Global Lysine Acetylation and 2-Hydroxyisobutyrylation Profiling Reveals the Metabolism Conversion Mechanism in *Giardia lamblia*

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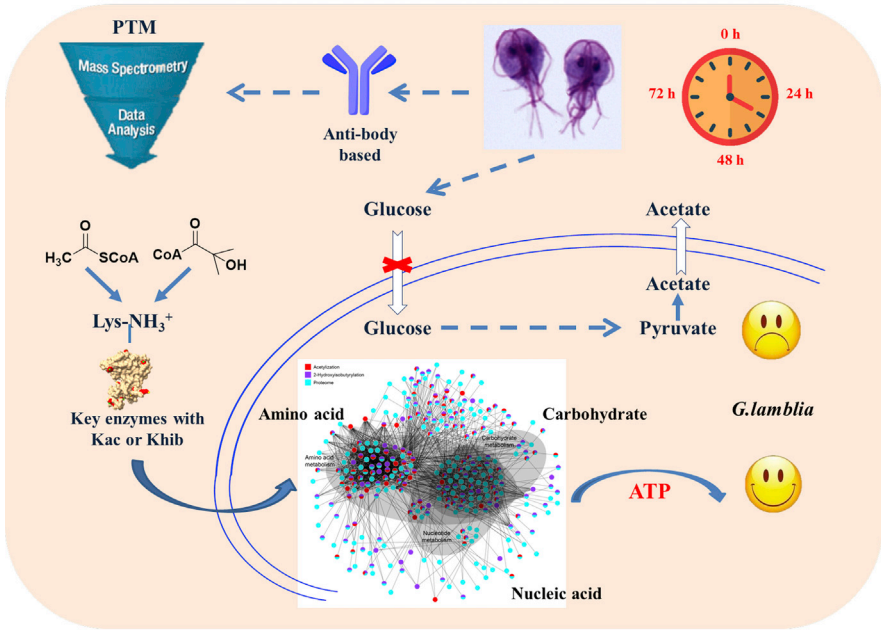
Graphical Abstract



In Brief

We performed a comprehensive lysine acetylation and 2-hydroxyisobutyrylation profiling to explore metabolism conversion in *G. lamblia*. A total of 2999 acetylation sites on 956 proteins and 8877 2-hydroxyisobutyryl sites on 1546 proteins were quantified under sugar starvation condition. Integrated data analysis revealed that glucose was not the only source of energy for *G. lamblia*, and a metabolism conversion occurred in *G. lamblia*. This study provides a valuable resource for understanding the molecular mechanism in metabolism conversion in *G. lamblia*.

Highlights

- A metabolism conversion occurs in *Giardia lamblia*.
- Glucose is not the only source of energy for *G. lamblia*.
- Kac and Khib provides insight into metabolism conversion mechanism in *G. lamblia*.
- Findings provide useful information of the evolutionary rule from prokaryote to eukaryote.

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Global Lysine Acetylation and 2-Hydroxyisobutyrylation Profiling Reveals the Metabolism Conversion Mechanism in *Giardia lamblia*

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***Giardia lamblia* (*G. lamblia*) is the cause of giardiasis, a common infection that affects the general population of the world. Despite the constant possibility of damage because of their own metabolism, *G. lamblia* has survived and evolved to adapt to various environments. However, research on energy-metabolism conversion in *G. lamblia* is limited. This study aimed to reveal the dynamic metabolism conversion mechanism in *G. lamblia* under sugar starvation by detecting global lysine acetylation (Kac) and 2-hydroxyisobutyrylation (Khib) sites combined with quantitative proteome analyses. A total of 2999 acetylation sites on 956 proteins and 8877 2-hydroxyisobutyryl sites on 1546 proteins were quantified under sugar starvation. Integrated Kac and Khib data revealed that modified proteins were associated with arginine biosynthesis, glycolysis/gluconeogenesis, and alanine, aspartate, and glutamate metabolisms. These findings suggest that Kac and Khib were ubiquitous and provide deep insight into the metabolism conversion mechanism in *G. lamblia* under sugar starvation. Overall, these results can help delineate the biology of *G. lamblia* infections and reveal the evolutionary rule from prokaryote to eukaryote.**

Giardia infection is a true zoonosis with a risk of infection for the general population, thereby posing a considerable threat to human and animal health globally (1, 2). Studies have reported that individuals with immune deficiencies, such as HIV/AIDS, and organ transplantation are more likely to have *Giardia* infection (3). The symptoms of giardiasis appear 6 to 15 days after infection, and it is characterized by diarrhea or greasy stool, fatigue, nausea, bloating, abdominal cramps, excessive gas, and headache (4, 5). Giardiasis infection leads to substantial production losses to the livestock industry and should be given increased attention. The classical therapeutic targets against *Giardia* are some points in the metabolic pathway or the key molecule in the signal-transduction pathway closely related to infection and pathogenicity (6). Despite the remarkable insights into energetic metabolism in different organisms from yeast to humans, research on energy metabolism conversion in protozoan parasites is limited.

Beyond its public health relevance, *Giardia* represents a valuable and fascinating model microorganism that provides a unique opportunity to define basal and ancestral eukaryotic functions (7). Genome analysis has verified that *Giardia* is an early diverging eukaryote and that the entire genome is nearly complete, rendering *Giardia* a suitable model organism (8). The life cycle of *Giardia* is relatively simple, involving two developmental stages of rapid trophozoite multiplication and infectious cyst formation, transmitted *via* the fecal–oral route (9). Sequencing of the genome contained in two diploid nuclei per trophozoite has revealed prokaryote-like features, such as short promoter sequences, a nearly complete absence of introns, and high similarities among some key enzymes of energy and intermediate metabolisms with prokaryotic enzymes, such as eubacterial-like pyruvate, ferredoxin oxidoreductase, NADP-dependent glutamate dehydrogenase, and fructose bisphosphate aldolase, suggesting acquisition by lateral transfer (10).

Because of lack of mitochondria and cytochrome-mediated oxidative phosphorylation, *Giardia* rely on fermentative metabolism (even when oxygen is present) for energy conservation (11, 12). Glucose is the only source of energy for *Giardia lamblia*. However, increasing published data reveal that glucose catabolism cannot account for all end products of *G. lamblia*, so other carbon sources must be utilized in mysterious organisms (13). Research has indicated that a number of amino acids are involved in energy metabolism during the proliferation of *G. lamblia* in culture (14, 15). This finding indicates possible imbalance in

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carbon fluxes when glucose availability is limited. However, the regulatory process in this metabolism conversion is unclear. Understanding this dynamic regulation is critical to explore the pathogenic mechanism and designing anti-giardial drugs. The findings may reveal the evolution rule from prokaryote to eukaryote from the perspective of energy metabolism.

Post-translational modifications (PTMs) of proteins influence cellular localization, stability, interaction, and enzymatic activity (16–18). For example, acylation has been identified to occur in thousands of proteins in cellular metabolisms and play important roles in metabolic regulation (19, 20). Cells under nutrient deprivation show a dramatic decrease in cellular acetyl-CoA levels owing to the restrained oxidation of pyruvate and fatty acids. Prolonged low levels of acetyl-CoA may cause increased NAD^+/NADH ratio to enhance NAD^+-dependent sirtuins' deacetylation activities and decrease nonenzymatic acetylation processes that affect the cellular acetylation status of most proteins (17, 21). Accordingly, the upregulated acetylation of proteins is critical for cell survival. Hydroxyisobutyrate is a precursor for the synthesis of 2-hydroxyisobutyryl-CoA and lysine 2-hydroxyisobutyrylation (Khib), a new type of histone PTM. This histone mark has unique features that differ from the extensively studied histone lysine acetylation (Kac) and methylation (Kme) marks. Khib has a unique chemical structure, specific genomic distributions, and varied dynamics among diverse model systems (22, 23). This newly identified chemical modification occurs in a variety of organisms and plays roles in the biosynthesis and degradation of proteins and energy metabolism (24, 25). However, how this modification is regulated remains unclear, and the biological consequences of this modification are almost completely unknown. Thus, exploring the proteins with acetylation and 2-hydroxyisobutyrylation regulation can provide effective targets for biological function.

The apparent successful control of *G. lamblia* infection is deceptive. Important challenges remain in the treatment of giardiasis. PTM biology clearly shows that the exploration of crucial regulatory proteins for a PTM pathway is a key point for investigating its biology. In the present study, we performed a comprehensive Kac and Khib study to explore the metabolism conversion in *G. lamblia*. A total of 2999 acetylation sites and 8877 2-hydroxyisobutyryl sites were identified for functional and pathway enrichment analyses and interactive networks under sugar starvation culturing of *G. lamblia*. The finding can help understand the biology of *G. lamblia* infections. The mechanism of starvation is a very complicate phenomenon, with huge difference from protozoan to mammals. Thus, identifying the key pathway is only one part of the phenomenon. It would be very useful to read the key references from different model cells or animals including human, laying a foundation for drug design.

EXPERIMENTAL PROCEDURES

Ethics Approval

This project was approved by the ethics committee of Jilin Medical University (number 2017-LW022).

