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Dissecting *Plasmodium yoelii* Pathobiology: Proteomic Approaches for Decoding Novel Translational and Post-Translational Modifications

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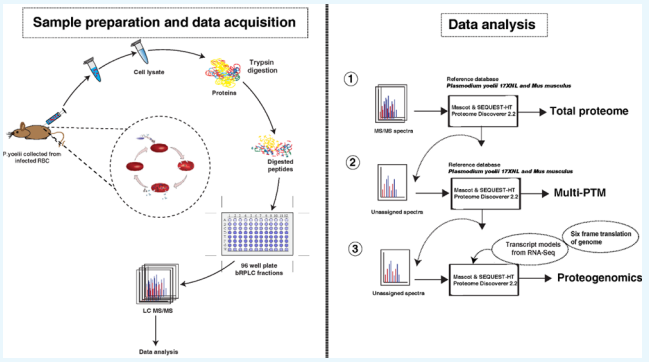
ABSTRACT: Malaria is a vector-borne disease. It is caused by *Plasmodium* parasites. *Plasmodium yoelii* is a rodent model parasite, primarily used for studying parasite development in liver cells and vectors. To better understand parasite biology, we carried out a high-throughput-based proteomic analysis of *P. yoelii*. From the same mass spectrometry (MS)/MS data set, we also captured several post-translational modified peptides by following a bioinformatics analysis without any prior enrichment. Further, we carried out a proteogenomic analysis, which resulted in improvements to some of the existing gene models along with the identification of several novel genes. Analysis of proteome and post-translational modifications (PTMs) together resulted in the identification of 3124 proteins. The identified PTMs were found to be enriched in mitochondrial metabolic pathways. Subsequent bioinformatics analysis provided an insight into proteins associated with metabolic regulatory mechanisms. Among these, the tricarboxylic acid (TCA) cycle and the isoprenoid synthesis pathway are found to be essential for parasite survival and drug resistance. The proteogenomic analysis discovered 43 novel protein-coding genes. The availability of an in-depth proteomic landscape of a malaria pathogen model will likely facilitate further molecular-level investigations on pre-erythrocytic stages of malaria.



INTRODUCTION

Malaria is an infectious disease with a significant global public health burden, responsible for nearly 409,000 deaths and 229 million new cases in 2019 (World Health Organization (WHO) World Malaria Report 2020). Malaria is caused by pathogens belonging to the genus *Plasmodium* and is transmitted by the female *Anopheles* species. The asexual development of malaria parasites in RBCs leads to high fever and shock, and, if not treated, it often results in morbidity and death.[1,2]

Plasmodium yoelii, *Plasmodium chabaudi*, and *Plasmodium berghei* are known to cause malaria in rodents and have been widely used as models to understand in vivo parasite development, pathogenesis, and host immunological responses. These species have similar biological characteristics to those of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. The *P. yoelii* strain 17XNL is considered an appropriate experimental model for malaria pathogenesis in humans and helps in understanding the host defense mechanisms.[3]

Significant efforts and resources have been utilized in the identification of malaria parasite proteins toward developing better diagnostics and vaccines. The emphasis has been on identifying protein targets and characterizing various cytoplasmic, cell surface, and secreted proteins.[4,5] Mass spectrometry-based quantitative phosphoproteomic analysis has been used to study specific phosphorylation-based molecular targets and associated signaling in apicomplexan parasites.[6−8] While several investigations involved prior enrichment of specific post-translational modifications (PTMs), a combination of advanced bioinformatics strategies can be adopted to fetch several PTMs from the large-scale proteomic data sets without a priori enrichment for those PTMs.[9,10] Although a set of protocols used for such a multi-PTM analysis appears to be

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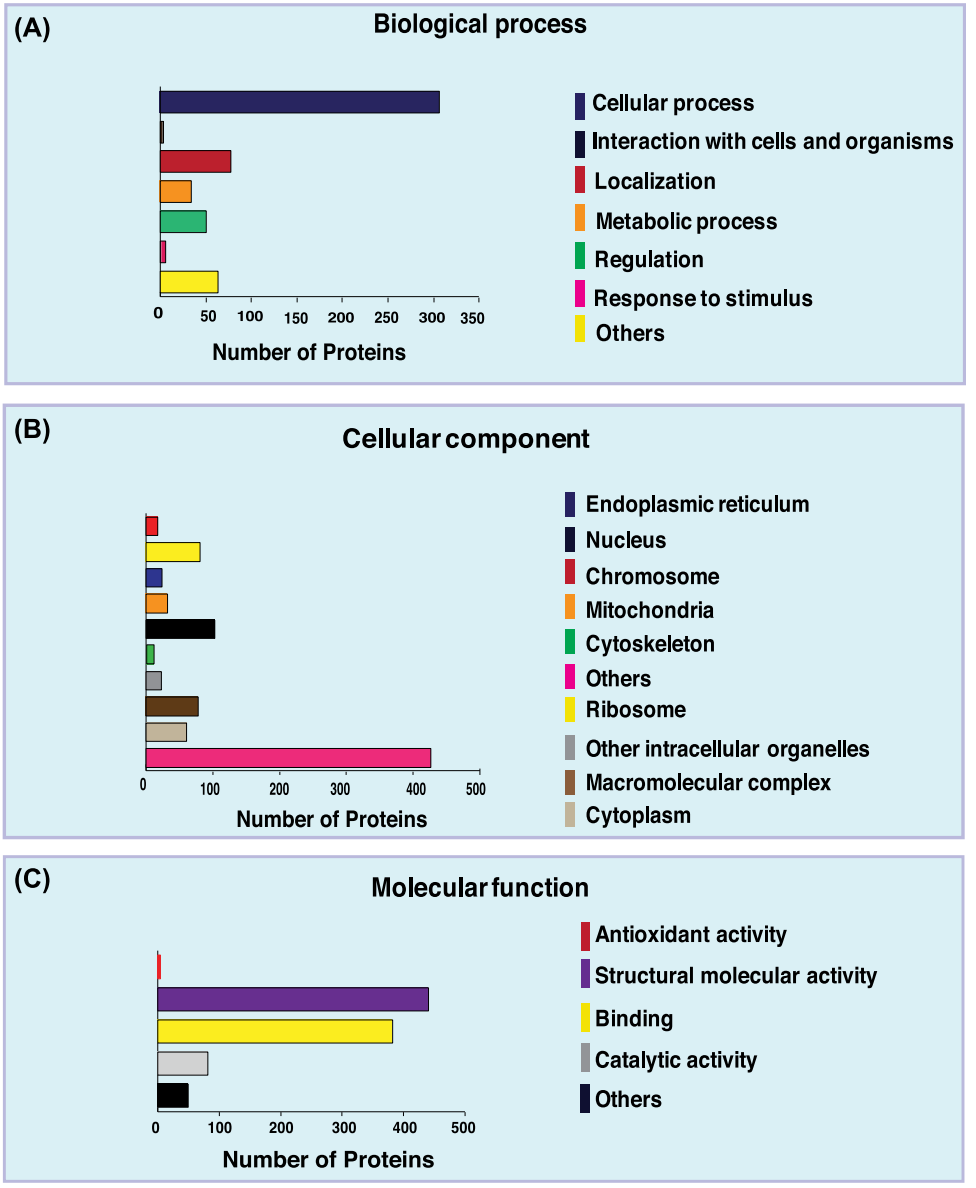

Figure 1. Gene ontology (GO) annotation: localization and functional annotation of the identified proteins in *P. yoelii* 17XNL. For the functional analysis of the identified proteins in *P. yoelii* 17XNL, GO information was fetched from the STRAP gene ontology annotation tool. (A) Biological processes, (B) cellular components, and (C) molecular functions are represented in order of their occurrence for each protein represented.

simple, it needs sophisticated computational infrastructure and takes more time when compared to routine proteomic analysis.[11]

PTMs are the result of either covalent linking of functional groups to amino acid residues or proteolytic cleavage.[12] Enzymatically attained PTMs are many, which include phosphorylation, glycosylation, acetylation, methylation, sumoylation, palmitoylation, biotinylation, ubiquitination, and nitration.[13,14] Other PTMs include glycation, nitrosylation, malonylation, oxidation/reduction, succinylation, and glutarylation.[15−19] PTMs play an essential part in modifying the protein conformation that imparts a unique feature to its activities. Therefore, PTM events are linked with cellular functions including receptor activation, signaling and metabolic pathways, enzyme activities, protein translocations, and cellular differentiation. Several PTMs have been known to be associated with host−pathogen interactions and pathogenesis.[20,21]

The metabolic pathways of malaria parasites are known to be significantly altered post infection from those of their hosts.[22] During intraerythrocytic stages, reversible PTMs of mitochondrial proteins have been shown to play an important role in response to differences in nutrient requirement and redox conditions. These modified proteins can also further affect protein−protein interaction networks, thereby altering mitochondrial functions,[15,23] including redox signaling via reactive oxygen/nitrogen species in the modulation of cellular activities, phosphorylation, and glutarylation. There has been a vast increase in the identification of mitochondrial PTMs and their protein targets in recent times.[24−26] The functional significance of these PTMs in disease etiology and the pathologic response is yet to be deciphered. However, many of these reversible PTMs have been known to act as regulatory mechanisms in mitochondria and can be avenues for the development of mitochondria-targeted therapeutic strategies.[27]

