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In vivo metabolic tracing demonstrates the site-specific contribution of hepatic ethanol metabolism to histone acetylation

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Abstract

Background: Epigenetic dysregulation through ethanol-induced changes in DNA methylation and histone modifications has been implicated in several alcohol-related disorders such as alcoholic liver disease (ALD). Ethanol metabolism in the liver results in the formation of acetate, a metabolite that can be converted to acetyl-CoA, which can then be used by histone acetyltransferases to acetylate lysine residues. Ethanol metabolism in the liver can also indirectly influence lysine acetylation through NAD⁺-dependent sirtuin activity that is altered due to increases in NADH as a result of ethanol metabolism. As a proof-of-concept study to determine the direct influence of hepatic ethanol metabolism on histone acetylation changes, we used heavy-labeled ethanol (¹³C₂) and mass spectrometry to site-specifically characterize lysine acetylation on histone proteins.

Methods: Eight-week male C57BL/6J mice were gavaged using a bolus dose of either ¹³C₂-labeled ethanol (5 g/kg), or maltose dextrin. Blood and livers were collected at 0, 4, and 24 hours followed by histone protein enrichment and derivatization using acid extraction and propionylation, respectively. Metabolic tracing and relative quantitation of acetylated histone proteins were performed using a hybrid quadrupole-Orbitrap mass spectrometer. Data were analyzed using MaxQuant, Xcalibur QualBrowser and the Bioconductor package “mzR”. The contribution of ethanol to histone acetylation was quantified using the change in relative abundance of stable isotope incorporation in acetylated peptides detected by mass spectrometry.

Results: Data show significant incorporation of the ethanol-derived ¹³C₂-label into N-terminal lysine acetylation sites on histones H3 and H4 after 4 h, with rapid turnover of labeled histone acetylation sites and return to endogenous levels at 24 h post-gavage. Moreover, site-specific

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Conflict of Interest

The authors declare no conflict of interest.

selectivity was observed in regards to label incorporation into certain lysine acetylation sites as determined by tandem mass spectrometry and comparison to isotope simulations.

Conclusions: These data provide the first quantitative evidence of how hepatic ethanol metabolism directly influences histone lysine acetylation in a site-specific manner and may influence ethanol-induced gene expression through these transcriptionally activating chromatin marks.

Keywords

Metabolic Tracing; Histone Acetylation; Epigenetics; Alcohol Liver Disease; Hepatocytes

Introduction

Chronic liver disease is consistently among the top 15 leading causes of death in the United States of which approximately 50% of those deaths are due to alcoholic liver disease (ALD) (WHO, 2014). In terms of global impact on human health, ALD, as well as other alcohol-related disorders, affect millions of people worldwide. Alcohol metabolism in the liver disrupts normal cellular metabolic pathways through various mechanisms including shifts in the redox potential and increases in reactive oxygen species (ROS). Interruption of redox homeostasis can ultimately lead to disruption in pathways such as lipid peroxidation, cellular signaling, inflammation as well as transcriptional reprogramming and epigenetic changes, all of which can lead to liver injury (Choudhary et al., 2014, Patel et al., 2011, Turner, 1998, Verdin and Ott, 2015). Recently, alcohol-induced epigenetic changes thought to play a role in liver injury have become of great interest; however, little is currently known about the underlying mechanisms relating to alcohol-induced epigenetic changes and liver injury.

Alcohol consumption has been demonstrated to induce changes in protein modifications such as acetylation, methylation, phosphorylation, DNA hypomethylation as well as affect miRNA expression levels and function (Mandrekar, 2011, Lieber et al., 2008, Lee and Shukla, 2007, Choudhury and Shukla, 2008, Park et al., 2003, Shukla et al., 2008). Histone modifications, in particular, are epigenetic marks that are recognized to be important for gene regulation, depending on the modification status of the N-terminal histone tail region (Zakhari, 2013, Ito et al., 2004, Choudhury et al., 2010, Noberini et al., 2016, Mandrekar, 2011, Lu and Thompson, 2012). Histones tails regulate transcriptional processes and alteration of their post-translational modifications by alcohol may result in differential gene expression, impaired recruitment of transcriptional proteins, and subsequently influence biological processes in the cell (Patel et al., 2011, Verdin and Ott, 2015, Choudhary et al., 2014). Understanding the underlying mechanisms of how alcohol metabolism influences epigenetic alterations on histones proteins, and furthermore, how these changes affect the progression of alcoholic liver disease has become increasingly important.