Experimental Design and Statistical Rationale

In this study, we performed quantitative proteomics based on tandem mass tag label–based quantitative analysis technique to test the difference between culturing *G. lamblia* under sugar starvation and normal culture. For protein quantitation, three biological replicate experiments were performed, and each experiment was separated 10 fractions by HPLC. For acetyl-proteomics and 2-hydroxyisobutyrylation-proteomics enrichment, we performed three biological replicate experiments, and each experiment was separated four fractions by HPLC. Student's *t* test and *F* test (joint hypotheses test) were used in the study of acetyl-proteomics and 2-hydroxyisobutyrylation-proteomics, and *p*-values smaller than 0.05 were considered as significantly regulated.

Cultivation of G. lamblia

Trophozoites of *G. lamblia* isolate C2, collected from a patient in Southwest China, were axenically cultured in modified trypticase yeast extract iron-serum-33 medium (TYI-S-33), pH 7.0, supplemented with 10% heat-inactivated bovine serum (Hangzhou Sijiqing Biological Engineering Materials Company) and 0.05% bovine bile (Sigma) in borosilicate glass screw-cap culture tubes, as described previously (26). The culture was started with 4×10^3 trophozoites per 4 ml per tube at 37 °C without shaking and subcultured three times a week.

Cultivation of G. lamblia *Under Sugar Starvation*

To collect the logarithmic *Giardia* trophozoites, the cultures were chilled on ice for 20 min to detach the trophozoites from the walls of the tubes. The parasites were pelleted by centrifuging at 367*g* at room temperature for 10 min and then washed with PBS twice. The freshly collected trophozoites were inoculated in TYI-S-33 without glucose as stated previously and subcultured every 24 h with TYI-S-33 without glucose.

Growth Curve

To establish the growth curves for culturing *G. lamblia* under different conditions, trophozoites in the logarithmic phase were initiated with trophozoites per 4 ml of medium per tube at 37 °C. Counts were performed each 24-h period for consecutive days. For each reading, one tube of culturing *G. lamblia* was placed on ice for 20 min and was later gently inverted to detach trophozoites. Tubes were centrifuged at 2000*g* for 10 min, supernatants were discarded, and pellets resuspended in 1 ml of PBS. After homogenization, aliquots were removed for parasite counts in Neubauer chamber. Three independent experiments were performed.

Determination of ATP Content

Logarithmic trophozoites of *G. lamblia* cultured with TYI-S-33 were transferred to sugar-free medium. Then, the parasites were harvested and lysed on ice with somatic cell ATP-releasing reagent at 24, 48, and 72 h, respectively. The ATP content was detected by ATP assay kits (Genmed Scientifics, Inc) according to the manufacturer's instructions (27).

Cultivation of G. lamblia *for Omics Analysis*

Logarithmic trophozoites of *G. lamblia* cultured with TYI-S-33 were transferred to sugar-free medium for 24, 48, and 72 h, respectively. The parasites were pelleted by centrifuging at 367*g* at room

temperature for 10 min and then washed with PBS, pH 7.4. The parasite pellets were resuspended in 1× PBS and were snap frozen at −80 °C for omics analysis. Three independent experiments were performed for omics analysis.

Acetyl-Proteomics

Western Blot Analysis—The *G. lamblia* was dissolved in SDS-PAGE loading buffer (250 mM Tris, 1.92 M glycine, and 1% SDS), electrophoresed on an SDS-PAGE gel, and transferred to polyvinylidene difluoride membrane. After being blocked with Tris-buffered saline containing Tween 20 and 5% skim milk for 1 h at 37 °C, the membrane was incubated in Tris-buffered saline containing Tween 20 containing 5% skim milk and the antiacetyl antibody (1:1000 dilution; PTM BIO) for 12 h at 4 °C. Next, the appropriate secondary antibody was added, and incubation was done for 1 h at room temperature. Immunoreactive protein bands were detected by using enhanced chemiluminescence reagents.

Enrichment of Acetylated Peptides and Sample Preparation—Four volumes of lysis buffer (8 M urea, 1% Triton-100, 10 mM dithiothreitol, and 1% Protease Inhibitor Cocktail) were added to the harvested parasites, followed by sonication three times on ice using a high-intensity ultrasonic processor (Scientz). The remaining debris was removed by centrifugation at 20,000*g* at 4 °C for 10 min. Finally, the protein was precipitated with cold 20% trichloroacetic acid for 2 h at −20 °C. After centrifugation at 12,000*g* at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with cold acetone for three times, and total protein was quantified by BCA kit. After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum dried. Peptide was reconstituted in 0.5 M triethylamine bicarbonate and mixed with tandem mass tag/iTRAQ reagent. Then incubated for 2 h at room temperature and pooled, desalted, and dried by vacuum centrifugation. The peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, and 250 mm length). Samples were first separated with a gradient of 8 to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into four fractions and dried by vacuum centrifuging.

To enrich acetyl-modified peptides, tryptic peptides dissolved in 100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, 0.5% nonyl phenoxypolyethoxylethanol-40, pH 8.0 buffer were incubated with prewashed antibody beads at 4 °C overnight with gentle shaking. Then the beads were washed four times with 100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, 0.5% nonyl phenoxypolyethoxylethanol-40, pH 8.0 buffer and twice with H_2O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. Finally, the eluted fractions were combined and desalted with C18 ZipTips (Millipore).

LC–MS/MS Analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a homemade reversed-phase analytical column (15-cm length, 75 μm i.d.). The gradient comprises an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min, and climbing to 80% in 3 min, then holding at 80% for the last 3 min, all at a constant flow rate of 400 μl/min on an EASY-nLC 1000 ultraperformance liquid chromatography system.

The peptides were subjected to nanospray ion source followed by tandem MS (MS/MS) in Q ExactiveTM Plus (Thermo Fisher Scientific, Inc) coupled online to the ultraperformace liquid chromatography. The electrospray voltage applied was 2.0 kV. The *m/z* scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using normalized collision energy setting as 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with a dynamic exclusion of 15.0 s. Automatic gain control was set at 5E4. Fixed first mass was set as 100 *m/z*.

The resulting MS/MS data were processed using MaxQuant search engine (version 1.5.2.8). Tandem mass spectra were searched against the UniProt Giardia intestinalis WB clone C6 (7154 entries) concatenated with reverse decoy database. Acetylation ($C_{(2)}H_{(2)}O$) on Lys was specified as variable modifications. Trypsin/P was specified as cleavage enzyme allowing up to four missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in first search and 5 ppm in main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification, and acetylated modification and oxidation on Met were specified as variable modifications. The modified peptides with their false discovery rate (FDR) smaller than 0.01 and minimum scores greater than 40 were extracted.

2-Hydroxyisobutyrylation-Proteomics

The samples were harvested as described in proteome analysis of Kac. The total proteins were isolated by 20% trichloroacetic acid and digested by trypsin. The 2-hydroxyisobutyrylated peptides were enriched with pan-anti-Khib–conjugated resin (PTM BioLabs; catalog no. PTM-804) and subjected to HPLC–MS/MS analysis. The HPLC–MS/MS analysis condition was the same as proteome analysis of Kac.

Bioinformatics Analysis

Reliability of the Quantification Between Biological/Technical Replicates—In order to evaluate reliability of the quantification among biological/technical replicates, we performed Pearson correlation analysis and coefficient of variation analysis.

Protein Function Annotation Analysis—Functional enrichment analysis for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and protein families database domain was performed by a hypergeometric test in using GOstats package in R (https://www.r-project.org/) (28). Significant enriched KEGG pathways ($p < 0.01$) and protein families database domains ($p < 0.01$) are selected. Protein complexes were enriched basing on that manually curated CORUM protein complex database (https://mips.helmholtz-muenchen.de/corum/) (29) for all mammals using a hypergeometric test. Unbiased Gene Ontology (GO) terms and UniProt keywords enrichment analyses were performed using a web tool with default parameters (https://agotool.sund.ku.dk).

Protein–Protein Interaction Network Analysis—Protein–protein interaction information was obtained from STRING database (version 10; http://www.string-db.org/). The protein–protein interaction network for the proteome analysis of Kac and 2-hydroxyisobutyrylation-proteomics was constructed. Only the interactions with highest confidence scores (above 0.9) were selected. The protein–protein interaction network was visualized by Cytoscape (version 3.2.1).

Clustering of Functions for Differentially Expressed Proteins and Modified Proteins—In order to explore the potential connections and differences in biological functions (GO, KEGG pathway, protein domains, etc.) of differentially expressed proteins or differentially modified proteins, we clustered the functional enrichment results of differentially expressed proteins and modified proteins.