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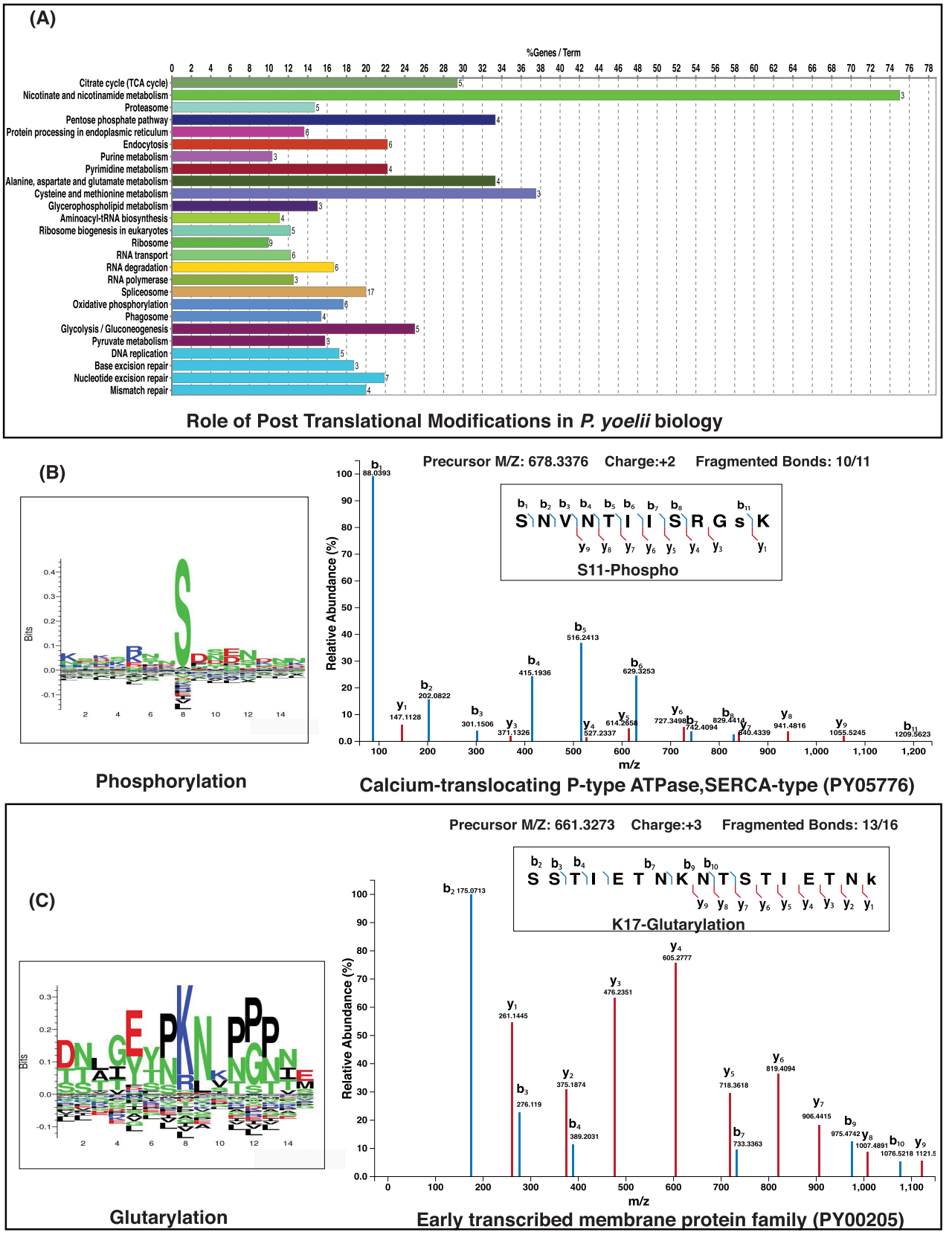

Figure 2. Pathway-wise distribution of PTMs in *P. yoelii* 17XNL, and the analysis of phosphorylation and glutarylation sites. (A) Pictorial diagram of gene ontology classification of genes with modified protein products from post-translationally modified proteins of *P. yoelii* 17XNL. (B) Motif images for motif conservation analysis of PTM types, including (a) phosphorylation and (b) glutarylation. Plots were generated with iceLogo and scaled for better data visualization. (c) Representative spectra of a phosphorylation and glutarylation peptide.

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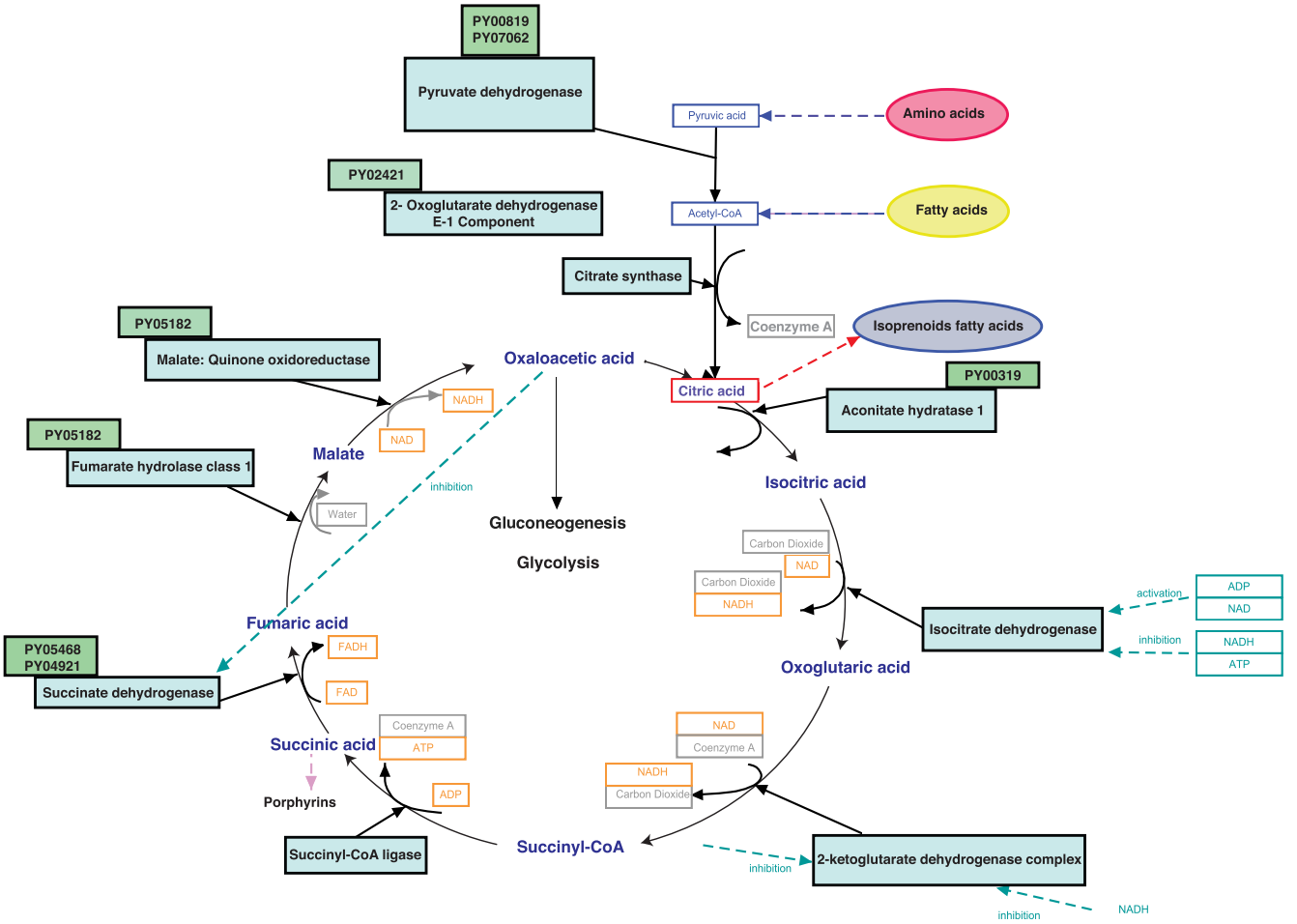

Figure 3. Tricarboxylic acid (TCA) cycle architecture in *P. yoelii* 17XNL. Schematic depiction of possible multiple PTMs involved in the TCA cycle architecture in *P. yoelii* 17XNL. Modified proteins are given in the green box: fumarate hydratase class I (PY05182, phosphorylated at S341), aerobic-related flavoprotein subunit of succinate dehydrogenase (PY05468, deamidated at R433), 2-oxoglutarate dehydrogenase, E1 (PY02421, phosphorylated at T104), pyruvate dehydrogenase E1 α subunit (PY00819, glutarylated at K130).

