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Quantitative measurement of histone tail acetylation reveals stage-specific regulation and response to environmental changes during *Drosophila* development

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Abstract

Histone modification plays a major role in regulating gene transcription and ensuring the healthy development of an organism. Numerous studies have suggested that histones are dynamically modified during developmental events to control gene expression levels in a temporal and spatial manner. However, the study of histone acetylation dynamics using currently available techniques is hindered by the difficulty of simultaneously measuring acetylation of the numerous potential sites of modification present in histones. Here, we present a methodology that allows us to combine mass spectrometry-based histone analysis with *Drosophila* developmental genetics. Using this system, we characterized histone acetylation patterns during multiple developmental stages of the fly. Additionally, we utilized this analysis to characterize how treatments with pharmacological agents or environmental changes such as gamma-irradiation altered histone acetylation patterns. Strikingly, gamma-irradiation dramatically increased acetylation at H3K18, a site linked to DNA repair via non-homologous end joining. In mutant fly strains deficient in DNA repair proteins, however, this increase in H3K18 acetylation was lost. These results demonstrate the efficacy of our combined mass spectrometry system with a *Drosophila* model system, and provide interesting insight into the changes in histone acetylation during development, as well as the effects of both pharmacological and environmental agents on global histone acetylation.

Dynamic control of gene expression is critical for developmental events and the maintenance of a healthy cellular state. Histones serve as one mechanism for limiting the access of transcriptional factors to DNA, a process that can be regulated through post-translational modification, such as methylation and acetylation, of histones. Thus gene expression can be controlled, in part, by post-translational modifications of the histones [1]. The effect of histone acetylation is largely dependent on the specific residue targeted for acetylation. For example, acetylation of lysine 18 of histone H3 (H3K18) has been associated with memory formation [2], while changes in H3K9 acetylation have been linked to cancer prognosis [3].

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Although specific modifications have been linked to biological events, our understanding of the role of individual histone modifications in cellular processes has been hampered by the complexity of measuring numerous lysines and their modification state at a given point in developmental time. This complexity is due to the fact that histones are composed of two copies of each of four subunits: H2A, H2B, H3, and H4. Together, these comprise a histone octamer, with each subunit containing between 10 to 20 lysines that can be targeted for acetylation [4] and over 100 potential sites of acetylation on the complete octamer. While examining each of these modifications individually by traditional means such as immunoblotting is possible, a multi-site analysis at multiple timepoints becomes time consuming and costly. To overcome this hurdle, our lab has developed a high throughput mass spectrometry-based assay that accurately detects acetylation of multiple residues simultaneously [5, 6]. We have successfully employed this methodology for a number of *in vitro* studies into the kinetics and targeting of lysine acetyltransferases, and have continued to expand its application into cellular systems [7].

The importance of studying histone acetylation is made clear by the numerous diseases that are associated with misregulation of this process: cancer [8], heart disease [9], and developmental disorders [10], to name a few. During development, acetylation plays a role, for example, during the maturation of neurons [11], stem cell differentiation [12], and cell death [13] by modifying specific gene expression [14, 15]. Transient increases in acetylation levels of H3 and H4 have been linked to the transition from proliferating progenitor cells in the ventricular zone of the developing rat cortex to differentiating neurons, suggesting a global epigenetic switch drives cellular differentiation in this system [14]. The utilization of the *Drosophila* model system has also played an important role in understanding the function of histone acetylation, including arresting neurodegeneration [16, 17], regulating X chromosome gene transcription [18], as well as a multitude of other processes [15, 19].

Here, in order to measure developmentally and environmentally regulated changes in *Drosophila* histone acetylation, we utilized a triple quadrupole (QqQ) mass spectrometry-based protocol that we previously developed for analysis of recombinant histones and histones extracted from cultured mammalian cells [5-7]. We utilized “selected reaction monitoring” (SRM), a quantitative approach where a parent ion of interest (in this case, an acetylated peptide) is filtered in the first quadrople, then fragmented in the second quadrupole (or collision cell). The specific product ions fragmenting from either acetylated or unacetylated histone peptides are then isolated in the third quadrupole, allowing us to quantify the amount of acetylation on specific residues based on the ratio of acetylated to total peptides. Using this protocol, multiple peptides can be analyzed simultaneously, enabling us to measure acetylation of multiple lysine residues at the same time. The quantitative and multi-site nature of this approach allows us to detect levels of histone acetylation at individual target sites relative to each other and measure changes in acetylation patterns as the system is altered. This quantitative analysis and comparison of acetylation at each site, and the quantitation of each site relative to each other, provides important information often not found with other methods.

In the past, we demonstrated the sensitivity of our assay *in vitro* by characterizing the residue-specific activity of the lysine acetyltransferases Gcn5 [5], p300 and CBP [6], as well as

characterizing how treatment with the p300-inhibitor C646 affects the histone acetylation pattern of p300 [7]. *In vitro*, our assays are conducted under steady-state conditions, meaning that the total amount of histone acetylation being observed is limited to the acetylation events that occur before 10% of the histone is acetylated. This means that our assay has been designed to be sensitive enough to detect changes in acetylation as low as 0.1%. For example, we found that the homologous acetyltransferases CBP and p300 acetylate many of the same residues, but CBP has a much higher preference for H3K18 than p300, while p300 is better able to acetylate H3K9 than CBP [6]. Thus, measuring changes in histone acetylation in a temporal context *in vitro* allows us to determine the kinetics of lysine acetyltransferases, and therefore determine their site-specific preferences.