First of all, we performed GO, KEGG pathway, and protein domain enrichment analyses on proteins, acetylated modifications, and 2-hydroxyisobutyryl modifications. Then, groups were divided according to different time points, which were 24 *versus* 0 h, 48 *versus* 0 h, and 72 *versus* 0 h. Each group contains three types of data: proteome, acetylation modification, and 2-hydroxyisobutyryl modification. Afterward, we collected the functional classification enriched by each group and the corresponding enriched *p*-value. Functional classifications that were significantly enriched ($p < 0.05$) in at least one protein group were

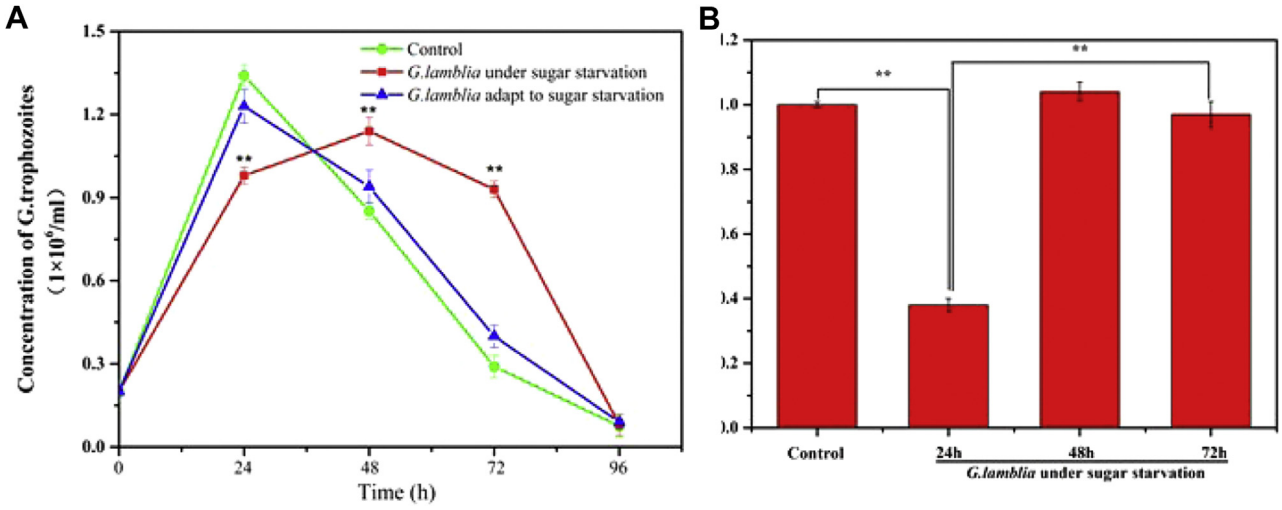

FIG. 1. **Effect of sugar starvation on growth and ATP production in *Giardia lamblia*.** *A*, growth curve under sugar starvation. *B*, effect of sugar starvation on ATP production in *G. lamblia*. *G. lamblia* cultured in trypticase yeast extract iron-serum-33 medium was used as control. Data are presented as mean ± SD, $n = 5$. Statistical significance: $^{**}p < 0.01$.

retained. The *p*-values of function enrichment data matrix undergo -ln and Z score transformation. Hierarchical clustering (Euclidean distance, average connected clustering) method was carried out for cluster analysis. The clustering relationship is visualized using the heat map package in R. The horizontal axis of the heat map represents different groups, and the vertical axis is a description of functions (GO, KEGG pathway, and protein domain). The color indicates the degree of enrichment. *Red* means strong enrichment, and *blue* means weak enrichment. The figure shows the top 50 significantly enriched features.

Analysis of Dichloroacetate and 2-Deoxy-D-Glucose on G. lamblia *Glycolysis*—Logarithmic trophozoites of *G. lamblia* cultured in TYI-S-33 with 200 mM dichloroacetate (DCA) or 10 mM 2-deoxy-D-glucose (2DG) and *G. lamblia* were cultured for 24 h. Then, the growth and ATP production was detected.

D-Ribose Activates Hexose Phosphate Bypass—*G. lamblia* was cultured in sugar-free medium at 24 h. Then, the D-ribose was added to the medium and cultured for another 24 h. The parasites were harvested and lysed on ice with somatic cell ATP-releasing reagent. The ATP content was detected by ATP assay kits (Genmed Scientifics, Inc) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± SEM. All data were analyzed by one-way ANOVA by using SPSS, version 13 software (SPSS Inc., Chicago, IL), and expressed in mean ± standard deviation. A *p*-value of <0.05 was considered significant.

RESULT

Growth Curve Under Sugar Starvation

The growth curves of *G. lamblia* under sugar starvation results revealed that *G. lamblia* grew quickly in TYI-S-33 medium and arrived to peak at 24 h. Then, *G. lamblia* started to show a downward trend and entered a growth recession period. After 72 h of culturing, the growth of *G. lamblia* rapidly declined. After 96 h of culturing, *G. lamblia* broke, and no live *G. lamblia* remained. However, upon culturing *G. lamblia* in TYI-S-33 medium without glucose, *G. lamblia* were grown slowly and maintained a relatively long period of stability from 24 to 72 h of culturing, after which they showed a downward trend (Fig. 1*A*). However, growth of the sugar-starved group declined more slowly than that of the normal culture group. After *G. lamblia* were subcultured in a sugar-free medium, we found that the *G. lamblia* adapted to under sugar starvation. The growth trend of the trophozoites was exactly the same as that in TYI-S-33 medium.

Effect of Sugar Starvation on ATP Production in G. lamblia

We detected the ATP generation of *G. lamblia* under sugar starvation by relevance using reagent kits. As shown in Figure 1*B*, after culturing under sugar starvation for 24 h, ATP generation of *G. lamblia* decreased quickly. However, it returned to the normal level after 48 h under sugar starvation. Although it slightly decreased for continuing culture, it remained higher than ATP production during the first 24 h and retained a stable level. The aforementioned results indicated that the sugar may not be the only energy supplied, and metabolism conversion probably existed in *G. lamblia*. For this purpose, we employed a comprehensive Kac and Khib study to explore the metabolism conversion process in *G. lamblia*.

Sugar Starvation Changes Acetylation Profile in G. lamblia

The Kac plays a crucial role in regulating central metabolism as the extensively acetylated enzymes responsible for metabolism have been found in both eukaryotes and prokaryotes (30). But no information was reported about the Kac in *G. lamblia* metabolism regulation. In this work, the acetylation level of lysine was investigated in response to the adaptability of *G. lamblia* to sugar starvation. We first tested effects of *G. lamblia* on Kac by Western blotting analysis. The result

