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RESEARCH

Global profiling of protein S-palmitoylation in the second-generation merozoites of *Eimeria tenella*

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Abstract

The intracellular protozoan *Eimeria tenella* is responsible for avian coccidiosis which is characterized by host intestinal damage. During developmental cycle, *E. tenella* undergoes versatile transitional stages such as oocyst, sporozoites, merozoites, and gametocytes. These developmental transitions involve changes in cell shape and cell size requiring cytoskeletal remodeling and changes in membrane proteins, which may require transcriptional and translational regulations as well as post-translational modification of proteins. Palmitoylation is a post-translational modification (PTM) of protein that orchestrates protein targeting, folding, stability, regulated enzymatic activity and even epigenetic regulation of gene expression. Previous research revealed that protein palmitoylation play essential role in *Toxoplasma gondii*, *Trypanosoma cruzi*, *Trichomonas vaginalis*, and several *Plasmodium* parasites. Until now, there is little information on the enzymes related to palmitoylation and role of protein acylation or palmitoylation in *E. tenella*. Therefore, palmitome of the second-generation merozoite of *E. tenella* was investigated. We identified a total of 2569 palmitoyl-sites that were assigned to 2145 palmitoyl-peptides belonging to 1561 protein-groups that participated in biological processes including parasite morphology, motility and host cell invasion. In addition, RNA biosynthesis, protein biosynthesis, folding, proteasome-ubiquitin degradation, and enzymes involved in PTMs, carbohydrate metabolism, glycan biosynthesis, and mitochondrial respiratory chain as well as vesicle trafficking were identified. The study allowed us to decipher the broad influence of palmitoylation in *E. tenella* biology, and its potential roles in the pathobiology of *E. tenella* infection. Raw data are publicly available at iProX with the dataset identifier PXD045061.

Keywords Protein acylation · *E. tenella* · Bioiological process · The second-generation merozoites

Introduction

Coccidiosis is a global and pervasive parasitic poultry disease caused by the genus *Eimeria* (Apicomplexan: Eimeriidae). *Eimeria* spp. especially *E. tenella* is highly pathogenic in chicken, causing substantial financial losses globally (Shirley et al. 2007; Blake et al. 2020). *E. tenella* undergoes complicated developmental life cycles that include sporogony, schizogony and gametogony. During the schizogony, trophozoites undergo multiple divisions to generate schizonts, followed by formation of numerous merozoites. The second-generation merozoites of *E. tenella* are the most pathogenic endogenous progenies of the parasite stages (Wan et al. 1999; Lal et al. 2009). The substantial increase in the second-generation merozoites of *E. tenella* causes severe destruction of the intestine, leading to massive caecal hemorrhage, and fatal consequences in infected poultry (Li et al. 2019). Thus, the second-generation merozoites stage can be a target stage for drug and vaccine development.

E. tenella undergoes an intricate life cycle involving multiple stages of oocyst, sporozoites, merozoites as well as gametocytes. These developmental transitions involve changes in cell shape and cell size requiring cytoskeletal remodeling and membrane changes, which may require transcriptional, translational and post-translational modifications. Post-translational modifications (PTMs), such as acylation, methylation, phosphorylation and glycosylation orchestrate several pathways that play vital roles in different life stages of *E. tenella* (Gong et al. 2017, 2023; Yakubu

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et al. 2018; Ma et al. 2021; Walker et al. 2010). Palmitoylation adds palmitic acid to a cysteine residue through a thioester linkage, orchestrating protein targeting, trafficking, structure, stability, regulation of enzymatic activities and even epigenetic regulation of gene expression (Brown et al. 2017; Frénal et al. 2014). It has been confirmed that a type of PTMs-palmitoylation modification exists in other apicomplexan parasites (Corvi et al. 2011; Brown et al. 2017; Foe et al. 2015; Caballero et al. 2016; Merino et al. 2014; Emmer et al. 2011; Jones et al. 2012; Tremp et al. 2017; Hodson et al. 2015; Santos et al. 2016; Hopp et al. 2016). In silico analysis of the genome in database (*Toxo*DB) predicts the existence of EtPATs enzymes with higher expression level in the second-generation merozoites suggesting that palmitoylation machinery might be functional in this protozoan parasite (Reid et al. 2014; Walker et al. 2015; Aunin et al. 2021). However, there has been no information on palmitoylation in *E. tenella.* Therefore, in this study, we examined the role of protein palmitoylation in *E. tenella* second-generation merozoite. A total of 1561 protein-groups were identified that participated in widespread biological processes. This indicates a broad function of PTMs in *E. tenella* biology, and thus lay a foundation for the significance of protein–protein interactions in biological process during parasite developments.

Materials and methods

Parasites

The oocysts of *Eimeria* spp. were obtained by propagation in coccidia-free chickens. Animals were reared in wire cages for 2 weeks before experimental infection. All experiments were performed in accordance with the animal care guidelines and approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

Coccidia-free 14-day-old chickens were inoculated with 5×10^4 sporulated oocysts of *E. tenella* (Guangdong strain). The second-generation merozoites were collected and purified from the cecal mucosa at 120 h post-infection of chickens (Xie et al. 1990).

Protein extraction and digestion

The substitution of S-palmitoylated Cys (S-PALM-Cys sites) with S-biotinylated Cys from the second-generation merozoites protein extracts as previously described Wan et al. (2007) and Rossin and Hueber (2017). In principle, the method relies on an acyl-biotinyl exchange chemistry in which thioesters-are replaced by biotin. It involves blockade of free thiols with N-ethylmaleimide (NEM), cleavage of Cys-palmitoyl thioester linkages by using hydroxylamine and labeling of thiols with biotin-HPDP (biotin-HPDP-N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide. After that, biotinylated proteins were captured by streptavidine-agarose and identified by mass spectrometry (MS/MS)-based proteomic technique.

Purified merozoites samples were ground into powder using liquid nitrogen. Approximately 20 mg of powder were resuspended in 200 µL lysis buffer (4% SDS, 100 mM DTT, 150 mM Tris–HCl pH 8.0) and 1% Protease Inhibitor Cocktail (Thermo Fisher Scientific, USA). The purified merozoites were sonicated on ice and separated by centrifugation at 16,000 rpm at 4℃ for 15 min. The supernatant was collected, and the protein concentration was measured with BCA kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK). Then each protein mixture was treated with 100 mM NEM in each sample at 4 °C for 16 h with agitation to block free thiols and excess NME was removed by trichloroacetic acid (TCA) precipitation method. The protein precipitate was resolved in 1mL PBS (pH 7.4) that contained 6 M urea and 2% SDS, and then the protein solutions was separated into two parts. One was treated with a mixture of 3ml 1M hydroxylamine (Sigma Aldrich, USA), 1 mL PBS, protease inhibitor, 5 mM EDTA and 500 µL 4 mM biotin-HPDP (Thermo Fisher Scientific, USA). The other part was treated with 1 ml PBS, protease inhibitor, 5 mM EDTA and 500 µL 4 mM biotin-HPDP (Thermo Fisher Scientific, USA) without hydroxylamine as negative control. Afterward, all the mixtures were incubated in the dark for 1.5 h at 25 °C. Then the protein mixture was concentrated by 5-kDa ultrafiltration protein concentrator (Sartorius, Germany) and precipitated with cold acetone overnight, washed, dried, and sonicated in an ice bath, gently mixed, and re-dissolved in 8M UA buffer (8M urea, 150 mM Tris–HCl (pH 8.0). An appropriate amount of 50 mM ammonium bicarbonate (NH_4HCO_3) (Sigma-Aldrich, USA) was added, followed by addition of trypsin for enzymatic hydrolysis overnight. After trypsin digestion, peptide was desalted by C_{18} cartridge (ThermoFisher Scientific, USA) and vacuum-dried.