Recent research has shifted to understanding how ethanol-induced changes in lysine acetylation affect epigenetic regulation through site-specific histone modifications. Histone acetylation, in particular, is regulated by the activities of various histone acetyltransferases (HATs) and histone deacetylases (HDACs), where the activities of these enzymes are altered in hepatocytes after ethanol exposure (Choudhury and Shukla, 2008, Kim et al., 2015, Park

et al., 2005, Park et al., 2012, Park et al., 2003, Shukla et al., 2015b). Further studies that aimed to determine the influence of ethanol, and ethanol metabolism, on histone acetylation showed that metabolically available acetyl-CoA from acetate is vital to the increased acetylation of histone marks associated with proinflammatory genes and subsequent proinflammatory response in ethanol-treated macrophages (Kendrick et al., 2010). Furthermore, Shukla *et.al.* (2015) reported that ethanol and acetate exhibit differential outcomes in terms of their metabolic effects on hepatic gene expression (Shukla et al., 2015b). However, the precise contribution of ethanol metabolism via acetate and subsequent acetyl-CoA production to histone acetylation is still unclear.

Over the past decade, new approaches in metabolic tracing using stable isotope labels and mass spectrometry have been developed to study changes in histone acetylation. Original work in this area demonstrated that ^{13}C -labeled glucose can be used to assess the contribution of the acetyl-CoA pool from glucose metabolism to histone acetylation utilizing quantitative mass spectrometry (Evertts et al., 2013). More recent studies show the use of ^{13}C -labeled glucose to study proteome-wide protein lysine acetylation following incorporation of the ^{13}C label into the acetyl-CoA pool (Kori et al., 2017). Here we build upon those experiments in the context of a novel stable isotope tracing approach using $^{13}\text{C}_2$ -labeled ethanol *in vivo*. Ethanol metabolism in the liver generates acetate, which contributes to the acetyl-CoA pool and, in conjunction with HAT's, can be used for histone protein acetylation. Our study aims to quantitatively determine the direct influence of ethanol metabolism on hepatic histone acetylation in an *in vivo* binge model of alcohol exposure. This study presents a novel approach for characterizing epigenetic marks directly influenced by ethanol metabolism that can be used to facilitate our understanding of molecular mechanisms underlying ALD pathogenesis and progression.

Materials and Methods:

Mice

C57BL/6J mice (Jackson Laboratory, Sacramento, California) were maintained at the University of South Florida animal facility and all procedures were performed following approval by the Institutional Animal Care and Use Committee.

In vivo acute bolus gavage by maltose-dextran or $^{13}\text{C}_2$ -labeled ethanol

Eight-week male C57BL/6J mice (n=3/group) weighing approximately 25 g, were administered an acute bolus dose of either $^{13}\text{C}_2$ -labeled ethanol (5g/kg) (Cambridge Isotope, Cat. # CLM-551-1, Tewksbury Massachusetts) or an isocaloric equivalent dose of maltose dextrin (BioServ, Cat. # 3653, Flemington, New Jersey) as previously described by Bertola *et. al.* (Bertola et al., 2013). Blood was collected and placed in a BD Microtainer (Cat. # 365967, Franklin Lakes, New Jersey) followed by liver tissue collection at 0 (baseline/no gavage), 4 and 24 hour post-gavage. Livers were snap frozen in liquid nitrogen for later processing.

Blood Alcohol Concentration

Microtainers containing whole blood were allowed to clot at room temperature for 30 minutes and then spun at $10,000 \times g$ for 10 minutes using a benchtop centrifuge. Serum was collected and stored in 1.7 μ l microcentrifuge tubes (Axygen, Cat. # MCT-175-A, Fisher Scientific, Hampton, New Hampshire). Ethanol concentration was measured in a 96-well plate, using Pointe Scientific diagnostic test (Cat. # A7504, Fisher Scientific, Hampton, New Hampshire) as per the manufacturer's instructions. Quality control was measured using Pointe Scientific standards (Cat. # A7504-STD, Fisher Scientific, Hampton, New Hampshire) and alcohol controls (Cat. #A7504-CTL, Fisher Scientific, Hampton, New Hampshire). Samples were read at 340nm using a Powerwave SX2 (BioTek, Winooski, Vermont) microplate spectrophotometer and exported to Excel for analysis. Serum collected from the first day was stored at -20°C and analyzed a second time with the 24 h collections where no change was observed between analyses.