To determine the identity and abundance of protein biomarkers, mass spectrometry (MS) data are frequently searched against protein databases. During the search, a substantial portion of MS/MS data gets matched to peptides represented in the protein database used for the search; however, a significant chunk of MS/MS spectra remains unassigned. These MS/MS data may contain different PTMs or novel peptides not represented in the protein database. The unassigned spectral data can be mined for fetching multiple post-translational modifications (multi-PTMs), including phosphorylation, glutarylation, succinylation, and acetylation, among others.[9−11] The unassigned MS/MS data can also be subjected to proteogenomic analysis to identify novel peptides, which can help define novel protein-coding regions. Several studies from across the world, including our group, have shown that proteogenomics helps refine genome annotation and identify taxonomically diverse reference proteomes.[28−31] In this study, we reported the discovery of 43 unique protein-coding genes from *P. yoelii* by this approach.

RESULTS

In the present study, we aimed to carry out in-depth proteomic profiling of *P. yoelii* 17XNL by performing a label-free proteomic analysis. We also subjected unassigned MS/MS spectra to a multi-PTM analysis and a proteogenomic analysis to identify multiple PTMs and novel protein-coding events in *P. yoelii*. Using high-resolution mass spectrometry as a discovery tool, we identified 3124 proteins, including possible PTMs and novel genes of *P. yoelii* 17XNL (Supporting Figure S1). The proteomic analysis of a total of 369,429 MS/MS spectra against a *P. yoelii* protein database resulted in 45,309 peptide-spectrum matches (PSMs) to 8456 unique peptides, which belonged to 1409 proteins (Supporting Table S1). The functional analysis of proteins identified in *P. yoelii* 17XNL was fetched from the Software tool for researching annotations of proteins (STRAP) database. Cellular processes, nuclei, and structural molecular activities were highly enriched in gene ontology (GO) analysis of the biological process, cellular components, and molecular functions, respectively (Figure 1).

In addition, a multi-PTM analysis resulted in the identification of 2324 proteins through 44,548 PSMs belonging to 24,944 unique PTM-containing peptides. The complete list of modified peptides identified along with site and residue information is provided in Supporting Table S2. Finally, a proteogenomic investigation helped us improve the possibility of novel protein identifications. We identified that 1892 PSMs belonged to 43 novel protein-coding genes with more than two PSMs (Supporting Table S3).

Post-Translational Modifications. To study the PTMs in *P. yoelii* 17XNL using proteomic data generated in this study,

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Table 1. Summary of the List of Genes Modified at the Protein Level and Involved in Metabolic Pathways

pathway ID	pathway	input Identifier(s)	pathway source	total pathway compounds	total pathway enzymes
ec00020	citrate cycle (TCA cycle)	PY01233,PY04573,PY03521,PY02619,PY02175,PY02160,PY02022,PY01291,PY00573,PY05233,PY02421,PY05468,PY07062,PY05567,PY05468,PY05182,PY05005,PY03887,PY00819,PY05175,PY04921	KEGG	31	56
ec00230	purine metabolism	PY00163,PY06466,PY06423,PY05109,PY04754,PY04439,PY03988,PY02022,PY01515,PY01480,PY01467,PY01287,PY01233,PY01231,PY00951,PY06858,PY06310,PY06200,PY05969,PY05353,PY04969,PY04622,PY04459,PY03547,PY03290,PY01847,PY01721,PY01291,PY01037,PY00920,PY00203,PY06559,PY06115,PY05727,PY05567,PY05402,PY04645,PY04450,PY03478,PY03255,PY02999,PY02858,PY02448,PY01488,PY01044,PY00206,PY05854,PY05002,PY04349,PY03601,PY03187,PY01438,PY00626,PY00349	KEGG	100	179
ec00240	pyrimidine metabolism	PY00160,PY06964,PY06466,PY06257,PY06115,PY04095,PY02448,PY01988,PY01291,PY00203,PY00163,PY06855,PY06210,PY05353,PY04969,PY04781,PY03441,PY02484,PY01480,PY00873,PY06423,PY06378,PY06200,PY05727,PY05596,PY05109,PY05046,PY05002,PY03547,PY03351,PY02925,PY02858,PY01847,PY01626,PY01438,PY01072,PY00858,PY00008,PY06559,PY06310,PY06281,PY05578,PY05567,PY04717,PY04622,PY04439,PY04160,PY04092,PY03885,PY03601,PY03255,PY03187,PY02580,PY02213,PY02022,PY01721,PY01515,PY01378,PY01037,PY00626	KEGG	75	114
POLYISOPRENSYN-PWY	polyisoprenoid biosynthesis	PY00446,PY05008,PY01291,PY00626,PY02448,PY01480,PY02022	KEGG	18	6
PRPP-PWY	superpathway of histidine, purine, and pyrimidine biosynthesis	PY00669,PY06310,PY05567,PY02448,PY02022,PY00920,PY00626,PY05596,PY05420,PY05102,PY02580,PY01826,PY01556,PY06257,PY06210,PY06050,PY03547,PY03290,PY01515,PY01480,PY01467,PY01438,PY01291,PY00962,PY00170,PY06546,PY05969,PY04969,PY04781,PY03768,PY03601,PY03087,PY02858,PY01721	MetaCyc	193	51
PWY-4202	arsenate detoxification I (glutaredoxin)	PY03441,PY02213,PY02022,PY01072,PY06964,PY06281,PY04095,PY00160,PY01988,PY01291,PY05046,PY04622,PY04160	MetaCyc	29	7
PWY-6111	mitochondrial L-carnitine shuttle	PY01291,PY02022,PY02160,PY05005,PY02619,PY03887	MetaCyc	6	2
PWY-7419	FR-900098 and FR-33289 antibiotic biosynthesis	PY00520,PY06310,PY05459,PY03988,PY02160,PY00941,PY00909,PY00385,PY04754,PY04573,PY03521,PY03001,PY01515,PY01233,PY00104,PY04969,PY04599,PY04349,PY03887,PY03309,PY02860,PY02679,PY02448,PY00897,PY00349,PY00206,PY06530,PY05854,PY05248,PY05005,PY04441,PY04257,PY03791,PY02619,PY02022,PY01438,PY01291,PY01256	MetaCyc	39	10
PWY-7560	methylerythritol phosphate pathway II	PY02340,PY06573,PY06556,PY06538,PY04970,PY04665,PY03319,PY01468,PY01198,PY07071,PY05578,PY03506,PY03298,PY00078,PY04544,PY04198,PY04026,PY03956,PY02339,PY00761,PY06874,PY04024,PY04005,PY02780,PY02022,PY05567,PY04665,PY01291	MetaCyc	35	9
NONMEVIPP-PWY	methylerythritol phosphate pathway I	PY01468,PY06874,PY06573,PY04970,PY04665,PY04544,PY04198,PY04005,PY03956,PY03319,PY02780,PY02340,PY00761,PY05578,PY04026,PY03298,PY02339,PY01198,PY06556,PY03506,PY00078,PY07071,PY06538,PY04024,PY05567,PY00446,PY01291	MetaCyc	33	9

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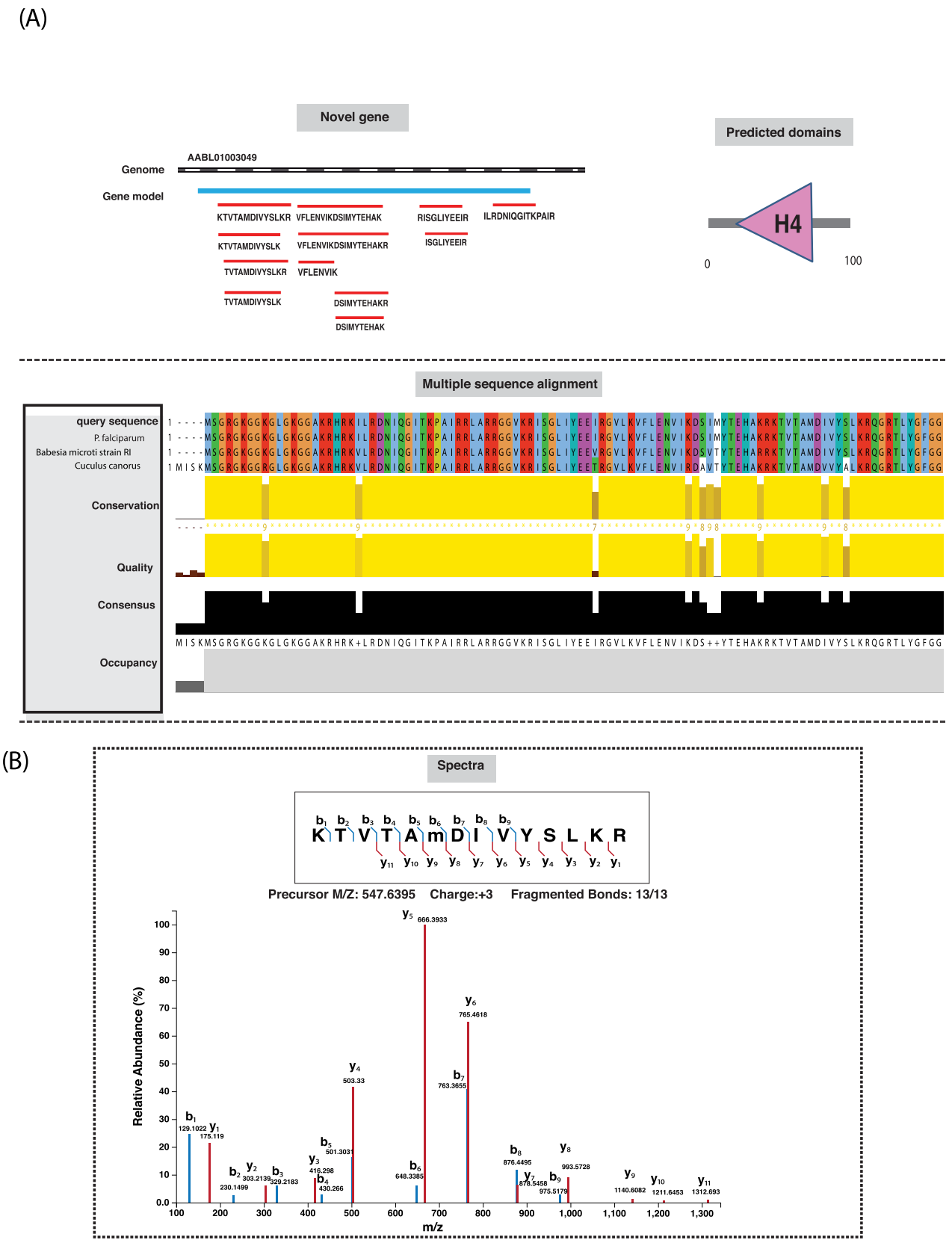

