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Global profiling of Lysine Acetylation in Human Histoplasmosis Pathogen *Histoplasma capsulatum*

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Abstract

Histoplasma capsulatum is the causative agent of human histoplasmosis, which can cause respiratory and systemic mycosis in immune-compromised individuals. Lysine acetylation, a protein posttranslational protein modification, is widespread in both eukaryotes and prokaryotes. Although increasing evidence suggests that lysine acetylation may play critical roles in fungus physiology, very little is known about its extent and function in *H. capsulatum*. To comprehensively profile protein lysine acetylation in *H. capsulatum*, we performed a global acetylome analysis through peptide prefractionation, antibody enrichment, and LC−MS/MS analysis, identifying 775 acetylation sites on 456 acetylated proteins; and functionally analysis showing their involvement in different biological processes. We defined six types of acetylation site motifs, and the results imply that lysine residue of polypeptide with tyrosine at the −1 and +1 positions, histidine at the +1 position, and phenylalanine (F) at the +1 and +2 position is a preferred substrate of lysine acetyltransferase. Moreover, some virulence factors candidates including calmodulin and DnaK are acetylated. In conclusion, our data set may serve as an important resource for the elucidation of associations between functional protein lysine acetylation and virulence in *H. capsulatum*.

Keywords: lysine acetylation; Mass spectrometry; *Histoplasma capsulatum*; virulence

Running Head: Proteome-wide lysine acetylation of Histoplasma capsulatum

Introduction

Histoplasma capsulatum, the causative agent of human histoplasmosis, is a major cause of respiratory and systemic mycosis, especially in immune-compromised individuals(Graybill, 1988). Histoplasmosis typically manifests as a benign respiratory infection and even entirely asymptomatic. In some individuals, especially immunocompromised persons, *H. capsulatum* readily disseminates, causing significant morbidity and mortality(Kauffman, 2008). Histoplasmosis is a common endemic mycosis throughout most of the world, which is endemic in the mid-western United States (such as in the Mississippi and Ohio River valleys) and Central

America(Kauffman, 2007). Sporadic cases of autochthonous histoplasmosis have been found in China, which was traditionally considered non-endemic for *H. capsulatum*. It is noteworthy that most cases of histoplasmosis occurred in regions through with the Yangtze River flows(Cao et al. , 2010, Ge et al. , 2010, Pan et al. , 2013). *H. capsulatum* thrives in soil in mycelial (mold) form, as with most other dimorphic fungal pathogens, conversion to a unicellular haploid yeast form occurs following inhalation and exposure to the warmer temperature of the respiratory tract(Eissenberg and Goldman, 1991).

Recent genetic approaches have begun to reveal the regulatory machinery that governs the conversion of Histoplasma to the yeast form and expression of the gene set involved in pathogenesis with the assumption that virulence factors are components unique to the yeast-phase(Batanghari and Goldman, 1997, Colonna-Romano et al. , 1998, Hwang et al. , 2003). Some virulence factors of *H. capsulatum* yeasts were identified to subvert or avoid activating macrophage antifungal defenses(Holbrook and Rappleye, 2008). Histoplasma HSP60 localized to the cell surface of yeast cells and functions as adhesin for macrophage binding and phagocytosis(Habich et al. , 2006). The β-glucan polysaccharides of all fungal cell walls were related to macrophages recognition, reactive oxygen compounds production and proinflammatory cytokines secretion(Robinson et al. , 2006, Willment and Brown, 2008). Histoplasma yeasts effectively conceal their immunostimulatory β-glucan signatures underneath a cell wall layer composed of α-glucan which was involved in the virulence of Histoplasma, as genetic loss of the α-(1,3)-glucan covering through mutation or RNAi of α-glucan synthase (AGS1) severely attenuates Histoplasma virulence(Rappleye et al. , 2004). Secreted factor YPS3 and calcium-binding protein CBP play an important roles in the virulence of Histoplasma yeast(Bohse and Woods, 2007), as YPS3 can decrease the pathogen burden in the internal organs of infected mice and CBP can impair the intracellular growth of Histoplasma yeast and attenuate the ability to colonize the lung(Sebghati et al. , 2000). However, the pathogenesis of *H. capsulatum* remains largely unknown.

The reversible lysine acetylation in proteins is now recognized as a common posttranslational modification (PTM) in both prokaryotes and eukaryotes(Thao and Escalante-Semerena, 2011, Xie et al. , 2012). Since the lysine acetylome revealed in mammalian cells(Kim et al. , 2006), global acetylation in eukaryotes has been reported and several biochemical studies showed that lysine acetylation may influence various cellular processes including metabolic pathway and transcriptional regulation(Rardin et al. , 2013, Still et al. , 2013, Weinert et al. , 2011). Recent advances in antibody-based affinity enrichment and high sensitive MS-based proteomics have made contributions to the global analysis of lysine acetylation in bacteria including *Mycobacteria tuberculosis* (Liu et al. , 2014, Xie et al. , 2015), *Escherichia coli*(Yu et al. , 2008), *Salmonella enteric* (Wang et al. , 2010b), *Bacillus subtilis*(Kim et al. , 2013), *Geobacillus kaustophilus* (Lee et al. , 2013), *Erwinia amylovora* (Wu et al. , 2013), and *Thermus thermophilus* (Okanishi et al. , 2013), *Saccharopolyspora erythraea* (Huang et al. , 2015), *Streptomyces roseosporus* (Liao et al. , 2014). Despite the popular studies of lysine acetylation in bacteria, the progress of lysine acetylome in fungus is relative limited and only one fungus species *Saccharomyces cerevisiae* have been examined.

In this study, we investigated the first acetylproteome of the *H. capsulatum* using a high-resolution mass spectrometry-based proteomics approach. Combining the affinity immuno-separation of acetylated peptides with nano-HPLC−MS/MS analysis, we identified a total of 775 unique lysine

acetylation sites on 456 proteins. Bioinformatics analysis showed that lysine-acetylated proteins are mainly involved in metabolic processes. Moreover, several sequence motifs including KacY, KacF, KacH, Kac*F, YKac and Kac***R were identified. Furthermore, a total of 116 acetylation sites on 86 proteins were also found to be succinylated, suggesting extensive overlap between acetylation and succinylation in this fungus. This first global acetyaltion profiling of *H. capsulatum* provides a basis for future interrogation of the roles of these acetylated proteins.

Materials and Methods

Strain culture and Protein Extraction

The *Histoplasma capsulatum* NAm1 cultured cells were harvested and washed twice with cold phosphate-buffered saline (PBS), then lysed in 8M urea supplemented with 1 mM DTT, 2 mM EDTA, protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem), and HDAC inhibitor (30mM nicotinamide, 50mM sodium butyrate, 3uM Trichostatin A). This was then sonicated with 12 short bursts of 10 s intervals followed by 30 s intervals for cooling. Unbroken cells and debris were removed by centrifugation at 4 °C for 10 min at 20,000 g. Protein content in supernatant was defined with 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions and precipitated with 20% trichloroacetic acid overnight at 4 °C. The resulting precipitate was then washed three times with ice-cold acetone. The air-dried precipitate was resuspended in 100mM NH4HCO3 and then digested with trypsin (Promega) at an enzyme-to-substrate ratio (1:50) at 37 °C for 12 h. The tryptic peptides were reduced with 5 mM dithiothreitol for 45 min at 56 °C and then alkylated with 15 mM iodoacetamide at room temperature for 30 min in complete darkness. The reaction was finally terminated with 15 mM cysteine for 30 min at room temperature. To ensure complete digestion, additional trypsin at an enzyme-to-substrate ratio (1:100) was added, and the mixture was incubated for an additional 4 h. The digested peptides were freeze-drying in a SpeedVac (Thermo Scientific).

Enrichment of Lysine Acetylated Peptides

The trypic digest was redissolved in NETN buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, pH 8.0) and incubated with anti-acetyllysine agarose beads (PTM Biolabs) at 4 °C overnight with a gentle oscillation. After incubation, the beads were carefully washed three times with NETN buffer, twice with ETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) and once with water. The bound peptides were eluted from the beads by 1% trifluoroacetic acid and dried in the SpeedVac. Prior to HPLC/MS/MS analysis, the obtained peptides were rinsed with C18 ZipTips (Millipore) according to the manufacturer's instructions.

