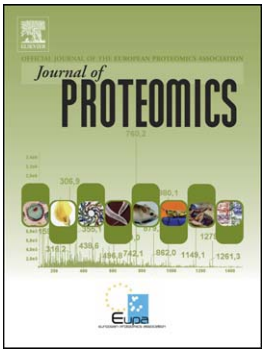

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PII: S1874-3919(16)30548-6
DOI: doi: 10.1016/j.jprot.2016.12.020
Reference: JPROT 2749

To appear in: *Journal of Proteomics*

Received date: 25 May 2016
Revised date: 28 December 2016
Accepted date: 30 December 2016

Please cite this article as: Xie Longxiang, Fang Wenjie, Deng Wanyan, Yu Zhaoxiao, Li Juan, Chen Min, Liao Wanqing, Xie Jianping, Pan Weihua, Proteomic analysis of lysine succinylation of the human pathogen *Histoplasma capsulatum*, *Journal of Proteomics* (2017), doi: 10.1016/j.jprot.2016.12.020

Proteomic Analysis of Lysine Succinylation of the Human Pathogen *Histoplasma capsulatum*

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Running title: lysine succinylated proteins of *H. capsulatum*

Key words: post-translational protein modification / lysine succinylation / histone proteins / metabolism / chaperone

ABSTRACT

Histoplasma capsulatum, the causative agent of histoplasmosis (also called "Darling's disease"), can affect both immunocompetent and immunocompromised hosts. Post-translational protein modification by lysine succinylation (Ksuc) is a frequent occurrence in eukaryote and prokaryote. Recently, the roles of succinylation and its regulatory enzymes in regulating metabolic pathway in bacteria, mammalian and fungus were highlighted. Here, we report the first global profiling of lysine succinylation, with 463 modification sites in 202 proteins from *H.capsulatum* NAM1 identified, coupling immune-affinity enrichment using an anti-succinyllysine antibody with mass spectrometry. The bioinformatics results including GO functional and enrichment analysis showed that these succinylated proteins are mainly involved in central metabolism and protein synthesis, consistent with previous reports. 13 lysine succinylation sites on histones including H2A, H2B, H3 and H4 in *H. capsulatum* were firstly reported. The data is a good resource for further functional characterization of lysine succinylation in *H. capsulatum*.

1 Introduction

Protein post-translational modification (PTM) is a dynamic and reversible protein chemical process which can regulate diverse aspects of protein properties and functions, and is involved in a range of cellular events[1]. Among all 20 amino acids, lysine residues are major targets. Several PTMs occur at lysine residues (also called lysine acylations), including acetylation [2, 3], malonylation[4], and succinylation[5]. There is increasing evidence that lysine acylation is crucial for protein function, and is associated with protein activity in both prokaryotes and eukaryotes [2, 5]. Among these lysine PTMs, lysine succinylation can transfer a larger structural moiety (a succinyl group, -CO-CH2-CH2-CO-) than that in lysine methylation or acetylation. This can change the charge status (+1 to −1) at physiological pH levels[5]. Lysine succinylation can potentially regulate the protein structure and function. For example, Xie *et al* demonstrated that lysine succinylation causes unique functional consequences and affects chromatin structure and gene expression[6]. Yang *et al* showed that the succinylation of acetyl-CoA synthetase has a negative effect on its enzymatic activity and affects its conformational stability[7]. In addition, Sadhukhana *et al* demonstrated that lysine succinylation plays an important role in regulating heart metabolism and function[8]. Cheng *et al* found that the alteration of succinylation level in rat may influence many metabolism processes and promote non-alcoholic fatty liver disease development (NAFLD)[9]. Lysine succinylation occurs in both bacterial and mammalian cells. In 2011, using affinity purification with an anti-succinyllysine antibody, Zhao *et al* firstly identified 69 succinylation sites from 14 succinylated proteins in *E. coli*[5]. Subsequently, comprehensive lysine succinylomes were characterized in bacteria[7, 10-12], parasites [13], yeasts [10], and human (HeLa) cells[10]. Fungal infections can be serious, particularly for immunocompromised and elderly individuals[14]. *H. capsulatum*, the fungus that causes histoplasmosis, can infect both immunocompromised and immunocompetent individuals[15]. In the United States, an estimated 200,000 infections occur every year through the inhalation of infectious particles[16].There are two morphological forms of the dimorphic *H. capsulatum:* a filamentous (or mycelial), and a yeast form [17]. Although dimorphism is a major virulence determinant of *H. capsulatum*, yeast cells are the main fungal elements leading to clinicopathological features of histoplasmosis[18]. Consequently, organisms locked in mycelial phase are avirulent[19]. Proteins involved in the *H. capsulatum* dimorphism include histidine kinase DRK1 and three proteins (RYP1, RYP2 and RYP3) related with yeast phase

growth[20, 21]. Vea1, the ortholog of *Aspergillus nidulans* Velvet A (VeA), is required for cleistothecia formation, and is a new virulence factor for *H. capsulatum*[22]. Iron acquisition is also critical for the virulence of *Histoplasma*[23].

PTMs and their regulatory significance have been extensively investigated in the model yeast *S. cerevisiae* and *Schizosaccharomyces pombe*. The relationship between PTMs and virulence in fungal pathogens including *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida albicans* is currently the focus of scientific investigation [24]. Recently, Zheng *et al* performed the systematic analysis of lysine succinylation in the human pathogen *C. albicans* and identified 1550 succinylation sites on 389 proteins which are involved in diverse cellular functions[25]. In addition, KEGG pathway analysis showed that lysine succinylation might play an important role in regulating the tricarboxylic acid cycle[25]. As *H. capsulatum* and *C. albicans* both belong to pathogenic dimorphic fungi, it is possible that this lysine modification may be also relevant in *H. capsulatum*.