Here, we demonstrate adaptation of our mass spectrometry based approach in order to map the histone acetylation dynamics during *Drosophila* development and in response to environmental stimuli. *Drosophila* have been used for decades to study genetic diseases, oncogenic signaling pathways, development and behavior, but a detailed analysis of the histone acetylation changes that occur during specific stages of development has not been reported. We show that our technique enables the analysis of these distinct stages, while also allowing us to study the effects of environmental factors including drug treatment, nutritional supplements, and DNA damaging events on histone acetylation. Thus our results uncover new patterns of histone acetylation that are distinguishable at specific developmental stages, allow us to identify acetylation sites that are particularly affected by factors such as drug and nutrient treatment, and demonstrate the effects of genetic mutation on histone acetylation levels in response to DNA damaging events.

Experimental Procedures

Reagents

All Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher (Pittsburgh, PA) and the purity is the highest commercial grade or meets LC/MS grade. Ultrapure water was generated from a Millipore Direct-Q 5 ultrapure water system (Bedford, MA).

UPLC-MS/MS analysis

A Waters Acquity H-class UPLC (Milford, MA) coupled to a Thermo TSQ Quantum Access (Waltham, MA) triple quadrupole (QqQ) mass spectrometer was used to quantify acetylated, acid-extracted histone as previously reported: selected reaction monitoring was used to monitor the elution of the acetylated and propionylated tryptic peptides. The detailed transitions of H3 and H4 have previously been reported [5, 6].

QqQ MS data analysis

Each acetylated and/or propionylated peak was identified by retention time and specific transitions [5, 6]. The resulting peak integration was done using Xcalibur software (version 2.1, Thermo). The fraction of a specific peptide (F_p) is calculated by Eq. 1, where I_s is the intensity of a specific peptide state and I_p is the intensity of any state of that peptide, and analyzed as previously described [5, 20].

$$F_P = I_S / \left(\sum I_P \right) \quad \text{Eq.1}$$

Fly strains

The fly strain $y^1\ w^{1118}$ was used as a wild-type control (Bloomington Stock Center). The following DNA damage repair mutant stocks were also obtained from the Bloomington Stock Center; $w^{1118}; Blm^{N1}$/Tm3Sb^1, w^{1118} ; PBac{RB}WRNexo^{e04496} and, $y^1\ w^{1118}; p53^{5A\text{-}1\text{-}4}$.

Developmental stages

Fly embryos were collected at 24 hours after egg laying on grape juice plates and collected via water washing on a sieve. Larvae were collected 1 day (1st instar), 2 days (2nd instar) and 3 days (3rd instar) after egg laying. Pupae were collected at 4 days, and adult flies were fertile flies of ~1 week of age. Ovaries were obtained from these adult flies.

Drug feeding assay

TSA was obtained from Sigma-Aldrich. To make fly food containing Trichostatin A (TSA), 7.5 μl of 5 mM stock solution was diluted in 3 ml of regular fly food to obtain an 8 μM final concentration. DMSO control food was obtained by mixing 7.5 μl DMSO in 3 ml of regular fly food. Pantothenic acid (Kalvitamins.com, 10 mg tablets) was used at 1.6 mg/ml of regular fly food to obtain final concentration of 1.3 mM [21]. Curcumin (Sigma, powder), 10 mg was directly dissolved in 1 ml of regular fly food (10 mg/ml) [22]. Flies were starved in empty vials for 3 hours and then transferred to either regular fly food or drug food for 3 days before collection.

Fly radiation assay for DNA damage induction

Twenty five adult female flies with genotypes: w^{1118}, $w^{1118}; Blm^{N1}$/Tm3Sb^1, w^{1118}; PBac{RB}WRNexo^{e04496} and, $y^1\ w^{1118}; p53^{5A\text{-}1\text{-}4}$ were gamma-irradiated with 36 Gy at a dose of 1 Gy/min using a Cesium-137 panoramic gamma irradiator Shepherd Model 81-14R [23]. Following irradiation, flies were allowed to recover for 30 minutes at 25°C. Subsequently these flies were kept at − 20°C for 30 minutes, followed by histone extraction.

Extraction of salivary glands and imaginal discs

Fly stocks were raised at 25°C on standard food. Eggs were collected from adult females for 1h on fruit juice plates at 25°C. Eggs were transferred to 3 ml of fly food supplemented with DMSO to obtain the optimum concentration as mentioned above. First set of wandering 3rd instar larvae were collected based on the timing of egg laying, just before pupation. Imaginal discs and salivary glands were dissected as previously illustrated [24-26].

Extraction of histone from Drosophila and preparation for mass spectrometry (MS) analysis

Several alternative protocols are available for the extraction of histones for analysis from cultured cells. Due to dramatic differences in cell numbers, morphology, and the presence of

protective structures such as cuticles at specific developmental stages in *Drosophila*, it was necessary to adapt available protocols in order to uniformly extract histones for MS analysis. The finalized procedure is based on the acid extraction protocol published by Abcam and is outlined in Figure 1. The detailed protocol is provided below. Critical adaptations to standard protocols were developed first using adult flies.