Enrichment of palmitoylated peptides

The same amount of peptide was added to 100-µL high capacity streptavidin agarose beads (Thermo Fisher Scientific, USA) and mixed thoroughly at room temperature for 1 h. Then the beads were washed with 1 mL wash buffer (0.2% SDS, 0.2% Triton X-100 and 500 mM NaCl) 5 times and then 100 µL of elution buffer (50 mM NH_4HCO_3) (pH 8.2) and 5 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP) were added for peptide elution. Finally, iodoacetamide (IAA) was added to a concentration of 20 mM and reaction was allowed for 1 h at room temperature without

light. Subsequently, drying, desalting and vacuum concentration were carried out to obtain peptide powder, and 10 µL 0.1% formic acid (FA) was added to dissolve powder for mass spectrometry analysis.

LC–MS/MS analysis

LC–MS analysis was performed on a Q Exactive HF-X mass spectrometer coupled to Easy nLC 1200 (Thermo Fisher Scientific, USA). The palmitoylated peptide were loaded into a chromatographic column (75 µm × 150 mm; 3 µm, C_{18}, Dr. Maisch GmbH, Germany) using buffer A. Peptide were eluted over 120 min with a linear gradient of buffer B from 5 to 8% at a flow rate of 300 nL/min. For MS data acquisition, full MS scans were surveyed from m/z 300 to m/z 1800 at a resolution of 60,000 at m/z 200 with an AGC target values of 1e6 and a maximum injection time 50 ms. Then data-Dependent top 20 MS/MS scans were applied by higher-energy collision dissociation (HCD) with normalized energy 28 at a resolution of 15,000 at m/z 200 with an AGC target value of 1e5 and a maximum injection time 50 ms. The isolation window was set to 1.6 Th. The procedure for palmitoylation modification is as depicted in Fig. 1.

Sequence database searching and data analysis

The palmitoylation proteome data were imported into MaxQuant software (version 1.6.1.0) for data interpretation. Protein identification against *E. tenella* was downloaded on 01/01/2022, from Uniprot database (https://www.uniprot.org/uniprot/?query=taxonomy:5802). For palmitoylation proteome, MS spectra were searched with maximal two missed cleavage sites. The modifications were variable oxidation (+ 15.9949) on methionine (M) and acetylation (+ 42.010565) on protein N terminus. The search results were filtered and exported with < 1% false discovery rate (FDR) at site level, peptide-spectrum-matched level, and protein level, respectively. MaxQuant analysis was filtered only for those palmitoylation sites that were confidently localized (class I, localization probability > 0.75). A site was considered as quantifiable when a minimum of 3 out of 5 replicates were observed in each condition. Label-free quantification was carried out in MaxQuant using intensity determination and normalization algorithm as previously described (Beer et al. 2017). The "LFQ intensity" of each site in different samples was calculated as the best estimate. The quantitative site ratios were weighted and normalized by the median ratio in Maxquant software.

Bioinformatics analysis

For palmitoylation proteomic data, the t-test (p value) analysis and fold change (FC) were followed and used to determine statistical significance ($p < 0.05$, and FC > 1.5 or < 0.83) for each comparison. Expression data were grouped together by hierarchical clustering according to the peptide level. To annotate the sequences, information was extracted from Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). GO and KEGG enrichment analyses were carried out with the Fisher's exact test, and FDR correction for multiple testing was also performed. GO terms were grouped into three categories: biological process (BP), molecular function (MF), and cellular component (CC). Enriched GO and KEGG pathways were nominally statistically significant at the $p < 0.05$ level. The model of protein sequence was analyzed by Motif-x. Construction of protein–protein interaction (PPI) networks were also conducted by using the STRING database with the cytoscape software (Szklarczyk et al. 2017).

Acyl-biotin exchange assay on immunoprecipitated proteins

Palmitoylated protein of interest in *E. tenella* SMGs was performed by an initial immunoprecipitation (IP) step using an antibody directed against the protein, followed by the ABE assay and western blotting. Immunoprecipitated protein (IP-ABE) Palmitoylation Kit (Cat: AM10314) (AIMS, China) was used for the ABE assay and the procedure was performed according to the manufacturer's instructions. In brief, the SMGs of *E. tenella* were first suspended in lysis buffer, then the lysate was incubated with anti-α-tubulin

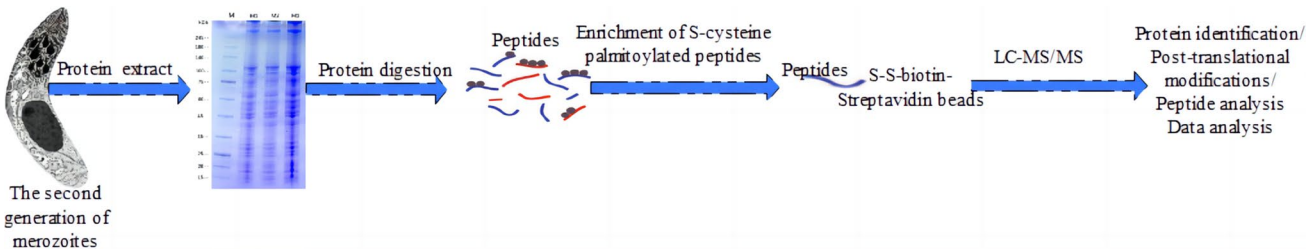

Fig. 1 The workflow for carrying out the experiment for decipering the palmitoylation modification of the second-generation of merozoites in *E. tenella*

monoclonal antibody (Solarbio Life Science, China) and Protein A beads overnight at 4°C. N-ethylmaleimide (NEM) was used for blocking unmodified cysteines for 30 min. Beads were washed and incubated with hydroxylamine for one hour at room temperature for specific cleavage and exposure of palmitoylated cysteine thiol group. For alpha tubulin, two groups namely one with HAM (+HAM) and the other without HAM (-HAM) was prepared. Beads were washed and exposed to thiol-reactive biotin molecules, biotin-BMCC, for one hour at room temperature. At the end, beads were washed to get the eluted proteins. The elute was subjected to SDS-PAGE gel separation and the proteins were transferred to PVDF membrane. Streptavidin-HRP antibody (dilution ratio 1:5000–1:10,000) was added to detect the immunoprecipitated proteins by chemiluminescence exposure.

