible to double stranded breaks.

Hydroxyurea, in contrast, affects DNA replication by depleting the dNTP pool. Without the dNTPs necessary for replication, the replication fork can stall. Since HU is not directly causing a DNA break at the replication fork, it is possible that different DNA protection mechanisms may be applied to recover stalled replication forks. Such mechanisms include cell cycle checkpoints to arrest the cell cycle, which is known to be activated in response to HU. Importantly, Swi1 has been shown to be vital in protecting the stalled replication fork by promoting cell cycle checkpoint. Consistently, *swi1* mutants were highly sensitive to HU. However, the checkpoint function of Swi1 might not contribute to the regulation of histone H4 acetylation because histone H4 mutants were not sensitive to HU. However, it is important to note that *swi1* mutants are also highly sensitive to camptothecin, indicating that Swi1 has a checkpoint-independent function that is important to protect the replication fork when it breaks. Since histone H4 mutants were only sensitive to camptothecin, we speculate that this checkpoint-independent function of Swi1 may modulate histone H4 acetylation by recruiting NuA4 at the replication fork.

Creation of $\Delta hhf1$ - $\Delta hhf2$ -*hhf3* point mutations

S. pombe has three genes encoding histone H4. Originally, it was attempted to create mutants that could not acetylate histone H4 by changing the

commonly acetylated lysine residues (K5, K8, K12 and K16) to arginines (Tanu Singh and Eishi Noguchi unpublished). However, mutating all of these four lysines proved to be lethal to *S. pombe*. Therefore, we have decided to create mutants with two of the histone H4 genes (*hhf1* and *hhf3*) deleted and the final H4 gene (*hhf2*) mutated at only one site. This way, it would be possible to determine which of the H4 acetylation sites was most vital for genomic stability during replication.

Currently it has been shown by DNA sequencing that plasmids have been obtained with *hhf2-K12R* and *hhf2-K16* mutant alleles and have been transformed into *S. pombe*. The *hhf2-K12R* and *hhf2-K16* mutants should be crossed with an *hhf1* Δ *hhf3* Δ strain to allow for the characterization of the importance of specific lysine residues. *hhf2-K5R*, *hhf-2K8R*, *hhf2-K5/12R* should also be obtained. These strains should be challenged with camptothecin and hydroxyurea to assess their ability to recover from DNA damage during S phase. These experiments will show which histone residues play the most vital roles in maintaining DNA integrity. Although further investigation is necessary, we speculate that these acetylated residues serve either as binding sites for DNA repair proteins, or to signal the cell that DNA damage has occurred.

Works Cited

- Agalioti, T., and G. Chen. "Deciphering the transcriptional histone acetylation code for a human gene." *Cell* 111 (2002): 381-92.
- Alberts, Bruce. *Molecular Biology of the Cell*. England: Garland Science, 2007
- Alfa, C., and P. Fantes. "Experiments with fission yeast." *Cold Spring Harbor Press* (1993).
- Allfrey, V., and A. Mirsky. "Acetylation and Methylation of Histones and their Possible Role in the Regulation of RNA Synthesis." *Proceedings of the National Academy of Sciences* 51 (1964): 786- 94.
- Aoki, F., and D. Worrad. "DNA replication in the I-cell mouse embryo: stimulatory effect of histone acetylation." *Zygote* 7 (1999): 165-72.
- Bernhard, Lehnertz, Derijck Alwin, and Braunschweig Ulrich. "Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin." *Current Biology* 13.14 (2003): 1192-200.
- Brand, M. "UV-damaged DNA-binding Protein in the TFTC Complex links DNA Damage Recognition to Nucleosome Acetylation." *EMBO* 20 (2001): 3187-196.
- Chen, Y., Y. Yang, and F. Wang. "Crystal structure of human histone lysine-specific demethylase 1 (LSD1)." *Proceedings of the National Academy of Sciences of the United States of America* 103 (2006): 13956-3961.
- Clarke, D., and L. O'Neill. "Selective use of H4 acetylation sites in the yeast *Saccharomyces cerevisiae*." *Biochemical Journal* 294 (1993): 557-61.