We began by operating under the assumption that histone extraction from flies should follow the same basic procedure as cells: lyse the cells, leave the nucleus intact, and then acid extract the histone from the nucleus. A scheme of the finalized procedure can be found in Figure 1. First, five adult female flies were collected, placed in 1.6 mL microfuge tubes, and euthanized by freezing at −20 °C for 30 minutes. We have tested the efficacy of 5, 10, and 20 adult flies, and found that 5 adult flies provide a sufficient amount of histone, while samples from 20 flies can become overly viscous and difficult to work with. All of the results that follow were obtained using groups of 5 flies unless otherwise specified.

To begin, 200 uL of Triton extraction buffer, or TEB (PBS containing 0.5% Triton × 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM sodium butyrate, and 0.02% (w/v) NaN_3) was added to each tube containing collected flies. The flies were then ground up in TEB with a disposable pestle. Both manual and mechanical means proved sufficient for grinding the flies for histone extraction. The mashed flies were then rocked at 4°C for 10 minutes to lyse the cells.

The mixture was spun for 5 minutes at 6,500 × g at 4°C to spin down the nuclei as well as the fly debris. Subsequently, the supernatant was removed and discarded. Note that it is not necessary to disturb the fly debris while removing the supernatant; leaving some of the TEB at the bottom of the tube is acceptable. The remaining debris was washed with 100 uL of TEB, and then centrifuged for another 5 minutes at 6,500 × g. Again, the supernatant was removed and discard.

At this point, there will still be some TEB trapped within the remaining fly debris. To compensate for this, we utilized a slightly higher concentration of HCl for the acid extraction than might be used for normal cell extractions. 30 uL of 0.4 N HCl was added to the spun down nucleus and fly debris. Histones were then extracted overnight at 4°C, with the samples placed on a rocker. We also determined that a significant amount of histone can be extracted with only 30 minutes of incubation on the rocker. However, the results that follow are all from overnight extractions.

The samples were spun down a final time for 5 minutes at 6,500 × g at 4°C. This time the supernatant was removed and saved: the supernatant at this point contains the histone. The histone can be aliquoted and stored at −20°C at this point, or it can be used immediately. While not required, if a cleaner sample is desired, a TCA (trichloroacetic acid) precipitation of histone can be performed. For MS analysis, we found that 10 uL of the extracted histone provided sufficient sample, although the entire sample can be concentrated and utilized as well.

Processing the histone at this point is similar to previously described methods [5]. 2 uL of propionic anhydride is added to each sample, followed by 6 uL of ammonium hydroxide to

adjust to a pH of ~8. The samples were incubated at 51 °C for 1 hour. As others have reported, we have also found that two rounds of propionylation can be effectively utilized to generate propionylated product [27]; this was accomplished by propionylating samples for 20 minutes, drying samples to ~5 μL in a SpeedVac, followed by resuspension in 5 μL of 100 mM ammonium bicarbonate (pH 8), and another 20 minutes of propionylation. With either method, the histone samples were then digested by adding 1 uL of 0.1 mg/mL trypsin, 30 uL of 50 mM ammonium bicarbonate, and then 2 uL of ammonium hydroxide. The pH of each sample was tested using a pH strip to ensure a pH of ~8. Ammonium hydroxide was added as needed to reach this target. Samples were then incubated at 37 °C overnight to complete trypsin digestion, and analyzed on the mass spectrometer as previously described [5].

Data and Statistical Analysis

For each experiment, 3 groups of flies were harvested (technical replicates), with each experiment repeated multiple times. Graphs are the mean values with error bars representing the standard error of the mean. Significance values were calculated by using two-tailed T-tests. Statistical analysis was performed using Prism v5.0 b.

Results

Changes in histone acetylation patterns during development

In order to demonstrate the utility of our system within developing organisms, we first set out to detect changes in histone acetylation across several different stages of development. While our mass spectrometry based method is able to detect each residue of H3 that can be targeted for acetylation [5], for the experiments that follow, we chose to focus on acetylation within the tail region of histone H3: the histone tail is an unstructured region of the histone that is still readily accessible even after DNA is wrapped around histones to form the nucleosome. Because of their relative ease of accessibility, the tail residues are often the most dynamically acetylated residues on histone H3, and their modifications have been associated with a number of regulatory pathways [28, 29]. For this reason, we focused our investigation on histone H3 lysine 9 (H3K9) as well as H3K14, H3K18, and H3K23, each of which is found on the tail of histone H3.

In vitro experiments utilizing purified histone generally require around 250 ng of protein for accurate quantitation. In cell culture, we utilize ~0.6 $\times 10^6$ cells to produce the amount of histones necessary for an accurate quantitative analysis. To characterize dynamics changes in histone acetylation *in vivo* over time, we analyzed histone acetylation in *Drosophila* at several different developmental stages: fly ovaries, embryo, 1st , 2nd, and 3rd instar larva, pupa, and adult flies. It was necessary to adjust the amount of starting material (flies collected) based on the size of animals collected at each stage. We found that histones extracted from five flies, five 3rd instar larvae, five pupae, or five pairs of ovaries provided adequate sample for MS analysis. We found that 40 embryos, 20 1st instar, and 10 2nd instar larvae provided adequate material as well. Comparing global histone acetylation patterns at the different stages of development, we found acetylation levels on the histone H3 tails were remarkably similar. A very moderate decrease in acetylation could be observed at H3K14 from ovaries to adult, while significant differences in acetylation were observed at H3K18,