In this study, combining immune-affinity enrichment using an anti-succinyllysine antibody with mass spectrometry, we successfully identified 463 unique lysine succinylation sites corresponding to 202 succinylated proteins with diverse biological functions and cellular localizations. To our knowledge, these results provide the first comprehensive analysis of the succinylome of *H. capsulatum.*

2 Materials and methods

2.1 Strain culture, Protein Extraction, Digestion and Enrichment of Ksuc Peptides

*H. capsulatum*NAm1 was cultured at 37°C in liquid HMM media at 200 rpm under 5% CO_2 as previously reported[26]. The harvested cells were carefully washed twice with cold phosphate-buffered saline (PBS) , then lysed in 8M urea with 1% Triton-100, 65 mM DTT and 0.1% Protease Inhibitor Cocktail Cell lysates were then sonicated on ice at 270W, followed by centrifugation at 4 ℃ for 10 min at 20,000g, to remove debris and unbroken cells. The resulting supernatant precipitated at 4 ℃ overnight by using cold 20% trichloroacetic acid (TCA). The obtained precipitate was then washed three times using ice-cold acetone, then dissolved in buffer ((8 M urea, 100 mM NH_4CO_3, pH 8.0)) and the protein concentration was quantified by 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions.

For digestion, the protein solution was reduced with 10 mM DTT for 1 h at 37 ℃ and alkylated with 20 mM iodoacetamide (IAA) for 45 min at room temperature in the complete dark. For trypsin digestion, the protein sample was diluted by adding 100 mM NH_4CO_3 to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

To enrich Ksu peptides, tryptic peptides dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) were incubated with pre-washed anti-succinyl-lysine antibody beads (PTM Biolabs) at 4℃ overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH_2O. The bound peptides were eluted from the beads with 0.1% TFA. The eluted fractions were combined and vacuum -dried. The resulting peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions, followed by LC-MS/MS analysis.

2.2 LC-ESI-MS/MS (Liquid chromatography electrospray ionisation tandem mass spectrometry)

The enriched Ksuc peptides were dissolved by adding buffer A containing 0.1% formic acid (FA). The resulting supernatant was loaded by centrifugation at 20000g for 2 min directly onto an Acclaim PepMap 100 C18 trap column (Dionex, 75μm×2cm) by EASY nLC1000 nanoUPLC (Thermo Scientific). It was then eluted onto an Acclaim PepMap RSLC C18 analytical column (Dionex, 50μm×15cm) for separation. A 34 min gradient starting with 5%-30% of buffer B (0.1% formic acid, 80% acetonitrile) for 30min (at a flow rate of 300nl/min),was then followed by constant gradient to 40% buffer B for 2 min, to 80% buffer Bfor 2 min, and maintained at 80% buffer B for 4 min.

The obtained peptides were firstly subjected to nanospray-ionization (NSI) source, followed by MS/MS in a Q Exactive (Thermo Scientific) mass spectrometer coupled online to UPLC. Intact peptides were detected at a resolution of 70000 in anOrbitrap analyzer (Thermo Scientific), and were then selected for MS/MS using normalized collision energy (NCE) setting as 30, followed by detection at a resolution of 17500 in the Orbitrap. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1.5E4 in the MS survey scan with 30.0s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800 Da.

2.3 Data manipulation and Bioinformatics Analysis

The obtained MS data were searched against the Uniprot *Histoplasma capsulatum* protein database (9251 sequences, 2015/01/09) using the MaxQuant search engine (v.1.3.0.5). Trypsin/P as the cleavage enzyme allows up to three missing cleavages, four modifications per peptide and five charges. The mass error for precursor ions and fragment ions was set to 10 ppm and 0.02 Da, respectively. Carbamidomethylation on cysteine (Cys) as a fixed modification was also included in the search, with oxidation on methionine, acetylation on protein N-terminal and succinylation on K (lysine +100.01604) as the variable modifications. The false discovery rate (FDR) thresholds were set at 1% for modification site, peptide and protein. Minimum peptide length was set at 7 and all other parameters in MaxQuant (Table S1) at default values.

The bio-informatics analysis including Gene Ontology annotation proteome, GO functional classification and GO enrichment of the lysine succinylated peptides and proteins was the same as previous study [12, 13].

3 Results and discussion

3.1 Systemic Identification of Ksuc Proteins in *H. capsulatum*

Recently, proteome-wide lysine succinylation has been examined in several species, including bacteria (*Escherichia coli*, *Mycobacterium tuberculosis* and *Vibrio parahaemolyticus*), parasites (*Toxoplasma gondii*), yeasts (*S. cerevisiae*), and human (HeLa) cells[7, 10, 13, 27]. This type of PTM has not been characterized in *H. capsulatum*, a human pathogenic fungus. Using the previously described method (Ksuc peptides enrichment combination with MS)[12], the succinylome of the *H.capsulatum* was characterized. The obtained MS raw data were searched against the UniProt *Histoplasma capsulatum* NAm1 protein database and 463 succinylation sites on 202 succinylated proteins were identified. The distribution of mass error was less than 2 ppm, which

fits the requirement of the mass accuracy of MS data (Figure S1A). The distribution of peptide length was mostly between 8 and 20 amino-acid residues, which agrees with the property of tryptic peptides (Figure S1B) and reaches the standard of sample preparation. The details of all identified succinylated peptides and succinylated proteins are shown in Table S1 and the representative MS/MS spectra of seven succinyl-peptide from HCAG_03525 (histone H2B), AVTKsucYSSSAK, HAVSEGTKsucAVTK, KsucETYSSYIYK, LAAYNKsucK, LILPGELAKsucHAVSEGTK, VATEASKsucLAAYNK, VLKsucQVHPDTGISNR with succinylated sites at K132, K128, K46, K97, K120, K91 and K58, respectively, are shown in Figure S2. As far as we know, this is the first reported evidence of protein lysine succinylation in *H. capsulatum*.