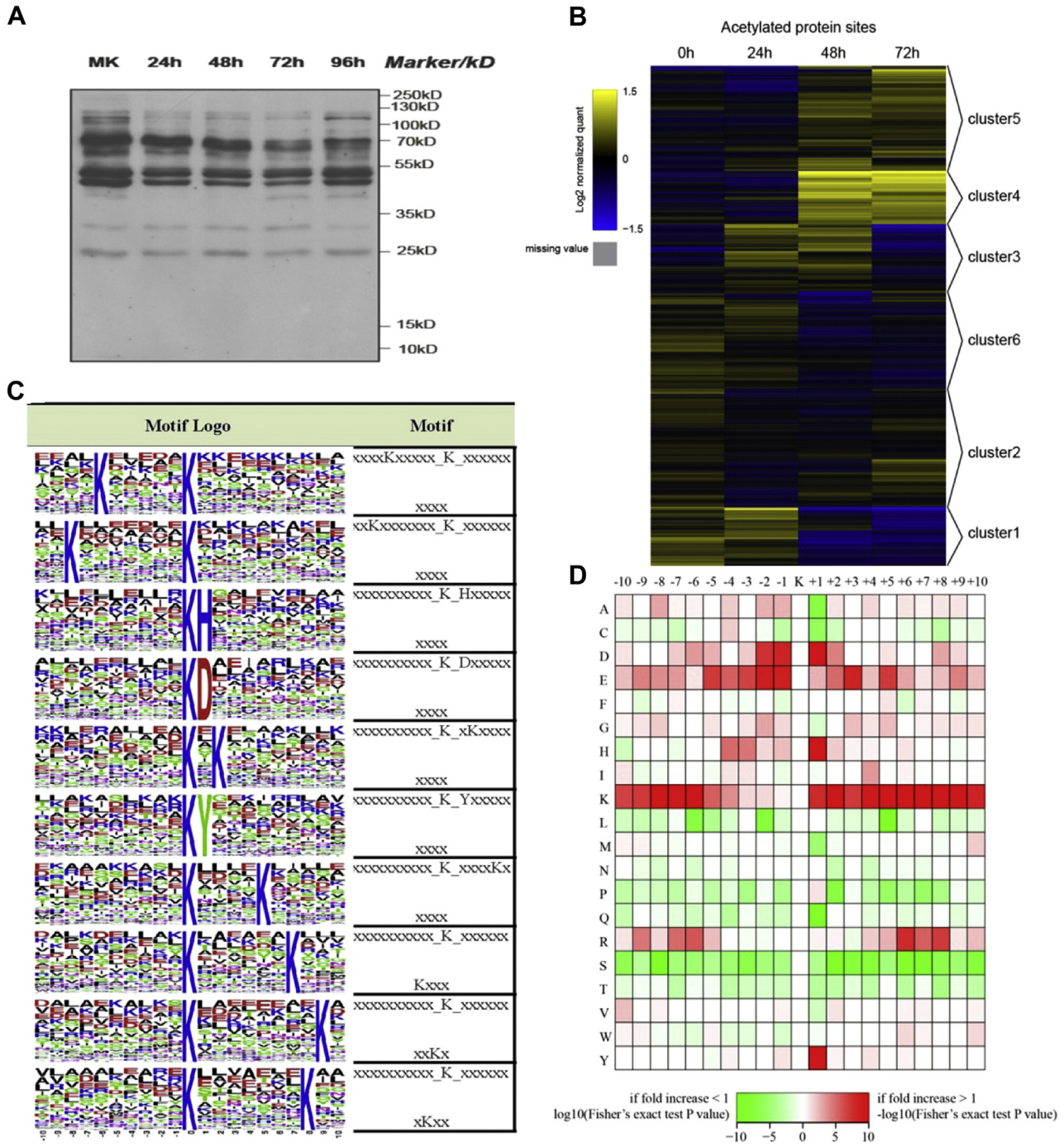

FIG. 2. **Dynamic regulation of Kac under sugar starvation culture *Giardia lamblia*.** *A*, effect of undergoing sugar starvation on Kac modification. *G. lamblia* cultured under sugar starvation were incubated from 0 to 96 h and probed for Kac by using Western blotting analysis. *B*, hierarchial clustering was conducted to cluster changes in peptide acetylation levels at different time points of sugar starvation. Whole-cell protein abundances were illustrated. Increased abundance, *yellow*; decreased abundance, *blue*; and not quantified in the proteome, *gray*. *C*, ten significantly enriched motifs were obtained from all the identified acetylated sites. The center K refers to the modified lysine. The enrichment of amino acids in positions around the modified lysine was done by MoMo based on Motif-X. *D*, heat map of the amino acids upstream and downstream of the acetylated sites shows significance change in *p*-value of frequency in different types of amino acids flanking the modified lysine.

showed a significant reduction of Kac in a time-dependent manner from 24 to 72 h. This result indicated that sugar starvation will lead to a sustained action on Kac in *G. lamblia* (Fig. 2*A*). Next, we detected the dynamic metabolism conversion process under sugar starvation changes of acetylation profile in *G. lamblia* from 24 to 72 h.

To characterize the dynamic change acetylation profile in *G. lamblia* under sugar starvation, we employed quantification of Kac and global quantification of protein expression. After culturing *G. lamblia* under sugar starvation for 24, 48, and 72 h, the parasites were harvested. The *G. lamblia* protein was extracted and digested with trypsin. Then, the lysine-acetylated peptides were enriched by immunoaffinity precipitation using pan antiacetylation antibodies, and analyses were performed in their biological replicates for all time points. The enriched peptides were subjected to HPLC–MS/MS analysis, followed by protein sequence database search for peptide identification, PTM site mapping, and quantification (supplemental Table S1). Mass error was set at 4.5 ppm for precursor ions and 0.02 Da for fragment ions. The assessments indicated that our data provide the depth, specificity, and efficiency of enrichment necessary for discovering changes in protein acetylation of *G. lamblia* during the process of adapting to sugar starvation.

To determine temporal changes in acetylation, we focused on identifications that were consistent between biological replicates of *G. lamblia* under sugar starvation at the different time points. In the present study, we identified 8534 peptides and 6844 acetylated peptides by spectral analysis. And 2999 acetylation sites on 956 proteins were identified, 2693 of them have quantitative information (supplemental Table S2).

Hierarchical clustering of quantified acetylated peptides highlighted distinct temporal profiles of either elevated or decreased acetylations during sugar starvation for *G. lamblia* culturing in different times (Fig. 2*B* and supplemental Table S3). From the result, we can see the dynamics change of acetylation modification during the energy metabolism conversion. Given the striking number of changes in acetylation levels and their specific temporal signatures, our results suggested that protein acetylation provided an important regulatory mechanism during the metabolism conversion in *G. lamblia*. Furthermore, by normalizing acetylation levels to protein abundance, we found that a majority of the identified alterations in site-specific acetylation were independent of protein abundance changes (Fig. 2*B*). This conclusion was reinforced by the low correlation between the changes in protein abundances and acetyl-peptide abundances. To determine if there were specific amino acids adjacent to acetylated lysine, we compared the sequences flanking acetylated sites by heat map. A strong bias of amino acid sequence, namely histidine (H), aspartic acid (D), glutamic acid (E), and arginine (R), was found in our data. These results indicated that lysine acetylation may be conserved and widespread (Fig. 2, *C* and *D*).

Functional Annotation and Enrichment Analysis of the Differentially Acetylated Proteins in G. lamblia

To determine the functions of acetylated proteins during the dynamic process, we analyzed GO functions and classified the identified proteins according to their biological processes (BPs), molecular functions, and cellular compartments. The result revealed a significant sign that the change of acetylation modification depends on the length of time under the sugar starvation. *G. lamblia* were under sugar starvation culture for 24 h, 63 proteins were involved in cellular processes, and 58 proteins were involved in metabolic processes. Identified acetylated protein was significantly enriched in GO BPs, including peptide biosynthetic process, amide biosynthetic process, peptide metabolic process, and single-organism carbohydrate catabolic process.

G. lamblia were under sugar starvation culture for 48 h, 145 proteins were involved in cellular processes, and 130 proteins were involved in metabolic processes. But, the identified acetylated proteins were not significantly enriched into any BP. After 72 h of culture, 136 proteins were involved in cellular processes, and 125 proteins were involved in enrichment in metabolic processes (supplemental Fig. S1*A*). Differentially acetylated proteins were significantly enriched in GO BPs, including nucleoside metabolic process, ribose phosphate metabolic process, and oxidoreduction coenzyme metabolic process (supplemental Fig. S1*B*).

The enriched KEGG pathways for acetylated proteins were analyzed (supplemental Fig. S1*C*). The results revealed that the acetylated proteins under 24 h of sugar starvation culture were enriched in the ribosome pathway. In 48 h of sugar starvation culture, the acetylated proteins were enriched in protein processing in endoplasmic reticulum pathway. In 72 h of sugar starvation culture, the acetylated proteins were enriched in metabolic pathways, including carbon metabolism, biosynthesis of antibiotics, biosynthesis of secondary metabolites, and biosynthesis of amino acids.

Khib is Dynamically Regulated Under Sugar Starvation in G. lamblia

Khib was identified in histone proteins from HeLa cells and *Saccharomyces cerevisiae* and was highly correlated with energy metabolism. However, it remained unclear whether the novel PTM exists in other organisms, especially in *G. lamblia*, a kind of valuable and fascinating model microorganism, and it was involved in the transformation of metabolic pathways. For this purpose, we first performed a detection of Khib in *G. lamblia* under sugar starvation to explore the function of Khib in energy conversion to examine the abundance and distribution of Khib in *G. lamblia* with anti-2-hydroxyisobutyryl lysine antibody by Western blot assay. As shown in Figure 3*A*, a number of 2-hydroxyisobutyrylated proteins were detected. Compared with control group where trophozoites were cultured in TYI-S-33(0 h), Khib of *G. lamblia* is more enriched in 25 to 55 KDa at different time points cultured in sugar starvation. The expression of 2-hydroxyisobutyrylated proteins was decreased in a time-dependent manner from 24 to 72 h and recovered to normal level in 96 h, which was consistent with Kac. These results suggested that sugar starvation may lead to a sustained action on Khib in