Tissue Lysis, Histone Extraction, and Peptide Derivatization

Livers were thawed on ice and 50 mg of tissue was cut and placed into 2 ml homogenization buffer containing 10 mM sodium butyrate (Alfa Aesar, Cat. #A1107922, Fisher Scientific, Hampton, New Hampshire) and protease inhibitor cocktail (Roche, Cat. #1183617001, Sigma Aldrich, St. Louis, Missouri). Tissues were broken down into a single cell suspension using a Dounce homogenizer with pestle A only. The whole cell lysate suspensions were placed into 50 ml conical tubes (Corning, Cat. # 430291, Fisher Scientific, Hampton, New Hampshire) and spun at $200 \times g$ for 10 minutes to form a pellet. The homogenization buffer was discarded and the pellets were washed again in homogenization buffer, the spin was repeated and wash was discarded. Nuclei were isolated using a nuclear extraction buffer, which was the homogenization buffer with the addition of 0.1% NP-40 (Thermo Scientific, Cat. # 28324, Fisher Scientific, Hampton, New Hampshire). Pellets were triturated five times using a wide-bore pipet tip and placed into microcentrifuge tubes on ice. Nuclei were centrifuged for 10 sec, using the manual spin button in a benchtop microcentrifuge, and the supernatant containing the cytoplasmic fraction was then discarded. Nuclei were again washed in nuclear isolation buffer and centrifuged two times using the manual spin option. Nuclei were then washed twice in homogenization buffer to remove excess detergent before histone isolation. Histone extraction buffer was prepared by adding 0.4N HCl (Fisher Chemical, Cat # A144-500, Fisher Scientific, Hampton, New Hampshire) to the homogenization buffer. Nuclei were resuspended and lysed by sonication (30% amplitude, 5s on/off for 1 min) while on ice and the supernatant was checked for complete DNA shearing by pipetting up and down. If DNA was still present after pipetting, then sonication was repeated until complete lysis and DNA shearing was achieved. Histone extract was placed at 4°C overnight, with rocking to ensure complete protein precipitation occurred. The following day, the histone extract was centrifuged at $17,000 \times g$ for 30 min and the supernatant containing the histones was placed into a clean microcentrifuge tube. Histone protein was quantified using the BCA kit (Pierce, Cat. # 23225, Thermo Scientific, Hampton, New Hampshire) according to the manufacturer's instructions. Histones were placed into 5 μ g aliquots and dried in a SpeedVac (Thermo Fisher Scientific, Hampton, New Hampshire) prior to chemical derivatization. Histones were derivatized by propionylation as previously described, except a 3:1 ratio of ACN (Fisher Chemical, Cat. # A998SK-4, Fisher

Scientific, Hampton, New Hampshire) to propionic anhydride (Sigma Aldrich, Cat. # 240311, St. Louis Missouri) was substituted in lieu of 2-propanol (Evertts et al., 2013).

LC-MS and Data Analysis

Lypholyzed samples were resuspended with 0.1% formic acid (Fisher Chemical, Cat. # A117-50, Hampton, New Hampshire) in HPLC-grade water (Fisher Chemical, Cat. # W5-4, Hampton, New Hampshire). Samples were next analyzed by LC-MS/MS using a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Hampton, New Hampshire). Samples were separated over a 1 h gradient (2–40% acetonitrile in 0.1% formic acid) using a 50 cm × 75 µm I.D. reversed phase column packed with 3 µm C18 material with 100 Å pore size (Thermo Fisher Scientific, Hampton, New Hampshire). A precursor MS scan encompassing the range m/z 350–1550 was performed at a resolution of 70,000 followed by data-dependent MS/MS acquisition of the top 10 most abundant ions performed at a resolution of 17,500. Precursor ion selection was performed using a larger isolation window (2.5 m/z) and a stepped normalized collision energy (NCE) of 29 and 32 in order to include both light and ¹³C-labeled acetylated peptides for MS/MS fragmentation as well as to induce efficient fragmentation of N-terminally propionylated peptides, respectively. Mass spectra were then processed using the MaxQuant software package (version 1.5.0.30, <http://maxquant.org>) employing the Andromeda search algorithm and searched against the SwissProt reference database for *Mus musculus* (UniProtKB, downloaded April 2017) as well as a second database of known contaminants provided by the MaxQuant suite. Parameters include a minimum peptide length of 7 amino acids, a maximum of 2 missed cleavages and a mass tolerance of 20 ppm for the initial search. A 1.0 % false discovery rate was used for both peptides and proteins with a mass error of less than 4 ppm for the second recalibrated search. Variable modifications included in the search consisted of lysine acetylation, heavy lysine acetylation (¹³C₂), lysine and N-terminal peptide propionylation, and mono- and tri-methylation of lysine and arginine. Manual data analysis by extracted ion chromatogram (XIC) and relative abundance analysis were performed using the Qual Browser program within the Xcalibur software package (version 3.0, Thermo Fisher Scientific, Hampton, New Hampshire). Reconstructed ion chromatograms were generated using a 5 ppm cutoff and precision to the 5th decimal based on the monoisotopic m/z value for each histone H3 peptide (containing H3K9/14 and H3K18/23) identified by MS and verified by MS/MS. Full scan mass spectra were averaged (n = 5 spectra surrounding the chromatographic peak apex) and the absolute intensities of the ¹²C and ¹³C₂ peaks were determined using the “Spectrum List” function in Qual Browser. ¹³C₂/¹²C ratios for histone peptides from the ethanol and maltose dextrin groups were normalized to the baseline control (0 h/no gavage). The normalized values were represented as a fold-change over baseline. Statistical analysis was performed using a Welch’s t-test (unrounded df) in order to compare the normalized values for the H3K9/14 and H3K18/23 peptides from the ethanol treatment group to corresponding time-matched (maltose dextrin) controls. Raw files are publicly available at Chorus, project #1485, <https://chorusproject.org>.