Next, we sorted the succinylated proteins according to the number of succinylation sites. Among these Ksuc proteins, approximately 50% had one Ksuc site. In addition, 12.9% (26) of total detected Ksuc proteins contained five or more lysine succinylation (Ksuc) sites. There were 7 highly intensively succinylated proteins having eight or more Ksuc modification sites. For instance, one heat shock protein (HCAG_04686) bears 16 succinylated lysine sites (Figure 1A).

In fungi, the global succinyl-proteome has only been constructed in yeast (*S.cerevisiae)*[10]. To investigate whether the identified succinylated proteins in this study are conserved among fungi, we compared the *H. capsulatum* succinyl-proteome to the yeast succinyl-proteome. Interestingly, 81 of 202 *H. capsulatum* lysine-succinylated proteins (40.6%) have succinylated homologs in *S.cerevisiae* (Figure 1B and Table S2). Most of these succinylated proteins involved in intermediary metabolism and protein synthesis were shown to be orthologous to *H. Capsulatum* and *S.cerevisiae*. We also searched the succinylated proteins in this study against the previously reported succinylomes of other species: *E. coli*[10], *V. parahemolyticus*[27], *T. gondii*[13], and Rice seed [28]. The results indicate that 33 succinylated proteins have homologs in *T. gondii*, 41 succinylated proteins have homologs in *E. coli*, 38 succinylated proteins have homologues in Rice seed, and 40 succinylated proteins have homologs in *V. parahemolyticus* (Table S2). This suggests that some succinylated proteins are conserved in fungi and bacteria.

3.2 Functional Taxonomies of Succinylated Proteins in *H. capsulatum*

To understand the potential role of lysine succinylation in *H. capsulatum*, we assigned them to a Gene Ontology category according to biological process and molecular function. GO classification results based on biological processes showed that the largest group of succinylated proteins was related to metabolic processes (52%), followed by cellular processes (30%) and single-organism processes (6%) (Figure 2 and Table S3). Most of succinylated proteins for the GO molecular function classification were associated with catalytic activity and binding of various targets. Approximately half of the lysine succinylated proteins were categorized as catalytic activity-related proteins (48%) and another large group as binding proteins (38%) (Figure 2 and Table S3). Enzyme binding to specific substrates and catalytic activity can control biochemical reactions in organisms, indicating that the majority of succinylated proteins in this study may be enzyme related proteins. When comparing our data with succinylation data obtained for other organisms, *M. tuberculosis* H37Rv, 50.8% and 45.5% of succinylated proteins were classified as having catalytic or binding activity, respectively [7]. Moreover, in extensively drug resistant (XDR) strains of *M. tuberculosis*, 49% of succinylated proteins were classified as having catalytic activity and 42% binding activity[12]. The classification results for molecular function were similar to our present study[12]. With respect to the sub-cellular localization, 143 of the identified *H. capsulatum* succinylated

proteins were located in the cytoplasm (71%), 39 on the cell membrane (19%), and 20 were extracellular (10%).

3.3 Functional Interaction Networks of Succinylated Proteins in *H. capsulatum*

In order to understand how these succinylated proteins might interact with diverse pathways, the protein-protein interaction (PPI) networks for all succinylated proteins in *H. capsulatum* were constructed and exhibited using Cytoscape software [29] and the STRING database[30]. A large network containing 194 succinylated proteins, connected by 2600 identified direct physical proteins based on the global network and two highly interconnected clusters of succinylated proteins was retrieved, through MCODE algorithm in Cytoscape (Figure 3 and Table S4)[31]. The first cluster consists of several succinylated proteins involved in ribosomal processes, whereas the second cluster consists of proteins associated with metabolic pathways. Owing to the identification of many ribosomal and metabolic process-related succinylated proteins, two additional interaction maps centered on these were constructed (Figure 3 and Table S4). This implies that the potential interactions among these succinylated proteins may be helpful for the regulation of protein synthesis machinery and metabolism in *H. capsulatum*.

3.4 Sequence Recognition Motifs

The motif of sequences in certain positions of succinyl-21-mers (10 residues upstream and downstream of the succinylation site) in all *H. capsulatum* protein sequences was characterized by using Soft motif-x. Three succinylation site motifs were defined (K_{suc} representing the succinylated lysine residue, and X representing any amino acid residue): K_{suc}******R, LK_{suc}, K_{suc}*******K, indicating that positively charged amino acids (arginine and lysine) were drastically overrepresented at K_{suc} position +7 and +8 (Figure S3). Then, we compared our data sets to the reported succinylome of *E. coli*[10, 11], *V. parahaemolyticus[27]*, *M. tuberculosis*[12], *T. gondii*[13], *S. cerevisiae*[10], *HeLa* cells[10], mouse liver tissue[10], and found that the succinylation pattern in *H. capsulatum* were quite different from site-specific succinylation motifs in *V. parahemolyticus*, *E. coli* BW25113 and *M. tuberculosis* XDR. However, L overrepresented frequently at the -1 position occurred in *Toxoplasma gondii, H. capsulatum, S. cerevisiae, HeLa cells and mouse liver tissue* (Table S5). Hence, it is not easy to make a conclusion that these three motifs (K_{suc}******R, LK_{suc}, K_{suc}*******K) are really motifs for lysine succinylation in *H. capsulatum* and there are conserved motifs present in all species.

3.5 Lysine Succinylated Proteins in Metabolic Pathway

In *V. parahemolyticus*, 40% of succinylated proteins are associated with metabolism [27]. Additionally, 377 succinylated proteins involved in metabolic processes have been identified in XDR *M. tuberculosis* [12]. In mouse liver tissue, lysine succinylation is associated with enzymes that catalyze intermediate metabolism such as in the citric acid (TCA) cycle, fatty acid oxidation, and the urea cycle. Here, we identified KEGG pathway enrichment of succinylated proteins in carbon metabolism, the TCA cycle, glyoxylate/dicarboxylate metabolism, and glycolysis/gluconeogenesis (Figure 4A and Table S3). Succinate and succinyl-CoA are the main derivatives of TCA cycle or the metabolism of fatty acid. Hence, it is not surprising that lysine succinylation in *H. capsulatum* mainly occurs on proteins involved in metabolism. We found that every enzyme involved in the TCA cycle, and four enzymes in the glycolytic pathway including

glucose-6-phosphate isomerase, fructose 1,6-biphosphate aldolase, pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were succinylated (Figure 4B). Most of these enzymes are also succinylated in *E. coli*[10], *V. parahemolyticus*[27], *T. gondii*[13], yeast[10], rice seed[28], and mammals[10], indicating that lysine succinylation may play a conserved function in the regulation of the glycolytic pathway and TCA cycle.

