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Characterization of novel mechanisms for steatosis from global protein hyperacetylation in ethanol-induced mouse hepatocytes

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ABSTRACT

Steatosis is the earliest and most common disease of the liver due to chronic ethanol consumption, and stems from alterations in the function of transcription factors related to lipid metabolism. Protein acetylation at the lysine residue (Kac) is known to have diverse functions in cell metabolism. Recent studies showed that ethanol exposure induces global protein hyperacetylation by reducing the deacetylase activities of SIRT1 and SIRT3. Although global acetylome analyses have revealed the involvement of a variety of lysine acetylation sites, the exact sites directly regulated by ethanol exposure are unknown. In this study, to elucidate the exact hyperacetylation sites that contribute to SIRT1 and SIRT3 down-regulation, we identified and quantified a total of 1285 Kac sites and 686 Kac proteins in AML-12 cells after ethanol treatment (100 mM) for 3 days. All quantified Kac sites were divided into four quantiles: Q1 (0–15%), Q2 (15–50%), Q3 (50–85%), and Q4 (85–100%). Q4 had 192 Kac sites indicating ethanol-induced hyperacetylation. Using the Motif-x program, the [LXKL], [KH], and [KW] motifs were included in the Q4 category, where [KW] was a specific residue for SIRT3. We also performed gene ontology term and KEGG pathway enrichment analyses. Hyperacetylation sites were significantly enriched in biosynthetic processes and ATPase activities within the biological process and molecular function categories, respectively. In conclusion, ethanol regulates the acetylation of proteins in a variety of metabolic pathways mediated by SIRT1 and SIRT3. As a result, ethanol stimulates increased de novo fatty acid synthesis in hepatocytes.

1. Introduction

Lysine acetylation (Kac) is a dynamic, reversible, and evolutionarily conserved protein post-translational modification (PTM) process. Kac is known to have diverse functions in biological systems, such as the regulation of metabolic pathways, cellular differentiation, cell survival, and cell signaling [6,31]. For the past two decades, non-histone acetylation targets have been discovered beyond histones targets, for study of the enzymatic regulation of acetylation and deacetylation. Kac is catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), and the equilibrium between protein acetylation and deacetylation controls diverse physiological and pathological cellular processes [18]. Kac is modified by HATs that use acetyl-CoA to transfer an acetyl group to the substrate. There are two classified groups (HAT A and HAT B), depending on the mechanism of catalysis and cellular localization [19]. The HAT A family can be further divided into other subclasses, including CBP, p300, PCAF, and TAF1, and are found in the nucleus. In the cytoplasm, the HAT B family of enzymes transfer the acetyl group to an ε–NH_2–lysine [18]. Lysine acetylation has an important direct regulatory potential in the control of protein stability, since a lysine blocking effect affects lysine ubiquitination negatively [4]. In humans, there are 18 potential deacetylase enzymes in four classes, which are responsible for the removal of acetyl groups [18]. Class III is NAD^+-dependent deacetylase, comprising seven silent mating-type information regulation (SIRT) isoforms; namely, SIRT1 to SIRT7 [18]. SIRT1 mainly plays a key role in the regulation of lipid and glucose homeostasis, which is located in cytoplasm [18]. SIRT3–5 are located in the mitochondria and play crucial roles in oxidative metabolism, apoptosis, and intracellular signaling [27].

Ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH) in the hepatic cytosol, and acetaldehyde is further

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metabolized to acetate by acetaldehyde dehydrogenase (ALDH) in mitochondria. The major site of ethanol metabolism is the liver, which means that this organ is highly susceptible to alcohol-induced injury such as fatty liver. Steatosis (the fatty liver) is the earliest and most common disease of the liver responding to chronic ethanol consumption [29]. The mechanism by which this occurs is that ethanol exposure alters the function of regulatory factors of lipid metabolism (viz., peroxisome proliferator-activated receptor alpha (PPAR-α) and steroid response element-binding protein-1 (SREBP-1)) as well as the action of adenosine monophosphate (AMP)-dependent protein kinase (AMPK) [2,30]. Furthermore, chronic ethanol consumption induces global protein hyperacetylation [8,14,21,22].