Isotopic precursor intensity extraction using R

Precursor intensity values were extracted using the Bioconductor package “mzR” (Chambers et al., 2012) and MaxQuant search results. Raw files were converted to .mzML format using

Proteowizard (Chambers et al., 2012, Martens et al., 2011). MaxQuant “Evidence.txt” search results were used to identify modified Histone H4 peptide-spectrum-matches (PSMs) for intensity extraction. MaxQuant results were filtered for modified histone PSMs containing values for ‘Raw file’, ‘MS/MS Scan Number’, and ‘MS/MS m/z’ value. These values were compared to the scan header information for each .mzML file to obtain precursor scan numbers. Intensity values for monoisotopic m/z peaks were extracted from the corresponding precursor scan number, using m/z values within 0.01 m/z units of the ‘MS/MS m/z’ value. The maximum intensity value within the given m/z window was used for each measurement. Intensity values for M + 1, M + 2, and M + 3, and M + 4 peak were extracted using the ‘MS/MS m/z’ value plus the mass difference of ^{13}C and ^{12}C divided by the charge state ($1.003355 / z * \text{isotope peak}$). Incorporation of ^{13}C -labeled ethanol was calculated using the ratio of [M + 2] peak to [M] peak. Incorporation values were plotted using ggplot2 from the Tidyverse package (Wickham, 2009).

Results

Blood alcohol levels after acute alcohol exposure correlate with $^{13}\text{C}_2$ incorporation into hepatic histone acetylation over 24 hours.

Eight-week-old male mice were separated into control or experimental groups based on equal weight. The mice were gavaged with an acute dose of either 5 g/kg $^{13}\text{C}_2$ -labeled ethanol or an isocaloric control of maltose dextrin. Serum BAC levels averaged 305 mg/dl at 4 h post-gavage for mice administered a bolus dose of $^{13}\text{C}_2$ -heavy labeled ethanol (Fig. 1A). Following 24 h, serum BAC levels were comparable to control levels (Fig. 1A). Mass spectrometric data analysis confirmed incorporation of the ethanol-derived stable isotope label into histone acetylation through increased relative abundance of the isotope peak corresponding to $^{13}\text{C}_2$. $^{13}\text{C}_2$ -heavy label ([M+2 peak]) incorporation after 4 h was apparent when compared to the time-matched control (Fig. 1 B-C), followed by return to basal levels at 24 h (Fig. 1 D-E). In order to assess the contribution of ethanol to N-terminal histone acetylation, $^{13}\text{C}_2$ -labeled ethanol incorporation was determined using extracted ion chromatogram analysis (XIC) to identify acetylated peptides followed by isotope ratio calculation using averaged absolute intensities of the $^{13}\text{C}_2$ and ^{12}C peaks. Stable isotope incorporation at each histone H3 lysine site within the N-terminal tail region was quantified independently in regards to the individual proteoform [doubly acetylated, singly acetylated (acetyl/propionyl), and doubly propionylated] of the identified peptides. XIC analysis was performed on two consecutive histone H3 peptides, the first is H3 (K9-R17) containing lysine residues H3K9 and H3K14, and the second peptide covers H3 (K18-R26) containing H3K18 and H3K23. Furthermore, chromatographic separation facilitated $^{13}\text{C}_2/^{12}\text{C}$ ratio calculation of the acetylated positional isomers containing H3K9 and H3K14 (Fig. 1F) as well as H3K18 and H3K23 (Fig. 1G), allowing for site-specific quantitation of $^{13}\text{C}_2$ incorporation. Chromatographic separation was confirmed by MS/MS analysis as demonstrated in Fig. 1F and G where the abundant y8 was used to show site localization in the singly acetylated positional isomers containing H3K9/14 and H3K18/23, respectively.