Results

The palmitoyl-protome of the second-generation of merozoites in *E. tenella*

In this study, 1561 protein-groups were confirmed in second generation merozoites of *E. tenella* and 2145 palmitoylated peptide segment (palmitoyl-peptide) were identified with 2569 independent palmitoylated peptide sites (probability ≥ 0.75) (Supplementary file 1, 2 and 3). Raw data are publicly available at iProX with the dataset identifier PXD045061. Several identified proteins were modified at multiple sites. Especially, 20 identified proteins were palmitoylated at more than 8 cysteine sites (Table 1). Microneme protein 4 (Uniprot No. U6L098) was the largest palmitoylated protein with 79 palmitoylated sites in the second generation of merozoites in *E. tenella*. Compared with the palmitoylated modified protein reported in *T. gondii* and *P. falciparum* (Table 2), the number of identified palmitoylated proteins in *E. tenella* from our results was much higher than that reported previously. Thus, it can be suggested that palmitoylation modification is widespread in apicomplexan parasites and plays an essential role in their growth and development.

Table 1 The top 20 putative proteins with the most S-palmitoylation modification sites in *E. tenella*

No	Uniprot no	Protein description	S-palmitoylated sites no
1	U6L098	Microneme protein 4	79
2	Q9U966	Micronemal protein MIC4, related	45
3	U6L1T3	EGF-like domain-containing protein	27
4	U6KM17	Uncharacterized protein	27
5	U6KXQ2	Microneme protein	18
6	U6KQ25	Microneme 1 (fragment)	15
7	U6KW83	Cysteine repeat modular protein	15
8	U6KVN8	Uncharacterized protein	14
9	U6L4S8	Microneme protein MIC3	13
10	U6KJB8	Zinc finger (CCCH type) protein	13
11	U6KWQ6	Dynein heavy chain protein, related	11
12	U6KNV2	Calcium-transporting ATPase	11
13	U6L470	Uncharacterized protein	10
14	U6KN00	Elongation factor 2	9
15	U6KTM1	Clathrin heavy chain	9
16	U6KV05	Membrane skeletal protein IMC1	9
17	U6KQK5	HECT domain-containing protein	9
18	U6KU66	Heat shock protein	8
19	U6KTP6	Sec63 domain-containing DEAD/DEAH box helicase	8
20	U6KT45	TCP-1/cpn60 family chaperonin	8

GO functional classification and pathway enrichment of palmitoylated proteome of the second-generation of merozoites in *E. tenella*

Palmitoylated proteomes were grouped into GO categories of biological processes, cellular components, and molecular functions. GO analysis of the palmitoylated proteome revealed that the palmitoylated proteins with a multiplicity of functions with GO terms that include RNA binding, structure molecular activity, structure constituent of ribosome, ligase activity, proton-transporting ATP synthase activity, proton channel activity, oxidoreductase activity, unfolded protein binding, cation channel activity, ubiquitin-protein transferase activity etc. Genes involved in organonitrogen compound metabolic process, amide biosynthetic process, translation, cellular amide metabolic process, peptide biosynthetic process, establishment of protein localization to organelle, peptide metabolic process, intracellular protein transport, and protein targeting (Fig. 2). Molecular interaction and reaction network of identified palmitoylated proteome were interpreted through KEGG pathway, and showed categories of palmitoylated proteome pathways including phagosome, ubiquitin mediated proteolysis, spliceosome, RNA degradation, ribosome biogenesis in eukaryotes, ribosome, protein processing in endoplasmic reticulum, protein export, proteasome, nucleocytoplasmic transport, mRNA surveillance pathway, aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, fatty acid metabolism, fatty acid biosynthesis, citrate cycle (TCA cycle), carbon metabolism, biosynthesis of amino acids, and synaptic vesicle cycle pathway etc. (Fig. 3).

Table 2 Number of putative S-palmitoylated proteins of *E. tenella* identified in this study compared with those found in other species

Species	Palmitoylated Proteins	Function	Reference
HeLa cell	551	Ion transmembrane transporter activity, G protein-coupled receptor activity and GTPase activity	Ji et al. 2023
Mus musculus testis	4883	Sperm morphology and motility	Gao et al. 2022
Human, dog and rat heart	454	Excitation–contraction coupling and intercellular communication of cardiomyocytes	Miles et al. 2021
Rat embryonic cortical neurons	68	Shape synapse morphology and function	Kang et al. 2008
Arabidopsis thaliana	582	Pathogen perception and response, essential for growth	Hemsley et al. 2013; Zhang et al. 2015
Trichomonas vaginalis	363	Protein transport, pathogenesis related, and signaling	Nievas et al. 2018
Toxoplasma gondii	401	Metabolic processes, gliding and host-cell invasion, regulation of transcription, and translation	Caballero et al. 2016
Toxoplasma gondii	282	Motility, cell morphology and host cell invasion	Foe et al. 2015
Trypanosoma brucei	124	Membrane attachment and protein sorting	Emmer et al. 2011
Plasmodium falciparum	409	Virulence and asexual-stage development	Jones et al. 2012
Yeast	47	Cellular signaling and membrane trafficking	Roth et al. 2006
Eimeria tenella	1561	Parasite morphology, motility, host cell invasion, RNA biosynthesis, protein biosynthesis, folding and degradation, carbohydrate metabolism, vesicle trafficking, and PTMs modification	

S-proteins involved in RNA biosynthesis, protein biosynthesis, machinery of vesicular trafficking, ubiquitin-dependent degradation machinery, and PTMs

Protein expression can be regulated at transcriptional, translational and PTMs levels in apicomplexan parasites (Bennink and Pradel 2019; Silmon de Monerri et al. 2015; Yakubu et al. 2018). In the current study, S-palmitoylated proteins participated in RNA biosynthesis, protein biosynthesis, protein folding, and ubiquitin–proteasome degradation (Supplementary file 4). Besides, several enzymes that are involved in palmitoylation and glycosylation were S-palmitoylated including the palmitoyl transferase (Uniprot No.U6KNC5), serine palmitoyl transferase (UniprotNo. U6L3L7), and UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase T3, (Uniprot No.U6KJV3). Apicomplexan parasites utilize organelles of the endomembrane system to transport protein and lipid, and this system is orchestrated by a conserved machinery of vesicular trafficking. This machinery contains SNAREs and Sec1/Munc18-like (SM) that are involved in vesicle fusion, Rab GTPases, COPII vesicles, and several vacuolar protein sorting proteins (Cao et al. 2020, 2021; Bisio et al. 2020). Besides, several transporters were palmitoylated including facilitative glucose transporter (Uniprot No. D7RXM4). The sec 1 family domain-containing protein and protein translocation complex SEC 23, 61, and 62; vacuolar protein sorting-associated protein 26, 35, 13, and 29 and coatomer alpha, beta, delta, epsilon, zeta-2 subunit, and coatomer protein complex subunit beta were all palmitoylated (Supplementary file 4), thus the palmitoylated proteins are essential for biogenesis, vesicle fusion and tethering of secretory organelles. In summary, the protein quality control system and protein vesicular trafficking system were regulated through S-palmitoylation PTMs. Besides, several phosphatase and kinase were also palmitoylated (Supplementary file 4).