While studies have focused on the protein hyperacetylation effect caused by ethanol exposure, the identity of the global acetylome affected by ethanol exposure is still not clear. In the present study, we aimed to clarify the target proteins changed by ethanol and to identify the global acetylome. To investigate the role of acetylation using ethanol-exposed hepatocytes progressing to fatty liver, we identified the exact hyperacetylation sites to confirm the dynamic acetylome in the mouse hepatocytes (AML-12). By applying the SILAC system and immunoprecipitation using anti-acetyl lysine antibodies, we were able to analyze the enriched acetylated-peptides by LC-MS/MS [15]. A total of 1515 Kac sites in 790 Kac proteins were identified, and 1285 Kac sites and 686 Kac proteins were quantified. Category Q4, indicating ethanol-induced hyperacetylation, had 192 Kac sites, and we identified the exact sites and characterized the dynamic acetylome.

2. Materials and methods

2.1. Cells, cell culture, and labeling

AML-12 cells were maintained and labeled using SILAC kits, with L-$^{13}C_6$-lysine ("Heavy") or L-$^{12}C_6$-lysine ("Light") labeling. After labeling completion and reaching the desirable amounts, cells with "Light" labeling were treated with ethanol (100 mM) for 72 h (treatment group). Cells with "Heavy" labeling were left untreated and used as the control group. After cell harvesting, equal numbers of cells from the two groups were mixed and crude proteins were extracted.

2.2. Lysine-acetylated peptide enrichment and mass spectrometry analysis

After cell harvesting, equal numbers of cells from the two groups were mixed and crude proteins were digested with trypsin. Kac peptides were affinity-enriched with an anti-acetyl lysine antibody (Jingjie PTM BioLab Co. Ltd, Hangzhou, China). The resulting peptides were analyzed by nano-HPLC-MS/MS on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Odense, Denmark).

2.3. MS/MS raw data search on databases

The resulting MS/MS data were searched by using MaxQuant with the integrated Andromeda search engine (v. 1.3.0.5) [7]. Tandem mass spectra were searched against the SwissProt Human database (20,274 sequences) concatenated with the reverse decoy database and protein sequences of common contaminants. Trypsin/P was specified as a cleavage enzyme, allowing up to three missing cleavages, four modifications per peptide, and five charges. The mass error was set to 6 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation of cysteine was specified as a fixed modification, whereas oxidation of methionine, acetylation of lysine, and acetylation of the protein N-terminal were specified as variable modifications. False discovery rate (FDR) thresholds for the protein, peptide, and modification site were specified at 0.01. The minimum peptide length was set at 7. All the other parameters in MaxQuant were set to default values. Lysine acetylation site identifications with a MaxQuant Score of <40, localization probability of <0.75 or from reverse or contaminant protein sequences were removed.

2.4. Bioinformatics

The gene ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Then, Kac proteins were further classified by GO annotation on the basis of three categories: biological process, cellular component, and molecular function. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate the protein pathway [13]. The software Motif-x was used to analyze the model of sequences comprising amino acids in specific positions of acetyl-13-mers (six amino acids upstream and downstream of the acetylation site) in all protein sequences [5]. All the Kac substrate categories obtained after enrichment were collated, along with their P values, and were then filtered for those categories that were at least enriched in one of the clusters with a P value of <0.05.

2.5. Western blot analysis

For western blot analysis, cells were lysed in RIPA buffer (50 mM Tris–HCl at pH 8.0, containing 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 10 μL/mL protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany), and the cellular debris was cleared by centrifugation. The protein concentration in the lysate was determined by using the BCA™ protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Total protein (40 μg) was separated by SDS-PAGE on an 8% gel and transferred to polyvinylidene difluoride membranes (Roche, Indianapolis, IL, USA). After overnight blocking in 5% bovine serum albumin, proteins were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish-peroxidase-conjugated secondary antibodies for 2 h. The proteins were detected by using an ImageQuant LAS-4000 mini system (GE Healthcare, Little Chalfont, UK).