where acetylation was lower in the larva than adult, and acetylation levels were even lower in the ovaries (Figure 2). H3K23 levels were largely unchanged across each stage, although a modest decrease was seen in embryos and 1st instar larva (Figure 2B). These results demonstrate the importance of quantitative site-specific analysis, as it provides greater insight into the relative dynamics of these acetylation marks. For example, H3K23 and H3K14 display the highest level of acetylation, which is largely maintained across these developmental stages. Meanwhile, H3K18 displays a lower level of acetylation but may be more dynamically regulated throughout development. This dynamic regulation of acetylation could be indicative of the necessity of careful gene regulation during growth and development [30]. Alternatively, the observed changes might be due to acetylation changes that correlate with specific developmental events. For example, the lysine acetyltransferases p300 and CBP play important roles in neurological development [31]. Both proteins preferentially acetylate H3K18 [6]. While *Drosophila* CBP (dCBP) serves the function of both p300 and CBP in flies [32], it could be that the increased levels of H3K18 acetylation in the larvae, and further increases in the adult flies, could be related to neurological development.

Manipulating histone acetylation in Drosophila

Having characterized the histone acetylation pattern of *Drosophila* across several stages of development, we next set out to determine how effectively we could manipulate histone acetylation levels in *Drosophila*, and also confirm our ability to detect these changes with our methodology.

In order to begin our site-specific analysis, we treated adult flies with Trichostatin A (TSA). TSA is a well-documented histone deacetylase inhibitor [33, 34]. Histone deacetylases are responsible for the removal of acetyl groups from histones; by preventing this removal, TSA treatment is characterized by a general increase in histone acetylation. We therefore predicted that we would see a large increase at each site of H3 in response to TSA treatment.

Adult flies were fed TSA-containing food or food containing a DMSO control for 3 days prior to collection. Fold-change in histone H3 acetylation of TSA fed flies was calculated in comparison to the DMSO fed flies. We observed that DMSO has only a weak effect on the acetylation of H3 (Figure 3A). As expected, TSA causes an increase in acetylation on several of the H3 residues (Figure 3B). The largest fold change was at H3K9, with a ~3.9-fold increase in histone acetylation. H3K14 acetylation increased by ~ 2.3-fold, and H3K18 increased by ~1.5-fold. However, acetylation of H3K23 changed very little as a result of TSA treatment. It is these relative amounts of acetylation that truly provide a novel view of what is occurring in response to TSA treatment. Although it has been known for some time that TSA has an effect on histone acetylation in general [34], and even though studies have looked at the ability of TSA to increase acetylation at certain sites, like H3K9 [35] or H3K18 [36], our methodology allows us to quickly and efficiently quantify these changes at multiple sites, allowing us to determine not only how the drug is affecting acetylation, but how it does so site-specifically.

Additionally, observing these specific changes is important to understanding how best to utilize TSA as a treatment in a living organism. For example, decreased H3K9 acetylation has been shown to correlate with prostate cancer [37]. Because we observe that TSA has a

stronger effect on H3K9 than other sites, this might help explain why TSA is utilized in the treatment of prostate cancer [38]. Similar analysis of the efficacy of other candidate compounds, which could be utilized for targeting specific histone acetylation sites important in other diseases, will be possible with our approach.

Altering histone acetylation through nutrient supplements

Our TSA treatment results indicate that predictable changes can be measured using this method and suggest its potential utility for quantitative analysis of histone acetylation changes upon treatment with uncharacterized drugs. We tested this idea by evaluating the effects of dietary supplements, a group of understudied compounds that are widely available and often used medicinally without a clear understanding of their effects on histone acetylation patterns *in vivo*. For these experiments, we utilized either pantothenic acid or curcumin as potential treatments for altering histone acetylation. Curcumin is a potent anti-inflammatory and inhibitor of the lysine acetyltransferases p300 and CBP, as well as dCBP [39, 40]. Because of its ability to inhibit these acetyltransferases, curcumin treatment has been shown to decrease levels of histone acetylation [39]. Pantothenic acid, meanwhile, is a precursor to coenzyme A [41], which in turn is used to synthesize acetyl-CoA, the source of the acetyl group used for histone acetylation. We hypothesized that more CoA would allow for greater acetyl-CoA production, which could in turn lead to higher levels of acetylation.

For these experiments, we dissolved 30 mg (~27 mM) of purified curcumin in 3 mL of regular fly food and fed the flies for 3 days, as described above. Histone acetylation in curcumin-treated flies was compared to flies fed only regular fly food (Figure 4A). As predicted, decreased histone acetylation was observed in the curcumin fed flies. We observed a modest decrease in acetylation at H3K14 and H3K23, while acetylation at H3K9 and H3K18 was cut approximately in half. Because curcumin inhibits dCBP, this inhibition is likely responsible for the observed decrease in acetylation: this result is consistent with previous work in which we demonstrated that both CBP and p300 target H3K18, with CBP showing a strong preference for acetylation of this site [6]. Additionally, we found previously that H3K9 is a target for both p300 and, to a lesser extent, CBP, supporting a model in which curcumin-mediated inhibition of dCBP accounts for the decrease in acetylation at this site.