Candidate protein whose palmitoylation may contribute to structural plasticity and cell invasion

The apicomplexan glideosome plays essential role in invasion and motility of the parasite aided by myosin. The inner membrane complex (IMC) in several parasites that belong to apicomplexan phylum exists as special structure, with the function of maintaining parasite stability as well as intracellular replication. IMC, glideosome, and cell skeleton structure (tubulin and actin) orchestrate and maintain structural plasticity in apicomplexan parasites (Harding et al. 2016; Frénal et al. 2017, 2010; Brown et al. 2017; Carmeille et al. 2021; Rompikuntal et al 2021). In the current study, several proteins orchestrating structural plasticity were S-palmitoylated, including membrane skeletal protein family member, myosin, actin, tubulin, kinesin (Table 3), and suggested that they may play important role in assembling the physiological mechanism responsible for motility.

Surface antigen (SAG) proteins, microneme family member, dense granular protein, and ROPs, especially rhoptry kinase, play vital roles in the attachment, invasion, gliding motility and parasite adhesion, and intracellular survival as

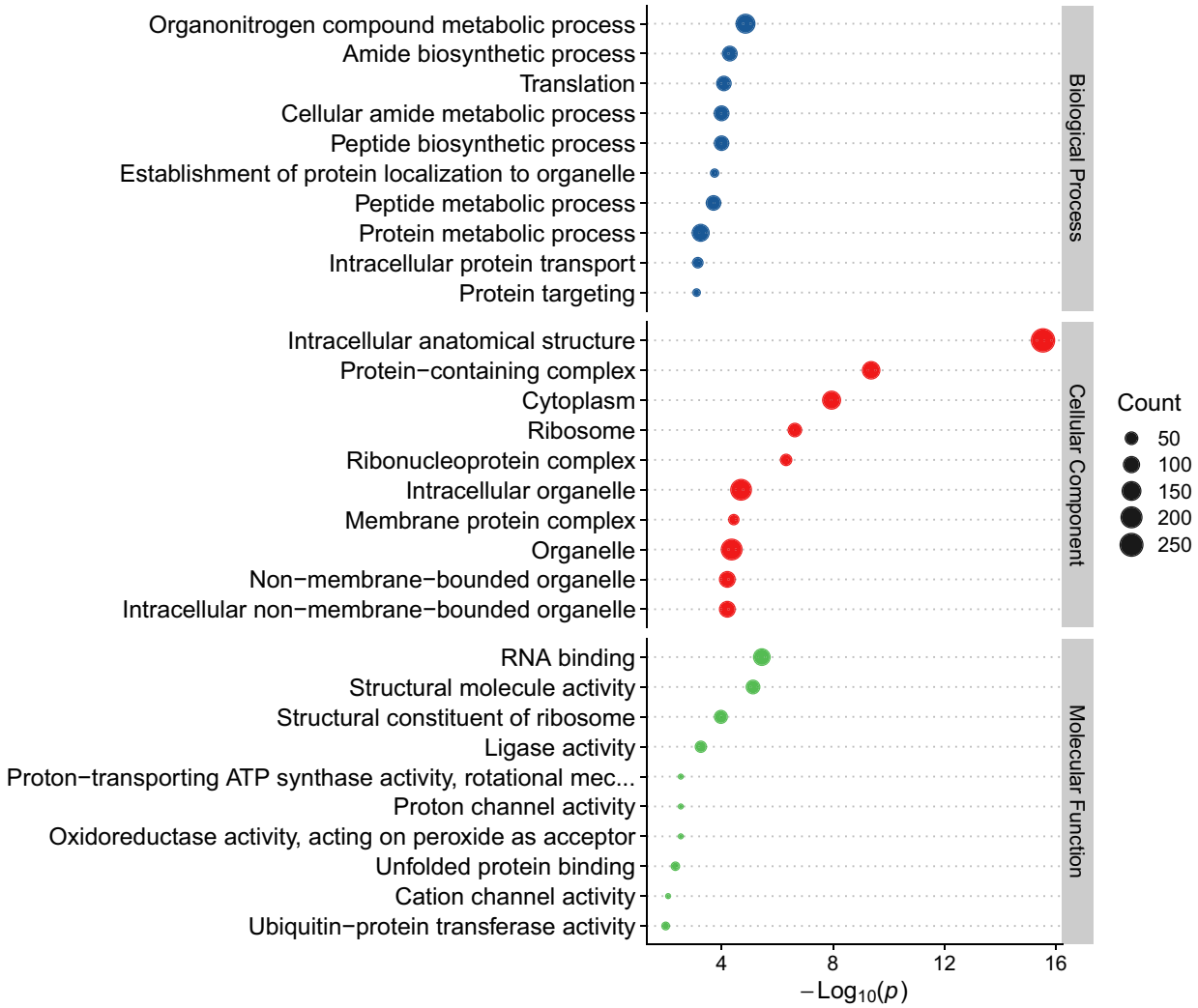

Fig. 2 Gene Ontology (GO) term analysis for the palmitoyl-protome of the second-generation of merozoites in *E. tenella*

well as modification of host cell membrane (Tabarés et al. 2004; Liu et al. 2019; Yan et al. 2018; Wang et al. 2020a, b; Li et al. 2020; Yin et al. 2013; Oakes et al. 2013; Song et al. 2020). Several SAGs, IMCs, ROPs, and even apical membrane antigen were palmitoylated (Table 4), the S-palmitoylation may therefore be a mechanism to regulate the interaction of parasite and the host during invasion process in the second-generation merozoites of *E. tenella*.

S-palmitoylation of enzymes involved in carbohydrate metabolism, glycan biosynthesis and metabolism as well as the respiratory chain. The carbohydrate metabolism is involved in citrate cycle (TCA Cycle), pentose-phosphate pathway, glycolysis/gluconeogenesis, and fructose and mannose etc. Previous report demonstrated that the intracellular carbohydrate amylopectin could produce ATP by using glycolysis and pentose-phosphate pathway. Besides, *E. tenell*a use a TCA cycle that is essential for aerobic respiratory (Matsubayashi et al. 2013). In this study, several proteins that are involved in citrate cycle (TCA Cycle) metabolism pathways were palmitoylated, including citrate synthase, fumarate hydratase, succinyl-CoA ligase, putative (fragment), pyruvate dehydrogenase E1 component subunit alpha, isocitrate dehydrogenase (NADP(+)), and a oxoglutarate dehydrogenase (succinyl-transferring); and 6-phosphogluconate dehydrogenase, putative, and transketolase as well as transaldolase that belong to pentose-phosphate pathway were palmitoylated (Table 5).