3. Results

3.1. Experimental strategy for assessing the dynamics of Kac

The impact of ethanol on hepatoma cells at the acetylated proteins level was assessed by a SILAC-based proteomics strategy for quantification of the acetylation dynamics (Fig. 1A). Control AML-12 cells were grown in "Heavy" SILAC (L-$^{12}C_6$-Lys), whereas ethanol-treated AML-12 cells were cultured in "Light" SILAC medium (L-$^{13}C_6$-Lys) medium. AML-12 cells are murine hepatocytes that express ADH and ALDH2, which metabolize ethanol to implement the effects of ethanol in biological systems [11]. The SILAC cell lysates were equally mixed and digested into peptides using trypsin. Acetylated peptides were enriched from the peptide mixture using specific Kac antibodies. The enriched acetylated peptides were analyzed by nano liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on an Orbitrap instrument (Q-Exactive). All obtained MS/MS spectra were analyzed using MaxQuant software, allowing a maximum FDR of 1% for the protein, peptide, and modification site. In this study, 1515 Kac sites in 790 Kac proteins were identified, of which 1285 Kac sites and 686 Kac proteins were quantifiable (Supplemental Table S1). The minimum

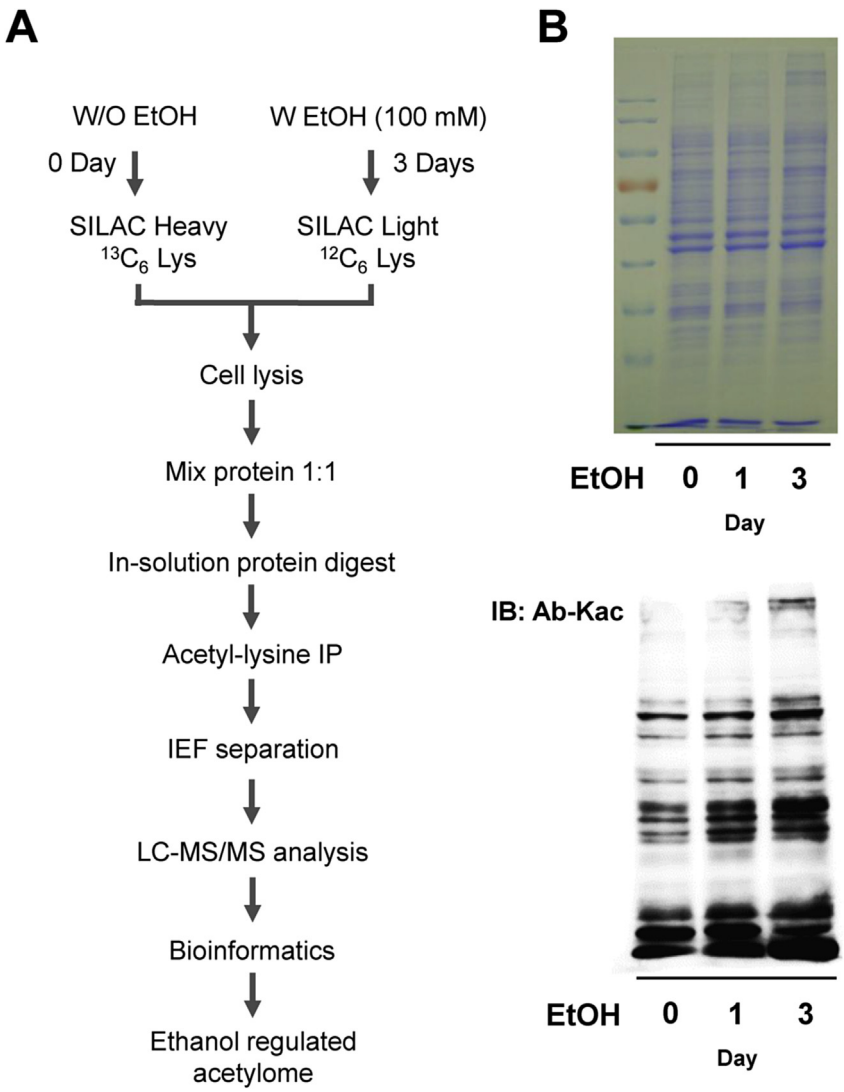

Fig. 1. Experimental strategy for elucidating the ethanol-induced global dynamics of lysine-acetylated proteins in AML-12 cells. (A) SILAC-based proteomics strategy for quantification of ethanol-induced acetylation sites. Control and ethanol-treated cells were grown in SILAC media with "Heavy" and "Light" amino acids, respectively. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis of the protein lysate from AML-12 cells treated with ethanol (100 mM) for 3 days. The acetyl lysine specific antibody used was from Jingjie PTM Biolabs Co. Ltd (Hangzhou, China).

peptide length was set at 7. Analysis of the peptide mass error and peptide length indicated that the MS analysis was of high quality (Supplemental Figure S1). The acetylated proteins were distributed among a variety of organelles, but mainly in the nucleoid (43%), mitochondria (19%), and cytoplasm (19%), as shown in (Supplemental Figure S2A). Our results showed that protein acetylations were assigned mainly to enrichment of the metabolic process (16%), cellular process (19%), cellular component organization or biogenesis (12%), and single-organism process (15%) (Supplemental Figure S2).