LC-ESI-MS/MS (Liquid chromatography electrospray ionisation tandem mass spectrometry) Analysis by Q Exactive

Peptides were resuspended in buffer A (0.1% FA, 2% ACN) and centrifuged at 20000g for 2 min. The supernatant was transferred into a sample tube and loaded onto an Acclaim PepMap 100 C18 trap column (Dionex, 75um×2cm) by EASY nLC1000 nanoUPLC (Thermo Scientific) and peptides were eluted onto an Acclaim PepMap RSLC C18 analytical column (Dionex, 50um×15cm). A 34 min gradient was run at 300 nl/min, starting from 5% to 30% B (80% ACN, 0.1% FA), followed by 2 min linear gradient to 40% B, 2 min to 80% B, and maintenance at 80% B for 4 min.

Peptides were subjected to nanospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo Scientific) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70000. Peptides were selected for MS/MS using 25% Normalized Collision Energy (NCE) with 4% stepped NCE. Ion fragments were detected in the Orbitrap at a resolution of 17500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the top 15 precursor ions above a threshold ion count of 4E4 in the MS survey scan with 2.5 s dynamic exclusion. The electrospray voltage applied was 1.8 kV. Automatic gain control was used to prevent overfilling of the ion trap; 2E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800 Da.

Data Processing

The identification of protein and acetylation site was performed by MaxQuant with integrated Andromeda search engine (v. 1.3.0.5). Tandem mass spectra were searched against Uniprot Histoplasma capsulatum protein database concatenated with reverse decoy database and protein sequences of common contaminants. Trypsin/P was specified as cleavage enzyme allowing up to 3 missing cleavages, 4 modifications per peptide and 5 charges. Mass error was set to 6 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on Cys was specified as fixed modification and oxidation on Met, acetylation on Lys and acetylation on protein N-terminal were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 0.01. Minimum peptide length was set at 7. All other parameters in MaxQuant were set to default values. Lys acetylation site identifications with localization probability less than 0.75 or from reverse or contaminant protein sequences were removed.

Bioinformatics Analysis

Protein functional annotation

Gene Ontology (GO) annotation proteome was performed using the UniProt-GOA Database (www.http://www.ebi.ac.uk/GOA/). Proteins were categorized into biological process, cellular compartment and molecular function according to Gene Ontology annotation. Kyoto Encyclopedia of Genes and Genomes (KEGG) were utilized to annotate pathways: firstly, using KEGG online service tools KAAS to annotate proteins, secondly, using KEGG online service tools KEGG mapper to map on the KEGG pathway database, finally, using InterPro database and InterProScan to annotate protein domains and applying CORUM database to annotate protein complex.

Functional enrichment analysis

We used the Fisher's exact test to check the enrichment or depletion (two-tailed test) of specific annotation terms among members of resulting protein clusters. Then through the method proposed by Benjamini and Hochberg, we further adjust the derived p-values to address multiple hypotheses. Any terms with adjusted p-values below 0.05 in any of the clusters were treated as significant.

Acetylated protein secondary structure prediction

The local secondary structures were carried out according to NetSurfP software. The different secondary structure probabilities of identified acetylated residues in this study were compared with the secondary structure probabilities at the position of control residues containing all Lys residues in our database. The distribution of acetylated and non-acetylated amino acids in protein secondary structures was analyzed.

Model of sequences around acetylation site analysis

The analysis of enrichment or depletion of amino acids at specific positions of acety-21-mers (10 amino acids upstream and downstream of acetylation sites) in all protein sequences were performed by software motif-X. The background database parameter was all protein sequences in the database.

Results and Discussion

Establishment of the Histoplasma acetylome

Lysine acetylation, conserved post translational modification (PTM) in both prokaryotic and eukaryotic cells, has been thought to play critical roles in regulating protein function in diverse ways. However, this PTM in *Histoplasma capsulatum* NAm1 has not yet been reported. The genome database of *H. capsulatum* has been established several year ago, which can promote the global analysis of the lysine acetylated sites and proteins in this species. To gain insights into the large-scale dataset of lysine acetylation in *H. capsulatum*, we used an integrated proteomic method to identify acetylated proteins and their modification sites. In total, we identified 775 acetylation sites with a peptide score greater than 40 from 456 proteins. To the best of our knowledge, none of these proteins have previously been associated with lysine acetylation in this pathogenic fungus, and this dataset provides the first global profiling of lysine acetylation in *H. capsulatum*. An example of the analysis of an acetylpeptide sequence from 40S ribosomal protein S25 and assignment of the acetylation site is provided in Figure 1.

Figure 1. Representative MS/MS spectra of acetyl peptides from 40S ribosomal protein S25. Acetylpeptide LYK(ac)DVQSYR with acetylation site at K41 in 40S ribosomal protein S25.

Functional annotation and cellular localization of acetylated proteins in *H. capsulatum*

To better understand the distribution and function of lysine acetylated proteins identified in H. capsulatum, we investigated the GO functional classification of all acetylated proteins based on their biological process, molecular function (Fig. 2 and Table S2). Among all the identified acetylated proteins, 285 (62.5%) were annotated on biological process (Fig. 2A), and a large proportion of identified proteins were involved in metabolic processes 132 (28.39%). In the molecular function classification, most acetylated proteins were related to the binding of various targets 111 (44.04%) and enzyme catalytic activity 113 (44.64%) (Fig.2B).Within the classification of subcellular localization, the majority of the identified acetylated proteins 307 (67.32%) were proposed to be located in the cytoplasm (Fig. 2C). Furthermore, to determine which types of proteins are preferred targets for acetylation, we conducted GO enrichment analyses (biological process, molecular function, and cellular component categories), KEGG pathway, and protein domain (Table S3). Our GO enrichment analysis showed that the acetylated proteins were markedly enriched in translation, generation of precursor metabolites and energy, organic acid metabolic process and hexose catabolic process. Consistently, the GO enrichment analysis of molecular functions further demonstrated that many functions were enriched in our set, including catalytic activity and binding. Likewise, in the GO cellular component category, we found that a large number of acetylated proteins we identified were significantly enriched in the intracellular part. Furthermore, the identified acetylated proteins were also mapped to KEGG metabolic pathways and protein domains (Table S3). We found that acetylation occurs on many

proteins involved in citrate cycle, ribosome, glyoxylate and dicarboxylate metabolism, carbon fixation in photosynthetic organisms, glycolysis/gluconeogenesis, and most acetylated proteins were enriched with P-loop containing nucleoside triphosphate hydrolase. In brief, these findings imply the crucial roles of lysine acetylation in most fundamental cellular processes of *H. capsulatum*.

Figure 2. Gene ontology functional classification of the identified acetylation proteins based on biological processes; molecular function; subcellular location (C).

Analysis of lysine acetylation sites

To investigate the preference of local secondary structures of acetylated lysine, we used the algorithm NetSurfP and found that the acetylation sites distribution was about 65.4% in coil, 27.9% in helix, and 6.4% in beta-strand (Figure 3A). To evaluate the nature of the acetylated lysine in *H. capsulatum*, we used Motif-X program, a soft developed to extract overrepresented patterns from any sequence dataset, to search the sequence motifs in all identified acetylated lysines. As shown in Figure 3B, six definitively conserved acetylation site motifs were defined according to the criteria of specific amino acid sequence from ten amino acids upstream and downstream of the acetylated lysine. These are $K^{ac}Y$, $K^{ac}F$, $K^{ac}H$, K^{ac}*F, YK^{ac} and K^{ac}***R. We observed that the residue preferences for acetylated peptides are tyrosine at the −1 and +1 positions, histidine at the +1 position, and phenylalanine (F) at the +1 and +2 position. Compared with the conserved motif sequence in plant and bacteria, we found the motifs KacH and KacY were also conserved in plant , human cell and bacteria(Fang et al. , 2015, Kim et al. , 2006, Pan et al. , 2014, Zhang et al. , 2009), which indicating that some common conserved motifs surrounding acetylated lysine sites may exist in both prokaryotes and eukaryotes.

To determine whether there is significant frequency of specific amino acids flanking the acetylated lysine site, these results were further demonstrated by a logo reflecting relative frequency of amino acids in specific positions of acetyl-21-mers (ten amino acids upstream and downstream of the modification site) compared with that of non-acetyl-21-mers (ten amino acids upstream and downstream of the non-modification site) (Figure 3C). Indeed, these results demonstrated by the intensity map are in accordance with above results. The different preference of amino acid residues surrounding lysine sites suggests unique substrate preferences in *H. capsulatum*.