The mitochondrial electron transport chain occurs in fumarate respiration and as classic oxygen-dependent respiration to initiate the process of oxidative phosphorylation in *E. tenella* (Matsubayashi et al. 2019). Several mitochondrial electron transport chain family members including ATP synthase, NADH, and dehydrogenase as well as a putative malate and quinone oxidoreductase were palmitoylated (Table 5). Thus, it is likely that mitochondrial electron transport chain may experience palmitoylation

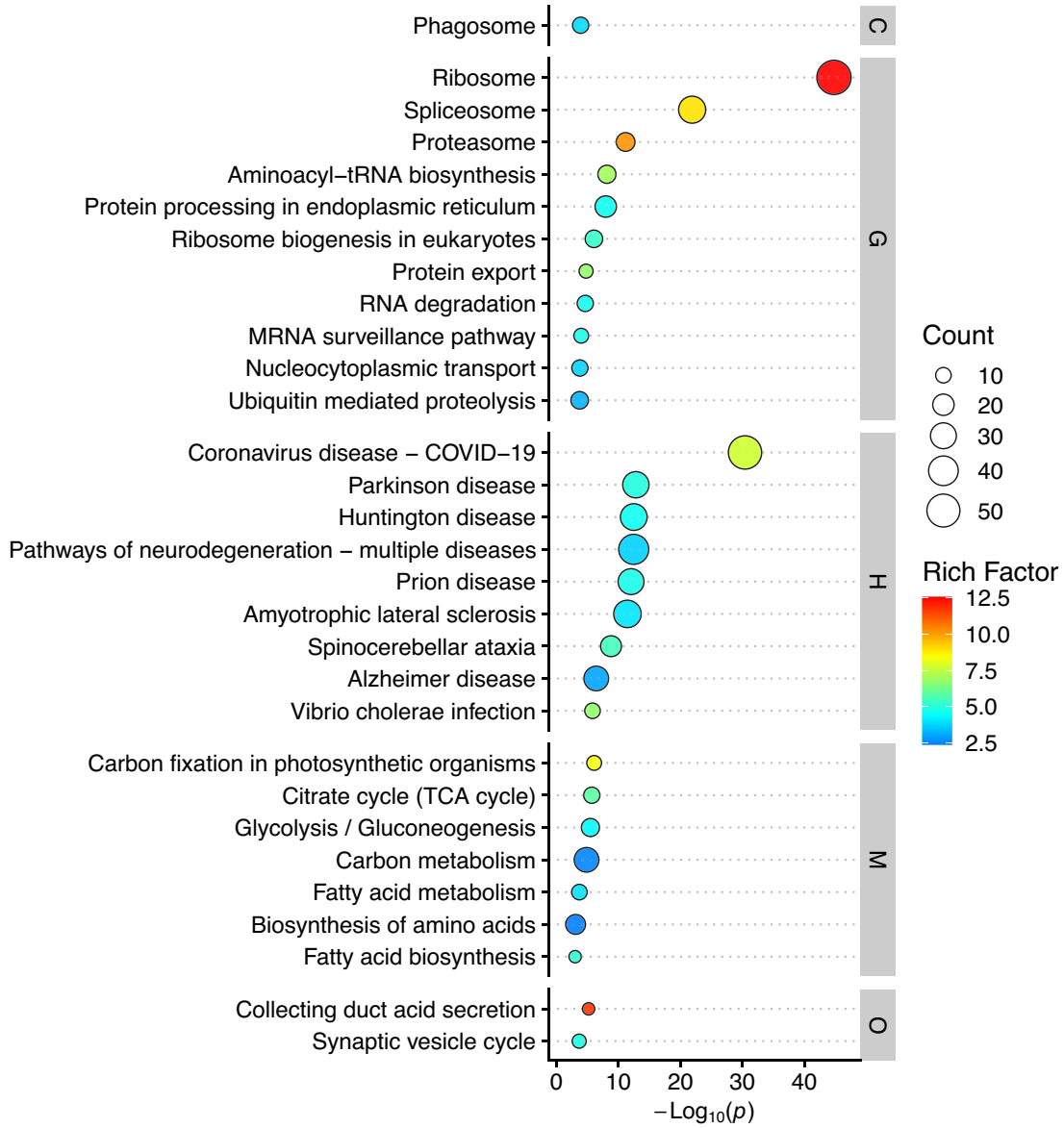

Fig. 3 Pathways enrichment of Palmitoylated proteome in the second generation of merozoites in *E. tenella* defined by KEGG pathway maps

modification to interact with the inner mitochondrial membrane during electron flow. Amylopectin can be degraded and synthesized according to the changing environment in apicomplexan parasite (Augustine 1980) and can be used as an energy supply coupled with glycolysis to fuel the organism and some imported glucose can be converted to glucan (Lyu et al. 2021). In the second-generation of merozoite of *E. tenella*, several pathways involved in glycolysis/gluconeogenesis, glycogen degradation, starch metabolism, glycan metabolism as well as mannose pathway were palmitoylated (Table 5, Fig. 4). Hence, carbohydrate metabolism, glycan biosynthesis, and metabolism process may be regulated by means of S-palmitoylation to adapt the parasite to the intracellular niche.

The protein–protein network in the second generation merozoites in *E. tenella*

Evidence suggests that PPIs form integral part of parasite survival and large PPI networks are useful for better understanding of multiple parasite biological pathways. The notable feature of PPI network was that several core modules were found to interact and play vital role for *E. tenella* invasion, and adhesion, energy supply, transport, degradations, and biogenesis of protein synthesis machinery etc. (Fig. 5). The network of the Top5 significantly enriched pathways ($p < 0.05$) were spliceosome, ribosome, Parkinson's disease, Huntington's disease, and coronavirus disease 2019 (COVID-19) (Fig. 5).

Table 3 Putative S-palmitoylated proteins involved in structure plasticity

No	Uniprot No	Protein description	S-palmitoylation sites
1	H9B970	Membrane skeletal protein IMC1	7, 8, 11, 12
2	U6KGQ6	Inner membrane complex protein IMC3	414, 554, 555
3	U6KV05	Membrane skeletal protein IMC1	350, 368, 551, 552, 555, 559, 566, 567, 569
4	U6KLV1	Myosin motor domain-containing protein	671, 966, 968
5	U6KMN7	Myosin motor domain-containing protein	428, 581, 701
6	U6KP52	Myosin motor domain-containing protein	86
7	U6KPS5	Myosin	192
8	U6KRU7	Myosin heavy chain	306, 1269
9	U6KT05	Myosin F (TgMyoF) protein	800, 1098
10	U6KUP6	Myosin A	53, 127, 334, 544, 664, 722, 733, 747
11	U6KZ69	Myosin heavy chain (Fragment)	346
12	U6L0N6	Myosin motor domain-containing protein	144, 217
13	A2TEQ1	Actin depolymerizing factor	11, 58, 78
14	H9BA70	Actin	53, 218, 258, 286
15	U6KLL3	Actin	37
16	U6KVP2	Dynactin subunit 4	744
17	U6KW14	Actin-like family protein ARP4a	334, 153
18	U6L342	Actin-like family protein ARP4a	97
19	U6L4X7	Actin	18, 20, 72
20	U6KL24	Tubulin beta chain	12, 238, 301, 314, 354
21	U6KWP4	Tubulin alpha chain	65, 347, 353
22	U6KT08	Kinesin heavy chain	448, 1067
23	U6KLQ3	Kinesin motor domain-containing protein	400
24	U6KYT4	AAA_9 domain-containing protein	167

Acyl-biotin exchange assay on immunoprecipitated protei

The IP-ABE Palmitoylation Kit (Cat: AM10314) (AIMS, China) was used for the ABE assay, according to the manufacturer's instructions. *E. tenella* SMGs were lysed for protein extraction, parasite lysate were then incubated with anti-α-tubulin monoclonal antibody and protein A beads, followed by blocking, reduction, labelling, elution, and detection. We found that palmitoylated α-tubulin was only detected in + HAM, and not in -HAM (Fig. 6).