Figure 4. Proteogenomic analysis map of histone H4 peptide evidence. (A) Comparative genomic analysis using BLAST showed evidence for histone H4 matching with other virulent *Plasmodium* parasites; sequences in red font indicate the peptides identified in this study. (B) Representative MS/MS spectrum of the peptide VFLENVIKDSIMYTEHAKR is shown in the lower panel.

we searched unassigned MS/MS spectra against *P. yoelii* 17XNL and *Mus musculus* RefSeq with possible modifications such as phosphorylation at serine, threonine, and tyrosine and glutarylation at lysine residues, deamidation at the arginine

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residue, and succinylation at the lysine residue. The PTM-Pro tool was used to summarize the high-confidence PTMs based on the statistical score provided by Proteome Discoverer search algorithms.[32] As a result, we identified 2906 unique PTM sites corresponding to 1807 proteins. These proteins containing PTMs were then subjected to downstream bioinformatics analysis. Figure 2**A** represents the potential role of PTMs in regulating the activity of different metabolic enzymes and their functions.

Phosphorylation and Glutarylation. Phosphorylation is one of the common PTMs in *Plasmodium*, which plays a key role in regulating the life cycle and fluctuates throughout the life cycle.[33] We identified 2490 unique phosphosites mapping to 1436 proteins. These phosphorylation sites include serine (S) (1329 sites), followed by threonine (T) (881 sites) and tyrosine (Y) (280 sites). We identified 60% of the phosphoproteins in the multi-PTM analysis.

This study identified several key phosphoproteins, including calcium-translocating P-type ATPase, SERCA-type (PY05776) phosphorylated at the T438 residue (Figure 2**B**) and the ABC transporter protein (PY07088) phosphorylated at S698 and S703, which are known to play a role in parasite development by second-messenger-mediated signaling and trafficking cascades.[34,35]

Lysine (K) glutarylation is known to be associated with cellular functions such as metabolic processes, oxidative damage, and other mitochondrial functions.[36,37] We identified 1058 unique glutarylation sites mapping to 836 proteins in this study. The examples include several proteins of the early transcribed membrane protein (ETRAMP) family, including erythrocyte membrane protein 3 (PY05500 at K15, PY04042 at K863, PY06125 at K770), erythrocyte membrane-associated antigen (PY06959 at K460), erythrocyte membrane-associated antigen, putative (PY06110 at K125), and ETRAMP (PY00205 at K17). Respective MS/MS spectra for ETRAMP (PY00205) SSTIETNKNTSTIETNK are shown in Figure 2**C**.

Role of PTMs in Metabolic Pathways in *P. yoelii* 17XNL. In addition to the detection of PTMs, to understand the role of PTMs in the pathways and interaction networks, we queried a list of PTM-containing proteins against the PlasmoDB to fetch the known role of these proteins in regulating *P. yoelii* 17XNL metabolic pathways. The resultant pathways included the TCA cycle, carbon metabolism, pyruvate, alanine, aspartate, and glutamate metabolism, and the biosynthesis of secondary metabolites. A list of modified proteins identified in metabolic pathways in *P. yoelii* 17XNL is made available in Supporting Table S4 and is depicted in Supporting Figure S2.

The TCA cycle, a fundamental metabolic pathway within the parasite mitochondrion, has not been fully explored as a potential drug target. We identified PTMs on 21 proteins among 32 proteins mapped to the TCA cycle. These proteins include enzymes such as fumarate hydratase class I (PY05182, phosphorylated at S341), 2-oxoglutarate dehydrogenase E1 (PY02421, phosphorylated at T104), and the pyruvate dehydrogenase E1 α subunit (PY00819, glutarylated at K130) (Figure 3).

Pathway enrichment analysis also showed that many PTMs act through the methylerythritol phosphate (MEP) pathway. Our analysis detected PTMs on three proteins among 16 proteins mapped to the MEP pathway. These included polyprenyl synthetase (PY00446, phosphorylated at S523), hypothetical protein (PY02448, phosphorylated at S504), and hypothetical protein (PY02022, phosphorylated at T29 and T30), which are post-translationally modified (Table 1). Orthology-based analysis showed that *P. yoelii* proteins identified in the TCA cycle and the MEP pathway show the same orthologue signature with functions with *P. falciparum* and *P. vivax* (Supporting Table S5).

Proteogenomic Analysis. Annotation of protein-coding genes in any sequenced genomes is generally carried out by gene prediction algorithms. Sometimes, transcriptomic data are used to provide an additional layer of confidence to the predicted genes. However, mass spectrometry data can derive real evidence for protein-coding genes. Therefore, when an MS/MS data of proteome is searched against the annotated proteome of *P. yoelii* 17XNL, a considerable portion of MS/MS data-validated peptides could be predicted in the protein database. Following established proteomic strategies, we searched the unassigned MS/MS spectra against a six-frame translated genome as well as the transcriptome of *P. yoelii*. Using this approach, we were able to identify 43 novel protein-coding genes of *P. yoelii*. We also gathered further evidence based on orthology for all of these newly discovered genes, which provided more confidence to the discovery. The list of peptides identified in the proteogenomic search for these 43 protein-coding genes is provided in Supporting Table S3. These novel genes included orthologues of histone H4, histone chaperone ASF1, and putative and 60S ribosomal protein *L15-1* of other virulent species of *Plasmodium* parasites. Peptide evidence for the predicted gene histone H4 shows the same structural relationship with *P. falciparum* and *Babesia microti* (Figure 4**A**). Representative MS/MS spectra of the peptide VFLENVIKDSIMYTEHAKR are given in Figure 4**B**. The sequences of these novel events along with orthologue evidence are provided in Supporting Table S6.

DISCUSSION

The *P. yoelii* 17XNL genome has an estimated genome size of 23.1 Mb, with more than 3300 protein-coding genes annotated. Previous proteomic investigations of *P. yoelii* 17XNL sporozoites by Lindner et al. and Siau et al. have reported the identification of 1876 and 1335 proteins.[38,39] However, through a series of OMICS approaches including proteomics, multi-PTM analysis, and proteogenomic analyses, we could identify 3124 protein-coding genes in the *P. yoelii* proteome.

In global proteome analysis, we identified several malarial drug target proteins including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and myosin A (MyoA). GAPDH is known to trigger the kinase activation and phosphorylation of other proteins.[40] A recent study has shown that covalent inhibitors of pfGAPDH can be a therapeutic target for malaria treatment.[41,42] The MyoA is a myosin motor, which implicates the gliding motility and is essential for efficient host red blood cell invasion.[43,44]

In addition to identifying evidence for proteins, we also identified several PTMs of *P. yoelii*. PTMs are likely to play key roles in apicomplexan biology and could affect protein activity, localization, and protein interactions. Therefore, the PTMs identified in this study may provide a platform for further investigation of their role in the context of the regulatory mechanisms in malaria transmission, virulence, and adaptations to multiple host environments.[45,46] The significant role of phosphorylation in regulating gametogenesis and exflagellation of male gametocytes has been studied in *P. falciparum*, *P. yoelii*,

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and *P. berghei*.[47−49] We identified several phosphoproteins, including several ribosomal proteins, such as Rps S2, Rps S9, Rps L3, and Rps L13e, which are known to be essential to maintain the structural and functional integrity of the mitochondrion. Ke et al. have reported that the mitochondrial ribosomal protein L13 is critical for the structural and functional integrity of the mitochondrion in *P. falciparum*.[50] We also identified phosphorylation of calcium-translocating P-type ATPase, SERCA-type (PY05776) and the ABC transporter protein (PY07088), which have been known to be involved in calcium transport and uptake by infected RBC.