Figure 3. Characterization of acetylated peptides. (A) Probabilities of localization to various secondary structures (α helix, beta strand, and coil). All the lysine residues of the proteins identified in this study were compared with acetylated lysines. (B) Probability sequence motifs of Histoplasma acetylation sites consisting of 20 residues surrounding the targeted lysine residue using Motif-X. Six significantly enriched acetylation site motifs were identified. (C) Heat map showing enrichment (red) or depletion (green) of amino acids in specific positions flanking the acetylated lysine in Histoplasma.

Protein interaction network of acetylated proteins in *H. capsulatum*

To further understand how these acetylated proteins involved diverse pathways crosslink to each other, we determined the protein interaction (PPI) network for all of the acetylated proteins using Cytoscape software. The detailed information of proteins involved in PPI network is presented in

Table S4. By using the MCODE plugin tool, we extracted several clusters of highly inter-connected networks from the whole interaction network. We found that the top two significantly enriched clusters were ribosome and proteasome (Figure 4), which supports the suggestion that lysine acetylation may play important roles in the machinery of protein synthesis.

Overlap between lysine acetylation and succinylation

To date, no PTMs have been reported in *H. capsulatum*. In *E. coli* and *M. tuberculosis*, many acetylated proteins are succinylated, even at the same lysine site (Colak et al. , 2013, Xie et al. , 2014a). To determine whether this phenomenon is also present in *H. capsulatum*, we compared the acetylation sites identified in our study to previous determined succinylation sites in Histoplasma (unpublished data) (Figure 5 and Table S5). The methods used to characterize succinylated proteins in *H. capsulatum* were almost similar to that used to characterize acetylated proteins in this article. Protein fractions from cells were firstly digested by trypsin, then the succinylated peptides were subjected to immune-affinity purification with specific succinyl-lysine antibody and finally the isolated succinylated peptides were analyzed by LC/MS/MS for peptide identification. We found that 116 acetylation sites on 86 proteins were succinylated at the same position, of which K109, K114, K196, K147 and K190 on peptidylprolyl isomerase (HCAG_04485) and K120, K107, K113, K62 and K89 on peptidyl-prolyl cis-trans isomerase B (HCAG_04215) identified in this study as lysine acetylated are also succinylated (Fig.4). Other overlap between succinylation and acetylation found in three lysine residues of one protein includes alkaline phosphatase M, ATP carrier protein. Besides, acetylation of K80, K57 on histone H3 and K132, K128, K120 on histone H4 are succinylated at the same site. This indicated that acetylation and succinylation in Histoplasma is a highly dynamic process.

Figure 5. Venn diagram outlined the overlap between acetylation and succinylation in Histoplasma. Overlapped sites between acetylation and succinylation. 116 acetylation sites identified were also succinylated at the same position. Overlapped proteins between acetylation and succinylation. 86 acetylation proteins identified were also succinylated.

Lysine acetylation on histone proteins and histone acetyltransferases in Histoplasma

Histone acetylation occurs in diverse organisms ranging from fungi to plants and mammals for differential regulation of cellular processes(Jeon et al. , 2014). For example, acetylation of lysine residues on histone 3 and histone 4 are commonly correlated with active transcription. The core histones of H. capsulatum include two H2A, H3, H2B and H4 subunits. This study identified 25 acetylation sites in H4, H2A, H2B and H3 proteins (Table 1). In recent reports, low levels of H3 K79 acetylation have been detected in HeLa cells, *Saccharomyces cerevisiae* yeast and *Toxoplasma gondii*, though the role of this mark has yet to be defined (Bheda et al. , 2012, Garcia et al. , 2006, Jeffers and Sullivan, 2012). H3 K79 acetylation was also found in our data, indicating that this histone acetylation is conserved in eukaryote.

Histone acetylation is usually catalyzed by protein complexes involving histone acetyltransferases (HATs) utilizing acetyl CoA as a cofactor. HATs are grouped into five families: GNAT (Gcn5-related N-acetyltransferases), MYST (MOZ, Ybf2/Sas3, Sas2, Tip60), p300/CBP, basal transcription factors (Xie et al. , 2012, Xie et al. , 2014b). In fungi, there exists a specific structural

homolog of p300/CBP, Rtt109, which is responsible for the acetylation of H3 K56, a histone residue at the DNA entry and exit point in the core of a nucleosome for transcriptional regulation. Rtt109 play important roles in maintaining genome stability and participating in DNA replication and DNA damage repair(Han et al. , 2007, Schneider et al. , 2006, Tang et al. , 2008). In the ubiquitous opportunistic pathogen *Candida albicans*, Rtt109 was demonstrated to be required for virulence and survival in host macrophages, where the pathogen is subjected to high oxidative stress(da Rosa et al. , 2010). In our data, we found that not only H3 K56 is acetylated but also the catalyzing enzyme Rtt109 (HCAG_00585) is acetylated at K273, implying that histone acetylation maintaining regulation of cellular processes is a complex network.

Acetylation of proteins involved in cellular progress

From our data, two acetylated proteins involved in vesicles trafficking, three acetylated proteins related to DNA replication (DNA replication licensing factor mcm2/HCAG_01107, mcm6/HCAG_00933 and mcm7/HCAG_06369) and one for chromatin remodeling (chromatin assembly factor 1 subunit C/HCAG_00486), 12 acetylated proteins were identified to be components of proteasome and 12 acetylated proteins are components of the 40S ribosome subunit and 22 acetylated proteins are components of the 60S ribosome subunit. In addition, 22 acetylated proteins involved in the different stage of protein translation, including aminoacyl-tRNA synthesis, translation initiation, elongation and peptide chain release (Figure 6).

Figure 6. The acetylated proteins were involved in vesicles trafficking, DNA replication, chromatin remodeling and protein synthesis and degradation.

Acetylated proteins related to vesicles trafficking

Intracellular protein transport in eukaryotic cells is mediated by small transport vesicles that are defined by their coat proteins(COPs). COPII-coated vesicles are thought to mediate the bulk flow of proteins from the endoplasmic reticulum (ER) to the early Golgi. COPI vesicles mediate the recycling of proteins from the Golgi to the ER, and clathrin-coated vesicles mediate transport from the trans-Golgi network, as well as endocytic transport from the plasma membrane(McMahon and Mills, 2004). Coat protein complex I (COPI) vesicles, coated by seven coatomer subunits including alpha-, beta-, beta'-, gamma-, delta-, epsilon-, and zeta-COP, are mainly responsible for Golgi apparatus toendoplasmaticreticulum (ER) transport (Wang et al. , 2010a). COPI is involved in other important physiological processes such as endosome, autophagy and posterior silk gland (PSG) tube expansion(Razi et al. , 2009, Whitney et al. , 1995). Recent paper has shown that coatomer beta subunit is related to the virulence of *Helicoverpa armigera* (Mao et al. , 2015). We found five proteins involving in vesicles trafficking were acetylated, including HCAG_01814 (coatomer beta), HCAG_03691 (coatomerzata), HCAG_04366 (coatomeralph), HCAG_05330 (coatomer gamma) and HCAG_04840 (small COPII coat GTPase sar1). The acetylation of these coatomer proteins suggests important role of acetylation in the pathogenesis of H.capsulatum.

Acetylated proteins involved in the components of organelle

Many components of proteasome were acetylated, including proteasome component PRE5/HCAG_08215, Y13/HCAG_05739, C5/HCAG_04101, PUP3/HCAG_04190, Pre1/HCAG_04198, Pup1/HCAG_03737, Pre8/HCAG_03939, and C7-alpha/HCAG_00053, three

26S protease regulatory subunits (6B/HCAG_00039, S10B/HCAG_04181 and 8/HCAG_07682) and a proteasome-activating nucleotidase HCAG_06316. The ubiquitin-proteosome system (UPS), is a non-lysosomal proteolysis system involved in the degradation of irrelevant/misfolded intracellular proteins, which is crucial for many diseases such as inflammatory and autoimmune diseases(Wang and Maldonado, 2006) and kidney diseases(Fukasawa, 2012, Lecker and Mitch, 2011). Multiply acetylated proteins were the components of another important organelle ribosome, which is important in the translation of proteins. 22 ribosome proteins from 60s subunit and 12 ribosome proteins from 40s subunit are found to be acetylated.