Discussion

Palmitoylation is a spatiotemporal PTM that converts palmitic acid to a cysteine residue through ligation with a thioester within the integral and peripheral membrane protein. Also, palmitoylation facilitates membrane localization, regulation of enzyme activity, stability, and trafficking of protein as well as epigenetic regulation of gene expression (Linder and Deschenes 2007; Brown et al. 2017; Fukata et al. 2016; Blaskovic et al. 2014; Corvi et al. 2011). Previous report demonstrated that palmitoylation exists in several protozoan, including *Toxoplasma gondii*, *Plasmodium falciparum*, *Trypanosoma brucei*, and *Trichomonas vaginalis* (Foe et al. 2015; Caballero et al. 2016; Frénal et al. 2014; Rashidi et al. 2021; Kilian et al. 2020; Jones et al. 2012; Emmer et al. 2011; Batista et al. 2018; Nievas et al. 2018). Specifically, protein palmitoylation plays important regulatory roles in pathophysiological states (Corvi and Turowski 2019), and palmitoylated proteins are known to play essential role in parasite life cycle progression, trafficking, structural remodeling, gliding motility, invasion and egress, and gametocytogenesis (Beck et al. 2013; Frénal et al. 2013; Santos et al. 2015; Santos et al. 2016; Tay et al. 2016). But protein palmitoylation in chicken-infecting pathogenic *E. tenella* remains to be fully characterized.

Several predictive algorithms and comparative proteomics analyses have been employed to study protein palmitoylation in protozoan parasites. Foremost among these are complementary acyl-biotinyl exchange chemistry (ABE) and metabolic labelling such as stable isotope labelling with amino acids in cell culture (SILAC) as well as quantification by mass spectrometry (Brown et al. 2017). Here, ABE method was used to study the palmitoylation within

Table 4 The putative main S-palmitoylation proteins groups involved in parasite-host interaction

Protein groups	Uniprot No	Protein description
Microne family protein	U6KXQ2	Microneme protein 3
	U6KQ25	Microneme 1 (Fragment)
	Q9U966	Micronemal protein MIC4, related
	Q9UAS0	Microneme protein etmic-2/7h
	U6L098	Microneme protein 4
	U6L204	Microneme protein 13, related
	U6L4S8	Microneme protein MIC3
Dense granular protein	U6KRQ5	Dense granular protein GRA10
Rhoptry protein	U6KQJ2	Rhoptry neck protein 2
	A7DZP7	Rhoptry neck protein
Apical membrane antigen	U6KGB8	Apical membrane antigen
SAG family protein	Q70CD4	SAG family member (Sag2)
	Q70CC1	SAG family member (Sag3)
	Q70CD9	SAG family member (Sag4)
	Q70CE2	SAG family member (Sag5)
	Q70CD8	SAG family member (Sag6)
	Q70CE1	SAG family member (Sag7)
	Q70CD7	SAG family member (Sag8)
	U6KW89	SAG family member (Sag9)
	Q70CC2	SAG family member (Sag10)
	Q70CD6	SAG family member (Sag11)
	Q70CE0	SAG family member (Sag12)
	Q70CD1	SAG family member (Sag13)
	A6YRZ5	Surface antigen 14
	Q70CC4	SAG family member (Sag15)
	Q70CD2	SAG family member (Sag16)
	Q70CD3	SAG family member (Sag17)
	Q70CC6	SAG family member (Sag18)
	Q70CC5	SAG family member (Sag20)
	Q70CC8	SAG family member (Sag21)
	Q70CC3	Surface antigen 22
	Q70CC7	SAG family member (Sag23)

E. tenella SMGs. This method was adopted to replace the palmitate with a biotin, followed by conjugation with streptavidin and identification of proteomes assemblage was performed with mass spectrometry. Nonetheless, identified palmitoylated protein in this study would require further validation with functional analysis. That said, several palmitoylated proteins have been shown to participate in parasite morphology, motility and host cell invasion, RNA and protein biosynthesis, folding, proteasome-ubiquitin degradation and enzymes involved in PTMs, carbohydrate metabolism, glycan biosynthesis, and mitochondrial respiratory chain as well as trafficking (Zhang and Hang 2017). In addition, this study suggests that S-palmitoylation is widespread in proteins of diverse functions and localization in *E. tenella*.

Eimeria merozoites structural components include apicoplasts, rhoptries, micronemes, conoids, dense granules, subpellicular microtubules, Golgi apparatus, and cytoskeleton-structure as well as inner membrane complexes (Olajide et al. 2022; Ferguson et al. 2007; López-Osorio et al. 2020). These organelles in *E. tenella* merozoite suggest the avalanche of palmitoylated protein identified in this study. This is because S-palmitoylation commonly target proteins associated with membranous compartments (Zhang and Hang 2017). Similarly, surface antigen proteins (SAGs) are membrane-bound and clasped by glycosylphosphatidylinositol (GPI) anchors to the surface of invasive merozoites in which at least 47 SAGs have been reported in *Eimeria* merozoites (Tabarés et al. 2004) where it played an essential role in attachment and invasion in host-parasite interaction just as micronemes, rhoptries, and dense granules (Song et al. 2020; Reid et al. 2014; Lal et al. 2009; Liu et al. 2019). After parasite gliding on cell adjacent to the interface of *Eimeria* and host, the micronemes released adhesion molecules to build the initial contact by binding with motor complex within the adhesion site. These proteins include the anonymous proteins related to thrombospondin as well as apical membrane antigen 1 (AMA1) (López-Osorio et al. 2020) found to undergo palmitoylation in this study. There is then the need to find out if palmitoylation of these proteins occurred before, during or after parasite transmigration.

Previous report demonstrated that the invasion process is coordinated by a successive secretion of micronemes, rhoptries and dense granules in *T. gondii* (López-Osorio et al. 2020). *E. tenella* may also follow the same principle with respect to evolutionary relatedness of apicomplexan parasites. Likewise, *E. tenella* MICs play essential role in gliding motility, migration and even parasite adhesion (Han et al. 2016; Olajide et al. 2022). Dense granules protein could reshape parasitophorous vacuole (PVs) for the survival of intracellular parasites (Yin et al. 2013). The palmitoylation of these proteins would likely have occurred during tethering on organellar membrane or compartment. Besides, the biological function of ROPs in *E. tenella* includes invasion,

Table 5 Putative S-palmitoylation of enzymes involved in carbohydrate metabolism, glycan biosynthesis, and metabolism