GAPDH, an enzyme which participates in the sixth step of glycolysis, is responsible for the phosphorylation and oxidation of glyceraldehyde 3-phosphate. It has been demonstrated that GAPDH, a cell surface protein in pathogenic fungus *Paracoccidioide brasiliensis*, is involved in fungal adhesion and invasion[32]. Recently, GAPDH has also been shown to be involved in several non-metabolic processes, such as the initiation of apoptosis, and activation of transcription[33, 34]. In our study, we analyzed six Ksuc sites (K4, K193, K214, K218, K250 and K258) on GAPDH that may be critical for catalysis. NADP-dependent isocitrate dehydrogenase (IDH), the rate-limiting enzyme of TCA cycle, can synthesize 2-oxoglutarote and CO2 from isocitrate. It has been demonstrated that two succinylated lysine sites, K100 and K242, are important for the enzyme activity of IDH in *E. coli*[5]. IDH, encoded by theHCAG_04358 gene, was succinylated on three lysine sites (K11, K35 and K356) in *H. capsulatum*.

The glyoxylate cycle, an anaplerotic metabolic route of TCA cycle, occurs in bacteria, fungi, and plants. Isocitratelyase (ICL), which is responsible for cleaving isocitrate to glyoxylate and succinate, and malate synthase (MSyn), responsible for converting glyoxylate and acetyl-CoA to malate, are the principal enzymes of this pathway. An increasing number of studieshas shown that ICL and MSyn play a critical role in the fungal virulence inplants and humans[35]. *Magnaporthe grisea*, the pathogen of rice blast, can also infect many other cereals containing rye, wheat and barley. Disruption of the *M. grisea* ICL1 gene leads to a decrease in appressorium formation and conidiogenesis, anda global reduction in damage to leaves of barley and rice[36]. *Candida albicans*, a dimorphic pathogenic fungus, can infect immune-compromised patients. Both ICL and MSyn are induced in *C.albicans* grown in macrophages or exposed to human neutrophils[37]. In mice, *C. albicans* ICL1 mutants were less virulent than the wild type strain [38]. *Stagonospora nodorum*, the necrotropic fungal pathogen, can cause glume and leaf blotch disease on wheat and other important cereals. Solomon *et al* demonstrated that deletion of MSyn in *S.nodorum* markedly reduces spore germination and decreases hyphae length[39]. In our study, we found that succinylation occurred at six lysine residues of HCAG_05089 (ICL, K55, K105, K185, K196, K226 and K400), and four lysine residues of HCAG_05084 (MSyn, K148, K319, K339 and K501). Of the six succinylated lysine residues in ICL, K226 is next to Mg^{2+}/Mn^{2+} binding site D231.

Additionally, we found that two proteins involved in the urea cycle, carbamoyl phosphate synthase 1 (CPS1) and argininosuccinate synthetase (ASS), are substrates of lysine succinylation. CPS1 plays a critical role in initiating the urea cycle by transferring an ammonia molecule from glutamine or glutamate to a molecule of bicarbonate. In mouse liver, CPS1 was also found to be succinylated. CPS1 showed reduced activity in mitochondrial sirtuin SIRT5 (responsible for catalyzing the desuccinylation of lysine residues) KO mice[40].

3.6 Lysine Succinylation on Histone Proteins in *H. capsulatum*

The core histones containing H2A, H2B, H3 and H4 are highly conserved in eukaryotes. Many amino acids in histones are subject to diverse PTMs including lysine acetylation, lysine succinylation, lysine ubiquitination, and serine/threonine phosphorylation. These are usually

associated with a wide range of biological processes and disease states[41]. In 2012, 7, 7, 10 and 13 lysine succinylation sites on histones were identified in *S. cerevisiae*, mouse embryonic fibroblast, *Drosophila* S2 and HeLa cells, respectively[6]. Recently, 7 lysine succinylation sites on histones were also found in *Toxoplasma*[13]. However, lysine succinylation sites on histones in *H. capsulatum* have not been previously identified. In our study, we were the first to identify13 lysine succinylation sites on histones in *H.capsulatum*, of which one site is on histone H2A (HCAG_04914), seven on histone H2B (HCAG_03525), three on histone H3 (HCAG_06701), andtwo on histone H4 (HCAG_03885), respectively (Table 1). Half of the identified succinylation sites on histones in our study are conserved among a variety of eukaryotic species (Figure 5 and Table 1). For example, H2B K58suc was found in *H. capsulatum*, *D. melanogaster*[6], *S. cerevisiae*[6] and *T. gondii*[13]. H3 K80suc occurred in *H. capsulatum*, *D. melanogaster*[6], *S. cerevisiae*[6], *M. musculus*[6], *Tomato* [42]and *H. sapiens*[6]. H4K32suc was also found in six species including *H. capsulatum*, *D. melanogaster*[6], *S. cerevisiae*[6], *M. musculus*[6], *T. gondii* [13] and *H. sapiens*[6] (Figure 5 and Table 1). In addition, only one lysine site was succinylated in *H. capsulatum* histone H2A, and the site is different from that in humans[6], *Drosophila*[6], and *S.cerevisiae*[6], indicating that it is a *Histoplasma*-specific H2A succinylation site.