Glutarylation, a PTM that occurs on a lysine residue, has been associated with mitochondrial functions and metabolic processes such as cellular respiration, fatty acid metabolism, and amino acid metabolism. We identified glutarylation of the early transcribed membrane protein family (ETRAMP) members, which play a significant role in parasite invasion into the host cell.[51] These ETRAMP family members are expressed throughout the life cycle of *Plasmodium* spp, particularly in the early intraerythrocytic cycle.[52] During the asexual blood-stage, these proteins are localized in the early parasitophorous vacuole membrane, where they act as an interface with the host erythrocyte.[53] The importance of glutarylation of ETRAMP proteins needs further investigation in the context of host−pathogen interactions.

CONCLUSIONS

A multipronged omics approach to analyze the proteome of *P. yoelii* 17XNL resulted in several discoveries of novel protein-coding genes and multiple PTMs. These findings provided substantial improvement for the genome annotation of this important model organism. Some of these novel genes have orthologues in other closely related species with very important roles in virulence and pathogenesis. PTMs identified were found to be on proteins with known roles in important metabolic and signaling pathways associated with host−pathogen interactions. Put together, these discoveries together will provide a platform for future investigation on molecular mechanisms of pathogenesis and potential drug targets.

MATERIALS AND METHODS

Parasite Culture and Protein Isolation. BALB/c female mice were housed in an individually ventilated caging (IVC) system at standard environmental conditions (22−25 °C, 40−70% humidity, and 12:12 h dark/light photoperiod). All animal experiments were performed according to the protocols (IAEC 5/2015) approved by the Institutional Animal Ethics Committees (IAEC) of the Centre for Cellular and Molecular Biology, Hyderabad. A frozen stock of *P. yoelii* 17XNL was injected intraperitoneally into three 3−6 week old BALB/c mice, and infection was monitored regularly by observing Giemsa-stained blood smears of the tail snips. The mice were euthanized at about 30% parasitemia, and blood was collected in Alsevier's solution by cardiac puncture. Parasites were purified by the saponin lysis method, and parasite pellets were frozen at −80 °C until use. The parasite pellets were lysed in 4% SDS in 50 mM triethyl ammonium bicarbonate (TEABC). The lysates were resuspended in twice the volume of lysis buffer, homogenized, sonicated, and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected, and protein concentration was estimated using the bicinchoninic acid assay (BCA) to estimate the concentration of proteins.

In-Solution Digestion. In-solution digestion was carried out as previously described.[54,55] Briefly, 250 μg of protein was reduced with 10 mM dithiothreitol (DTT) and alkylated using 20 mM iodoacetamide (IAA). The lysate was further subjected to acetone precipitation to remove sodium dodecyl sulfate (SDS). The protein pellet was dissolved in 50 mM TEABC and digested with trypsin (1:20) (modified sequencing grade; Promega, Madison, WI) at 37 °C for 16 h. The reaction was stopped by adding 0.1% formic acid. The peptides were lyophilized and stored at −80 °C until further use.

Basic pH RPLC-Based Fractionation. The lyophilized peptides were subjected to basic pH reverse-phase chromatography (bRPLC). The samples were reconstituted in bRPLC solvent A (10 mM TEABC) and separated on a Waters XBridge C18 column (Waters Corporation, Milford, MA; 130 Å, 5 μm, 250 mm × 4.6 mm) attached to a Hitachi LaChrom Elite HPLC system. The flow rate was set at 0.5 mL/min. The fractionation was carried out using a linear increase in gradient from 5 to 100% of the solvent B (10 mM TEABC buffer, 90% ACN, pH ∼ 8.5) for 130 min. A total of 96 fractions were initially collected and later concatenated to six fractions and dried in a SpeedVac concentrator. The dried fractions were further desalted using SCX cartridges. Desalted peptides were vacuum-dried and stored at −80 °C until LC-MS/MS analysis as described previously.[54,55]

LC-MS/MS Analysis. In the present study, we performed LC-MS/MS analysis of six bRPLC fractions in technical replicates in an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) connected to the Easy-nLC-1200 nanoflow liquid chromatography system (Thermo Scientific). The peptides were reconstituted in 0.1% formic acid and loaded into a trap column (Thermo Scientific, 75 μm × 2 cm, nanoViper, 3 μm); peptides were separated using a 15 cm analytical column (EASY-SPRAY RSLC C18 2 μm 15 μm × 50 μm; Thermo Scientific) at a flow rate of 300 nL/min. The solvent gradients were set as the linear gradient of 5−35% solvent B (80% acetonitrile in 0.1% formic acid) over 90 min and a total run time of 120 min. MS analysis was carried out at a scan range of 400−1600 m/z mass range (120,000 mass resolution at 200 m/z) in the data-dependent mode using an Orbitrap mass analyzer. The maximum injection time was 10 ms. Data for MS/MS were acquired at the top speed mode with 3 s cycles and subjected to higher collision energy dissociation (HCD) with 32% normalized collision energy. MS/MS scans were carried out in the range of 100−1600 m/z using an Orbitrap mass analyzer at a resolution of 30,000 at 200 m/z with an injection time of 200 ms.

MS/MS Data Analysis. Mass spectrometry-derived data were searched against the reference protein database of *P. yoelii* 17XNL (consisting of 7724 protein entries) from PlasmoDB version 39 and *Mus musculus* RefSeq release 89 (comprising 29,938 protein entries along with common contaminants). The mass spectrometry data was analyzed with SEQUEST-HT and Mascot (version 2.5.1; Matrix Science, London, United Kingdom) search algorithms in the Proteome Discoverer (PD) software suite, version 2.2 (Thermo Fischer Scientific, Bremen, Germany). The search parameters used were (a) trypsin as the proteolytic enzyme (with up to one missed cleavage), (b) fragment mass error tolerance of 0.05 Da, (c) peptide mass error tolerance of 10 ppm, (d) oxidation at methionine (+15.995 Da on M) as a dynamic modification, and (e) carbamidomethylation at cysteine (+57.021 Da on C)

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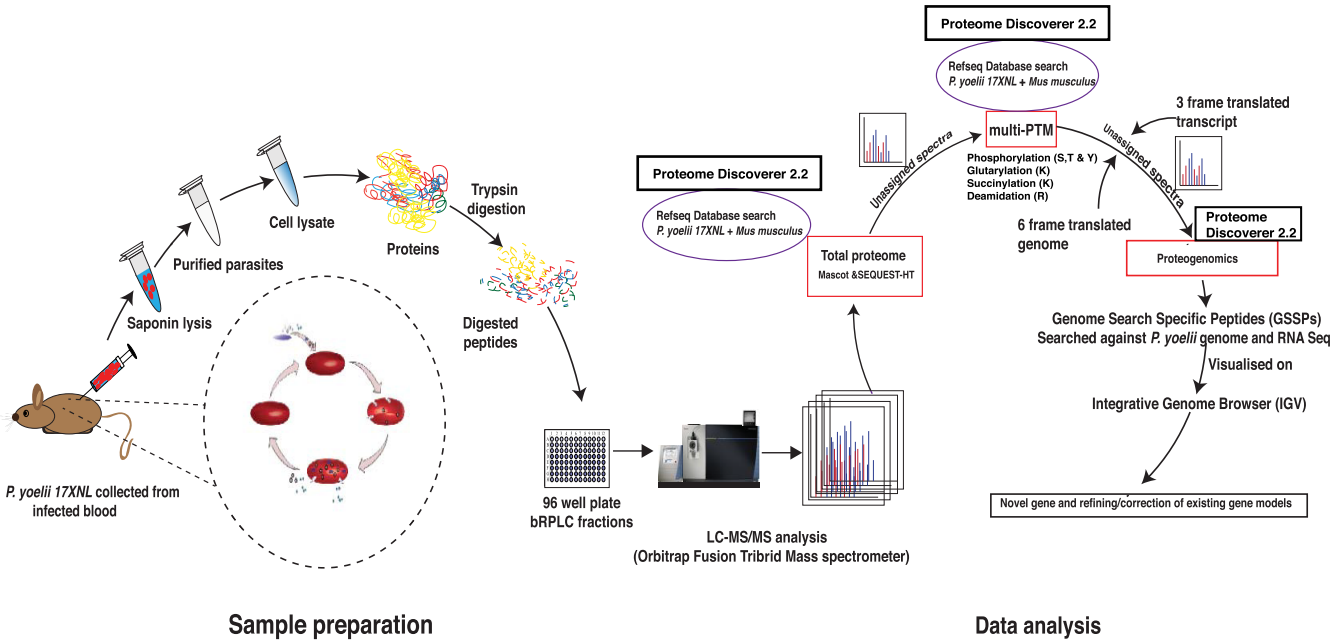

Figure 5. Schematic of the workflow illustrating the proteomic analysis of *P. yoelii* 17XNL. *P. yoelii* 17XNL was injected intraperitoneally into 3−6 week old BALB/c mice, infected blood was collected, and parasites were isolated by the saponin lysis method. Protein extracts of parasite pellets were subjected to in-solution digestion, and peptides were fractionated and collected from basic pH reversed-phase liquid chromatography. The peptide fractions obtained were analyzed using Fourier transform mass spectrometry, and the data obtained were searched against the known protein database of *P. yoelii* 17XNL to confirm the predicted genes. The data were also searched against three-frame transcript models from RNA-Seq and the six-frame translated genome database to identify novel gene models and make refinements to the current annotation of the genome.

as a fixed modification. The false discovery rate (FDR) was set to 1% at PSM and peptide levels.