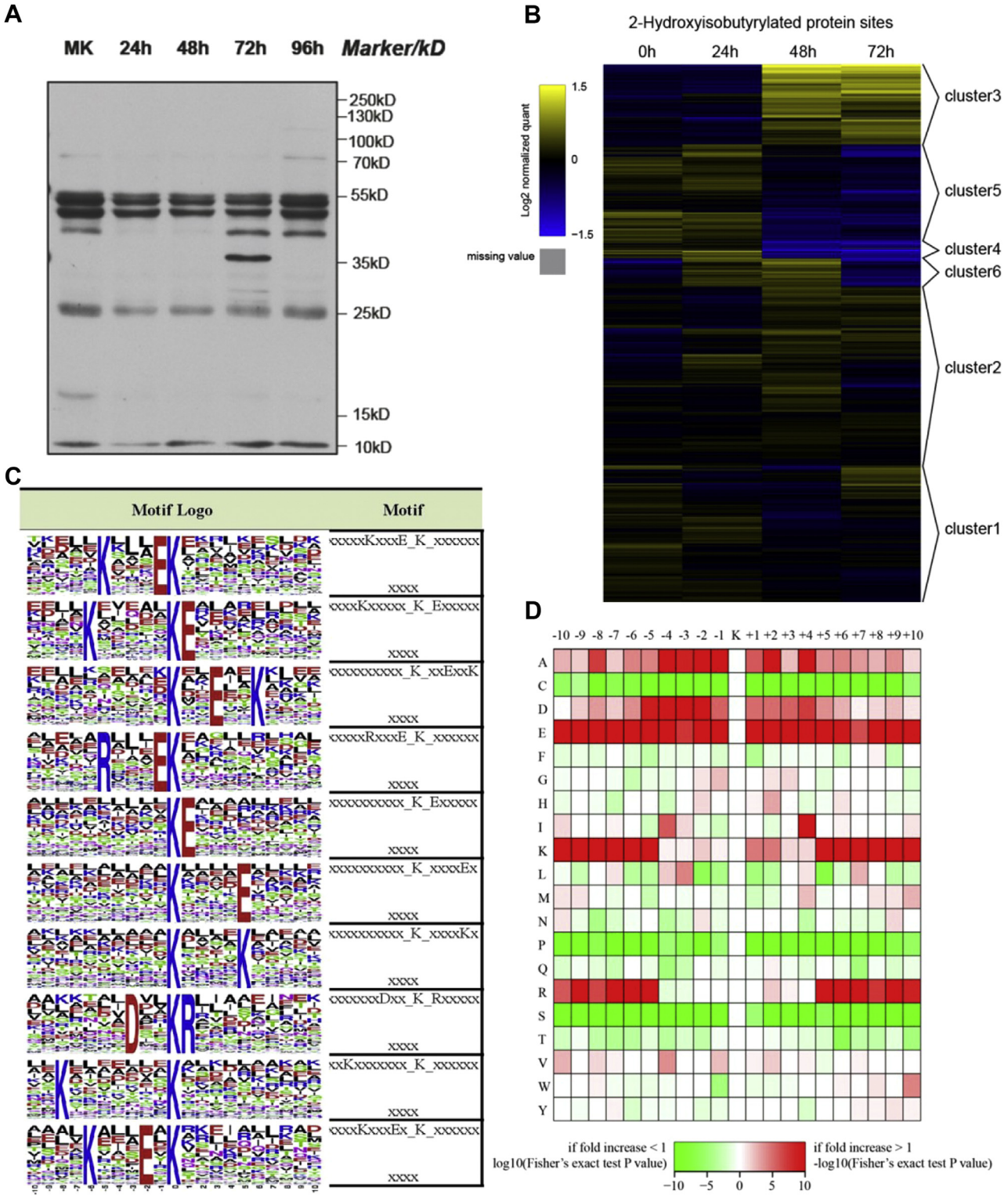

FIG. 3. **Dynamic regulation of Khib under sugar starvation culture *Giardia lamblia*.** *A*, detection of Khib by Western blot analysis. Effect of undergoing sugar starvation on Kac modification. *G. lamblia* cultured under sugar starvation were incubated from 0 to 96 h and probed for Kac by using Western blotting analysis. *B*, hierarchical clustering was conducted to cluster changes in peptide 2-hydroxyisobutyrylation levels at different time points of sugar starvation. Whole-cell protein abundances were illustrated. Increased abundance, *yellow*; decreased abundance, *blue*; and not quantified in the proteome, *gray*. *C*, ten significantly enriched motifs were obtained from all the identified Khib sites. The center K refers to the modified lysine. The enrichment of amino acids in positions around the modified lysine was done by MoMo based on Motif-X software. *D*, heat map of the amino acids upstream and downstream of the acetylated sites shows significance *p*-value of frequency change in different types of amino acids flanking the modified lysine.

G. lamblia. Following tryptic digestion, affinity enrichment and HPLC–MS/MS analysis, a total of 8877 2-hydroxyisobutyryl lysine sites across 1546 proteins were identified with FDR of ≤1% using MaxQuant software (supplemental Tables S4 and S5).

Hierarchical clustering of quantified 2-hydroxyisobutyrylated proteins highlighted distinct temporal profiles of either elevated or decreased 2-hydroxyisobutyrylation during sugar starvation for *G. lamblia* culturing in different time points (Fig. 3*B*). The majority of the 2-hydroxyisobutyryl proteins carried one to three Khib sites, while around 9.2% proteins carried more than 10 Khib sites. That suggesting that lysine 2-hydroxyisobutyrylaton is an abundant PTM and may have more profound roles on substrate protein regulations in *Giardia*. To determine if there were specific amino acids adjacent to 2-hydroxyisobutyrylation lysine, we compared the sequences flanking 2-hydroxyisobutyrylation sites by heat map. A strong bias of amino acid sequence, namely glutamic acid (E) and arginine (R) was found in our data (Fig. 3, *C* and *D*).

Functional Enrichment Analyses and Cellular Localization of 2-hydroxyisobutyrylated Proteins

To better understand the potential function of 2-hydroxyisobutyrylated proteins, we performed functional annotation analysis. First, we investigated the GO functional classification of all the 2-hydroxyisobutyrylated proteins based on their BP, cellular component, and molecular function. As shown in the supplemental Fig. S2*A*. At the 24th h of sugar starvation, in the GO BP, 104 proteins were involved in cellular processes and 94 in metabolic processes. At the 48th h of sugar starvation, 265 proteins were involved in cellular processes and 245 in metabolic processes. At the 72nd h of sugar starvation, 226 proteins were involved in cellular processes and 227 in metabolic processes. Furthermore, the GO enrichment analysis was performed to identify the BPs of 2-hydroxyisobutyrylated proteins. The results showed that the identified 2-hydroxyisobutyrylated proteins at the 24th h of sugar starvation were significantly enriched in peptide biosynthetic process, amide biosynthetic process, peptide metabolic process, cellular macromolecule biosynthetic process, oxoacid metabolic process, carboxylic acid metabolic process, single-organism carbohydrate catabolic process, and oxidoreduction coenzyme metabolic process. At the 48th h of sugar starvation, the 2-hydroxyisobutyrylated proteins were enriched into oxoacid metabolic process, carboxylic acid metabolic process, single-organism carbohydrate catabolic process, nucleoside metabolic process, hexose metabolic process, carbohydrate biosynthetic process, monosaccharide metabolic process, and ribose phosphate metabolic process. At the 72nd h of sugar starvation, the 2-hydroxyisobutyrylated proteins were significantly enriched in GO BPs, including oxoacid metabolic process, carboxylic acid metabolic process, single-organism carbohydrate catabolic process, nucleoside metabolic process, ribose phosphate metabolic process, hexose metabolic process, monosaccharide metabolic process, oxidoreduction coenzyme metabolic process, monosaccharide biosynthetic process, lipid transport, peptide biosynthetic process, amide biosynthetic process, peptide metabolic process, and single-organism carbohydrate catabolic process (supplemental Fig. S2*B*).

The 2-hydroxyisobutyrylated proteins involved were analyzed in the KEGG pathways (supplemental Fig. S2*C*). The results revealed that the 2-hydroxyisobutyrylated proteins were enriched in the pathway of ribosome and carbon metabolism at the 24th sugar starvation, in the pathway of biosynthesis of antibiotics at 48th sugar starvation, and in the pathway of biosynthesis of antibiotics, carbon metabolism, biosynthesis of secondary metabolites, metabolic pathways, glycolysis/gluconeogenesis, biosynthesis of amino acids, protein processing in endoplasmic reticulum, and arginine biosynthesis at the 72nd h of sugar starvation.

Crosstalk Between Acetylation and 2-Hydroxyisobutyrylation

Each PTM can reportedly crosstalk with at least one other PTM, and the co-occurrences of different PTMs at the same site are abundant. Thus, under the sugar starvation culturing *G. lamblia*, responsive acetylation and 2-hydroxyisobutyrylation were examined to determine their relationship. A total of 8877 2-hydroxyisobutyryl sites and 2999 acetylated sites were identified. Among them, 2401 lysine sites were modified with 2-hydroxyisobutyryl and by acetylation. The correlation between the L/H ratios of acetylation and 2-hydroxyisobutyrylation, as well as the scatter diagram, is shown in Figure 4*A*. Pearson's correlation coefficient was 0.734 (Fig. 4*B*). Notably, some important modified proteins participated in the following pathways: biosynthesis of amino acids, carbon metabolism, arginine biosynthesis, metabolic pathways, alanine, aspartate, and glutamate metabolisms, glycolysis/gluconeogenesis, and pentose phosphate pathway (Fig. 4*C*). Thus, the two modifications were involved in the metabolism conversion and positively correlated under the sugar starvation culturing *G. lamblia*.