Acetylated proteins involved in protein translation

A total of 192 lysine-acetylated proteins were found in this functional group, accounting for 42.1% of total lysine-acetylated proteins. These proteins include ribosome proteins, translation initiation factors, elongation factors, aminoacyl-tRNA synthetases and peptide chain release, suggesting that acetylation might play a profound role in regulating translation in Histoplasma. Among them, elongation factor 1-α was heavily acetylated at 9 lysine residues, similar to its orthologs in other eukaryotes. Of the eight cytosolic aminoacyl-tRNA synthetases (Table S6), isoleucine-tRNA synthetase is acetylated at only one lysine site, which is consistent with the monoacetylation detected in the homologs of human and Toxoplasma. In addition, two peptide chain release factors were acetylated, including peptide chain release factor GTP-binding subunit/ HCAG_05778 and peptide chain release factor subunit 1/HCAG_03051.

Acetylation of enzymes involved in metabolism

Many enzymes involved in central metabolic pathways (glycolysis/gluconeogenesis and citric acid (TCA) cycle) are acetylated in both prokaryotes and eukaryotes (Fang et al. ,2015, Henriksen et al. , 2012, Kim et al. , 2013, Weinert et al. , 2013, Wu et al. , 2013, Xie et al. , 2015, Zhang et al. , 2013, Zhao et al. , 2010) (Table S6). We investigated the acetylation of metabolic enzymes in pathogenic fungus *H. capsulatum* by mapping acetylated proteins to KEGG metabolic pathways(Kanehisa and Goto, 2000). A large proportion of metabolic enzymes that are involved in glycolysis/gluconeogenesis, pyruvate metabolism, citric acid (TCA) cycle and amino acid metabolisms are acetylated (Kanehisa and Goto, 2000) (Figure 7). Most of these acetylated proteins related to glycolysis/gluconeogenesis, pyruvate metabolism and citric acid (TCA) cycle were also found to be acetylated in *B. subtilis*, *E. coli*　*S. erythraea*, *Fragaria vesca*，*S. cerevisiae*, and human cell, suggesting that acetylation may control activity or stability of these enzymes and be potential conserved function of the modification in the regulation of central metabolism. There are eight acetylated enzymes involved in glycolysis/gluconeogenesis that convert glucose to pyruvate, eleven enzymes involved in the pyruvate metabolism and nine enzymes involved in TCA (Table S6).

Previous studies have shown that the acetylated proteins of *S. cerevisiae* in amino acid metabolisms are mainly involved in glycine/serine/threonine biosynthesis(Henriksen et al. , 2012) and the enzymes related to leucine/isoleucine/valine/tyrosine biosynthesis were identified by acetylproteomics of *S. erythraea* (Huang et al., 2015). Acetylated enzymes in amino acid metabolisms of *H. capsulatum* are difference with other known bacteria *S. erythraea* or yeast *S. cerevisiae* by comparison with the lysine acylation profiling. As shown in the Figure 7, many enzymes involved in valine/leucine/isoleucine degradation were lysine acetylated in *H.*

capsulatum. Some acetylated enzymes identified in this study were not reported in previous acetylomes, including branched-chain amino acid aminotransferase (HCAG_08945), 2-oxoisovalerate dehydrogenase alpha subunit(HCAG_05761), dihydrolipoamide dehydrogenase (HCAG_08825), isovaleryl-CoA dehydrogenase (HCAG_00677), methylcrotonoyl-CoA carboxylase beta chain(HCAG_00676), hydroxymethylglutaryl-CoA synthase (HCAG_08805), succinyl-CoA:3-ketoacid-coenzymeA transferase (HCAG_05311), acetyl-CoA acetyltransferase(HCAG_08621), methylmalonate-semialdehyde dehydrogenase(HCAG_06059), aldehyde dehydrogenase(HCAG_08367).

Figure 7. Acetylation of metabolic enzymes identified in the glycolysis/gluconeogenesis, pyruvate metabolism, citric acid (TCA) cycle and amino acid degradation in H. capsulatum. The identified lysine-acetylated proteins are shown in red.

The virulence factors candidates were acetylated

The proteins related to intracellular Ca2+ were acetylated

H. capsulatum is readily engulfed by macrophages, in which the yeasts survive and proliferate within the normally hostile environment of phagolysosomes(Eissenberg et al. , 1988). The characteristics of this particular intracellular compartment are poorly understood, although we have previously demonstrated that Histoplasma laden phagolysosomes fail to acidify(Eissenberg et al. , 1993). Some observations have shown that the survival of intracellular pathogens depends on the surrounding poor in calcium(Sibley et al. , 1986, Véscovi et al. , 1997). *Toxoplasma gondii* appears to have a mechanism for survival within a potentially calcium-poor environment. *Salmonella typhimurium*, which also survives within phagolysosomes of macrophages low in Ca2+ concentration. *H. capsulatum* yeasts are capable of growing in a calcium-deprived environment and secreting a calcium-binding protein (CBP), suggesting that intracellular Ca2+ are important to the survival of *H. capsulatum*(Kügler et al. , 2000, West Batanghari et al. , 1998). Several proteins involved in intracellular Ca2+ signaling were acetylated in our data. The calmodulin encoded by HCAG_01325 was acetylated. The calmodulin served as bacterial virulence factor candidates through activating bacterial adenylatecyclase(Arias‐Negrete et al. , 1999, Ullmann and Mock, 1994). Two calcium/calmodulin-dependent protein kinases HCAG_04620 and HCAG_05417 were also acetylated in *H. capsulatum*. Some proteins directly or in directly related to intracellular Ca2+ signaling were acetylated, such as classical protein kinase HCAG_10055 and several peptidyl-prolylisomerase family proteins, such as HCAG_08833, HCAG_07345, HCAG_04215, HCAG_04485 and HCAG_03630.

The virulence related molecular chaperones were acetylated

Molecular chaperones are both constitutive and stress-inducible, and comprise a diverse group of proteins that possess the ability to transiently assist in the non-covalent assembly or disassembly of other macromolecular structures(Hemmingsen et al. , 1988). Previous studies have shown that molecular chaperones were related to the virulence of many pathogen(Lewthwaite et al. , 1998, Neckers and Tatu, 2008, Tatu and Neckers, 2014). Except for heat shock proteins HSP60 and HSP70, DnaK is a well-characterized bacterial chaperone critical for the survival of *Salmonella entericasero* var Typhimurium in host environments(Takaya et al. , 2004). T-complex family protein were reported to weakly, but significantly related to GroEL chaperonin family, which is

inducible in nature and have been implicated in helping bacteria override stressful environmental conditions(Arnold et al. , 2007). T-complex protein 1 (HCAG_05423) and its subunit eta (HCAG_05524), epsilon (HCAG_05697) and delta (HCAG_06315) were acetylated. Another two acetylated T-complex proteins are HCAG_06628 and HCAG_07282. Previous study has shown that serine/threonine protein phosphatase with potential chaperone activities, which involved in the stress resistance of *Arabidopsis thaliana*(Park et al. , 2012, Park et al. , 2011). Four serine/threonine protein phosphatases were acetylated, including 2A/HCAG_00424, cot-1/HCAG_01501, chk2/HCAG_04049 and srk1/ HCAG_04507.

Acetylation of virulence factors

Heat shock proteins may therefore form a first line of attack and help consolidate pathogen virulence(Kamiya et al. , 1998). Heat shock protein family member normally found in the cytosol where it helps promote proper folding and assembly of proteins and protein complexes. Three HSP-like proteins, including HCAG_00783 (HSP88), HCAG_01398 (HSP70) and HCAG_06961 (HSP60) were acetylated in our data. Hsp70 has been most commonly implicated in microbial virulence (Kamiya et al. , 1998, Sullivan and Pipas, 2002). HSP60 appears to constitute the Histoplasma adhesin that interacts with CR3 molecules on host phagocytes by identifying in vitro and in vivo interaction between CR3 and HSP60 (Long et al. , 2003). Further study has shown that HSP60 involved in Histoplasma attachment to host macrophages, as coating polystyrene beads with HSP60 is sufficient to promote their attachment to macrophages. Blocking human HSP60 binding to macrophages with fragments of Histoplasma HSP60 has broadly delineated a region containing the macrophage-binding epitope on Histoplasma HSP60 between amino acids 214 and 484. How Histoplasma HSP60 becomes localized to the cell surface of yeast cells and whether it functions as the sole adhesin for macrophage binding and phagocytosis remains unknown.