PTM Analysis. Unassigned MS/MS spectra from total proteome analysis were extracted and searched against the same database for specific PTMs such as phosphorylation at serine, threonine, and tyrosine (+79.966 Da on S/T/Y), glutarylation at lysine (114.031 Da on K), deamidation at arginine (+0.984 Da on R), succinylation at lysine (+100.016 Da on K), and N-terminal protein acetylation (+42.011 Da) as dynamic modifications. Carbamidomethylation at cysteine (+57.021 Da on C) was selected as a fixed modifications along with default parameters and FDR as mentioned above using PD 2.2. The post-translational modification-profiling (PTM-Pro, version 2.0) tool was used for summarizing the high-confident PTM sites and residue information at the peptide and protein levels with biological inferences based on the *P. yoelii* 17XNL protein database.[32,56] The spectral quality of the modified peptides was manually verified to ensure the correct assignments of PTMs. The PTM localization was assigned with a probabilistic score using the ptmRS node of PD 2.2,[57] and the *q*-value was calculated using Percolator.[58] Further, the unassigned MS/MS spectra from PTM searches were exported and used to carry out proteogenomic analysis (Figure 5).

Proteogenomic Analysis Workflow. Custom databases were generated by translating transcripts from next-generation sequencing-based transcriptome data in three frames. The genomic sequence of *P. yoelii* 17XNL was translated in six frames, and sequences between stop−stop codon, with a minimum length of seven amino acids, were retained. The RNA-sequencing data sets for the rodent model of *Plasmodium* species were downloaded from the NCBI-SRA repository. The SRA accessions for *P. yoelii* were PRJEB15102 and PRJEB2511, while accessions such as PRJEB4572, PRJNA72437, PRJNA178363, and PRJNA304182, which correspond to *P. chabaudi*, *P. yoelii 17X*, *P. yoelii* 17XNL, and *P. yoelii Nigeriensis*, respectively, were also obtained. These data sets were downloaded in the FASTQ format using the SRA toolkit (https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/), and the quality of each data set was accessed using the FASTQC tool [http://www.bioinformatics.babraham.ac.uk/projects/fastqc]. The data sets passing the quality were further aligned to the reference genome *P. yoelii* 17XNL using HISAT2. The aligned reads in the BAM format were used to build the transcript assembly using StringTie, and the assemblies from each data set were merged using StringTie-merge. The merged assembly annotations of gene transfer format (GTF) transcripts were used to extract genomic sequences and further translate them into three frames. These custom databases were searched using the unassigned spectra MS/MS with previously mentioned default parameters similar to that of total proteome using the PD 2.2 suite. Genome search-specific peptides (GSSPs) were categorized as novel protein-coding genes and gene corrections using in-house python scripts. MS/MS spectra of all of the peptides providing evidence of novel genes or gene structure changes were manually verified, and spectra with good quality were considered novel events.

Bioinformatics Analysis. *Mus musculus* proteins were filtered out prior to bioinformatics analysis. The resultant proteins with PTMs that qualify the ptmRS probability threshold of ≥75 were chosen for downstream analysis. Gene ontology (GO) annotation for the *P. yoelii* 17XNL proteome was obtained from the Gene Ontology Annotation (GOA) database. Comparative GO for different modifications was achieved using STRAP.[59] Statistically significant motif

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enrichment was performed using IceLogo.[60] The modified proteins were analyzed in the plasmoDB portal for metabolic pathways.[61] Novel events from proteogenomics analysis were validated through comparative genome analysis by mapping to PlasmoDB and NCBI databases through the Basic Local Alignment Search Tool Blastp (protein−protein BLAST).[61,62] ClustalW was used to identify novel proteins from the orthologous region in other species.[63] Sequence similarity of proteins was performed with BioEdit software (https://bioedit.software.informer.com/7.2/). Integrative Genomic Viewer (IGV) was used for visualizing peptides and transcript mapping to the genome (software.broadinstitute.org/software/igv).[64] TDR Targets 6 was used for orthologue-based analysis (https://www.tdrtargets.org/).[65]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03892.

Table S1, list of total proteins, peptides, and PSMs identified in P. yoelii 17XNL using LC-MS/MS; Table S2, list of post-translationally modified proteins, peptides, and PSMs in P. yoelii 17XNL from unassigned spectra; Table S3, list of peptides identified from proteogenomic analysis of P. yoelii 17XNL using the PlasmoDB database; Table S4, list of P. yoelii 17XNL proteins mapped with metabolic pathway analysis; Table S5, orthologous-based target analysis of P. yoelii 17XNL protein signature identified from the TCA cycle and the MEP pathway; Table S6, list of novel peptides identified from P. yoelii 17XNL with orthologue evidence from NCBI BLASTp; Figure S1, Venn diagram depicting the number of proteins identified in proteome, multi-PTM, and proteogenomic analysis; Figure S2, bar diagram depicting the contribution of protein phosphorylation and glutarylation in metabolic pathways (PDF)

(PDF)

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Author Contributions

‖D.A.B.R. and A.H.P. contributed equally. T.S.K.P., S.M.P., and P.S.S. conceived the study and designed experiments. P.S.S. and N.T. developed the parasites in mice, isolated from the blood. D.A.B.R., S.M.P., and P.K.M. isolated, estimated, and ran the QC for protein extraction from parasites. D.A.B.R. processed the parasite proteins for mass spectrometry. S.M.P. performed mass spectrometry analysis. D.A.B.R., P.K.M., A.H.P., R.C., and S.K. performed data analysis, prepared figures and tables, and wrote the manuscript. P.S.S. and T.S.K.P. edited and revised the manuscript with critical review. All authors read and approved the final version of this manuscript.

Notes

The authors declare no competing financial interest.

The mass spectrometry proteomic data have been deposited to the Proteome Xchange Consortium[66] (http://www.proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data-set identifier PXD013932.

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■ LIST OF ABBREVIATIONS

PTMs:post-translational modifications
PSM:peptide-spectrum match
MEP:methylerythritol phosphate
FDR:false discovery rate
STRAP:software tool for researching annotations of proteins
GSSPs:genome search-specific peptides
ETRAMP:early transcribed membrane protein
multi-PTMs:multiple post-translational modifications

REFERENCES

(1) Kwenti, T. E.; Kwenti, T. D. B.; Njunda, L. A.; Latz, A.; Tufon, K. A.; Nkuo-Akenji, T. Identification of the *Plasmodium* species in clinical samples from children residing in five epidemiological strata of malaria in Cameroon. *Trop. Med. Health* **2017**, *45*, No. 14.

(2) Cowman, A. F.; Healer, J.; Marapana, D.; Marsh, K. Malaria: Biology and Disease. *Cell* **2016**, *167*, 610−624.

(3) Wykes, M. N.; Good, M. F. What have we learnt from mouse models for the study of malaria? *Eur. J. Immunol.* **2009**, *39*, 2004−2007.

(4) Davies, D. H.; Duffy, P.; Bodmer, J. L.; Felgner, P. L.; Doolan, D. L. Large screen approaches to identify novel malaria vaccine candidates. *Vaccine* **2015**, *33*, 7496−7505.

(5) Sims, P. F.; Hyde, J. E. Proteomics of the human malaria parasite *Plasmodium falciparum*. *Expert Rev. Proteomics* **2006**, *3*, 87−95.

(6) Bansal, P.; Antil, N.; Kumar, M.; Yamaryo-Botte, Y.; Rawat, R. S.; Pinto, S.; Datta, K. K.; Katris, N. J.; Botte, C. Y.; Prasad, T. S. K.; Sharma, P. Protein kinase TgCDPK7 regulates vesicular trafficking and phospholipid synthesis in Toxoplasma gondii. *PLoS Pathog.* **2021**, *17*, No. e1009325.

(7) Kumar, S.; Kumar, M.; Ekka, R.; Dvorin, J. D.; Paul, A. S.; Madugundu, A. K.; Gilberger, T.; Gowda, H.; Duraisingh, M. T.; Keshava Prasad, T. S.; Sharma, P. PfCDPK1 mediated signaling in erythrocytic stages of *Plasmodium falciparum*. *Nat. Commun.* **2017**, *8*, No. 63.

(8) Maurya, R.; Tripathi, A.; Kumar, M.; Antil, N.; Yamaryo-Botte, Y.; Kumar, P.; Bansal, P.; Doerig, C.; Botte, C. Y.; Prasad, T. S. K.; Sharma, P. PI4-kinase and PfCDPK7 signaling regulate phospholipid biosynthesis in *Plasmodium falciparum*. *EMBO Rep* **2021**, *23*, No. e54022.

(9) Deolankar, S. C.; Patil, A. H.; Rex, D. A. B.; Subba, P.; Mahadevan, A.; Prasad, T. S. K. Mapping Post-Translational Modifications in Brain Regions in Alzheimer's Disease Using Proteomics Data Mining. *OMICS: J. Integr. Biol.* **2021**, *25*, 525−536.