3.2. Quantitative analysis of Kac in ethanol-induced AML-12 cells

It is well known from many previous reports that ethanol-induced hyperacetylation of hepatic proteins leads to major physiological consequences that contribute to ethanol-induced hepatotoxicity [8,21,22]. We also confirmed the acetylome dynamics by western blot analysis after ethanol treatment (100 mM) of AML-12 cells for 3 days [28]. As shown in Fig. 1B, the density of Kac proteins on the western blot was significantly increased after 3 days of ethanol treatment, indicating the hyperacetylation of hepatic protein in AML-12 cells. Among all identified Kac proteins, the 1285 Kac sites quantified were divided into four quantiles (Supplemental Figure S3). The L/H SILAC ratio of each acetylated peptide was then transformed to a Z score, based on the equation $Z = (\log2Ratio - x)/y$, where Ratio is the L/H SILAC ratio. Each acetylated peptide was then allocated to the quantiles on the basis of the transformed Z score. In this way, the four quantiles generated were Q1 (0–15%), Q2 (15–50%), Q3 (50–85%), and Q4 (85–100%). Q1 was calculated to have 195 Kac sites, which were decreased by ethanol treatment (normalized L/H ratio, <0.749). Q2 and Q3 had 450 and 448 Kac sites, respectively. Q4 had 192 Kac sites (normalized L/H ratio, >1.278), which indicated an increase in the hyperacetylation intensity of the Kac sites after ethanol treatment (Supplemental Table S1).

Using the Motif-x program [20], we extracted a total of 11 motifs by comparing the relative abundance, which was calculated and schematically represented by an intensity map (Fig. 2A). All the motifs, including [KH], [KY], [KF], [KXF], and [FXK], were previously known as preferred residues for Kac [15,18]. To discover the potential consensus motifs for ethanol-regulated Kac sites, we calculated the enrichment of each motif in all quantiles, and the P value was transformed into a Z score for hierarchical clustering