Site-specific quantitation of lysine acetylation affected by ethanol metabolism

Histone H3K9 and H3K14 proteoforms were analyzed and graphed to show fold change associated with $^{13}\text{C}_2$ incorporation (Fig. 2A). Incorporation of the $^{13}\text{C}_2$ label derived from ethanol metabolism was determined through comparison of the $^{13}\text{C}_2/^{12}\text{C}$ ratio (normalized to the 0h baseline control) of each peptide from the ethanol-treated group to the ratio of the matching peptides from the corresponding time-matched control group (maltose dextrin gavage). Significant incorporation ($p < 0.05$, Welch's t-test) of $^{13}\text{C}_2$ was observed 4 h after ethanol gavage compared to time-matched gavage control for the doubly acetylated peptides containing H3K9/14 (Fig. 2A) and H3K18/23 (Fig 2B) as well as the singly acetylated positional isomers containing the same sites. Next, relative total acetylation change was quantified as a ratio of ratios, which is equal to the ratio of the sum of acetylated to non-acetylated peptide (considering both $^{13}\text{C}_2 + ^{12}\text{C}$ contribution) for the $^{13}\text{C}_2$ -ethanol-treated mice divided by the sum of acetylated to non-acetylated peptide (considering both $^{13}\text{C}_2 + ^{12}\text{C}$ contribution) from the control mice (Fig. 2 C-D). Total acetylation was normalized to the baseline control group. Upon comparison of the ethanol treatment group to the corresponding time-matched controls, no significant ethanol treatment-specific increase in total acetylation was observed for either the H3K9/14- or H3K18/23-containing peptides (Fig. 2 C-D). However, a significant increase in total acetylation was observed for both H3K9/14- and H3K18/23-containing peptides at 24 h compared to the baseline control group, suggesting the experimental procedure (gavage) in addition to ethanol treatment may result in increases in histone H3 acetylation at these sites.

Isotope simulation determines mixture of $^{13}\text{C}_2$ incorporation in the doubly acetylated tryptic peptides containing H3K9/K14- and H3K18/K23 after 4 h

Mass spectra obtained from the doubly acetylated H3K9/K14 and H3K18/K23 peptides were compared to isotope simulations of the naturally occurring peptide (Fig. 3 and Fig. 4, respectively). Figure 3A shows the predicted isotope simulation of the naturally occurring doubly acetylated peptide containing H3K9_(ac)/H3K14_(ac). The simulated isotope distribution was found to be comparable, with no noticeable increases in the $^{13}\text{C}_2$ peak when compared to the mass spectra obtained for the 4 h maltose dextrin control mice (Fig. 3A-B). However, the mice gavaged with heavy-labeled ethanol (Fig. 3C) show a marked increase at 4 h in the relative abundance of $^{13}\text{C}_2$ and $^{13}\text{C}_4$ peaks when compared to the control spectrum (Fig. 3B). Next, a mixture of isotopes was simulated to match the isotopic distribution of the ethanol-treated mice (Fig. 3D). The simulation predicted a mixture of isotopes and found that approximately 41% of peptides were found to incorporate $^{13}\text{C}_2$ at a single acetyl site, whereas 19% of peptides show incorporation at both sites, leaving 40% of the peptide unlabeled (Fig. 3C-D). The same isotopic distribution analysis was completed for doubly acetylated peptide containing H3K18_(ac)/K23_(ac) (Fig. 4 A-D). Results were comparable for the control and the ^{12}C simulation, where both the 4 h control mice and isotope simulation distributions closely resemble each other (Fig. 4 A-B). A mixture of label incorporation was again simulated to assess site-selectivity of $^{13}\text{C}_2$ incorporation in ethanol-treated mice (Fig. 4D). Results show the doubly acetylated peptide, H3K18_(ac)/K23_(ac), was observed to contain approximately 44% of peptides with $^{13}\text{C}_2$ incorporation only at a single acetylation site, 23% of peptides show incorporation occurring at both acetyl sites ($^{13}\text{C}_4$), and 33% of the peptides remain unlabeled when compared to the 4-h ethanol-treated mice (Fig. 4 C-D).

These data suggest that after acute ethanol exposure, label incorporation could be selective at a single acetylation site before being incorporated at both sites (Fig. 3 C-D & Fig. 4 C-D).

Stable isotope incorporation occurs for histone H4 acetylation after $^{13}\text{C}_2$ -ethanol exposure

The N-terminal region of histone H4 consists of four lysine residues, all of which can be acetylated or methylated (data not shown). Figure 5 illustrates all of the multiple combinations of lysine acetylation identified in our experimental samples by MaxQuant pertaining to the tryptic peptide containing H4K5/K8/K12/K16. Incorporation of the heavy label was observed in the ethanol-gavaged mice following 4 h at each available H4 acetylation site independently, as well as in other numerous patterns of lysine acetylation (Fig. 5). For example, we observed $^{13}\text{C}_2$ incorporation on the doubly acetylated peptide H4K12_(ac) and H4K14_(ac) as well as numerous other proteoforms of the triple and tetra-acetylated peptide (Fig. 5). Heavy-label incorporation was observed after 4 h in all identified H4-acetyl proteoforms, with the exception of the K5_(Ac),K12_(Ac),K16_(Ac) peptide, which did not show label incorporation until 24 h (Fig. 5). Quantitation of site-specific acetylation on multiply acetylated peptides is confounded by differential incorporation and turnover rates of each acetyl site, and further confounded by the isobaric and isomeric nature of these peptides. Once multiple acetylation sites have incorporated the label at different rates, combined with acetylation turnover, in addition to the number of lysine residues on a single tryptic peptide, quantitation of acetylation is difficult to determine using precursor intensity values. In cases like histone H4, where the N-terminal tail sequence is lysine-rich, tandem mass spectrometry was used to gain insight into heavy-label incorporation at specific sites (Fig. 6, top). Upon annotation, MS/MS spectra display a mass shift of +2 Da when a particular fragment ion containing an acetylated residue also contains the heavy label (Fig. 6, top). As an example, when the y7 fragment peak is magnified, the isotope distribution of the y7 monoisotopic peak shows a significant contribution of $^{13}\text{C}_2$ from ethanol as well as some contribution of $^{13}\text{C}_4$ (Fig. 6, bottom). In contrast, fragment ions (e.g., y5) that do not contain K12 but only K16 show a lower extent of modification. These data present challenges to quantifying label incorporation at one site given the number and proximity of lysine residues within the N-terminal tail region of histone H4, although MS/MS-based fragment ion quantitation could facilitate assessment of site-selective incorporation into lysine acetylation in cases where multiple lysine residues reside on a single peptide.