We next investigated the effect of pantothenic acid on histone acetylation. We began these experiments by adding 1.3 mM of pantothenic acid to fly food [21]. While we predicted that the role of pantothenic acid in CoA synthesis might have caused increased acetyl-CoA levels, and subsequent increased acetylation, we found that pantothenic acid had no significant effect on histone acetylation (Figure 4B). One possible reason for this lack of change could be due to the fact that synthesizing coenzyme A in itself is not enough to increase acetyl-CoA concentrations, nor lead to increased acetylation. In fact, recent studies suggest the existence of a feedback loop whereby acetylation is regulated by the ratio of acetyl-CoA and CoA existing in the cell [42]. It is also possible that CoA is already saturating in the fly, and the production of excess does nothing towards increasing acetyl-CoA levels themselves. While these neutral results might normally be uninteresting, the lack of change with pantothenic acid demonstrates the ability of our assay to distinguish effective

treatments from ineffective treatments, and shows the consistency of histone acetylation levels between different treatment groups.

Monitoring the histone acetylation response to gamma-irradiation

The intention of utilizing our mass spectrometry-based assay in *Drosophila* was to create a system where we can track changes in histone acetylation on multiple lysine residues, allowing us a more detailed analysis of events that alter histone acetylation patterns. Therefore, we performed experiments to determine whether we could observe changes in acetylation in response to DNA damage, and whether this response was altered in repair deficient mutants. After a damaging event, acetylation of the H3 tails provides important markers for initiating DNA damage repair [43] and signaling the presence of DNA strand breaks [44]. We therefore predicted that the histone acetylation response to DNA damage could be altered in DNA repair deficient mutants.

Because of the number of sites that have been reported to have changes in histone acetylation in response to damage, understanding how each of these sites change relative to each other by traditional means of detection can become a daunting task. On top of concerns about throughput and sensitivity, study of this topic has also suffered from issues with antibody cross-reactivity [45]. Because our mass spectrometry based system addresses these concerns, we feel it is well suited to this type of study, while the existence of DNA repair deficient mutants in *Drosophila* provides a means for investigating how specific changes in repair pathways correlate with changes in histone acetylation patterns.

For this investigation, we utilized flies bearing mutations in three known DNA repair genes, WRN, BLM or p53. BLM is a helicase involved in preventing inappropriate homologous recombination [46], WRN has helicase and exonuclease activity important to homologous recombination [46], and p53 is an important regulator of base excision repair [47]. We were interested in whether we would observe any disruption to histone signaling in response to DNA damage. Additionally, we hypothesized that there could be changes in the basal level of histone acetylation in the mutants as well: the very nature of these proteins and their association with histone acetylating proteins [48, 49] means that, even independent of a damaging event, histone acetylation levels may be different from that of wild-type flies. To test these predictions, we characterized acetylation patterns of WRN, BLM, and p53 mutant flies along with wild-type. We studied the effect of a DNA damaging event by subjecting adult flies to gamma-irradiation. After irradiation, the flies were then allowed to recover for 30 minutes at 25°C before being harvested for histone extraction. We compared acetylation patterns of the mutant flies to wild type, as well as the response of each to the irradiation (Figure 5 A & B).

In untreated flies, we saw only slight variations between wild-type flies and the mutants on H3K14, and H3K23 (Figure 5A). However, both BLM and p53 mutants exhibited significantly increased histone acetylation at H3K18. The mechanisms that mediate changes in H3K18 acetylation upon mutation of BLM and p53 are unclear. However, both BLM and p53 play a role in the resolution of stalled replication forks [50], suggesting that the observed changes in H3K18 may be linked to this molecular process, while dCBP, which targets H3K18, has also been shown to affect stalled DNA replication [51].

Gamma-irradiated wild-type flies exhibited significant differences in the acetylation response compared to mutant flies (Figure 5B). All three mutants displayed a decreased H3K18 acetylation while the WRN and p53 mutants showed decreased H3K23 acetylation. As acetylation of the H3 tails marks locations for initiating DNA damage repair [43], mutants deficient in damage repair would potentially display less change in histone acetylation in response to a damaging event. Interestingly, BLM mutants, which are known to display hyperactive non-homologous end joining (NHEJ) [52], displayed a significantly greater increase in H3K14 compared to wild type. This suggests the possibility that H3K14 acetylation may recruit proteins that act in the NHEJ-mediated mechanism of double stranded DNA repair. This mechanism contrasts with homologous recombination (HR), a mutually exclusive mechanism of DNA repair that is facilitated by WRN [46]. As H3K14 acetylation was not affected in the WRN mutants, it is possible that H3K14 acetylation may be an important mark to signal for NHEJ but dispensable for HR. It is precisely this type of investigative lead that helps to illustrate the power of coupling MS analysis with a *Drosophila* model.

Mass spectrometer analysis of imaginal discs, salivary glands, and histone H4 acetylation

One of the overarching goals of this investigation, in addition to better understanding changes in histone acetylation in *Drosophila*, was to explore the capabilities of our mass spectrometry analysis within the *Drosophila* system. For example, although the previous data have largely focused on analysis of acetylation in whole fly extracts, we have found that we are also able to quantitate histone acetylation in a tissue specific manner. This ability was touched on above when we studied ovaries during our developmental experiments. However, it is worth noting that we are able to quantitate acetylation of a number of other organs within the fly, as well. Doing so utilizes the exact same protocol (histone extraction and preparation for mass spectrometry analysis) that we used for adult flies, simply with organs that are first removed from the flies. To demonstrate this, we have characterized the acetylation pattern of histones extracted from imaginal discs and salivary glands (Figure 6 A&B). These organs in particular were chosen due to their prevalence and utility in *Drosophila* studies [53-55]. In these experiments, the data is quantitated from 10 imaginal discs or 10 salivary glands. Here we see similar patterns of H3 acetylation in the imaginal discs and the salivary glands, with moderately higher acetylation at H3K14 and H3K23 in the salivary glands (~1.3-fold and ~1.2-fold, respectively). This higher acetylation in the salivary glands could be related to endoreplication in the salivary glands [56], which requires a more consistently open conformation of the chromatin.