Protein groups	Uniprot No	Protein description
TCA cycle	U6KQ81	Citrate synthase
	H9BA08	Isocitrate dehydrogenase (NADP(+))
	U6L4E6	Oxoglutarate dehydrogenase (succinyl-transferring)
	U6KQM5	Pyruvate dehydrogenase E1 component subunit alpha
	U6KV19	Succinyl-CoA ligase
	U6KZM0	Fumarate hydratase
Pentose-phosphate pathway	U6KWG0	6-phosphogluconate dehydrogenase
	U6KUY8	Transketolase
	U6L0I8	Transaldolase
Glycolysis	U6KUE1	Hexokinase
	U6L3G0	Pyrophosphate–fructose 6-phosphate 1-phosphotransferase
	D7P8J6	Glucose-6-phosphate isomerase
	U6KHR5	Probable ATP-dependent 6-phosphofructokinase
	U6KYI1	Fructose-bisphosphate aldolase
	H9BA04	Triosephosphate isomerase
	E3VWM6, U6KT28	Glyceraldehyde -3-phosphate dehydrogenase
	H9B9A9, U6L4Y7	Phosphoglycerate kinase
	U6L3D6	Phosphoglycerate mutase
	Q967Y8	Enolase
	O44006	Pyruvate kinase
	Q8I8U4	L-lactate dehydrogenase
Opine production	U6KWV4	Opine dehydrogenase
Gluconeogenesis	U6L092,U6KWM0	Fructose-bisphosphatase
Glycogen degradation	U6L804	Glycogen debranching enzyme
Starch metabolism	U6L3I4	Alpha-amylase_C domain-containing protein
	U6L514,U6L7Q2	hDGE_amylase domain-containing protein
	U6KU12, U6KS58	glucan water dikinase
	U6KZC7	1,4-alpha-glucan branching enzyme
Glycan metabolism	U6KPC3	Glycan synthetase, putative
Mannose metabolism	U6KHT7	GDP-mannose 4,6-dehydratase
	U6L658	Mannose-6-phosphate isomerase
The respiratory chain	U6KQ85	Mitochondrial alternative NADH dehydrogenase 1
	U6KP16	Malate:quinone oxidoreductase
	U6KQU4,U6KJT4,U6KT21, U6LCQ7	Vacuolar ATP synthase subunit e, ATP synthase subunit alpha, epsilon and gama chain

modification of the vacuolar environment as well as remodelling of host cell membrane architecture (Oakes et al. 2013). Additionally, in conjunction with MICs, *E. tenella* sporozoite apical membrane antigen 1 (AMA1) plays a vital role in parasite invasion as well as formation of MJ (Li et al. 2020; Wang et al. 2020a, b; Han et al. 2016). Thus, the palmitoylation machinery may function during the invasion and adhesion process in *E. tenella*. Previous research demonstrated that AMA1 in *T. gondii* was a validated palmitoylation protein (Foe et al. 2015). In this study, AMA protein homologous to *T. gondii* AMA1 was also palmitoylated in *E. tenella* and may perform the same role in *E. tenella* as reported in *T. gondii*. Also, several proteins involved in assembling the machinery powering motility were palmitoylated. These evidence suggest that palmitoylation could play essential roles in the function of glideosome. However, previous report demonstrated that the palmitoylated myosin light chain 1 was unrelated with parasite motility (Rompikuntal et al. 2021). Thus the role of palmitoylation in glideosome component and interaction with other proteins

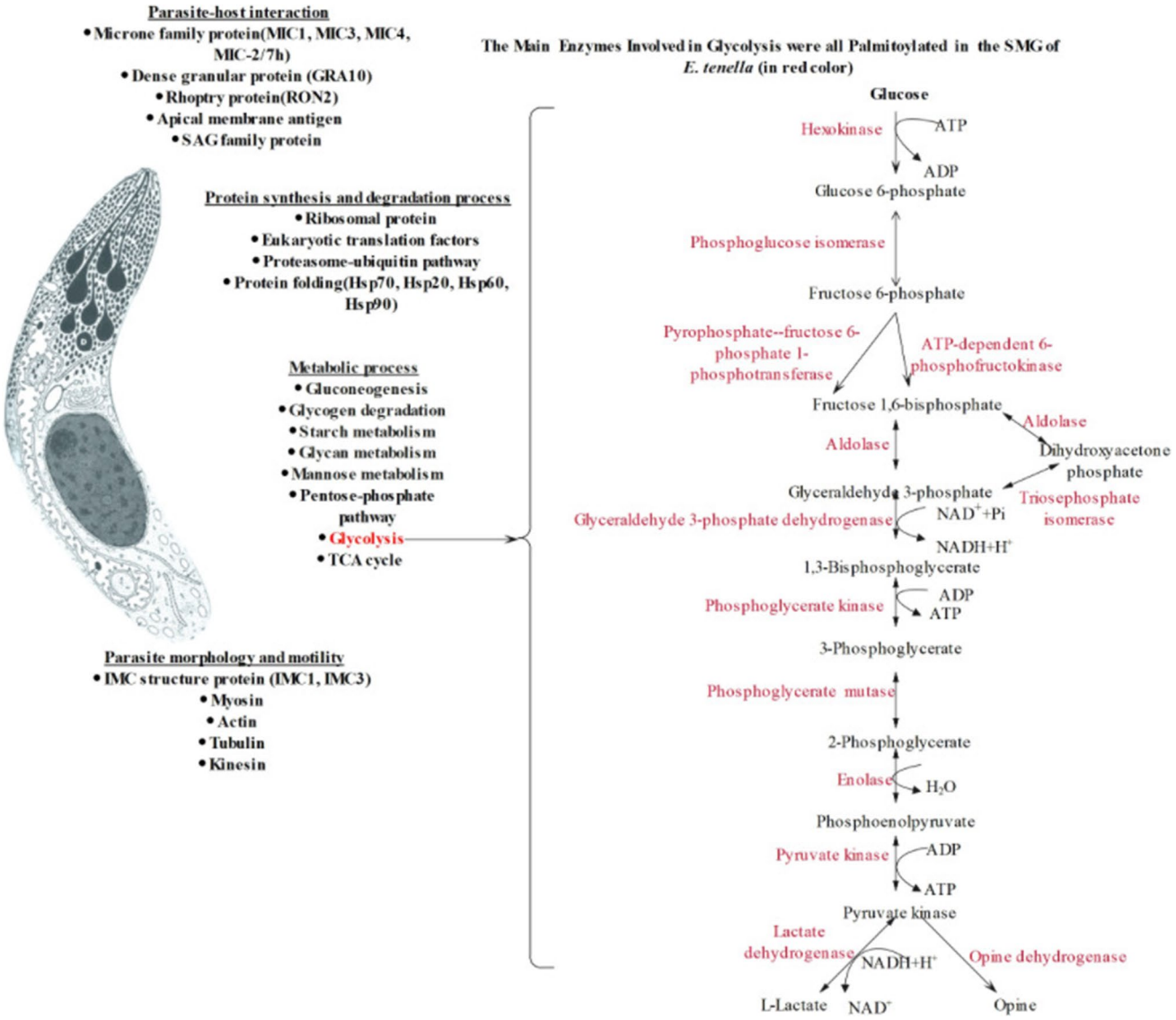

Fig. 4 Selected *E. tenella* palmitoyl proteins important for the second generation merozoites (left panel). Schematic representation of the *E. tenella* palmitoyl proteins important for the second-generation merozoites, including parasite-host interaction, protein synthesis and degradation process, and parasite morphology and motility as well as glycolysis. Several enzymes that participated in glycolysis were palmitoylated (in red color) right panel

involved in motility of *E. tenella* merozoite worth further study. Morphogenesis is facilitated by cytoskeletal structures composed of membrane skeletal proteins (IMC) and the underlying subpellicular microtubules. In previous research, it was demonstrated that palmitoylation of ISP1/ISP3 proteins was crucial during zygote-to-ookinete differentiation in *Plasmodium* species (Wang et al. 2020a, b) and location *in T. gondii* (Fung et al. 2012). Also, palmitoylation at the inner membrane complex is essential for gliding motility and host cell invasion (Brown et al. 2017). It is reasonable to speculate that adjacent palmitoylation of IMC protein and tubulin may be orchestrated to maintain the proper structure of subpellicular microtubules in *E. tenella* SMGs.