- Ding, D., and A. Yamamoto. "Dynamics of homologous chromosome pairing during meiotic prophase in fission yeast." *Developmental Cell* 6 (2004): 329-41.
- Dion, M., and O. Rando. "Genomic characterization reveals a simple histone H4 acetylation code". *Proceedings of the National Academy of Sciences* 102 (2005): 5501-5506.
- Downs, J. "Histone H3 K56 acetylation, chromatin assembly, and the DNA damage checkpoint." *DNA Repair* 7.12 (2008): 2020-024.
- "Forsburg Lab pombe Pages: Introduction." *Forsburg Lab*. University of Southern California. Web. 25 Feb. 2010. <<http://www-rcf.usc.edu/%7eforsburg/main.html>>.
- Fukuda, H., and N. Sano. "Simple histone acetylation plays a complex role in the regulation of gene expression." *Briefings in Functional Genomics and Proteomics* 5.3 (2006): 190-208.
- Groth, A. "Replicating Chromatin: a tale of histones." *Biochem. Cell Biol.* 87 (2009): 51-63.
- Hans, F. "Histone H3 Phosphorylation and Cell Division." *Oncogene* 20 (2001): 3021-027.
- Huang, Y., and E. Greene. "Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes." *Proceedings of the National Academy of Sciences of the United States of America* 104.19 (2007): 8023-028.
- Ikura, T. "Involvement of the TOP60 Histone Acetylase Complex in DNA Repair

- and Apoptosis." *Cell* 102 (2000): 463-73.
- Imbalzano, A. H., and H. Kwon. "Facilitated binding of TATA-binding protein to nucleosomal DNA." *Nature* 370 (1994): 481-85.
- Imai, S., and C. Armstrong. "Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase." *Nature* 403 (2000): 795-800.
- Jazayeri, A., A. McAlinsh, and Stephen Jackson. "Saccharomyces cerevisiae Sin3p facilitates DNA double-strand break repair." *Proceedings of the National Academy of Sciences of the United States of America* 101.6 (2004): 1644-649.
- Jenuwein, T. "Translating the Histone Code." *Science* 293 (2001): 1074-080.
- Kimura, A., and K. Matsubara. "A Decade of Histone Acetylation: Marking Eukaryotic Chromosomes with Specific Codes." *Cell* 84 (1996): 843-51.
- Kouzarides, T. "Chromatin modifications and their function." *Cell* 128 (2007): 683-92.
- Krebs, J. "Moving marks: Dynamic histone modifications in yeast." *Molecular BioSystems* 3 (2007): 590-597
- Kunkel, T. "Rapid and efficient site-specific mutagenesis without phenotypic selection." *Proceedings of the National Academy of Sciences* 82 (1985): 488-92.
- Kusch, T. "Acetylation by Tip60 is Required for Selective Histone Variant Exchange at DNA Lesions." *Science* 306 (2004): 2084-087.
- Lee, K., and J. Workman. "Histone Acetyltransferase Complexes: One Size

- Doesn't Fit All." *Nature* 8 (2007): 284-95.
- Li, Q., and H. Zhou. "Acetylation of histone H3 lysine 56 regulates replication-coupled nucleosome assembly." *Cell* 134 (2008): 244-55.
- Lipford, J., and S. Bell. "Nucleosomes positioned by ORC facilitate the initiation of DNA replication." *Molecular Cell* 7 (2001): 21-30.
- Maile, T., and Simona Kwoczyński. "TAF1 Activates Transcription by Phosphorylation of Serine 33 in Histone H2B." *Science* 304 (2004): 1010-014.
- Mirzabekov, Andrei. "Asymmetric lateral distribution of unshielded phosphate groups in nucleosomal DNA and its role in DNA bending." *Proceedings of the National Academy of Sciences of the United States of America* 76.3 (1979): 1118-121.
- Moreno, S., and A. Klar. "Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*." *Methods in Enzymology* 194 (1991): 488-92.
- Motamedi, M., E. Hong, X. Li, S. Gerber. "HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms." *Molecular Cell* 32 (2008): 778-90
- Noguchi, E., C. Noguchi, W. McDonald, J. Yates, and P. Russell. "Swi1 and Swi3 are components of the replication fork protection complex in fission yeast." *Molecular and Cellular Biology* 24 (2004): 8342- 355.
- Piacentini, L., L. Fanti, M. Berloco, B. Perrini and S. Pimpinelli. "Heterochromatin protein 1 (HP1) is associated with induced gene expression in *Drosophila*