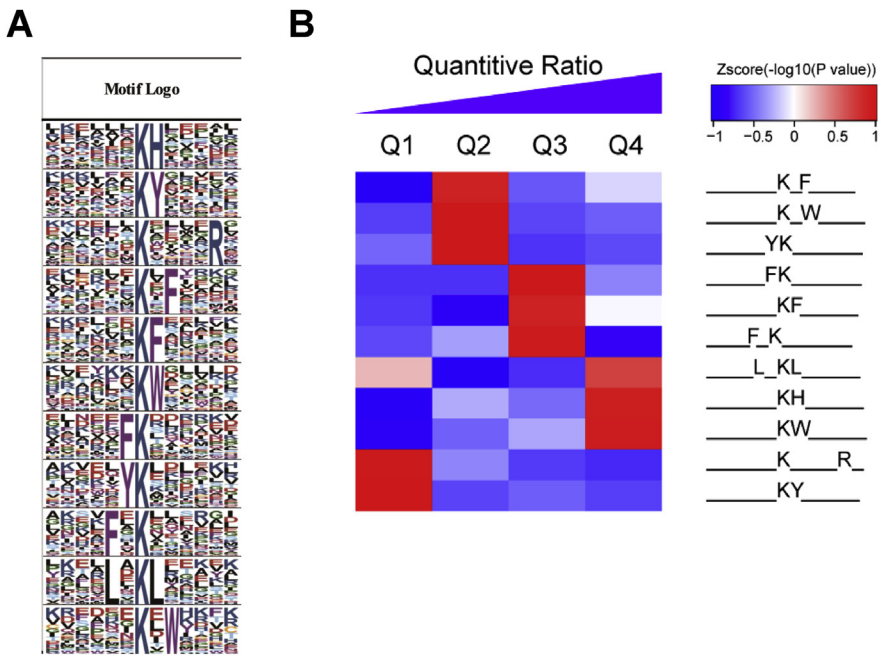

Fig. 2. Motif-X analysis of acetylation motifs. (A) Motif-x analysis of identified lysine-acetylated peptides. (B) Clustering analysis of lysine-acetylation motif logos among different quantitations of acetylated peptides.

analysis (Fig. 2B). In our study, the motifs [LXKL], [KH], and [KW] were included in the Q4 category. [KW] is known to be a specific residue for SIRT 3 [23], and [KH] is a known mitochondrial Kac motif [18].

3.3. *Biological functions regulated by ethanol treatment in AML-12 cells*

To further characterize the ethanol-induced Kac dynamics, we performed GO term and KEGG pathway enrichment analyses to identify the biological processes, cellular components, and molecular functions with significantly increased Kac in ethanol-treated AML-12 cells (Figs. 3 and 4). The results for the biological process category showed that a large portion of acetylated proteins associated with biosynthetic processes were within Q4 (Fig. 3). In the cellular component category, Q4 was annotated with the endoplasmic reticulum and Golgi apparatus (Fig. 3). In addition, Kac proteins involved with several ATPase activities were significantly enriched in quantile Q4, with high L/H SILAC ratios in the molecular function category (Fig. 3). In Table 1, we have summarized the 16 acetylation sites in 14 proteins that were concluded to be in the metabolic process category assigned by the KEGG database. In the