Concluding remarks

Combining high-affinity enrichment of lysine acetylated peptides with high-sensitivity mass spectrometry and bio-informatics tools, we presented the first lysine-acetylated proteome dataset for a pathogenic fungus *H. capsulatum*, identifying 775 lysine acetylation sites in 456 acetylated proteins including 5 histones and 451 non-histone proteins. In addition, with the extensive characterization of the acetylation, we found that acetylation occurred on a large number of Histoplasma proteins targeting diverse functions ranging from the control of metabolic process to virulence regulation, suggesting that protein acetylation may be very important in regulating Histoplasma cellular physiology and virulence. However, the identification of the acetyl-proteome in Histoplasma raises several questions. We identified 25 acetylation sites in core histones, but eight putative histone acetyltransferases (HCAG_08549, HCAG_04276, HCAG_02224, HCAG_05978, HCAG_00975, HCAG_08722, HCAG_06079, HCAG_00585) are present in Histoplasma. It will be very interested for us to study which one is responsible for these histone acetylation. In addition, whether the large number of acetylated nonhistone proteins is catalyzed by these histone acetyltransferases or chemical acetylation in *H. capsulatum* remain to be determined. This dataset may serve as a rich source for future characterization of the roles of protein acetylation in *H. capsulatum*.

Supporting Information

Table S1 showing the identified acetylated protein annotation summary. Table S2 showing the

functional analysis of biological process, molecular function and domain. Table S3 showing the GO enrichment analysis of biological process, molecular function, cellular component, pathway enrichment, and protein domain. Table S4 showing the detail of protein-protein interaction (PPI) network of all the lysine acetylated proteins. Table S5 showing the overlap between acetylation and succinylation in Histoplasma. Table S6 showing common acetylated enzymes involved in central metabolic pathways in both prokaryotes and eukaryotes, and acetylated enzymes and aminoacyl-tRNA synthetases in *H. capsulatum*.

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Declaration

The authors have declared no conflict of interest.

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Table 1. Detected lysine acetylation on *Histoplasma* Histone proteins and Histone acetyltransferases

Protein name	Accession no.	Acetylation sites
Histone		
Histone H2A	HCAG_03524	4, 8
Histone H2A	HCAG_04914	4, 6, 11
Histone H2B	HCAG_03525	7, **8, 14**, 19, 131, 54, 127, 119
Histone H3	HCAG_06701	**79**, 27, 36, **18, 23, 9, 14,** 56
Histone H4	HCAG_03885	5, 8, **12, 16,** 91
Histone acetyltransferases		
Rtt109	HCAG_00585	273

Highlights:

1. Our data provides the first global survey of acetylation in *Histoplasma capsulatum* and we identified 775 acetylation sites on 456 acetylated proteins.
2. Acetylated proteins are involved in the regulation of different cellular processes including metabolism and protein synthesis.
3. Six types of acetylated peptide sequence motif were revealed from the acetylome.
4. Some virulence factors candidates including calmodulin and DnaK are acetylated.

Figure 1.Representative MS/MS spectra of acetyl peptides from 40S ribosomal protein S25. Acetyl-peptide LYK(ac)DVQSYR with acetylation site at K41 in 40S ribosomal protein S25.

Figure 2.Gene ontology functional classification of the identified acetylation proteins based on biological processes; molecular function; subcellular location.

Figure 3.Characterization of acetylated peptides. (A) Probabilities of localization to various secondary structures (α helix, beta strand, and coil). All the lysine residues of the proteins identified in this study were compared with acetylated lysines. (B) Probability sequence motifs of Histoplasma acetylation sites consisting of 20 residues surrounding the targeted lysine residue using Motif-X. Six significantly enriched acetylation site motifs were identified. (C) Heat map showing enrichment (red) or depletion (green) of amino acids in specific positions flanking the acetylated lysine in Histoplasma.

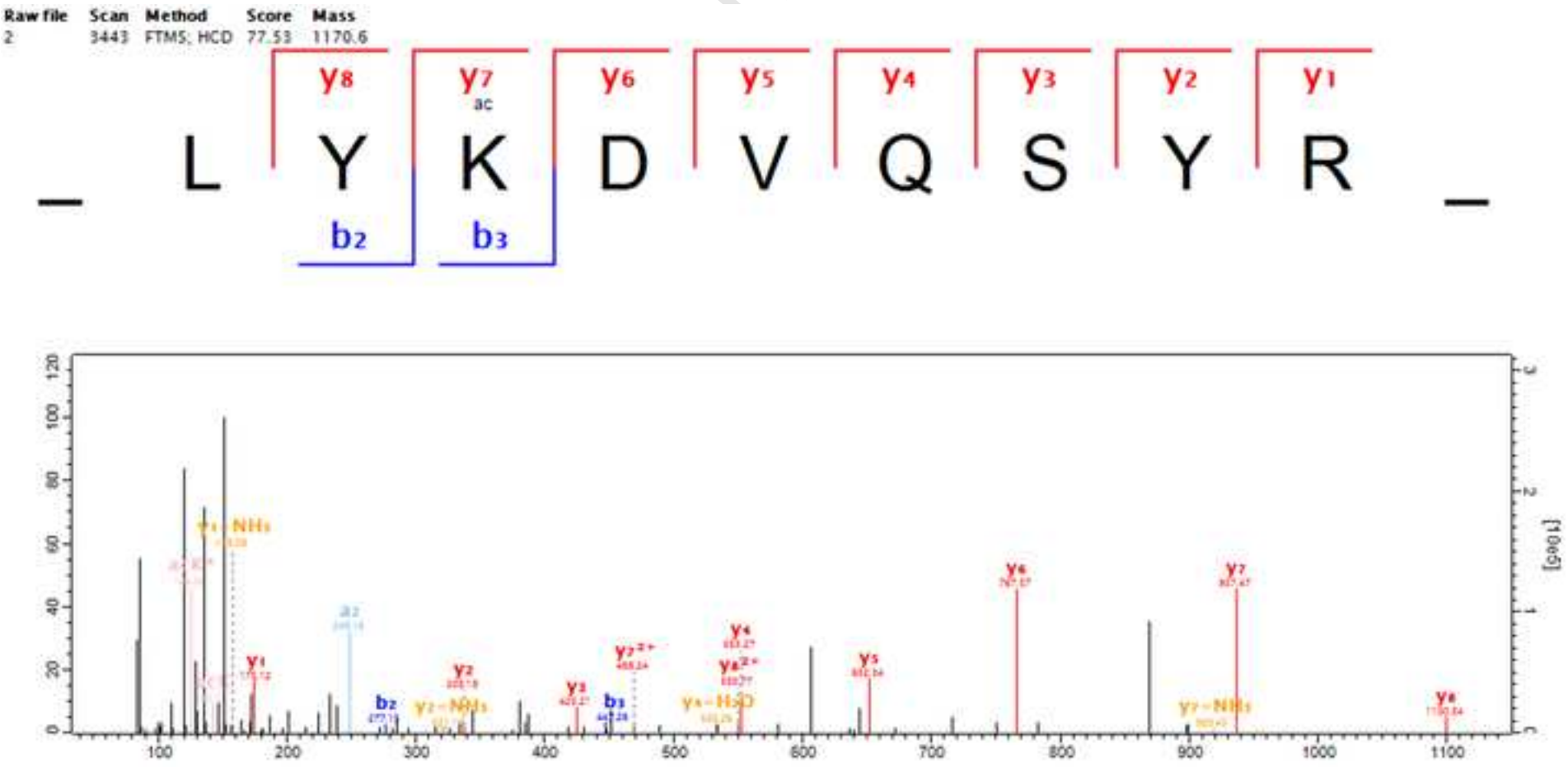
Figure 4.Top two clusters of highly interconnected lysine-acetyalted protein networks.

Figure 5.Venn diagram outlined the overlap between acetylation and succinylation in Histoplasma. Overlap sites between acetylation and succinylation. 116 acetylation sites identified were also succinylated at the same position. Overlap proteins between acetylation and succinylation. 86 acetylation proteins identified were also succinylated.

Figure 6.The acetylated proteins were involved in vesicles trafficking, DNA replication, chromatin remodeling and protein synthesis and degradation.

Figure 7.Acetylation of metabolic enzymes identified in the glycolysis/gluconeogenesis, pyruvate metabolism, citric acid (TCA) cycle and amino acid degradation in *H. capsulatum*. The identified lysine-acetylated proteins are shown in red.