To better understand the crosstalk between acetylation and 2-hydroxyisobutyrylation accompanying metabolism conversion under the sugar starvation culturing *G. lamblia*, the correlation among acetylation and 2-hydroxyisobutyrylation expressed proteins was performed by GO analysis (supplemental Fig. S3). Under the BP, after 24 h of sugar starvation culture, the correlation of acetylation and 2-hydroxyisobutyrylation expressed proteins was classified as follows: peptide metabolic process, amide biosynthetic process, peptide biosynthetic process, oxidoreduction coenzyme metabolic process, and carboxylic acid metabolic process. After 48 h of sugar starvation culture, the correlation of acetylation and 2-hydroxyisobutyrylation expressed proteins was classified as single-organism carbohydrate catabolic process,

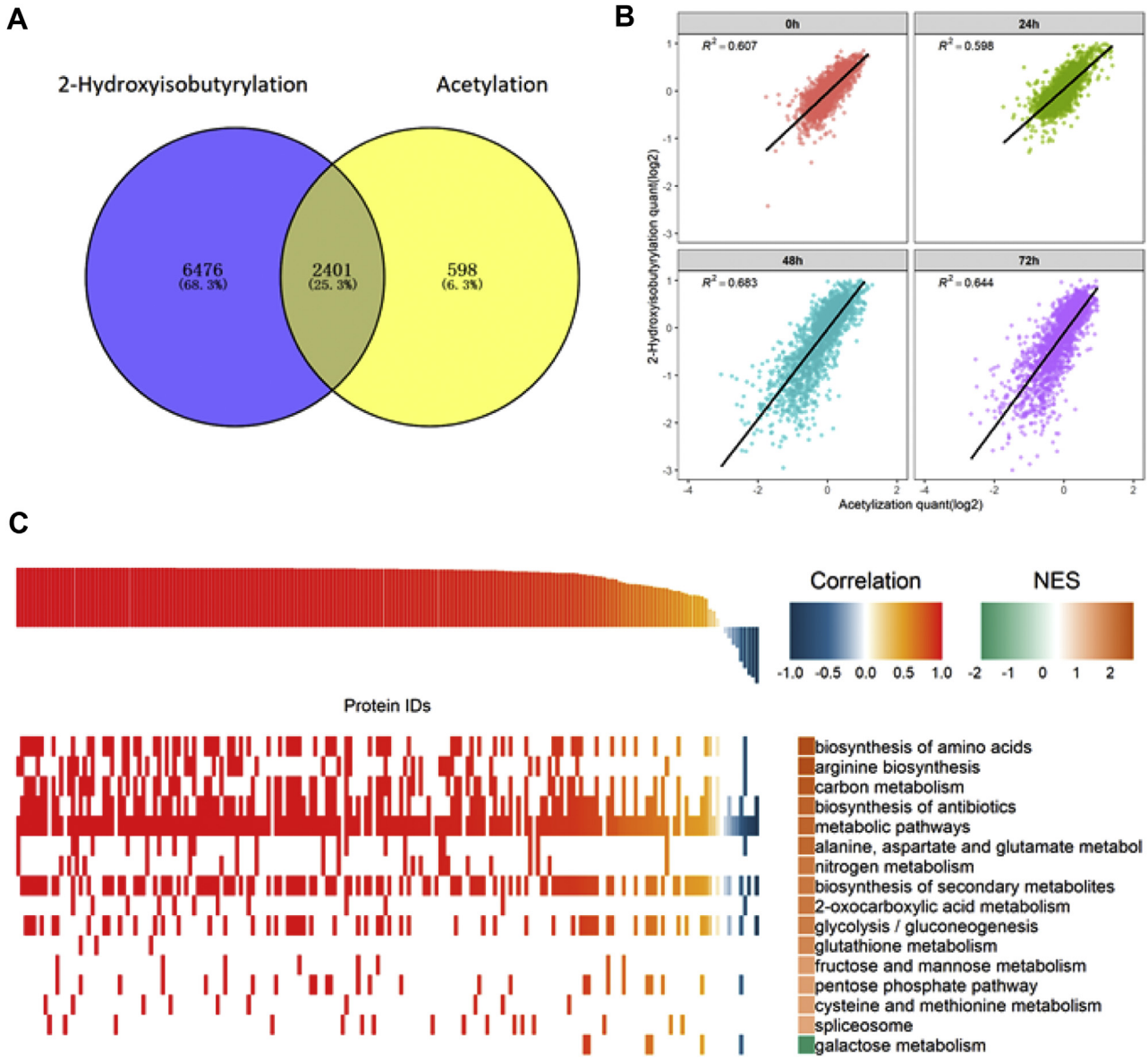

FIG. 4. **Correlation of acetylation and 2-hydroxyisobutyrylation.** *A*, overlap between acetylated acetylation and 2-hydroxyisobutyrylation proteins. *B*, correlation of acetylation and 2-hydroxyisobutyrylation. *C*, gene set enrichment analysis of Kyoto Encyclopedia of Genes and Genomes pathway between 2-hydroxyisobutyrylation and acetylation modification.

carboxylic acid metabolic process, oxoacid metabolic process, carbohydrate biosynthetic process, monovalent inorganic protein catabolic process, regulation of protein catabolic process, regulation of proteolysis, monosaccharide metabolic process, oxidoreduction coenzyme, and metabolic process.

After 72 h of the sugar starvation culture, the correlation of acetylation and 2-hydroxyisobutyrylation expressed proteins was classified to cellular protein catabolic process, protein catabolic process, regulation of protein catabolic process, regulation of proteolysis, carboxylic acid metabolic process, monosaccharide metabolic process, nucleoside phosphate metabolic process, and ribose phosphate metabolic process.

During the dynamic process of culturing *G. lamblia* under sugar starvation, in the first phase, the correlation of acetylation and 2-hydroxyisobutyrylation expressed proteins was primarily related to amino acid metabolism. Thus, amino acid metabolism process supplied the energy for *G. lamblia* growth instead of sugar. However, after 72 h of *G. lamblia* culture, analysis results indicated that the hexose metabolic process, carboxylic acid metabolic process, oxoacid metabolic process, monosaccharide metabolic process, and ribose phosphate metabolic process were also involved in energy supply.

Enrichment of KEGG pathway indicated that the crosstalk between acetylation and 2-hydroxyisobutyrylation proteins

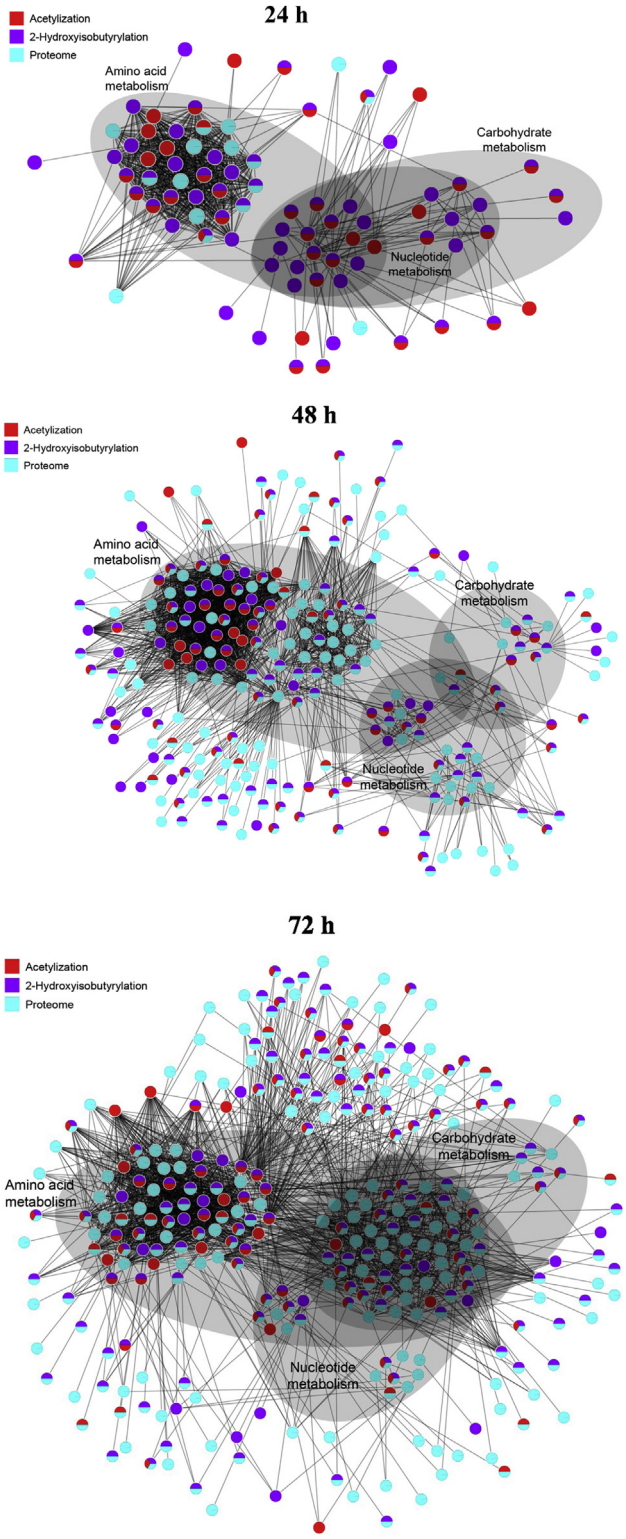

FIG. 5. **Protein–protein interaction network for proteins corresponding to differentially expressed modification sites between 2-hydroxyisobutyrylation and acetylation.** Proteins associated with metabolism were selected to construct the correlation network diagram.

was involved in carboxylic acid metabolic process, oxoacid metabolic process, single-organism carbohydrate catabolic process, and oxidoreduction coenzyme metabolic process after 48 h of sugar starvation culture. However, the 72 h sugar starvation culture was in major enriched in the proteasome, protein processing in endoplasmic reticulum, aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, and arginine biosynthesis (supplemental Fig. S4). The aforementioned results revealed the dynamic process of metabolism conversion under sugar starvation culturing *G. lamblia*.