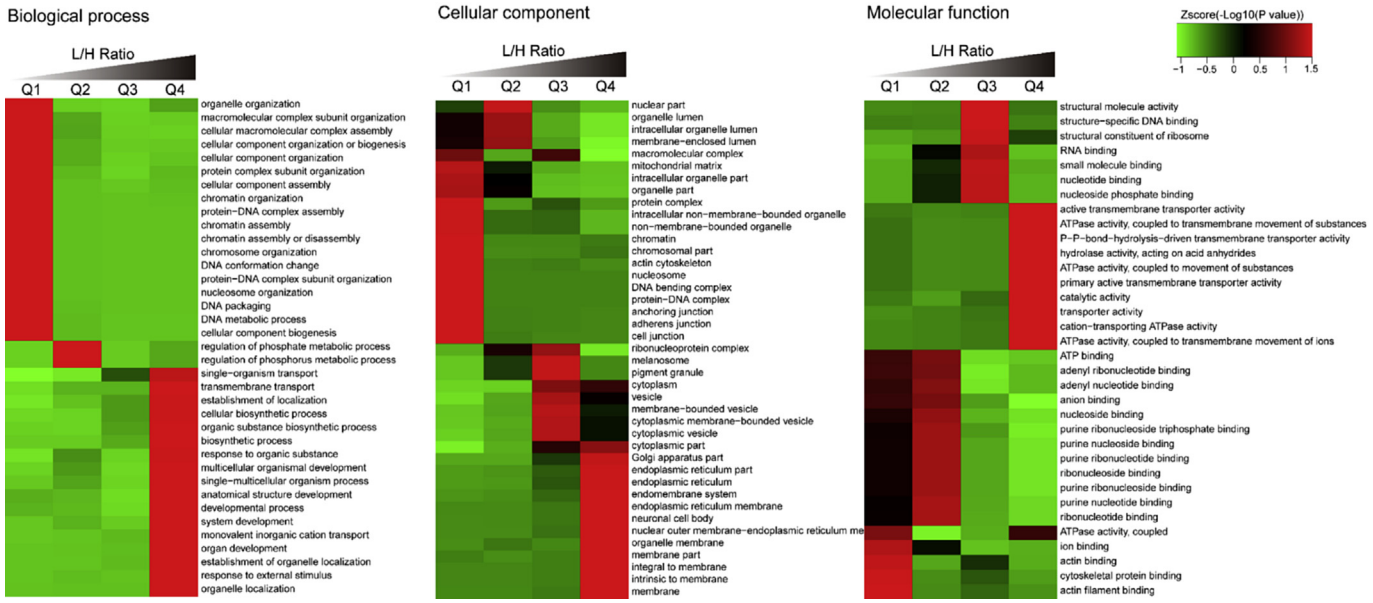

Fig. 3. Acetylated protein functional enrichment. Gene ontology functional classification of the quantified acetylated proteins, based on biological process, molecular function, and subcellular location.

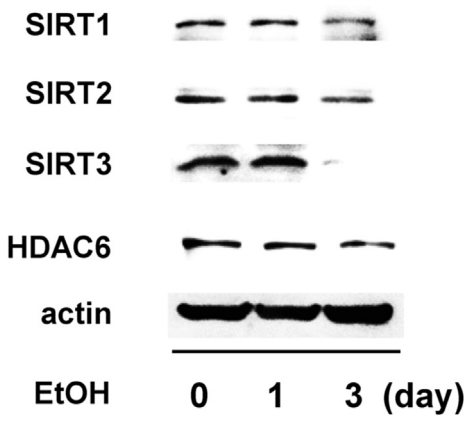

Fig. 4. Western blots of deacetylases in ethanol-induced AML-12 cells. SIRT2, silent mating-type information regulation 2 (also sirtuin); SIRT1 and SIRT3, sirtuin homologs 1 and 3; HDAC6, histone deacetylase 6; EtOH, ethanol.

KEGG pathway analysis, the proteins included in the Q4 category were significantly enriched in metabolic pathways and endocytosis (Supplemental Figure S4A). Among the diverse metabolic pathways, the Kac of fatty acid synthase (FASN) was significantly enriched in the fatty acid biosynthesis pathway (Supplemental Figure S4B). We identified two Kac sites on FASN (K673 and K1276) that were within Q4 (Table 1).

To further characterize the ethanol-induced physiological changes, we used the immunoblotting assay to confirm the expression of the deacetylases in AML-12 cells after ethanol treatment (100 mM) for 3 days. The activities of several cytosolic and mitochondrial acetyl deacetylases that are involved in ethanol-induced hyperacetylation or hepatotoxicity were decreased after ethanol treatment [8,16] (Fig. 4 and Supplemental Figure S5). Specifically, western blotting analysis showed SIRT3 to be significantly decreased in the ethanol-treated AML-12 cells. The expression of SIRT1 was also slightly decreased; however, the other protein expression levels were unchanged.