Figure 1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ac
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b2 b3
[1065]

Figure 2

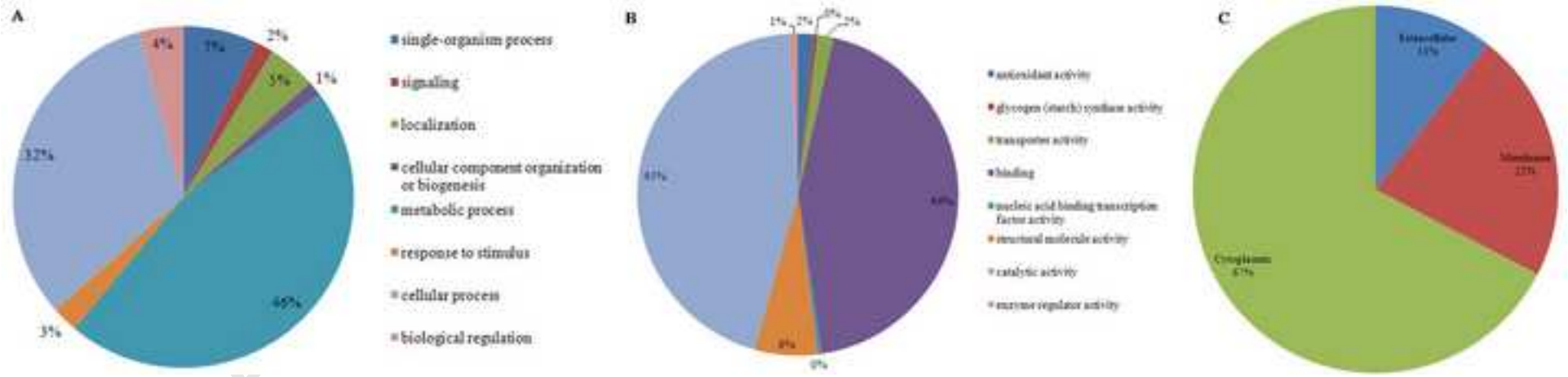


Figure 3

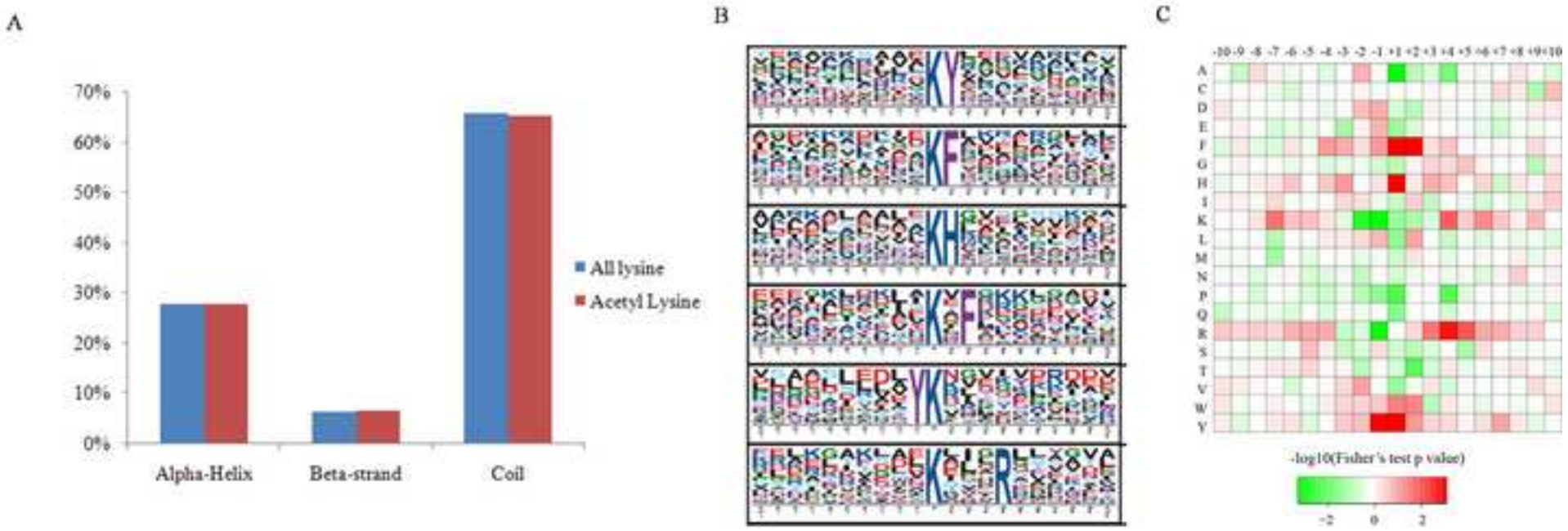
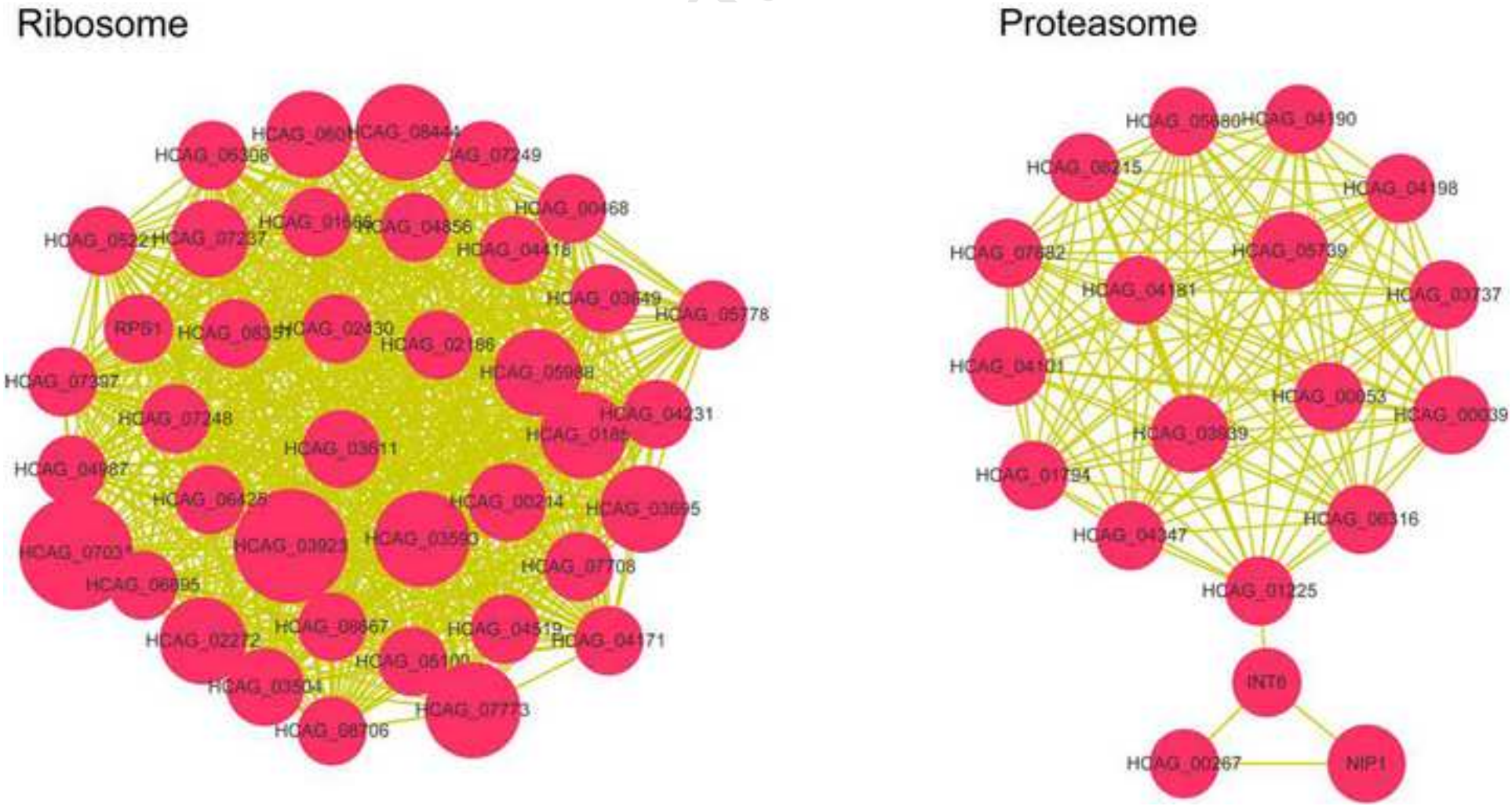