Discussion

Chronic ethanol exposure has been shown to induce hyperacetylation of proteins within the mitochondria and cytoplasm in the liver (Harris et al., 2015, Shepard et al., 2010, Shepard and Tuma, 2009, Fritz et al., 2013, Picklo, 2008). Furthermore, dysregulation of lysine acetylation has been found to have numerous implications in pathophysiological processes such as oncogenesis, DNA damage, and inflammation; however, its role in alcoholic liver disease is still largely unexplored (Kori et al., 2017, Kim and Yang, 2011, Simpson et al., 2009, Spange et al., 2009, Kendrick et al., 2010). Emerging evidence now demonstrates a link between alcohol use and changes to histone acetylation (Park et al., 2003, Mandrekar, 2011, Starkman et al., 2012, Shukla et al., 2015a, Choudhury and Shukla, 2008). Currently, mass spectrometry-based identification and metabolic tracing techniques of histone

modifications are becoming more prevalent in the identification of epigenetic changes induced by the external environment, (Mandrekar, 2011, Starkman et al., 2012, Nieratschker et al., 2013, Shukla et al., 2015a, Park et al., 2005, Aroor et al., 2012, Choudhury and Shukla, 2008, Lin et al., 2014, Shukla et al., 2015b, Zee et al., 2010, Kori et al., 2017, Li et al., 2013, Zee and Garcia, 2013, Everitts et al., 2013); however, little is quantitatively known regarding site-specific histone acetylation that occurs directly from ethanol and ethanol metabolites. This study is the first of its kind to show a mass spectrometry-based stable isotope tracing approach for ethanol *in vivo*. We show a correlation between BAC levels and $^{13}\text{C}_2$ -label incorporation on histone proteins over the course of 24 hours (Fig. 1A and Fig. 2 A-B) after a binge dose (5g/kg) of ethanol in mice. Lysine acetylation sites, in particular, have a relatively fast turnover, typically between 1–2 hours, which is faster than methylation; however, acetylation turnover is still slower than phosphorylation (Kori et al., 2017). Our data are consistent with the faster turnover of acetylated histones where we observe a rapid decrease in stable isotope-labeled histone acetylation upon metabolic clearance of ethanol in our mouse model of acute ethanol exposure.

Histone acetylation turnover in the liver is related to the activity of HAT and HDAC enzymes, where ethanol-induced modulation of expression and/or activity of these enzymes has been reported (Choudhury and Shukla, 2008, Kim et al., 2015, Park et al., 2005, Park et al., 2012, Park et al., 2003, Shukla et al., 2015b). Currently, there are 18 known HDAC proteins in humans, which are classified into four classes based on sequence and cofactor dependence (Shepard and Tuma, 2009). Class III HDACs (sirtuins) target a broad array of substrates including nonhistone proteins and are NAD^+ -dependent. Therefore, alteration of the NADH/NAD^+ ratio from ethanol metabolism in the liver could consequently affect their activities. Several studies have investigated the role of, for example, SIRT3 and SIRT1 in ethanol-induced liver injury based on modulating the acetylation status of several nonhistone substrates (Fritz et al., 2013) (Picklo, 2008) (You et al., 2008). Further research into the role of other HATs/HDACs (Class I, II, and IV) have become increasingly important in understanding transcriptional regulation in the progression of alcohol-induced liver injury (Kirpich et al., 2012, Choudhury and Shukla, 2008). For example, using a multi-binge mouse model of ethanol exposure, down-regulation of Hdac 1,7,9,10, and 11 mRNA levels and the upregulation of Hdac3 in conjunction with increased histone acetylation was reported (Kirpich et al., 2012). HDAC3 upregulation has been linked to the expression of carnitine palmitoyltransferase 1 α (CPT1 α), a gene important in alcohol-induced steatosis whose expression is mediated by increased H3K9 acetylation (Kirpich et al., 2013). There have also been numerous reports of alcohol, or surrogate alcohols, influencing the acetylation status of H3K9, through both alteration of HAT or HDAC activity (Choudhury and Shukla, 2008, Park et al., 2003, Park et al., 2012). These studies suggest that HAT/HDAC-mediated histone acetylation is influenced by both alcohol and alcohol metabolites, and can largely depend on the dose and time of alcohol exposure.