- euchromatin." *The Journal of Cell Biology* 161 (2003): 707-14
- Rea, S., F. Eisenhaber, D. O'Carroll, B. Strahl, Z. Sun, "Regulation of chromatin structure by site-specific histone H3 methyltransferases." *Nature* 406 (2000): 593-9
- Shukla, Vivek, Thomas Vaissiere, and Zdenko Herceg. "Histone acetylation and chromatin signature in stem cell identity and cancer." *European Journal of Cancer* 45 (2008): 442-44.
- Simpson, R. T., S. Y. Roth, and R. H. Morse. "Nucleosome positioning and transcription." *Cold Spring Harbor Symposia on Quantitative Biology* 58 (1993): 237-45.
- Smale, S. T., and A. G. Fisher. "Chromatin structure and gene regulation in the immune system." *Annual Review of Immunology* 20 (2002): 427-62.
- Teng, Y., and R. Walters. "The *Saccharomyces Cerevisiae* Histone Acetyltransferase Gcn5 has a Role in the Photoreactivation and nucleotide excision repair of UV-induced cyclobutane Pyridine Dimers in the MFA2 gene." *Journal of Molecular Biology* 316 (2002): 489-99.
- Utley, R., and N. Lacoste. "Regulation of NuA4 Histone Acetyltransferase Activity in Transcription and DNA Repair by Phosphorylation of Histone H4." *Molecular and Cellular Biology* 25 (2005): 8179-190.
- Venter, Craig, Mark D. Adams, Eugene W. Myers, Peter W. Li, Richard J. Mural, Granger G. Sutton, and Hamilton O. Smith. "The Sequence of the Human Genome." *Science* 291.5507 (2001): 1304-351.

- Verreault, A. "Clothing up DNA for all seasons: Histone chaperones and nucleosome assembly pathways." *Federation of European Biochemical Societies* 582 (2008): 1938-949.
- Wood, V., R. Gwilliam, M. Rajandream, M. Lyne, R. Lyne, and A. Stewart. "The genome sequence of *Schizosaccharomyces Pombe*." *Nature* 415 (2002): 871-80.
- You, J., V. Srinivasan, G. Denis, and W. Harrington. "Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen Interacts with Bromodomain Protein Brd4 on Host Mitotic Chromosomes." *Journal of Virology* 80.18 (2006): 8909-919.
- Yu, Y., and R. Walters. "Histone Acetylation, Chromatin Remodeling and Nucleotide Excision Repair: Hint From the Study on MFA2 in *Saccharomyces Cerevisiae*." *Cell Cycle* 4 (2005): 1043-045.

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EDUCATION

- Schreyer Honors College, Pennsylvania State University,
- Microbiology (BS), Spanish (BA), International Studies (BS)
- Minor in Biochemistry and Molecular Biology

RESEARCH AND WORK EXPERIENCE

SURF Internship at Drexel College of Medicine *Summer 2008 and 2009*

- Researched histone acetylation, elucidating pathways in genetic disease
- Honors thesis in progress
- Honorable mention at Drexel Discovery Day for poster on “The Role of H4 Acetylation in DNA Replication”

Independent Study at Pennsylvania State University *Fall 2007-Spring 2008*

- Investigated antibiotic resistance in *E. coli*
- Assisted in multiple cloning projects and detergent sensitivity assays

Cardiac Cath/EP Lab Aide at Lankenau Hospital *Summer 2004-2008*

- Oriented new aides, assisted in patient preparation for surgery
- Volunteered free time to assist inventory manager
- Developed time management and leadership skills

INTERNATIONAL EXPERIENCE

Study Abroad in Salamanca, Spain *Spring 2009*

- Expanded spanish with 19 credit hours of culture and history classes in spanish
- Integrated into culture by living with a host family as well as extensive travel

Volunteer Work with Makarios in Puerta Plata, Dominican Republic *Summer 2007*

- Independently arranged trip to do volunteer work with Makarios,
- Assisted with farm work, building a school and preparation for future service trips

Group Leader at Children’s Camp in Puebla, Mexico *Summer 2006*

- Oversaw and led a group of 6 multi-aged children for a 5 day camp
- Developed friendships with high school students at Torre Fuerte church

ACTIVITIES

- No Refund Theater: Music Director for Musical “Waiting for Guffman”
- Jazz Ensemble: Piano player for two semesters
- Navigators: Worship leader for two semesters