4. Discussion

Hepatic protein hyperacetylation is well known to result from chronic ethanol exposure, contributing to overall metabolic regulation and resulting in alcoholic liver disease [8,21,22]. In this study, acetylation sites included in category Q4 were shown to be involved in ethanol-induced hyperacetylation in hepatocytes. A total of 192 acetylation sites were included in Q4, 13 of which were significantly increased by ethanol exposure (normalized L/H ratio, >2). SIRT1 and SIRT3, as the NAD^+-dependent sirtuins, are mainly related to protein hyperacetylation leading to fatty acid oxidation and the fatty liver [8,10]. Therefore, NAD^+ depletion will decrease the SIRT1 and SIRT3 activities during ethanol metabolism [16]. SIRT1 is located in the nucleus and cytoplasm and is involved in glucose metabolism and differentiation, whereas SIRT3 is a mitochondrial enzyme contributing to ATP production and fatty acid oxidation [3]. In our study, SIRT3 expression was significantly decreased by ethanol treatment. SIRT1 was decreased by ethanol treatment to a lesser extent, whereas the other protein expression levels were unchanged. This means that SIRT1 and SIRT3 might be the main coordinators for protein hyperacetylation in the cytoplasm and mitochondria, respectively.

Fatty acid synthase (FAS) is a multienzyme protein that catalyzes the de novo synthesis of fatty acids [12]. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA (in the presence of NADPH) into long-chain saturated fatty acids. As a housekeeping protein in the liver, FAS contributes to fat production for energy storage when nutrients are present in excess [12]. FAS is not a single enzyme but a multienzymatic system composed of two identical 272 kDa multifunctional polypeptides, in which substrates are handed from one functional domain to the next [1,26]. Here, we identified a total of five acetylation sites in FAS that were increased by ethanol exposure, among which the K673 and K1276 sites were identified as belonging to category Q4 (Table 1). In a previous study, FAS was one of a large number of hepatic metabolic enzymes found to be lysine acetylated [32]. The K673 and K1276 sites have been identified as targets for ubiquitination, and the acetylation at K1276 overlapped with the succinylation site [17]. Although further study should be done to confirm these findings, the interplay between acetylation and ubiquitination at K673 and K1276 could be suggested as a novel mechanism for the dynamic regulation of FAS [9].

Lysine-acetylation-mediated regulation is conserved among central metabolic enzymes in the tricarboxylic acid (TCA) cycle. Here, we identified two Kac sites within the Q4 category; namely, isocitrate dehydrogenase [NAD] subunit gamma 1 (IDH) at position

Table 1
Ethanol-induced hyperacetylated proteins included in category Q4. (Abbreviations: cyto, cytosol; mito, mitochondria).