The new data presented here now propose that alcohol can be incorporated, site-specifically into known N-terminal histone acetylation sites that are important in chromatin remodeling and transcriptional regulation (Fig. 2 A-D). Furthermore, when two or more acetylation sites are available, incorporation of heavy-label occurs as a site-specific mixture (Fig. 3 and Fig. 4). Site-specific incorporation of ethanol through its metabolite, acetate (converted to

Acetyl-CoA), into histone protein acetylation could suggest a specific and transient transcriptional regulation response based on the immediate reaction to the environment (acute ethanol exposure). Our findings suggest that during times of alcohol-induced stress, acetylation of histones is site-specific and could regulate a selective transcriptional response related to ethanol stimulation and liver injury. Future work will investigate chronic ethanol exposure, given this particular model results in hyperacetylation, and enable us to quantify the exact contribution of ethanol metabolism on histone acetylation with focus on identifying and characterizing the functional impact of acetylation sites that are highly affected by ethanol directly.

Recent advances in mass-spectrometry-based post-translational modification identification and quantitation have enhanced the understanding of epigenetic mechanisms associated with disease progression (Britton et al., 2011, Li et al., 2013, Zee and Garcia, 2013, Everitt et al., 2013). Our study is the first to show metabolic tracing of ethanol metabolism to histone acetylation using mass spectrometry-based quantitation. Interestingly, these new data demonstrate that incorporation and site-specificity of acetylation are time-dependent where significant labeling of histone acetylation occurred at 4 hours and returned to baseline levels at 24 hours. The degree of total acetylation does not seem to change significantly and highlights the advantage of our method to determine specific sub-populations of acetylated histones, generated by either the endogenous or ethanol-derived acetyl-CoA pools. The limitation of our approach, however, is that only global histone acetylation changes can be quantified, which restricts insight on regional influences at the gene and/or promoter level.

We also found that manual data analysis was limited when analyzing peptides such as H4 K5-R17, which contain multiple acetylation sites and sequences rich in lysine residues (Figs. 4 and 5). The need for the development of computational approaches that use chromatography, MS and MS/MS information is apparent. Our automated approach utilizes output from database search algorithms such as MaxQuant (used in this study), where intensities of the various proteoforms can be used to compare the modified and unmodified forms (Fig. 5) (Yuan et al., 2014). As with any computational approach, data must be validated and confirmed through manual raw data evaluation (Fig. 6) (Yuan et al., 2014). Furthermore, histone analysis by mass spectrometry is often complicated by co-elution of species related to other histone proteoforms (Zee and Garcia, 2013, Zee et al., 2010). Co-elution of simultaneous proteoforms or partially labeled peptides makes quantitation accuracy difficult when studying overall changes in alcohol-induced lysine acetylation. However, this proof-of-concept study clearly demonstrates that ethanol-derived metabolites directly influence histone acetylation. Future work will focus on delineating the impact of ethanol in both acute and chronic ethanol exposure models on histone acetylation in the liver using a larger sample size and addressing sex as a biological variable. Moreover, additional insight is needed into how other tissues may be affected based on acetate redistribution and utilization in vivo. Further research using stable isotope labeling techniques and high-resolution mass spectrometry will facilitate identification of novel targets and pathways directly affected by alcohol consumption and could provide new insights into the pathogenesis of alcoholic liver disease and other alcohol-related disorders.

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Fig. 1.

Site-specific incorporation of heavy-labeled ethanol into histone acetylation and correlation to BAC levels. (A) Blood was collected from 8-week-old mice (n=3) for BAC analysis at 0 (baseline/no gavage), 4, and 24 h. Error bars represent SD with triplicate replicates. (B-D) Representative mass spectra of the acetylated peptide containing H3K9 and H3K14 were used to metabolically trace ¹³C₂-heavy label incorporation based on changes in the [M+2] peak intensity over the course of 24 h following ¹³C₂ ethanol or maltose dextrin administration. Endogenous ¹²C is represented by the monoisotopic peak ([M+2H]²⁺) and

combination of endogenous $^{13}\text{C}_2$ and $^{13}\text{C}_2$ from label incorporation is depicted by the [M +2] peak (dashed ovals). Chromatographic separation of the singly acetylated positional peptide isomers representing (F) Histone 3 (K9-R17) and (G) Histone 3 (K18-R26) as well as the corresponding MS/MS spectra used for sequence confirmation and acetylation site localization.