Several enzymes and proteins involved in protein biosynthesis and degradation, protein synthesis machinery as well as palmitoylation and glycosylation modification were S-palmitoylated in *E. tenella* merozoites. It is interesting that the enzyme involved in palmitoylation, including the palmitoyltransferase (Uniprot No. U6KNC5) and serine palmitoyltransferase (Uniprot No. U6L3L7) were

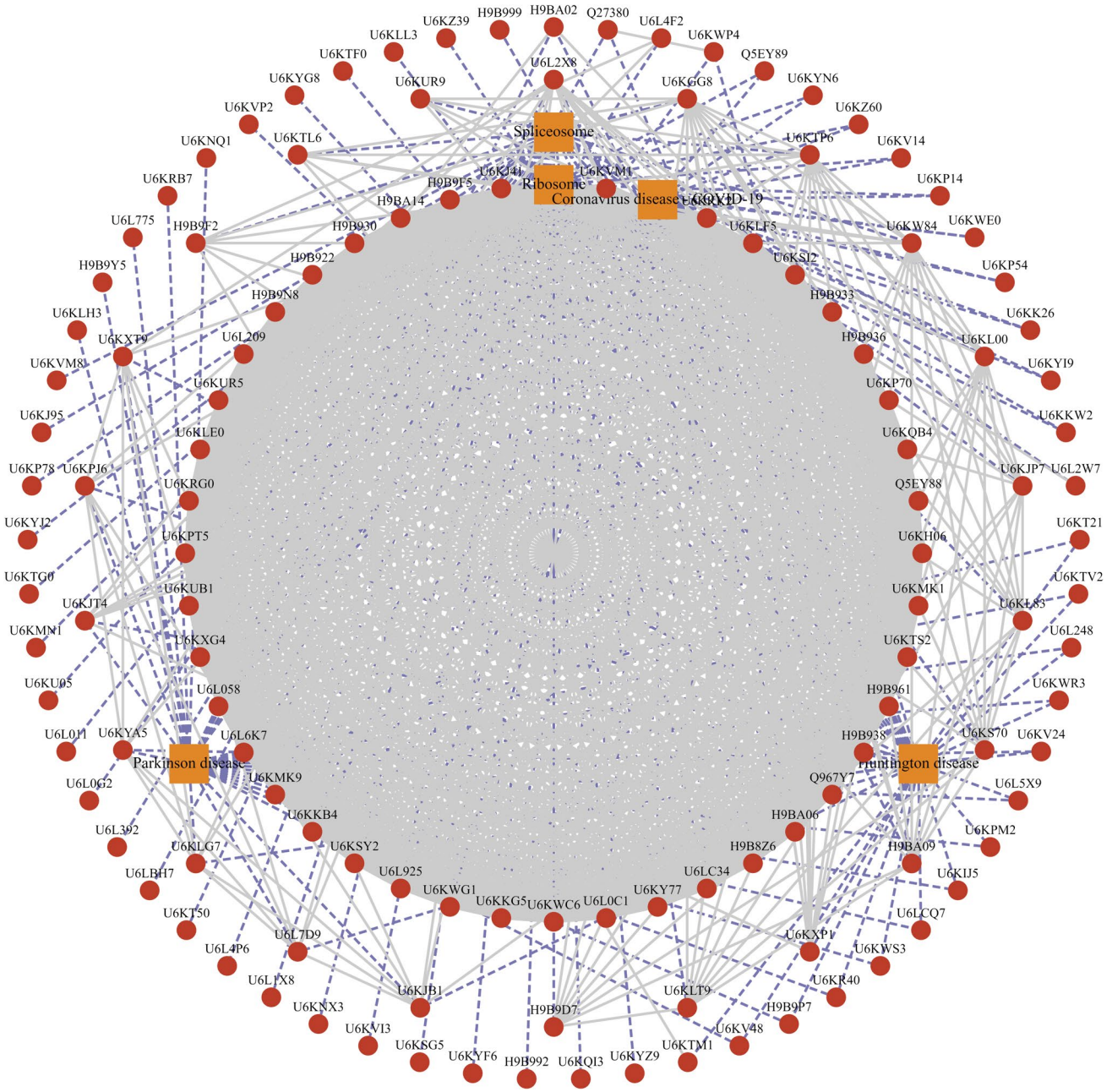

Fig. 5 The protein–protein interaction network in *E. tenella* second generation merozoites. Protein gene (red circle) and related pathway (brown square)

suggested to be palmitoylated. Previous report revealed similar phenomenon by targeting plasma membrane localization in APT1 and APT2 (Kong et al. 2013). The palmitoylated metabolism involves in citrate cycle (TCA Cycle), pentose-phosphate pathway, glycolysis/gluconeogenesis, and amylopectin, fructose, and mannose metabolism, besides vesicular trafficking, were regulated by palmitoylation in *E. tenella*. Thus, palmitoylation cycles may now be considered as a potential novel regulatory architecture worth further study for its core significance in *E. tenella* biology and development.

Global studies have shown S-palmitoylation as important and pervasive post-translational process in parasites with potential to synchronize diverse biological processes.

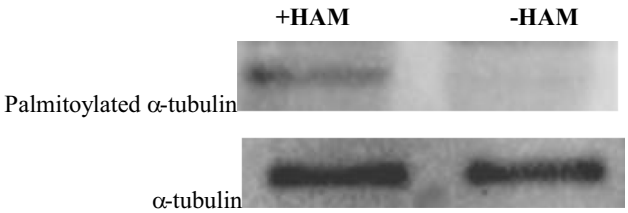

Fig. 6 Acyl-biotin exchange assay on immunoprecipitated α-tubulin (IP-ABE). The SMGs of *E. tenella* were subjected to lysis for protein extraction, then the parasite lysate was incubated with anti-α-tubulin monoclonal antibody and protein A beads, followed by blocking, reduction, labelling, elution, and detection. Palmitoylated α-tubulin was detected in + HAM but not in -HAMw

In this study, palmitoylated proteome was revealed in the second-generation merozoite of *E. tenella* to illustrate the broad impact of this post-translational modification in parasite biology, including trafficking, host cell invasion, carbohydrate metabolism, and gliding motility. This study presents experiential foundation for in-depth study of palmitoylation in the pathobiology of *E. tenella*.

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Author contributions Z.Q. and Y.L. performed the experiment and drafted the manuscript. W.L., N.Z. assisted in data analysis, X.M. designed the study, J.S.O. and B.F. revised the manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Ethics approval Animals used for this study were treated in accordance with protocols reviewed and approved by the Research Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All animals were handled strictly according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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