Most lysine succinylation sites are located in the C terminus of histones but not in the N-terminal tail, which are among the amino acid residues readily accessible by some reactive chemicals. For example, the succincylation site of histone H2A (K44) and one of two succinylation sites in histone H4 (K78) were the DNA binding sites. The K57 of *H. capsulatum* histone H3 is equivalent to K56 in yeast H3,whichis located next to the site of nucleosome entry and to the DNA double helix[43]. In yeast, K56 acetylation of H3 is important for histone deposition. K56 substitution of H3 with glutamic acid, mimicking succinylation, can result in cell lethality[44]. This implies that *H. capsulatum* histone H3 K57 succinylation might be very important for cell viability.

Table 1 Succinylation sites of histone in different organisms

Organism	H2A	H2B	H3	H4
H. capsulatum	K44	K8, K46, K58, K91, K97, K120, K128, K132	K43, K57, K80	K32, K78
Drosophila S2 cells	K35	K43, K113, K117	K56, K79	K31, K77, K79, K91
T. gondii		K37, K70, K99, K107	K56, K122	K31
S. cerevisiae cells	K13, K21	K37, K49	K79	K31, K77
mouse MEF cells		K120	K56, K79	K31, K77, K79, K91
HeLa cells	K9, K95	K34, K116, K120	K14, K56, K79, K122	K12, K31, K77, K91
Tomato (*Solanum lycopersicum*)		K16	K80	

4 Discussion and Conclusion

In this work, we identified a total of 463 succinylation sites from 202 succinylated proteinsusing proteomics. Technological advances may unveil further sites of lysine succinylation in *H. capsulatum*, as shown in other microorganisms. For example, in 2011,a total of 69 succinylation sites in 14 succinylated proteins from *E. coli* cells were mapped[5]. Two years later, a large-scale identification of succinylated proteins was achieved by succinyllysine enrichment, strong cation exchange (SCX)-based peptide fractionation and MS identification, resulting in the identification of more than 2572 succinylation sites on 990 proteins[10]. It is likely that the catalog of succinylated proteins in *H. capsulatum* will continue to expand with the development of technology. However, our study represents the first lysine succinylome dataset for *H. capsulatum*, which not only increases the known PTM types of *H. capsulatum* (the first global file of PTM-lysine acetylation in *Histoplasma* was recently reported) but also greatly expands the knowledge of lysine succinylation sites and succinylated proteins in this fungus.

During comparison of the *H. capsulatum* succinylome with the previously reported succinylome of *E. coli*, we found that the number of succinylated proteins in *H. capsulatum* is smaller than that in *E. coli*. Colak and co-workers found more than 2500 succinylation sites in *E. coli*[11], but we just found 463 succinylation sites in *H. capsulatum*. Studies have shown that during the evolution from bacteria to eukaryotes, the number of succinylated proteins appears to have decreased[10].

GO functional and enrichment analyses revealed that the identified succinylated proteins in *H. capsulatum* are involved in diverse cellular processes, particularly in metabolic pathways. Recent succinylome studies indicate that some enzymes involved in metabolic process are over-represented [7, 12, 13, 28]. Our data are in agreement with previous studies concerning various bacteria and eukaryotes, implying that there is a common mechanism for succinylation involved in regulating metabolic pathways. KEGG pathway analysis showed that more than 100 succinylated proteins were involved in diverse metabolic processes including glycolysis, the TCA cycle, glyoxylate and dicarboxylate metabolism.

PPIs are important for various biological processes. In former studies, the PPI network of lysine succinylated proteins was only constructed in bacteria and plants. For example, in*M. tuberculosis*[7] and *Taxus* × media[45]. Our study provided the first global PPI network of succinylated proteins in fungi. Interestingly, the sub network of ribosomal and metabolic pathways presented comparatively high enrichment in *H. capsulatum*, which confirmed the critical regulatory role of lysine succinylation in ribosomal and metabolic pathways.

Increasing evidence indicates that histone modification may have an important role in epigenetic gene regulation. In some eukaryotic species including *S. cerevisiae*, *T. gondii*[13] and *S.lycopersicum*[42], histone succinylation has previously been reported. H2B K58suc, H3 K80suc and H4K32suc were confirmed in our study, which is in agreement with recently published results in other species, indicating the three Ksuc sites are highly conserved.

It will be meaningful to explore the detailed biological function of these succinylated proteins using several biochemical and molecular methods, such as co-immunoprecipitation, knock out, and site-mutation. It will be interesting to characterize the enzyme responsible for succinylation and the crosstalk between lysine acetylation and succinylation in *H. capsulatum* in the future.

Notes

The authors have declared no conflict of interest.

ACKNOWLEDGMENTS

The work was supported by the National Basic Research Program of China (2013CB531606), National Science and Technology Major Projects of China Grant (2013ZX10004612001), National Natural Science Fund (31270180, 81371851) and Shanghai Key Laboratory of Molecular Medical Mycology Fund (14DZ2272900).We thank Rachel Kandt (Rutgers University, USA) for critical reading and grammar checking of this manuscript.

Captions for Figures

Figure 1. 16 lysine succinylation sites present in HCAG_04686. Red sequences indicate the Ksuc sites. Blue sequences indicate the active sites. Underlined sequences indicate histidine kinase-like ATPase domains. (B) Comparison of succinylated proteins in *H. capsulatum* and *S. cerevisiae*.

Figure 2. Go classification of the identified succinylation proteins based on biological processes; molecular function.

Figure 3.Protein-protein interaction network of all the lysine succinylated proteins in *H. capsulatum*. The whole PPI network, bubble size represents that the number of succinylation sites; (B) Two clusters of highly interconnected lysine-succinylated protein networks. Interaction network of lysine-succinylated proteins (listed in gene names) was analyzed by the Cytoscape software (version 3.0.1).

Figure 4. KEGG pathway enrichment of all identified Ksuc proteins; (B) Succinylated proteins are marked red in TCA cycle, glycolysis/ gluconeogenesis, and glyoxylate cycle.