(10) Rex, D. A. B.; Kumar, S. T. A.; Rai, A. B.; Kotimoole, C. N.; Modi, P. K.; Prasad, T. S. K. Novel Post-Translational Modifications and Molecular Substrates in Glioma Identified by Bioinformatics. *OMICS: J. Integr. Biol.* **2021**, *25*, 463−473.

(11) Griss, J.; Perez-Riverol, Y.; Lewis, S.; Tabb, D. L.; Dianes, J. A.; Del-Toro, N.; Rurik, M.; Walzer, M. W.; Kohlbacher, O.; Hermjakob, H.; Wang, R.; Vizcaino, J. A. Recognizing millions of consistently unidentified spectra across hundreds of shotgun proteomics datasets. *Nat. Methods* **2016**, *13*, 651−656.

(12) Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J., Jr. Protein posttranslational modifications: the chemistry of proteome diversifications. *Angew. Chem., Int. Ed.* **2005**, *44*, 7342−7372.

(13) Omenn, G. S.; Lane, L.; Lundberg, E. K.; Beavis, R. C.; Overall, C. M.; Deutsch, E. W. Metrics for the Human Proteome Project 2016: Progress on Identifying and Characterizing the Human Proteome, Including Post-Translational Modifications. *J. Proteome Res.* **2016**, *15*, 3951−3960.

(14) Karve, T. M.; Cheema, A. K. Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease. *J. Amino Acids* **2011**, *2011*, No. 207691.

(15) Stram, A. R.; Payne, R. M. Post-translational modifications in mitochondria: protein signaling in the powerhouse. *Cell. Mol. Life Sci.* **2016**, *73*, 4063−4073.

(16) Tan, M.; Peng, C.; Anderson, K. A.; Chhoy, P.; Xie, Z.; Dai, L.; Park, J.; Chen, Y.; Huang, H.; Zhang, Y.; Ro, J.; Wagner, G. R.; Green, M. F.; Madsen, A. S.; Schmiesing, J.; Peterson, B. S.; Xu, G.; Ilkayeva, O. R.; Muehlbauer, M. J.; Braulke, T.; Muhlhausen, C.; Backos, D. S.; Olsen, C. A.; McGuire, P. J.; Pletcher, S. D.; Lombard, D. B.; Hirschey, M. D.; Zhao, Y. Lysine glutarylation is a protein posttranslational modification regulated by SIRT5. *Cell Metab.* **2014**, *19*, 605−617.

(17) Xie, Z.; Dai, J.; Dai, L.; Tan, M.; Cheng, Z.; Wu, Y.; Boeke, J. D.; Zhao, Y. Lysine succinylation and lysine malonylation in histones. *Mol. Cell. Proteomics* **2012**, *11*, 100−107.

(18) Zhang, Z.; Tan, M.; Xie, Z.; Dai, L.; Chen, Y.; Zhao, Y. Identification of lysine succinylation as a new post-translational modification. *Nat. Chem. Biol.* **2011**, *7*, 58−63.

(19) Chen, Y.; Sprung, R.; Tang, Y.; Ball, H.; Sangras, B.; Kim, S. C.; Falck, J. R.; Peng, J.; Gu, W.; Zhao, Y. Lysine propionylation and butyrylation are novel post-translational modifications in histones. *Mol. Cell. Proteomics* **2007**, *6*, 812−819.

(20) Bautista, J. M.; Marin-Garcia, P.; Diez, A.; Azcarate, I. G.; Puyet, A. Malaria proteomics: insights into the parasite-host interactions in the pathogenic space. *J. Proteomics* **2014**, *97*, 107−125.

(21) Pascovici, D.; Wu, J. X.; McKay, M. J.; Joseph, C.; Noor, Z.; Kamath, K.; Wu, Y.; Ranganathan, S.; Gupta, V.; Mirzaei, M. Clinically Relevant Post-Translational Modification Analyses-Maturing Workflows and Bioinformatics Tools. *Int. J. Mol. Sci.* **2018**, *20*, No. 16.

(22) Xu, T.; Ping, J.; Yu, Y.; Yu, F.; Yu, Y.; Hao, P.; Li, X. Revealing parasite influence in metabolic pathways in Apicomplexa infected patients. *BMC Bioinf.* **2010**, *11*, No. S13.

(23) Doerig, C.; Rayner, J. C.; Scherf, A.; Tobin, A. B. Post-translational protein modifications in malaria parasites. *Nat. Rev. Microbiol.* **2015**, *13*, 160−172.

(24) Audagnotto, M.; Dal Peraro, M. Protein post-translational modifications: In silico prediction tools and molecular modeling. *Comput. Struct. Biotechnol. J.* **2017**, *15*, 307−319.

(25) Jensen, O. N. Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Curr. Opin. Chem. Biol.* **2004**, *8*, 33−41.

(26) Distler, A. M.; Kerner, J.; Lee, K.; Hoppel, C. L. Post-translational modifications of mitochondrial outer membrane proteins. *Methods Enzymol.* **2009**, *457*, 97−115.

(27) Koc, E. C.; Koc, H. Regulation of mammalian mitochondrial translation by post-translational modifications. *Biochim. Biophys. Acta* **2012**, *1819*, 1055−1066.

(28) Krug, K.; Nahnsen, S.; Macek, B. Mass spectrometry at the interface of proteomics and genomics. *Mol. BioSyst.* **2011**, *7*, 284−291.

(29) Imrie, L.; Le Bihan, T.; O'Toole, A.; Hickner, P. V.; Dunn, W. A.; Weise, B.; Rund, S. S. C. Genome annotation improvements from cross-phyla proteogenomics and time-of-day differences in malaria mosquito proteins using untargeted quantitative proteomics. *PLoS One* **2019**, *14*, No. e0220225.

(30) Datta, K. K.; Patil, A. H.; Patel, K.; Dey, G.; Madugundu, A. K.; Renuse, S.; Kaviyil, J. E.; Sekhar, R.; Arunima, A.; Daswani, B.; Kaur, I.; Mohanty, J.; Sinha, R.; Jaiswal, S.; Sivapriya, S.; Sonnathi, Y.; Chattoo, B. B.; Gowda, H.; Ravikumar, R.; Prasad, T. S. Proteogenomics of Candida tropicalis–An Opportunistic Pathogen with Importance for Global Health. *OMICS: J. Integr. Biol.* **2016**, *20*, 239−247.

(31) Prasad, T. S. K.; Mohanty, A. K.; Kumar, M.; Sreenivasamurthy, S. K.; Dey, G.; Nirujogi, R. S.; Pinto, S. M.; Madugundu, A. K.; Patil, A. H.; Advani, J.; Manda, S. S.; Gupta, M. K.; Dwivedi, S. B.; Kelkar, D. S.; Hall, B.; Jiang, X.; Peery, A.; Rajagopalan, P.; Yelamanchi, S. D.; Solanki, H. S.; Raja, R.; Sathe, G. J.; Chavan, S.; Verma, R.; Patel, K. M.; Jain, A. P.; Syed, N.; Datta, K. K.; Khan, A. A.; Dammalli, M.; Jayaram, S.; Radhakrishnan, A.; Mitchell, C. J.; Na, C. H.; Kumar, N.; Sinnis, P.; Sharakhov, I. V.; Wang, C.; Gowda, H.; Tu, Z.; Kumar, A.; Pandey, A. Integrating transcriptomic and proteomic data for accurate assembly and annotation of genomes. *Genome Res.* **2017**, *27*, 133−144.

(32) Patil, A. H.; Datta, K. K.; Behera, S. K.; Kasaragod, S.; Pinto, S. M.; Koyangana, S. G.; Mathur, P. P.; Gowda, H.; Pandey, A.; Prasad, T. S. K. Dissecting *Candida* Pathobiology: Post-Translational Modifications on the *Candida tropicalis* Proteome. *OMICS: J. Integr. Biol.* **2018**, *22*, 544−552.