Figure 4


Ribosome
HCAG_05306
HCAG_08444
HCAG_07249
HCAG_00468
HCAG_04856
HCAG_04418
HCAG_03649
HCAG_05778
RPS1
HCAG_02430
HCAG_02186
HCAG_05088
HCAG_07397
HCAG_07248
HCAG_04231
HCAG_03611
HCAG_04987
HCAG_06428
HCAG_00214
HCAG_03695
HCAG_03923
HCAG_03590
HCAG_07708
HCAG_06895
HCAG_02272
HCAG_04519
HCAG_04171
HCAG_05100
HCAG_03504
HCAG_07773
HCAG_08706
Proteasome
HCAG_04190
HCAG_04198
HCAG_07682
HCAG_05739
HCAG_04181
HCAG_03737
HCAG_04101
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INT6
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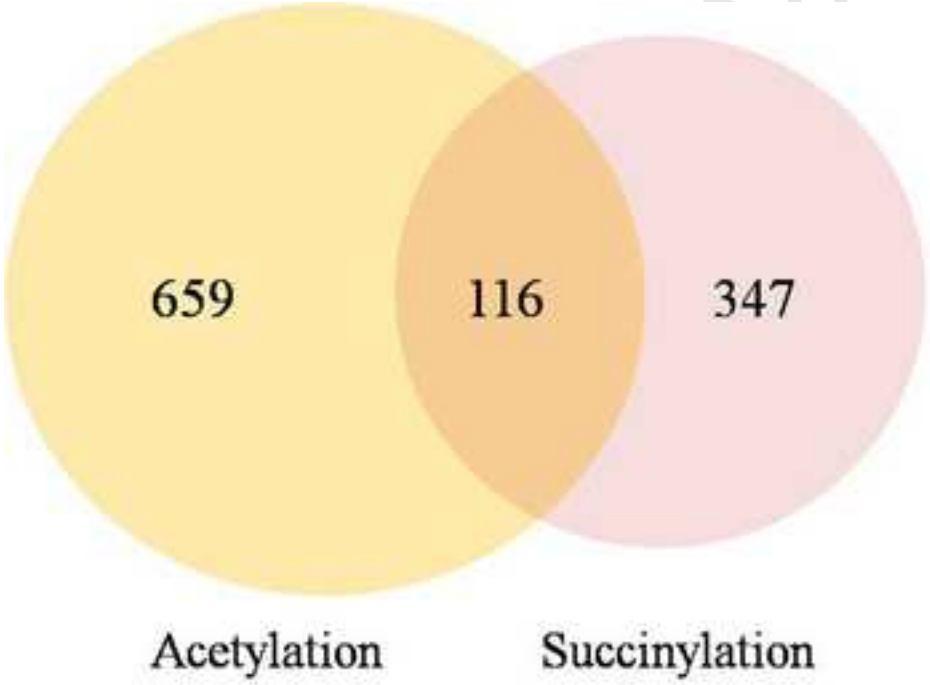
NIP1