So far, this study has focused on the H3 tails of histones, as these are very dynamically modified regions of the histone, and are relatively easier to detect with the mass spectrometer than the H4 tails. H4 detection is less efficient due to ionization efficiency of this particular peptide: that is, any given peptide has an inherent ionization efficiency, which will determine the intensity of the peak detected via mass spectrometry. Especially when a peptide has a lower ionization efficiency, it can be harder to reliably quantitate, if the readings are below the level of sensitivity of the machine. In the past, it has been our experience that the intensity of H4 is often ~1/10 of that of H4 (data not shown). However, this discrepancy can be overcome by both increasing the starting material by ~two-fold,

TCA precipitating the sample, and concentrating the entirety of the sample before processing for mass spectrometry analysis. Prepared in this manner, we were able to accurately quantify acetylation of histone H4 in adult *Drosophila* (Figure 6C). Together, these results demonstrate that histone modifications in tissues and on H4 tails can be measured if appropriate steps are taken to accommodate the sensitivity of this approach.

Discussion

In summary, we have demonstrated the efficacy of combining a *Drosophila* model system with a mass spectrometry-based detection method in order to investigate the dynamics of histone acetylation in a living organism. While this investigation dealt exclusively with histone acetylation, in the future this methodology could easily be coupled with other assays for a full investigation into acetylation and, for example, gene expression. The site-by-site analysis provided by our assay would allow for deeper investigation into the role of individual lysine acetylation on proper gene expression, which presents a clear advantage over the analysis of overall acetylation levels in a non-site specific manner. Indeed, as we have demonstrated, not all sites respond equally to drug treatment (Figure 3 & 4), and understanding these site-specific differences could be the key to understanding the effects of altered histone acetylation on an organism. Additionally, we have demonstrated that this type of analysis can also be utilized for quantitating histone acetylation on an organ specific level, and to see differences throughout development.

Over the past two decades, great progress has been made in integrating mass spectrometry into the analysis of biological system, from biological tissue samples to work done in cellular systems [57, 58]. A push has also been made to move this type of analysis into model organisms, with important information gleaned from sources ranging from tetrahymena and yeast [59], to plants[60], and mouse [61]. Thus, building on these inroads into the use of mass spectrometry in living systems, it is our intention that utilizing our analysis in a whole fly system will provide even further insight into how altering histone acetylation in a site-specific manner affects various biological processes.

By moving our analysis into *Drosophila*, we can create a larger picture of how treatment of a full organism affects histone acetylation, and the feasibility of correcting aberrant histone acetylation in that living organism. This system allows us to create a model for human disease, whether through gene mutation, or perturbations to environmental factors, which will in turn allow us to test the efficacy of altering histone acetylation in order to correct acetylation-related defects. These initial experiments have allowed us to begin creating a toolbox of factors (drugs, nutrients) that alter histone acetylation, which may provide a potential means for treating those diseases that arise due to misregulation of histone acetylation. Therefore, the ability to alter histone acetylation, as well as characterizing such changes on a site-specific level, holds promise for disease treatment. Additionally, our study highlights interesting changes in histone acetylation in several DNA damage repair mutants in flies, as well as in their response to gamma irradiation (Figure 5). These observations set the groundwork for further investigation into the factors bringing about these changes, and further illustrates the benefits of developing and utilizing this system.

Finally, the combination of a *Drosophila* model with mass spectrometry analysis presented here provides many advantages in terms of time, effort, and materials. For those who already work with flies, the high throughput nature of the mass spectrometry assay allows for large amounts of data to be collected simultaneously, and allows for large sample sizes to be run on an automated system. The increase in efficiency cannot be overstated: although traditional methods are capable of similar detection, our method can accomplish in a 17.5 minute run what would take days to accomplish through techniques like Western blotting. Our methodology is also a quantitative approach, which we have shown allows us to distinguish even small changes in acetylation. Meanwhile, for those studying histone acetylation or utilizing mass spectrometry approaches, the *Drosophila* system is a well-characterized model system, with a number of genetic mutants available for the study of histone acetylation in DNA damage response and during development, just to name a few applications. The *Drosophila* system has a low upkeep cost compared to some of the other potential model organisms, and the number of flies required for this analysis is very low: performing an experiment in triplicate would require a total of only 15 adult flies per treatment group. All of these factors contribute towards making this combination of mass spectrometry-based analysis with a *Drosophila* model system a time and cost efficient system for the quantitative study of histone modification and the numerous biological processes in which these modifications are involved.