Protein accession number	Protein names	Position	Modified sequence	KEGG pathway	Subcellular location
P17751	Triosephosphate isomerase	K56	_K(ac)FFVGGNWK_	Fructose and mannose metabolism	cyto
P05064	Fructose-bisphosphate aldolase A	K230	_ALSDHHVYLEGTLLK(ac)PNMVTPGHACTQK_	Fructose and mannose metabolism	cyto
P19096	Fatty acid synthase	K673	_QEGVFAK(ac)EVR_	Fatty acid biosynthesis	cyto
P19096	Fatty acid synthase	K1276	_HPQALK(ac)DVQTK_	Fatty acid biosynthesis	cyto
P06151	L-Lactate dehydrogenase	K243	_QVVDSAYEVIK(ac)LK_	Pyruvate metabolism	cyto
Q60597	2-Oxoglutarate dehydrogenase	K947	_EAQK(ac)YPNAELAWCQEEHK_	Citric acid cycle	mito
P70404	Isocitrate dehydrogenase [NAD]	K221	_VTAVHK(ac)ANIMK_	Citric acid cycle	mito
Q8C2Q8	ATP synthase	K66	_K(ac)HLIIGVSSDR_	Oxidative phosphorylation	mito
Q8C2Q8	ATP synthase	K130	_THSDQFLVSFK(ac)DVGR_	Oxidative phosphorylation	mito
Q9DB20	ATP synthase subunit *O*	K192	_IGEK(ac)YVDMSAK_	Oxidative phosphorylation	mito
Q03265	ATP synthase subunit alpha	K230	_TSIAIDTIINQK(ac)R_	Oxidative phosphorylation	mito
Q9EQ20	Methylmalonate-semialdehyde dehydrogenase	K113	_YQQLIK(ac)ENLK_	Metabolic pathways	mito
Q8QZT1	Acetyl-CoA acetyltransferase	K335	_VLK(ac)YAGLK_	Pyruvate metabolism	mito
P35486	Pyruvate dehydrogenase E1 component subunit alpha	K321	_SKSDPIMLLK(ac)DR_	Pyruvate metabolism	mito
P38060	Hydroxymethylglutaryl-CoA lyase	K48	_DGLQNEK(ac)SIVPTPVK_	Metabolic pathways	mito
P53395	Lipoamide acyltransferase	K435	_FDQK(ac)GDVYK_	Metabolic pathways	mito

K221, and 2-oxoglutarate dehydrogenase E1 component (OGDH) at position K947 (Table 1). IDH catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate and CO_2 in the TCA cycle. OGDH, as an oxoglutarate dehydrogenase complex (OGDC), converts 2-oxoglutarate to succinyl-CoA in the TCA cycle. In previous studies, two enzymes had been identified as endogenous substrates of the mitochondrial SIRT3 [8,24]. Moreover, SIRT3 has been shown to directly regulate IDH2 activity through deacetylation, as its hyperacetylation is known to be inhibitory [8,25]. Therefore, the hyperacetylation of IDH and OGDH by the ethanol-induced decreased activity of SIRT3 results in downregulation of the TCA cycle and upregulation of de novo fatty acid synthesis for the accumulation of fatty acids.

The Kac at position K321 of pyruvate dehydrogenase E1 and at position K335 of acetyl-CoA acetyltransferase were identified as SIRT3 substrates (Table 1) [8,14]. These two sites are included in category Q4, meaning that they are involved in ethanol-induced hyperacetylation in hepatocytes. Pyruvate can be converted to acetyl-CoA for use in lipid synthesis. Pyruvate dehydrogenase E1, a member of the pyruvate dehydrogenase complex (PDC), binds with pyruvate and thiamine pyrophosphate to produce acetyl-CoA, the final product of the PDC. Acetyl-CoA acetyltransferase is an acyltransferase, transferring acyl groups from two acetyl-CoAs to acetoacetyl-CoA. SIRT3 activity has been shown to be reduced in the liver of mice fed a high-fat diet, leading to the hyperacetylation of proteins involved in gluconeogenesis and mitochondrial oxidation [14]. Although the modulation of those enzyme activities by hyperacetylation has not been demonstrated, the ethanol-induced hyperacetylation-mediated SIRT3 reduction of pyruvate dehydrogenase and acetyl-CoA acetyltransferase might be related to the modulation of fatty acid synthesis, resulting in fat accumulation in hepatocytes.

Alcohol-induced oxidative stress is a direct result of ethanol metabolism through a number of pathways regulated by microsomal and mitochondrial systems. Here, we have investigated the exact hyperacetylation sites induced by ethanol in AML-12 cells. Along with the hepatic lipid metabolism controlled by transcription factors, a novel mechanism has been suggested. In summary, ethanol can deplete NAD^+ during the metabolic pathway, which induces the decrease of SIRT1 and SIRT3 activities. Since SIRT1 and SIRT3 modulate the key regulators of fatty acid metabolism and synthesis in the cytosol and mitochondria, ethanol stimulation increases the de novo synthesis of fat acids in hepatocytes as a result.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.154.

Transparency document

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