Holistic Views of Acetylation and 2-Hydroxyisobutyrylation

To understand the complete mechanism of the metabolism conversion under sugar starvation, we performed a strategy to integrate the acetylation and 2-hydroxyisobutyrylation data of PTMs at the protein level. In the present study, a number of acetylation and 2-hydroxyisobutyrylation proteins were detected, and further attention should be given to explore their function. Using Metscape software, we investigated the latent relationships of these acetylation and 2-hydroxyisobutyrylation proteins by constructing a protein and protein interaction network (Fig. 5). We discovered that the acetylated and 2-hydroxyisobutyrylation proteins had a close crosstalk in the network during the sugar starvation culturing of *G. lamblia*, indicating that some important proteins underwent both acetylated and 2-hydroxyisobutyrylation proteins. The correlation of acetylated and 2-hydroxyisobutyrylation proteins was enriched mostly in the following pathways: arginine biosynthesis, glycolysis/gluconeogenesis, alanine, aspartate, and glutamate metabolisms, and ribosome. Analysis of the correlation from the dynamic process revealed that during the first 24 h of sugar starvation, the major enrichment processes were peptide metabolic and amide biosynthetic process, indicating the proteins primarily supplied the energy for *G. lamblia*. From 48 to 72 h, the correlation between acetylation and 2-hydroxyisobutyrylation proteins was enriched in protein catabolic process, regulation of proteolysis, hexose metabolic process, ribose phosphate metabolic process, nucleoside metabolic process, and lipid transport. This finding indicated that multiple bypasses including the hexose pathway were also activated and involved in supplying the energy for *G. lamblia* to increase tolerance to sugar starvation. Remarkably, the lipid transport indicated that lipid metabolism may also be mobilized to be involved in metabolic changes.

Amino acids are becoming increasingly recognized as important components of the energy metabolism of *G. lamblia*. Our data confirmed that alanine and arginine may be involved in the metabolism conversion under sugar starvation. The uptake of aspartate, alanine, and arginine from the extracellular medium, as well as the documentation of glucose-independent metabolism, suggested the potential importance of amino acid metabolism for energy production in

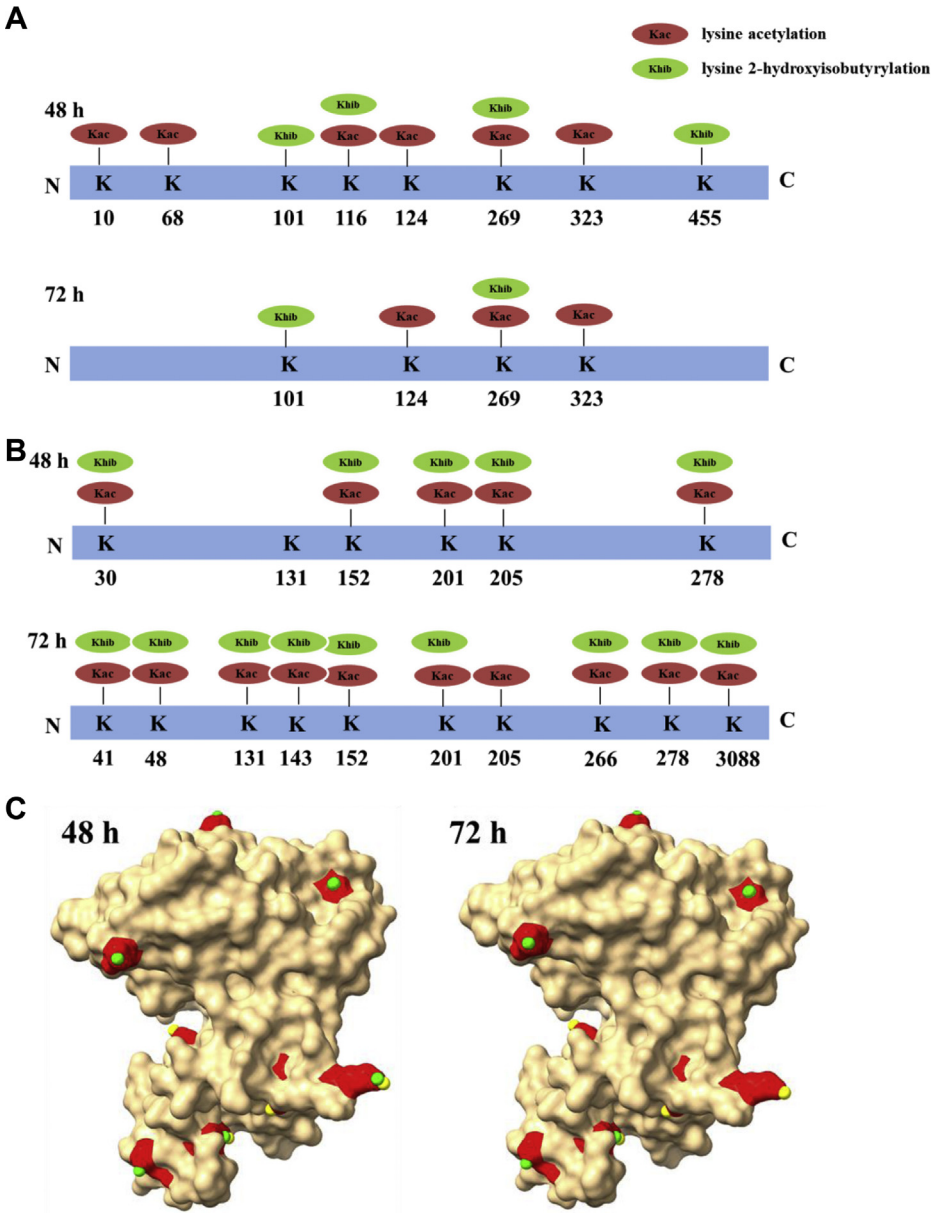

FIG. 6. **Site-specific 2-hydroxyisobutyrylation and acetylation on arginine deiminase and carbamate kinase.** *A*, site-specific modification on arginine deiminase. *B*, site-specific modification on carbamate kinase. *C*, differential Kac and Khib sites on carbamate kinase are exhibited. The *red* region represents the lysine site where the modification occurred. The Kac site is shown in *yellow* and the Khib site is show in *green*, respectively.

Giardia. The arginine dihydrolase pathway is a potential source of energy for *G. lamblia* undergoing sugar starvation. However, the mechanism that regulates the energy conversion process is unclear. In the current research, we integrated the acetylation and 2-hydroxyisobutyrylation data of PTMs at protein level and found that arginine deiminase and carbamate kinase were changed obviously after 48 and 72 h of sugar starvation (Fig. 6). Some spectra of lysine sites that underwent both acetylation and ubiquitination are presented in supplemental Figs. S5 and S6. Thus, by analyzing the Kac and 2-hydroxyisobutyrylation data, we found an intricate link between the PTMs at protein level and metabolism conversion in *G. lamblia*.

D-Ribose Reversed the Energy Supply Under Sugar Starvation

To examine metabolism conversion under sugar starvation, we initially added modulators of energetic metabolism, namely DCA and 2DG to detect the growth and ATP production in *G. lamblia*. We found that DCA and 2DG had no effect on

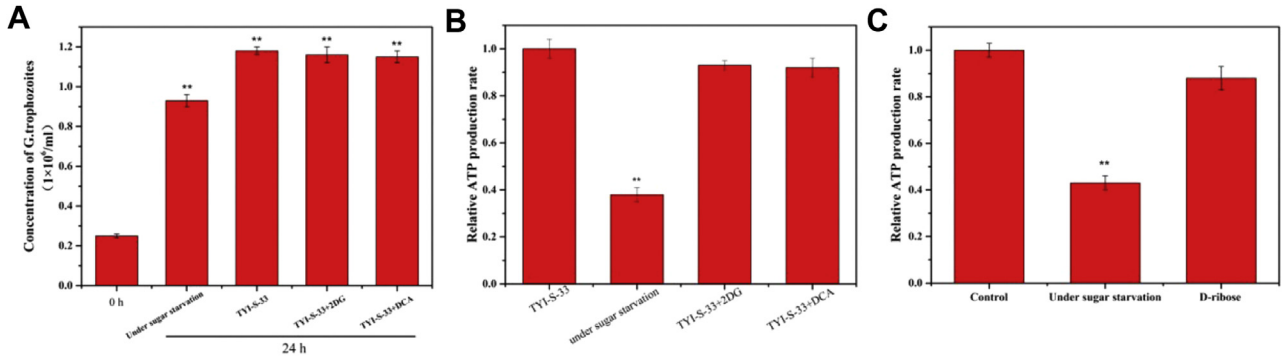

FIG. 7. **D-ribose reversed energy supply under sugar starvation.** *A* and *B*, effect of DCA and 2DG on growth and ATP production in *Giardia lamblia*. *C*, D-ribose reversed energy in *G. lamblia*. DCA, dichloroacetate; 2DG, 2-deoxy-D-glucose; TYI-S-33, trypticase yeast extract iron-serum-33 medium.

suppressing growth and ATP production in *G. lamblia* (Fig. 7, *A* and *B*). We then added the D-ribose to the medium after 24 h of culturing to detect ATP generation. The ATP generation results revealed that generations of ATP were decreased under sugar starvation. However, the generation of ATP was upregulated whether or not supplied with D-ribose after 24 h of sugar starvation (Fig. 7*C*). The aforementioned results indicated that the hexose phosphate shunt pathway participated in metabolism conversion in *G. lamblia* under sugar starvation and were consistent with the omics results.