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Abbreviations and Textual Footnotes

acetyl-CoA	acetyl coenzyme A
CBP	CREB-binding protein
dCBP	*Drosophila* CBP
DMSO	Dimethyl Sulfoxide
TCA	trichloroacetic acid
TEB	triton extraction buffer
TSA	Trichostatin A

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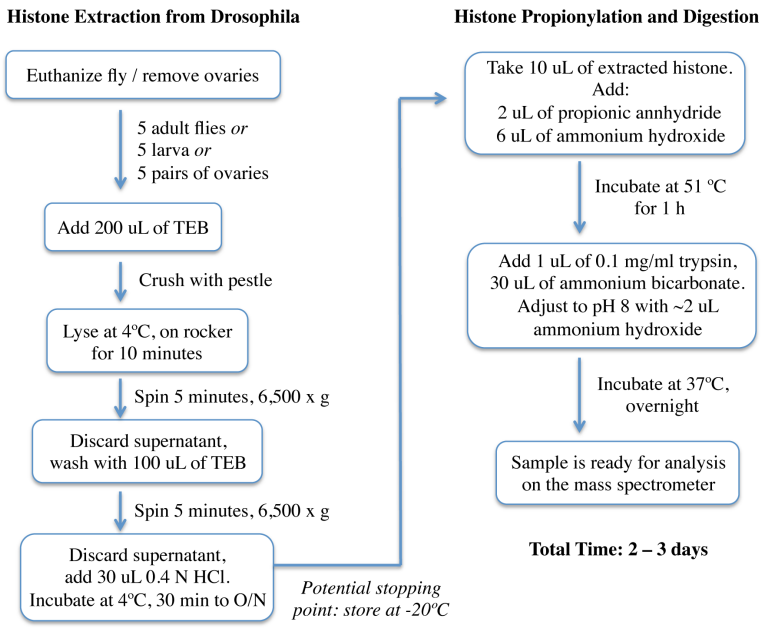

FIGURE 1.
Workflow for extracting and processing histone from *Drosophila*.

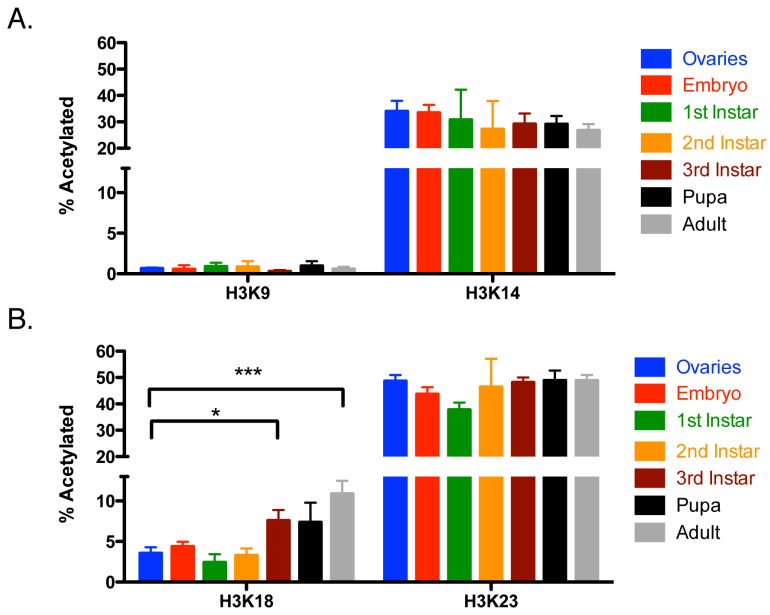

FIGURE 2. Comparison of histone H3 acetylation during *Drosophila* development

Histones extracted from either 5 pairs of ovaries (blue), 40 embryos (red), 20 first instar larvae (green), 10 second instar larvae (yellow), 5 third instar larvae (brown), 10 pupae (black), or 5 adult *Drosophila* (gray) were analyzed on the mass spectrometer. Triplicate samples were prepared for each. The percentage of acetylation of each site is calculated as the relative intensity of acetylation divided by the relative intensity of the unacetylated peaks. Lysines 9, 14, 18 and 23 on histone H3 were analyzed. * denotes $p < .05$, *** denotes $p < .001$

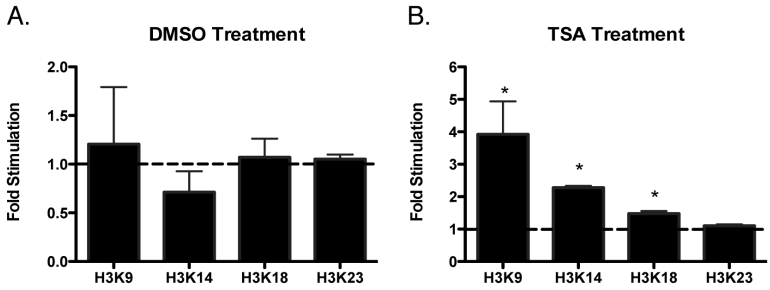

FIGURE 3. Increased histone acetylation in TSA treated *Drosophila*

Adult flies were fed in vials containing either 3 mL untreated food, food containing 7.5 uL of DMSO, or food containing 7.5 uL of TSA dissolved in DMSO (8 uM final concentration). A) Fold change in acetylation for DMSO treated flies compared to untreated. B) Fold change in acetylation of TSA treated flies compared to DMSO treated flies. * denotes $p < .05$ when comparing the level of acetylation of the treated to untreated flies, for the indicated site.