Figure 5


659
116
347
Acetylation
Succinylation

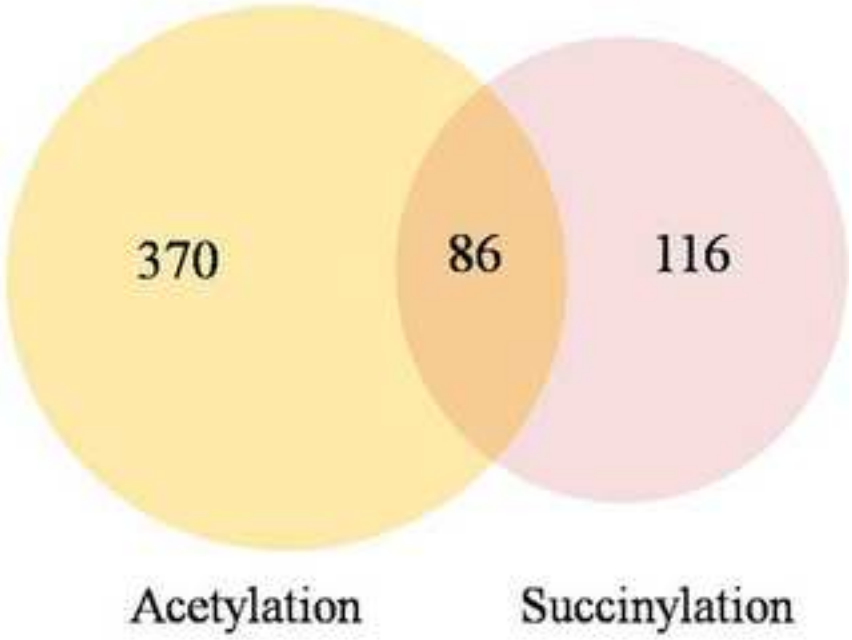

370
86
116
Acetylation
Succinylation

Figure 6

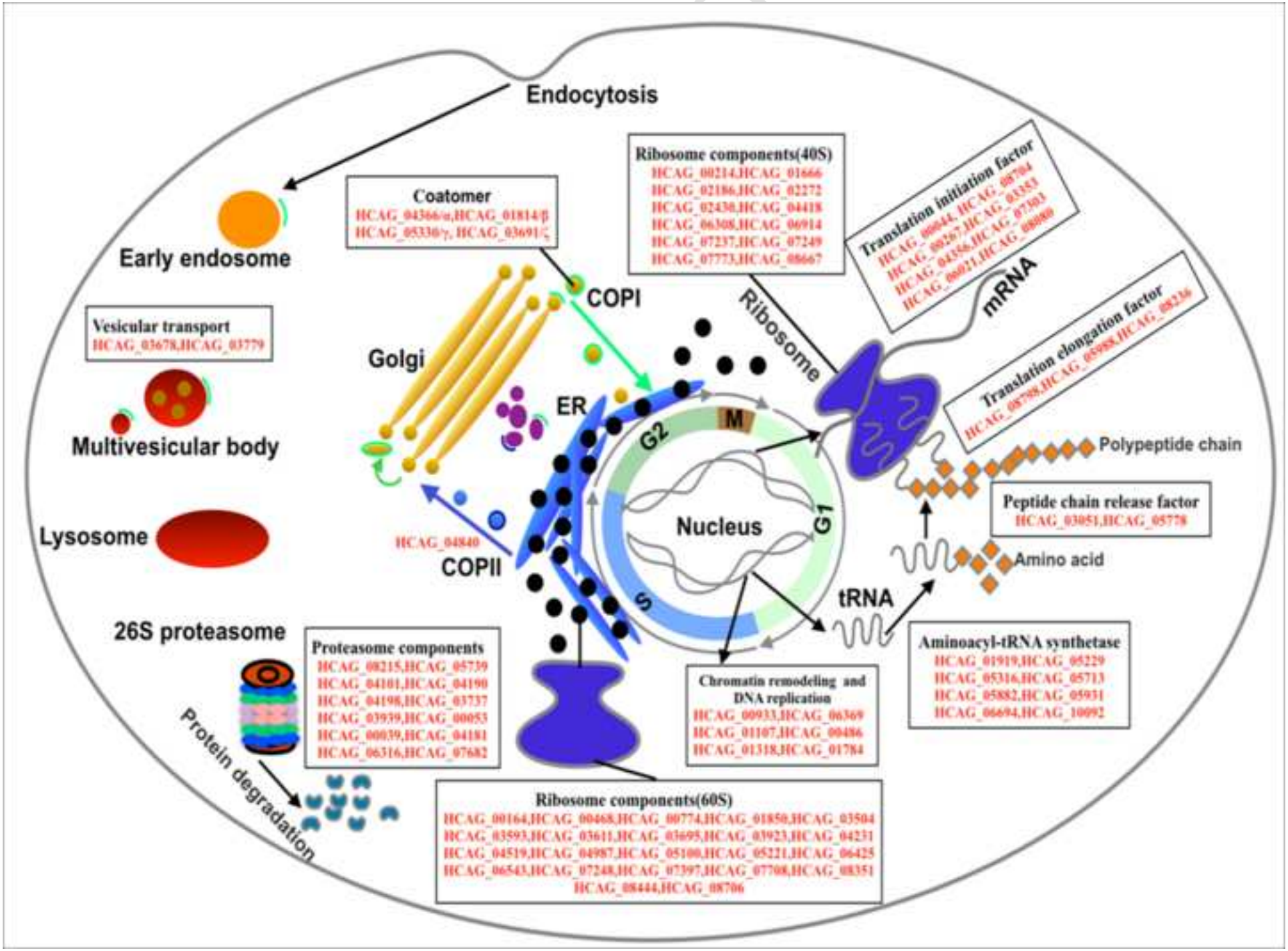
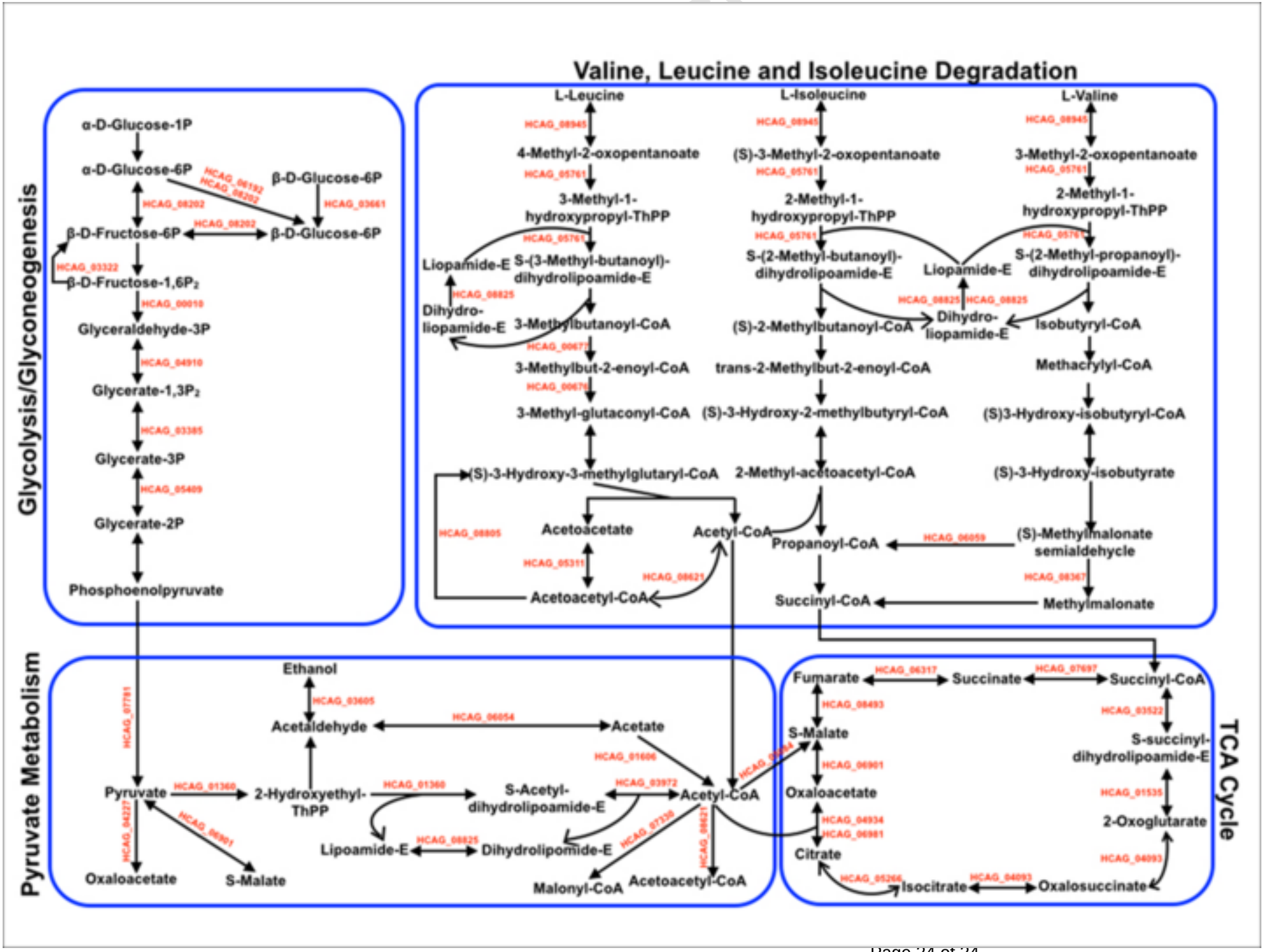

Endocytosis
Early endosome
Vesicular transport
HCAG_03678,HCAG_03779
Multivesicular body
Lysosome
26S proteasome
Protein degradation
Coatomer
HCAG_04366/α,HCAG_01814/β
HCAG_05330/γ, HCAG_03691/ζ
COPI
Golgi
ER
HCAG_04840
COPII
Ribosome components(40S)
HCAG_00214,HCAG_01666
HCAG_02186,HCAG_02272
HCAG_02438,HCAG_04418
HCAG_06308,HCAG_06914
HCAG_07237,HCAG_07249
HCAG_07773,HCAG_08667
Ribosome
mRNA
Translation initiation factor
HCAG_00044,HCAG_08704
HCAG_00267,HCAG_03353
HCAG_04358,HCAG_07503
HCAG_06021,HCAG_08080
Translation elongation factor
HCAG_08788,HCAG_05988,HCAG_08236
Polypeptide chain
Peptide chain release factor
HCAG_03051,HCAG_05778
Amino acid
tRNA
Nucleus
G1
S
G2
M
Chromatin remodeling and DNA replication
HCAG_00933,HCAG_06369
HCAG_01107,HCAG_00486
HCAG_01318,HCAG_01784
Aminoacyl-tRNA synthetase
HCAG_01919,HCAG_05229
HCAG_05316,HCAG_05713
HCAG_05882,HCAG_05931
HCAG_06694,HCAG_10092
Proteasome components
HCAG_08215,HCAG_05739
HCAG_04101,HCAG_04190
HCAG_04198,HCAG_03737
HCAG_03939,HCAG_00053
HCAG_00039,HCAG_04181
HCAG_06316,HCAG_07682
Ribosome components(60S)
HCAG_00164,HCAG_00468,HCAG_00774,HCAG_01850,HCAG_03504
HCAG_03593,HCAG_03611,HCAG_03695,HCAG_03923,HCAG_04231
HCAG_04519,HCAG_04987,HCAG_05100,HCAG_05221,HCAG_06425
HCAG_06543,HCAG_07248,HCAG_07397,HCAG_07708,HCAG_08351
HCAG_08444,HCAG_08706

Figure 7


Valine, Leucine and Isoleucine Degradation
Glycolysis/Glyconeogenesis
Pyruvate Metabolism
TCA Cycle
α-D-Glucose-1P
α-D-Glucose-6P
β-D-Glucose-6P
β-D-Fructose-6P
β-D-Fructose-1,6P2
Glyceraldehyde-3P
Glycerate-1,3P2
Glycerate-3P
Glycerate-2P
Phosphoenolpyruvate
L-Leucine
L-Isoleucine
L-Valine
4-Methyl-2-oxopentanoate
(S)-3-Methyl-2-oxopentanoate
3-Methyl-2-oxopentanoate
3-Methyl-1-hydroxypropyl-ThPP
2-Methyl-1-hydroxypropyl-ThPP
S-(3-Methyl-butanoyl)-dihydrolipoamide-E
S-(2-Methyl-butanoyl)-dihydrolipoamide-E
S-(2-Methyl-propanoyl)-dihydrolipoamide-E
Liopamide-E
Dihydro-liopamide-E
3-Methylbutanoyl-CoA
(S)-2-Methylbutanoyl-CoA
Isobutyryl-CoA
3-Methylbut-2-enoyl-CoA
trans-2-Methylbut-2-enoyl-CoA
Methacrylyl-CoA
3-Methyl-glutaconyl-CoA
(S)-3-Hydroxy-2-methylbutyryl-CoA
(S)3-Hydroxy-isobutyryl-CoA
(S)-3-Hydroxy-3-methylglutaryl-CoA
2-Methyl-acetoacetyl-CoA
(S)-3-Hydroxy-isobutyrate
Acetoacetate
Acetyl-CoA
Propanoyl-CoA
(S)-Methylmalonate semialdehycle
Acetoacetyl-CoA
Succinyl-CoA
Methylmalonate
Ethanol
Acetaldehyde
Acetate
Pyruvate
2-Hydroxyethyl-ThPP
S-Acetyl-dihydrolipoamide-E
Lipoamide-E
Dihydrolipomide-E
Malonyl-CoA
Acetoacetyl-CoA
Oxaloacetate
S-Malate
Fumarate
Succinate
Succinyl-CoA
S-Malate
S-succinyl-dihydrolipoamide-E
Oxaloacetate
2-Oxoglutarate
Citrate
Isocitrate
Oxalosuccinate