Figure 5.Comparison of succinylated residues in histone proteins of *H. capsulatum* (Hc) that of *T. gondii*[13], *S. cerevisiae* (Sc) [6], *D. melanogaster* (Dm) [6], *M. musculus* (Mm) [6], *S. lycopersicum* (*Sl*) [42] and *H. sapiens* (Hs) [6]. Red sequences indicate the succinylation sites. Numbers below the sequences represent the amino acid position.

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A

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SDPSKLDSNK DLRIDIIPDK ENKTLTISDT GIGMTKADLV NNLGTIARSG
        110        120        130        140        150
TKQFMEALTA GADISMIGQF GVGFYSAYLV ADKVTVISKH NDDEQYMWES
        160        170        180        190        200
SAGGTFKITQ DTDGEPLGRG TKMILHLKDE QTEYLNESKI KEVVKKHSEF
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ISYPIYLHVL KETEKEVPDE DAEEVKDEGD DKAPKVEEVD DDEEDKTKEK
        260        270        280        290        300
KTKKIKETKI EEEELNKTKP IWTRNPADIT QEEYASFYKT LSNDWEDHLA
        310        320        330        340        350
VKHFSVEGQL EFRAILFVPK RAPFDLFETK KTKNNIKLYV RRVFITDDAT
        360        370        380        390        400
DLIPEWLSFI KGVVDSEDLP LNLSRETLQQ NKIMKVIKKN IVKKTLELFN
        410        420        430        440        450
EIAEDREQFD KFYSAFSKNI KLGIHEDAQN RPALAKLLRF NSTKSGDETT
        460        470        480        490        500
SLTDYVTRMQ EHQKQMYYIT GESLKAVQKS PFLDTLKEKN FEVLFLVDPI
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DEYAMTQLKE FDGKKLVDIT KDFELEETEE EKKAREAEEK EYEGLAKSLK
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VD
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B