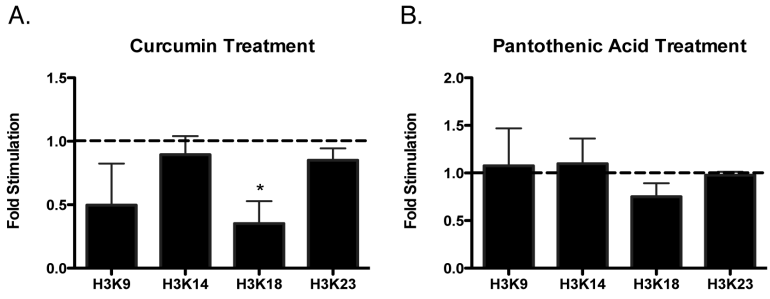

FIGURE 4. Curcumin but not pantothenic acid alters histone acetylation patterns in *Drosophila*

A) Adult flies were fed either 3 mL untreated food, or food containing 30 mg of curcumin acid. Fold change in acetylation for curcumin treated flies compared to control is shown. B) Flies were fed in vials containing either 3 mL untreated food, or food containing 1.3 mM of pantothenic acid. Fold change in acetylation for pantothenic acid treated flies compared to control is shown. * indicates $p < 0.05$ when comparing the level of acetylation of the treated to untreated flies, for the indicated site.

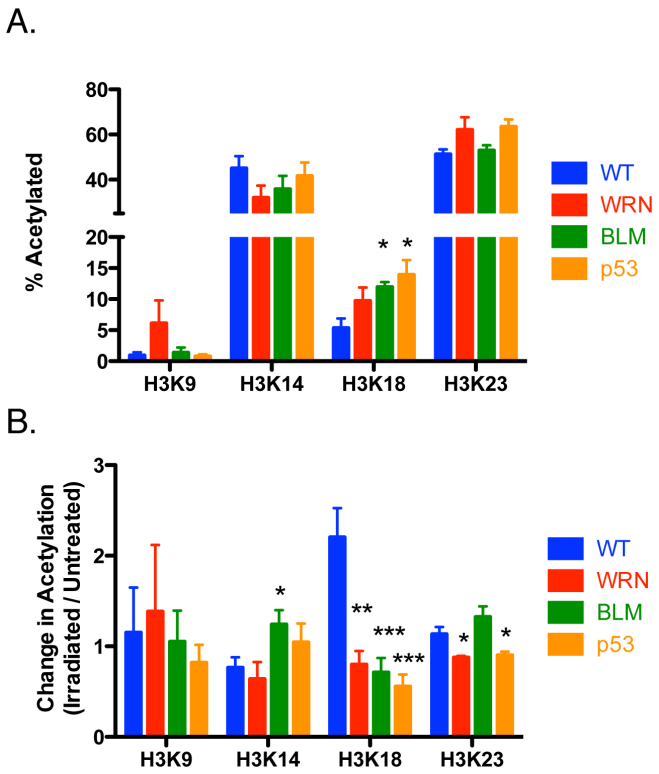

FIGURE 5. Altered histone acetylation in DNA damage repair deficient mutants in response to gamma irradiation

4 groups of adult flies (wild-type (blue), or flies deficient in WRN (red), BLM (green), or p53 (yellow) were exposed to gamma radiation as described in the Materials and Methods. A) The basal level of acetylation in the untreated flies. * denotes p<.05 for the basal acetylation of mutant compared to the wild-type at the given site. B) The change in acetylation of the treated flies compared to the untreated flies is shown. * denotes p<.05, ** p<.01, and *** p<.001 for the change in acetylation of mutant compared to the wild-type at the given site.

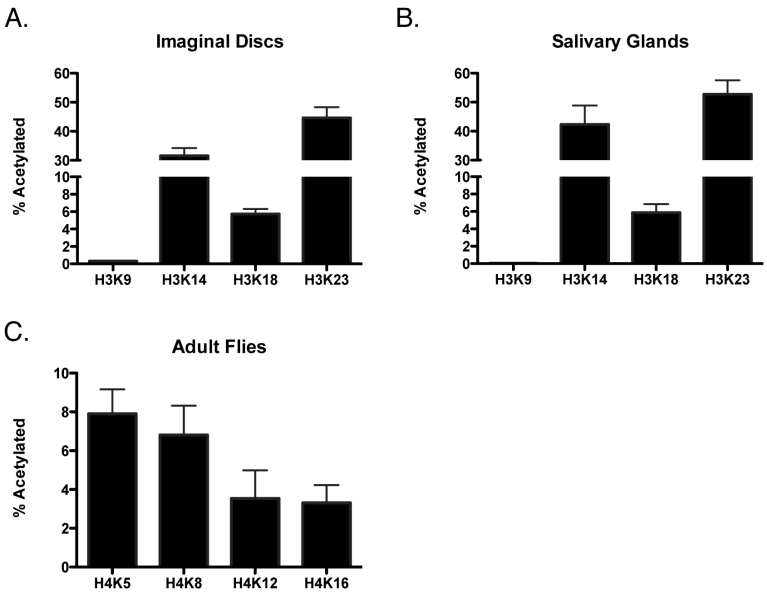

FIGURE 6. Detection of H3 acetylation in individual organs and H4 acetylation in adult flies

Sample analysis of histone H3 acetylation from A) 10 imaginal discs harvested from 3rd instar larva and B) 10 salivary glands harvested from 3rd instar larva. C) Analysis of H4 acetylation of histone extracted from 20 adult flies.