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Fig. 2. Quantitation of $^{13}\text{C}_2$ label incorporation and overall changes in acetylation. (A-B) Fold change in $^{13}\text{C}_2$ incorporation was calculated using the $^{13}\text{C}_2/^{12}\text{C}$ ratio for peptides containing H3K9/K14 (A) and H3K18/K23 (B) and normalizing to the baseline control group. (A) Fold change of $^{13}\text{C}_2$ incorporation for the peptide containing H3K9/K14 at 4 and 24 hours for the doubly acetylated, either H3K9 or K14 single acetylation and the non-acetylated forms. (B) Fold change of $^{13}\text{C}_2$ incorporation for the peptide containing H3K18/K23 was calculated as stated above. Ratios were graphed for the doubly acetylated, singly acetylated, and non-

acetylated peptides containing H3K18/K23. (C) Overall acetylation was calculated for the peptide corresponding to H3K9/K14 and (D) H3K18/K23. Significance was determined using a Welch's t-test (unrounded df) upon comparison of the ethanol-treatment time points to the corresponding time-matched (maltose dextrin) controls and denoted by * $p < 0.05$ or ** $p < 0.01$. Error bars represent +SD.

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Fig. 3.

Mass spectra of the H3 doubly acetylated peptide containing H3K9/K14 show site-specific and time-dependent label incorporation from ¹³C₂-ethanol. Increases in heavy-label incorporation can be determined by peak comparison of the ¹³C₂ [M+2], and ¹³C₄ [M+4] peaks (dashed ovals). (A) Isotope simulation showing the expected isotopic distribution of the doubly acetylated peptide corresponding to H3K9/K14 (m/z 521.2880) under normal conditions. (B) Mass spectrum obtained for the 4 h (maltose dextrin) control time point demonstrated no label incorporation. (C) Mass spectrum showing isotopic distribution after

4 h following $^{13}\text{C}_2$ -labeled ethanol gavage in 8-week male mice with clear incorporation at the $^{13}\text{C}_2$ [M+2], and $^{13}\text{C}_4$ [M+4] peaks. (D) Mass spectrum showing simulated isotopic distribution corresponding to a mixture of stable isotope labeled mixture of acetylated peptides that match the experimental mass spectrum in (C). The simulation shown represents a mixture of 40:41:19% (unlabeled, one acetylation site labeled, and both acetylation sites labeled).

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Fig. 4. Mass spectra of the H3 doubly acetylated peptide containing H3K18/K23 show site-specific and time-dependent label incorporation from $^{13}C_2$ -ethanol. Increases in heavy-label incorporation can be determined by peak comparison of the $^{13}C_2$ [M+2], and $^{13}C_4$ [M+4] peaks (dashed ovals). (A) Isotope simulation showing the expected isotopic distribution of the doubly acetylated peptide corresponding to H3K9/K14 (m/z 563.8326) under normal conditions. (B) Mass spectrum obtained for the 4 h (maltose dextrin) control time point demonstrating no label incorporation. (C) Mass spectrum showing isotopic distribution after

4 hours following $^{13}\text{C}_2$ -labeled ethanol gavage with clear incorporation at the $^{13}\text{C}_2$ [M+2], and $^{13}\text{C}_4$ [M+4] peaks. (D) Mass spectrum showing simulated isotopic distribution corresponding to a mixture of stable isotope labeled mixture of acetylated peptides that match the experimental mass spectrum in (C). The simulation shown represents a mixture of 33:44:23% (unlabeled, one acetylation site labeled, and both acetylation sites labeled).

H4: Propionyl-GK(5)GGK(8)GLGK(12)GGAK(16)R

Fig. 5.

The Bioconductor package “mzR” facilitates quantitation of $^{13}\text{C}_2$ incorporation in the multiple acetylation sites of Histone H4 (G4-R17). Precursor intensity values for $^{13}\text{C}_2$ and ^{12}C isotopic peaks were extracted using the Bioconductor package “mzR”. Plot values represent the ratio of $^{13}\text{C}_2/^{12}\text{C}$ peak intensities for each identified Histone H4 proteoform after $^{13}\text{C}_2$ -labeled ethanol gavage or maltose dextrin gavage at 4 and 24 h. The points represent average incorporation for each mouse, and line ranges represent 0.5 standard deviations above and below the average.

Fig. 6. Analysis of MS/MS spectrum shows site-selective $^{13}\text{C}_2$ -label incorporation into the acetylation sites on Histone H4 (G4-R17). (Top) MS/MS spectrum showing fragment ion annotation of the H4 tryptic peptide, in which all four lysine residues (5, 8, 12, and 14) are acetylated. Annotation was performed using MS-Product (UCSF, Protein Prospector v. 5.20.0, <http://prospector.ucsf.edu>). A mass shift of +2 Da on either the b- or y-ion series demonstrates incorporation of the $^{13}\text{C}_2$ on the respective lysine residue(s). (Bottom)

Expanded m/z region showing the y7 monoisotopic peak (m/z= 757.4315) as well as $^{13}\text{C}_2$ incorporation as shown by increase in the [M+2] peak.

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