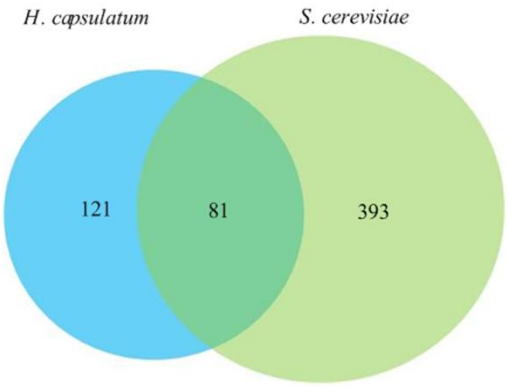


Figure 1

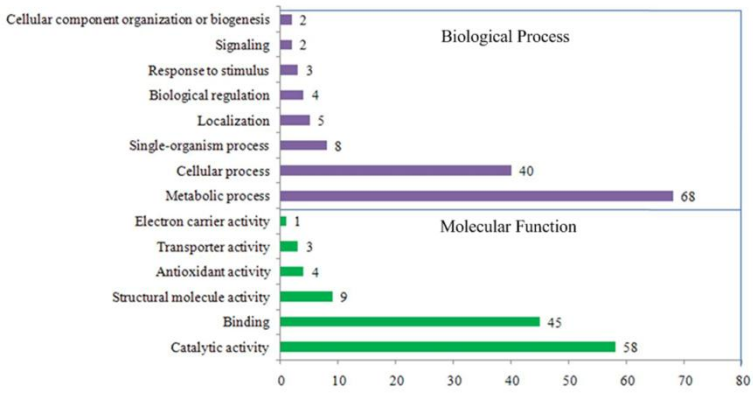


Figure 2

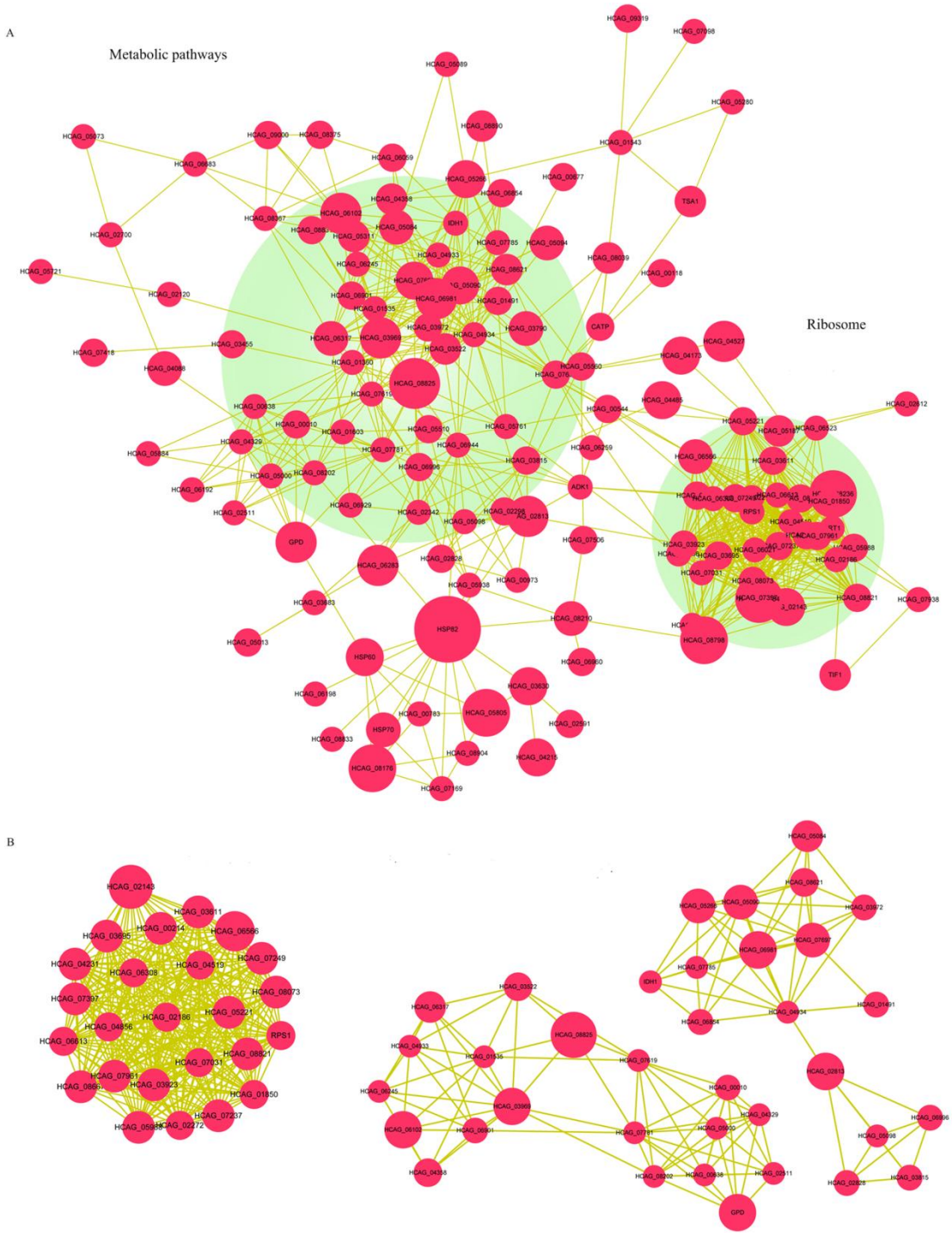


Figure 3

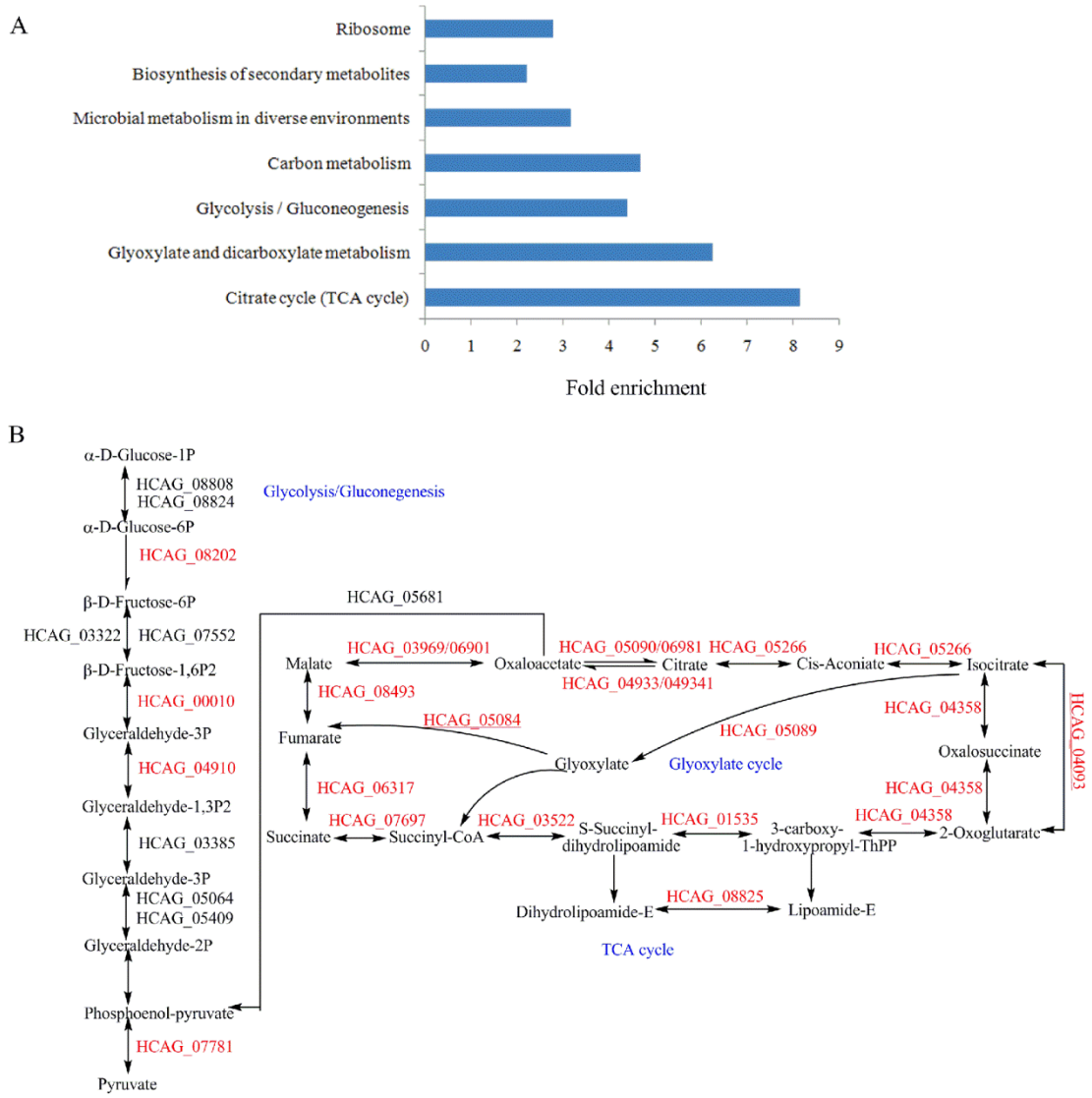


Figure 4

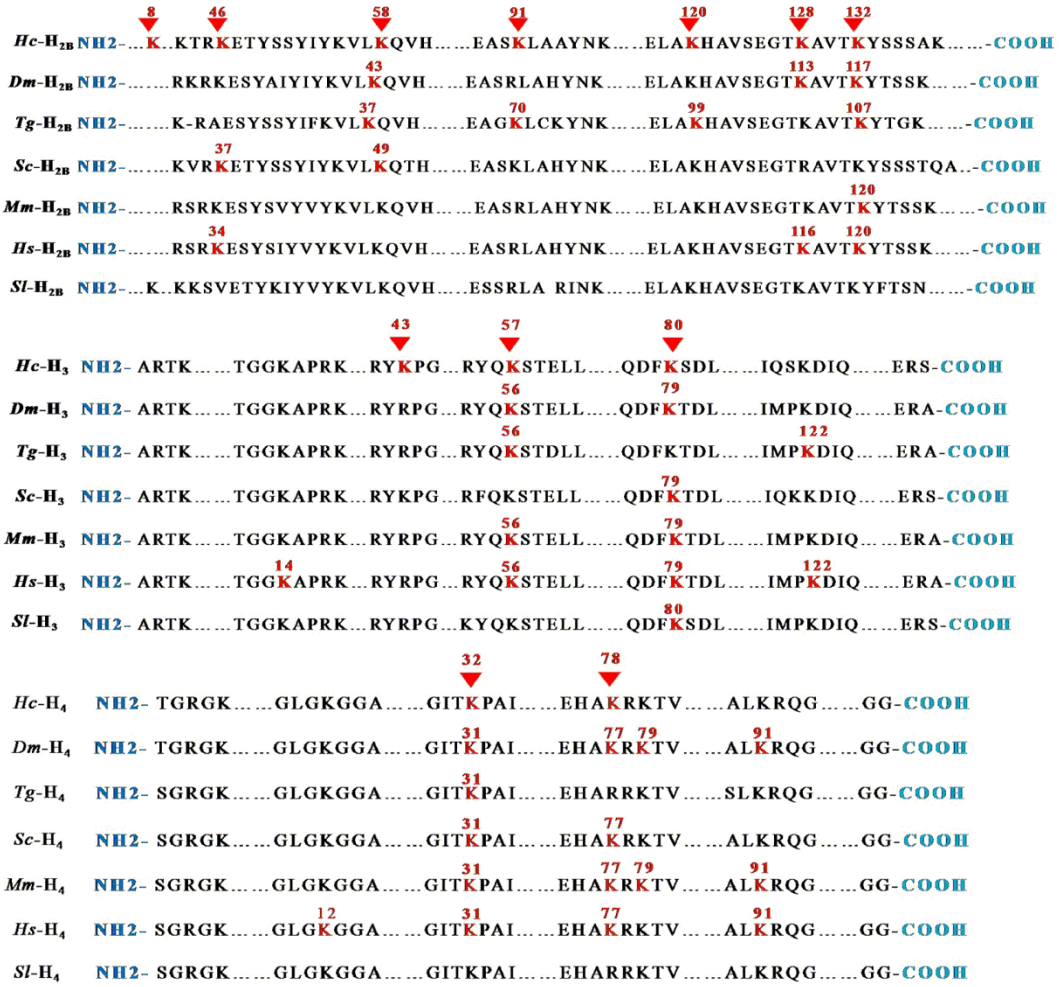
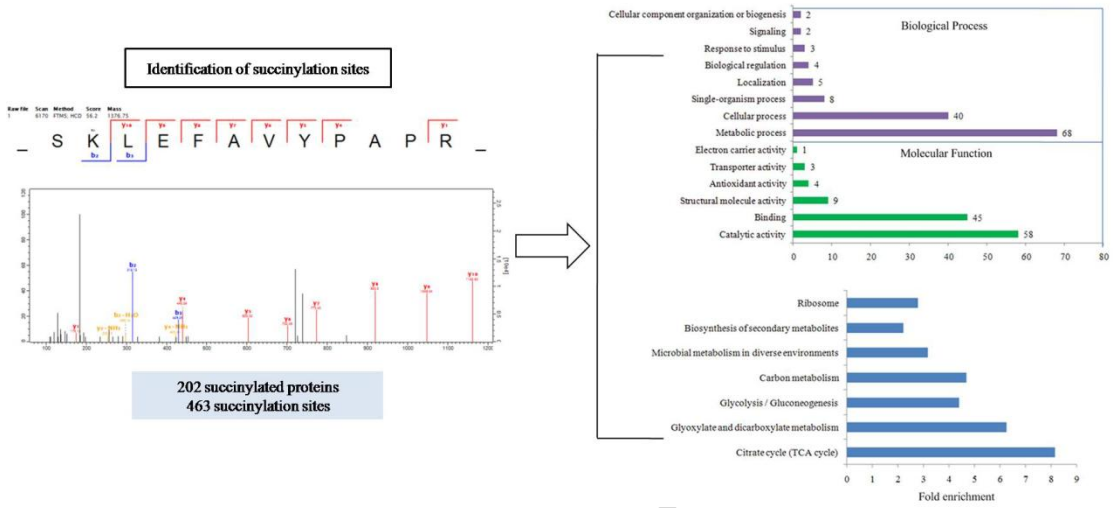


Figure 5

Significance

H. capsulatum is the causative agent of lung disease histoplasmosis. The ability of *H. capsulatum* yeasts to infect and proliferate within macrophages as an intracellular pathogen can be contributed to several virulence factors and metabolic regulation. Lysine succinylation was recently shown to play a critical role in the metabolism regulation of *Candida albicans*. *H. capsulatum* succinylated proteins were firstly characterized in this work, and bioinformatics results showed that this modification may also be relevant with central metabolism in *H. capsulatum*. New succinylation sites on histones were reported. This represents an important resource to address the function of *H. capsulatum* lysine succinylation.



Graphical abstract

Highlights:

1. Our data provides the first global survey of succinylation in *Histoplasma capsulatum* and we identified a total of 463 lysine sites in 202 proteins.
2. More than 52% of the succinylated proteins are metabolic enzymes, which is consistent with previous reports.
3. Thirteen lysine succinylation sites on histones including H2A, H2B, H3 and H4 in *H. capsulatum*.