DISCUSSION

Energy is necessary for the growth of *G. lamblia* cells. Despite the constant possibility of damage because of their own metabolism, *Giardia* have survived and evolved to adapt to various environments (31, 32). However, environments outside a laboratory setting are rarely constant, and *G. lamblia* often face adverse conditions. One of the major challenges is the acquisition of nutrients necessary to grow and divide. Cells respond to environmental challenges with appropriate regulation of their transcriptional and translational programs (33, 34). Although transcription and translation are time- and energy-intensive processes, post-translational control over the function of existing proteins can rapidly alter cellular physiology and permit fitness or survival under otherwise suboptimal or lethal conditions.

The history of PTM biology clearly shows that the exploration of crucial regulatory proteins for a PTM pathway is key point to studying its biology (35–38). PTM is a key regulatory mechanism for cell function, such as transcription and metabolism. Many new acylation forms, such as histone butyrylation, crotonylation, and β-hydroxybutyrylation, have been identified (39). Compared with the aforementioned PTMs, little is known about the function and regulation of Kac and 2-hydroxyisobutyrylation. No study has attempted to define the role of global regulation of acetylation and 2-hydroxyisobutyrylation in the energy conversion mechanism of *G. lamblia* when sugar, the main source of energy, is in short supply (40, 41). Interestingly, when the *G. lamblia* experienced the stress of sugar starvation, ATP generation decreased in the first phase and increased to the normal level after a long time. We speculated that glucose was not the only source of energy for *G. lamblia* and that a metabolism conversion occurred in *G. lamblia* during this process. Here, we performed a comprehensive Kac and 2-hydroxyisobutyrylation study to explore the metabolism conversion in *G. lamblia*.

Acetylation is a modification that can dramatically change the function of a protein by altering its properties, including hydrophobicity, solubility, and surface properties, all of which may influence protein conformation and interactions with substrates, cofactors, and other macromolecules (42, 43). Researches have shown that most central metabolic enzymes possess lysine that was acetylated in a regulated fashion (44). The interconnectedness of acetylation and central metabolism suggested that acetylation may be a response to nutrient availability or the energy status of the cells. Our comprehensive study of global Kac provided a dataset of 2999 acetylation sites on 956 proteins in *G. lamblia* during the dynamic process. These datasets represent the first acetylation proteome in *G. lamblia* to date and illustrate the broad landscape of the acetylation function. The acetylation proteins were enriched in the GO BPs of peptide biosynthetic process, amide biosynthetic process, and peptide metabolic process. This finding indicated that amino acids may be important components of the energy metabolism for *G. lamblia* instead of the perspective glucose are not the only source of energy for *G. lamblia*. Meanwhile, our data showed that protein acetylation may be involved in metabolism conversion in *G. lamblia*.

Kac occurs in core histones and numerous other proteins in nuclei, cytosol, and mitochondria, many of which are metabolic enzymes (45). Previous studies have suggested that the Khib pathway is also highly likely to function similar to Kac (46, 47). Our comprehensive study of global lysine 2-hydroxyisobutyrylome provided a dataset of 8877 2-hydroxyisobutyryl lysine sites across 1546 proteins identified

with FDR. The data represented the first Khib proteome in *G. lamblia* to date and illustrate the broad landscape of the Khib pathway. The 2-hydroxyisobutyrylation proteins were enriched in the GO BPs of metabolism process, including peptide biosynthetic process, amide biosynthetic process, peptide metabolic process, and carbohydrate biosynthetic process. The KEGG pathways involving the 2-hydroxyisobutyrylated proteins showed that the 2-hydroxyisobutyrylated proteins were enriched in the pathway of carbon metabolism, biosynthesis of secondary metabolites, metabolic pathways, glycolysis/gluconeogenesis, biosynthesis of amino acids, protein processing in endoplasmic reticulum, and arginine biosynthesis when undersugar starvation. Interestingly, the metabolite changes in the arginine biosynthesis pathway suggested that the operation of the arginine dihydrolase pathway is a significant energy source during the growth in *G. lamblia* (48). After 72 h of sugar starvation, carboxylic acid metabolic process, ribose phosphate metabolic process, and hexose metabolism were also participated in energy supplying of *G. lamblia*. This finding may be because of the fact that *Giardia* mobilized as many metabolic pathways as possible to adapt to sugar starvation to guarantee their reproduction.

The evidence suggested that acetylation and 2-hydroxyisobutyrylation may have a close relationship (49, 50), so we focused on the full mechanism of the metabolism conversion under sugar starvation by integrating the acetylation and 2-hydroxyisobutyrylation data of PTMs at protein level. The correlation of acetylation and 2-hydroxyisobutyrylation proteins was mainly enriched in the pathway of arginine biosynthesis, glycolysis/gluconeogenesis, alanine, aspartate and glutamate metabolisms, and ribosome. This finding indicated that alanine and arginine may be involved in the metabolism conversion under sugar starvation. Analysis of the dynamic process further revealed interesting results. In the first phase, the correlation proteins were related mostly to protein metabolism. Thus, the proteins were primarily supplied the energy when *G. lamblia* was cultured under sugar starvation. When *G. lamblia* adapted to the sugar-free culture, protein metabolism was not the only source of energy, and we speculated that the metabolic processes in *G. lamblia* changed. Proteomics data analysis showed that the expression of proteins associated with metabolic pathways was downregulated. However, the acetylated and 2-hydroxyisobutyrylation levels of some pathway were upregulated. These metabolic pathways included monosaccharide metabolism process, hexose metabolic process, ribose phosphate metabolic process, and carboxylic acid metabolic process. The aforementioned data indicated that the decomposition of fatty acid, phosphate pathway, and other monosaccharide metabolism may enhance to supply the energy for *G. lamblia*. Our results further revealed the dynamic process of energy conversion in *G. lamblia*. Amino acids are involved in the energy metabolism supplied during the first phase of *G. lamblia* proliferation under sugar starvation. However, when *G. lamblia* adapted to the sugar-free culture condition, metabolic changes occurred again. Monosaccharide metabolic process, hexose metabolic process, ribose phosphate metabolic process, and carboxylic acid metabolic process also participated in the energy conversion. When we added the D-ribose to the sugar-free medium at the 48th h of sugar starvation, the ATP generation level dramatically increased. This finding supported our omics results, although and further experiments are still needed.

From the aforementioned data, we can conclude that glucose was not the only source of energy for *G. lamblia*, and a metabolism conversion occurred in *G. lamblia*. This study also provided a valuable resource for understanding the molecular mechanism in which acetylation and Khib were associated with metabolism conversion in *G. lamblia*. *G. lamblia* is the earliest diverging eukaryotic lineage. Although it is a true eukaryotic cell, it displays several prokaryotic properties, such as lack of mitochondria and peroxisomes (51, 52). In evolutionary terms, *G. lamblia* is more divergent from yeast than yeast is from man, and elements that are conserved from *G. lamblia* to man are therefore likely to reflect universal eukaryotic cell functions (53). *G. lamblia* is the most divergent eukaryote examined to date, and tools have been recently developed to study genes in detail (54). Every cell requires adequate levels of high-energy phosphates to maintain their integrity and function. ATP is produced by intracellular pathways, such as glycolysis and the tricarboxylic acid cycle, with glucose as the starting substrate. When cells experience ischemia or hypoxia, the normal production of energy compounds is reduced. For example, myocardial ischemia depletes ATP levels, which can affect intracellular reactions and cell functions (55). D-ribose has been shown to enhance the recovery of ATP levels and aid in alleviating ischemia through preclinical and pilot clinical studies (56).

This finding is consistent with our results about *G. lamblia* metabolic changes under sugar starvation culture. Our research clarified the metabolic changes in *G. lamblia* in acetylation and 2-hydroxyisobutyrylation levels and reflected universal eukaryotic cell functions. Thus, the evolution rule from prokaryote to eukaryote from the perspective of energy metabolism was revealed using modified omics method, which may also provide instruction for treatment of clinical diseases such as myocardial ischemia and cerebral ischemia.

DATA AVAILABILITY

Data are available *via* ProteomeXchange with via the PRIDE partner repository with the dataset identifier PXD022317.

DATA SHARING

All data discussed in the article will be made available to readers.

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Conflict of interest—The authors declare no competing interests.

Abbreviations—The abbreviations used are: 2DG, 2-deoxy-D-glucose; BPs, biological processes; DCA, dichloroacetate; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PTMs, post-translational modifications; TYI-S-33, trypticase yeast extract iron-serum-33 medium.

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