(33) Pease, B. N.; Huttlin, E. L.; Jedrychowski, M. P.; Talevich, E.; Harmon, J.; Dillman, T.; Kannan, N.; Doerig, C.; Chakrabarti, R.; Gygi, S. P.; Chakrabarti, D. Global analysis of protein expression and phosphorylation of three stages of *Plasmodium falciparum* intra-erythrocytic development. *J. Proteome Res.* **2013**, *12*, 4028−4045.

https://doi.org/10.1021/acsomega.1c03892
ACS Omega 2022, 7, 8246−8257

(34) Arnou, B.; Montigny, C.; Morth, J. P.; Nissen, P.; Jaxel, C.; Moller, J. V.; Maire, M. The *Plasmodium falciparum* Ca(2+)-ATPase PfATP6: insensitive to artemisinin, but a potential drug target. *Biochem. Soc. Trans.* **2011**, *39*, 823−831.
(35) Peel, S. A. The ABC transporter genes of *Plasmodium falciparum* and drug resistance. *Drug Resist. Updates* **2001**, *4*, 66−74.
(36) Huang, K.-Y.; Kao, H.-J.; Hsu, J. B.-K.; Weng, S.-L.; Lee, T.-Y. Characterization and identification of lysine glutarylation based on intrinsic interdependence between positions in the substrate sites. *BMC Bioinf.* **2019**, *19*, No. 384.
(37) Xie, L.; Xiao, Y.; Meng, F.; Li, Y.; Shi, Z.; Qian, K. Functions and Mechanisms of Lysine Glutarylation in Eukaryotes. *Front. Cell Dev. Biol.* **2021**, *9*, No. 667684.
(38) Lindner, S. E.; Swearingen, K. E.; Harupa, A.; Vaughan, A. M.; Sinnis, P.; Moritz, R. L.; Kappe, S. H. Total and putative surface proteomics of malaria parasite salivary gland sporozoites. *Mol. Cell. Proteomics* **2013**, *12*, 1127−1143.
(39) Siau, A.; Huang, X.; Weng, M.; Sze, S. K.; Preiser, P. R. Proteome mapping of *Plasmodium*: identification of the P. yoelii remodellome. *Sci. Rep.* **2016**, *6*, No. 31055.
(40) Kawamoto, R. M.; Caswell, A. H. Autophosphorylation of glyceraldehydephosphate dehydrogenase and phosphorylation of protein from skeletal muscle microsomes. *Biochemistry* **1986**, *25*, 657−661.
(41) Krause, R. G. E.; Hurdayal, R.; Choveaux, D.; Przyborski, J. M.; Coetzer, T. H. T.; Goldring, J. P. D. *Plasmodium* glyceraldehyde-3-phosphate dehydrogenase: A potential malaria diagnostic target. *Exp. Parasitol.* **2017**, *179*, 7−19.
(42) Bruno, S.; Pinto, A.; Paredi, G.; Tamborini, L.; De Micheli, C.; La Pietra, V.; Marinelli, L.; Novellino, E.; Conti, P.; Mozzarelli, A. Discovery of covalent inhibitors of glyceraldehyde-3-phosphate dehydrogenase, a target for the treatment of malaria. *J. Med. Chem.* **2014**, *57*, 7465−7471.
(43) Saunders, C. N.; Cota, E.; Baum, J.; Tate, E. W. Peptide Probes for *Plasmodium falciparum* MyoA Tail Interacting Protein (MTIP): Exploring the Druggability of the Malaria Parasite Motor Complex. *ACS Chem. Biol.* **2020**, *15*, 1313−1320.
(44) Green, J. L.; Wall, R. J.; Vahokoski, J.; Yusuf, N. A.; Ridzuan, M. A. M.; Stanway, R. R.; Stock, J.; Knuepfer, E.; Brady, D.; Martin, S. R.; Howell, S. A.; Pires, I. P.; Moon, R. W.; Molloy, J. E.; Kursula, I.; Tewari, R.; Holder, A. A. Compositional and expression analyses of the glideosome during the *Plasmodium* life cycle reveal an additional myosin light chain required for maximum motility. *J. Biol. Chem.* **2017**, *292*, 17857−17875.
(45) Yakubu, R. R.; Weiss, L. M.; Silmon de Monerri, N. C. Post-translational modifications as key regulators of apicomplexan biology: insights from proteome-wide studies. *Mol. Microbiol.* **2018**, *107*, 1−23.
(46) Willger, S. D.; Liu, Z.; Olarte, R. A.; Adamo, M. E.; Stajich, J. E.; Myers, L. C.; Kettenbach, A. N.; Hogan, D. A. Analysis of the *Candida albicans* Phosphoproteome. *Eukaryotic Cells* **2015**, *14*, 474−485.
(47) Invergo, B. M.; Brochet, M.; Yu, L.; Choudhary, J.; Beltrao, P.; Billker, O. Sub-minute Phosphoregulation of Cell Cycle Systems during *Plasmodium* Gamete Formation. *Cell Rep.* **2017**, *21*, 2017−2029.
(48) Bansal, A.; Molina-Cruz, A.; Brzostowski, J.; Mu, J.; Miller, L. H. *Plasmodium falciparum* Calcium-Dependent Protein Kinase 2 Is Critical for Male Gametocyte Exflagellation but Not Essential for Asexual Proliferation. *mBio* **2017**, *8*, No. e01656-17.
(49) Hitz, E.; Balestra, A. C.; Brochet, M.; Voss, T. S. PfMAP-2 is essential for male gametogenesis in the malaria parasite *Plasmodium falciparum*. *Sci. Rep.* **2020**, *10*, No. 11930.
(50) Ke, H.; Dass, S.; Morrisey, J. M.; Mather, M. W.; Vaidya, A. B. The mitochondrial ribosomal protein L13 is critical for the structural and functional integrity of the mitochondrion in *Plasmodium falciparum*. *J. Biol. Chem.* **2018**, *293*, 8128−8137.
(51) Hirschey, M. D.; Zhao, Y. Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation. *Mol. Cell. Proteomics* **2015**, *14*, 2308−2315.
(52) MacKellar, D. C.; Vaughan, A. M.; Aly, A. S.; DeLeon, S.; Kappe, S. H. A systematic analysis of the early transcribed membrane protein family throughout the life cycle of *Plasmodium yoelii*. *Cell. Microbiol.* **2011**, *13*, 1755−1767.
(53) Spielmann, T.; Fergusen, D. J.; Beck, H. P. etramps, a new *Plasmodium falciparum* gene family coding for developmentally regulated and highly charged membrane proteins located at the parasite-host cell interface. *Mol. Biol. Cell* **2003**, *14*, 1529−1544.
(54) Selvan, L. D.; Renuse, S.; Kaviyil, J. E.; Sharma, J.; Pinto, S. M.; Yelamanchi, S. D.; Puttamallesh, V. N.; Ravikumar, R.; Pandey, A.; Prasad, T. S.; Harsha, H. C. Phosphoproteome of *Cryptococcus neoformans*. *J. Proteomics* **2014**, *97*, 287−295.
(55) Prasad, T. S.; Harsha, H. C.; Keerthikumar, S.; Sekhar, N. R.; Selvan, L. D.; Kumar, P.; Pinto, S. M.; Muthusamy, B.; Subbannayya, Y.; Renuse, S.; Chaerkady, R.; Mathur, P. P.; Ravikumar, R.; Pandey, A. Proteogenomic analysis of *Candida glabrata* using high resolution mass spectrometry. *J. Proteome Res.* **2012**, *11*, 247−260.
(56) Deolankar, S. C.; Patil, A. H.; Koyangana, S. G.; Subbannayya, Y.; Prasad, T. S. K.; Modi, P. K. Dissecting Alzheimer's Disease Molecular Substrates by Proteomics and Discovery of Novel Post-translational Modifications. *OMICS: J. Integr. Biol.* **2019**, *23*, 350−361.
(57) Taus, T.; Kocher, T.; Pichler, P.; Paschke, C.; Schmidt, A.; Henrich, C.; Mechtler, K. Universal and confident phosphorylation site localization using phosphoRS. *J. Proteome Res.* **2011**, *10*, 5354−5362.
(58) Käll, L.; Canterbury, J. D.; Weston, J.; Noble, W. S.; MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* **2007**, *4*, 923−925.
(59) Bhatia, V. N.; Perlman, D. H.; Costello, C. E.; McComb, M. E. Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Anal. Chem.* **2009**, *81*, 9819−9823.
(60) Colaert, N.; Helsens, K.; Martens, L.; Vandekerckhove, J.; Gevaert, K. Improved visualization of protein consensus sequences by iceLogo. *Nat. Methods* **2009**, *6*, 786−787.
(61) Aurrecoechea, C.; Brestelli, J.; Brunk, B. P.; Dommer, J.; Fischer, S.; Gajria, B.; Gao, X.; Gingle, A.; Grant, G.; Harb, O. S.; Heiges, M.; Innamorato, F.; Iodice, J.; Kissinger, J. C.; Kraemer, E.; Li, W.; Miller, J. A.; Nayak, V.; Pennington, C.; Pinney, D. F.; Roos, D. S.; Ross, C.; Stoeckert, C. J., Jr.; Treatman, C.; Wang, H. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res.* **2009**, *37*, D539−D543.
(62) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403−410.
(63) Chenna, R.; Sugawara, H.; Koike, T.; Lopez, R.; Gibson, T. J.; Higgins, D. G.; Thompson, J. D. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **2003**, *31*, 3497−3500.
(64) Robinson, J. T.; Thorvaldsdottir, H.; Winckler, W.; Guttman, M.; Lander, E. S.; Getz, G.; Mesirov, J. P. Integrative genomics viewer. *Nat. Biotechnol.* **2011**, *29*, 24−26.
(65) Landaburu, L. U.; Berenstein, A. J.; Videla, S.; Maru, P.; Shanmugam, D.; Chernomoretz, A.; Aguero, F. TDR Targets 6: driving drug discovery for human pathogens through intensive chemogenomic data integration. *Nucleic Acids Res.* **2020**, *48*, D992−D1005.
(66) Martens, L.; Hermjakob, H.; Jones, P.; Adamski, M.; Taylor, C.; States, D.; Gevaert, K.; Vandekerckhove, J.; Apweiler, R. PRIDE: the proteomics identifications database. *Proteomics* **2005**, *5*, 3537−3545.

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