Post-translational modifications in Plasmodium: More than you think!

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Review

Post-translational modifications in *Plasmodium*: More than you think!

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A B S T R A C T

Recent evidences indicate that transcription in *Plasmodium* may be hard-wired and rigid, deviating from the classical model of transcriptional gene regulation. Thus, it is important that other regulatory pathways be investigated as a comprehensive effort to curb the deadly malarial parasite. Research in post-translational modifications in *Plasmodium* is an emerging field that may provide new venues for drug discovery and potential new insights into how parasitic protozoans regulate their life cycle. Here, we discuss the recent findings of post-translational modifications in *Plasmodium*.

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1. Introduction

Malaria is one of the deadliest infectious diseases of the world. Each year, malaria infects over 300 million people world-wide and causes an estimated one to two million deaths [1–3]. With the increase of drug resistance to most of the widely used anti-malarial drugs, it is imperative to better understand key regulatory elements driving the *Plasmodium* life cycle, eventually leading to the discovery of new drug targets.

The sequencing of the *Plasmodia* genomes, along with subsequent comparative bioinformatics approaches, transcriptome and proteome analyses, have created a vast amount of information regarding protein prediction and their hypothetical functions in both the human host and mosquito vector [4–7]. While microarray analyses have demonstrated a remarkable change in steady-state mRNA levels during parasite development, only relatively few regulatory motifs and transcription regulators have been uncovered so far [8]. In addition, unlike other organisms, there seems to be few transcriptional changes in *Plasmodium* following exposure to external stimuli [3,9,10]. These findings imply that parasite transcription could be hard-wired [10]. This rigidity in transcription suggests that post-transcriptional and post-translational mechanisms are likely to play major roles in regulating the parasite life cycle.

By definition, post-translational modifications (PTMs) must add or subtract a specific mass difference and not be particular to any one protein [11]. In addition to changing protein mass, PTMs can also alter the protein charge and conformation. Such changes modify the protein's enzyme activity, binding affinity and hydrophobicity [12]. The spectrum of the PTMs within a cell is immense and varies with respect to specificity and abundance. For example, some types of PTMs such as phosphorylation and ubiquitination are universally employed to regulate a broad host of functions, have relatively high abundance, and have a wide range of target substrates. On the other hand, some PTMs such as acetylation are highly specific in their roles, have a relatively low abundance, and may target only a few proteins or even one target substrate at a time. Furthermore, PTMs can either modify a protein at one specific amino acid residue or be associated with numerous different residues. The potential diversity of protein modifications seem limitless due to the fact that an individual protein can undergo a single modification or multiple types of modifications at several sites, possibly producing multiple protein isoforms, each with a unique biological activity. One can easily see why PTMs are responsible for a major increase in complexity from genome to proteome. For example, the human genome contains approximately 30,000 open reading frames but is predicted to give rise to roughly 1.8 million different protein variants [13].

Furthermore PTMs are reversible and thus provide the flexibility and adaptability that are essential for mediating rapid cellular responses to the cell's constantly changing conditions. Thus, post-translational modifications are vital for the survival of all kinds of cells, with *Plasmodium* not being an exception. Due to their diversity, essentiality, and wide-spread roles, post-translational modifications could present new major targets for effective and specific therapeutic intervention against the malarial parasite. Here we present an overview of the major post-translational modifications found so far in *Plasmodium* and discuss the recent developments of this rapidly expanding field. A summary of the major proteins involved in regulating PTMs in *Plasmodium* is also presented in Fig. 1 and Table 1.

2. Phosphorylation/dephosphorylation

Phosphorylation involves the reversible esterification of a phosphate group to an amino acid residue by protein kinases (PKs) that transfer a phosphoryl group from an ATP to hydroxyamino acid residues, mostly serine, threonine and tyrosine [14]. As the most highly studied covalent modification of proteins in eukaryotic cells, phosphorylation events can be linked to practically most functions within a cell: cell growth, cell differentiation, receptor activations, metabolic pathways, enzyme activities, cytoskeletal organization, chromatin remodeling, protein activations/inhibitions and protein–protein interactions. Approximately 1.5–2% of genes in an eukaryotic genome are PK family genes, which reflects the importance of phosphorylation events. While protein kinases catalyze the phosphorylation of protein residues, phosphatases hydrolyze the phosphoester bond of the modified amino acid, restoring the hydroxyamino acid to its unphosphorylated state [15]. In eukaryotes, the number of protein phosphatases is relatively small when compared to that of protein kinases. However, additional phosphatase regulatory proteins mediate specific regulation of these enzymes and it is speculated that the number of phosphatase complexes involved in regulatory pathways may exceed the protein kinases repertoires [16].

2.1. Kinases

Several kinase inhibitors have been shown to inhibit *Plasmodium* development at different stages of the life cycle [9,17–20] validating the importance of phosphorylation in maintaining the parasite. Depending on the stringency applied, computational analyses retrieved 86–99 PK-related enzymes from the *Plasmodium falciparum* (*Pf*) genome [21,22]. Phylogenetic studies have demonstrated that most of the eukaryotic families of PKs are present in the *Plasmodium* genome with the exception of two groups, ste-20 (STE) and the tyrosine protein kinases (TyrK) families. The STE family includes PKs involved in Mitogen-activated protein kinase (MAPK) cascades and plays a central role in transduction signals (see reference Ward et al., 2004 for further details). This group is evolutionarily conserved and their absence in the parasite genome may indicate that the mode of activation of the MAPK pathway in the parasite differs from other eukaryotes. The TyrKs are known to function in hormone-response receptor-linked pathways essential for intercellular communication in multicellular organisms and are therefore not expected in the malaria parasite.

Several "orphan" PKs have also been identified in the *Plasmodium* kinome. They display only limited similarities with yeast or mammalian kinases. Examples of these atypical kinases include the NIMA-related kinase Nek1 (PFL1370W) [17], twenty FIKK PK-related proteins, found only in apicomplexan parasites [22,23], and a family of calcium-dependant kinases (CDPKs) that are usually found in plants and alveolate but not in metazoans [24]. In the following section we briefly describe the key biological functions identified so far for a few selected *Plasmodium* kinases.

2.1.1. Cell-cycle regulation

In eukaryotes, kinases are known to play a major role in cell-cycle progression. This is particularly the case for cyclin-dependent kinases (CDKs), several of which have been identified in the *Pf* genome [22]. Two *Pf* CDKs, PfPK5 (MAL13P1.279) and Pfmrk (PF10_0141), have been shown to be positively regulated by the binding of *Plasmodium* and mammalian cyclins as well as negatively regulated by CDK inhibitors [25–27]. These results demonstrate that regulatory activities of CDKs are well-conserved across species. While the targets of these *Plasmodium* kinases have not yet been identified, it is speculated that such proteins might play a major role in the regulation of the parasite schizogony and its nuclear division cycles [28]. *In vitro* biochemical characterizations and crystal structure analyses have uncovered possible unique regulatory mechanisms of *Plasmodium* CDKs [27,29]. Such particularities indicate an atypical mode of DNA replication/mitosis in the parasite [30]. Furthermore, an atypical *Pf* orphan protein kinase, PfPK7
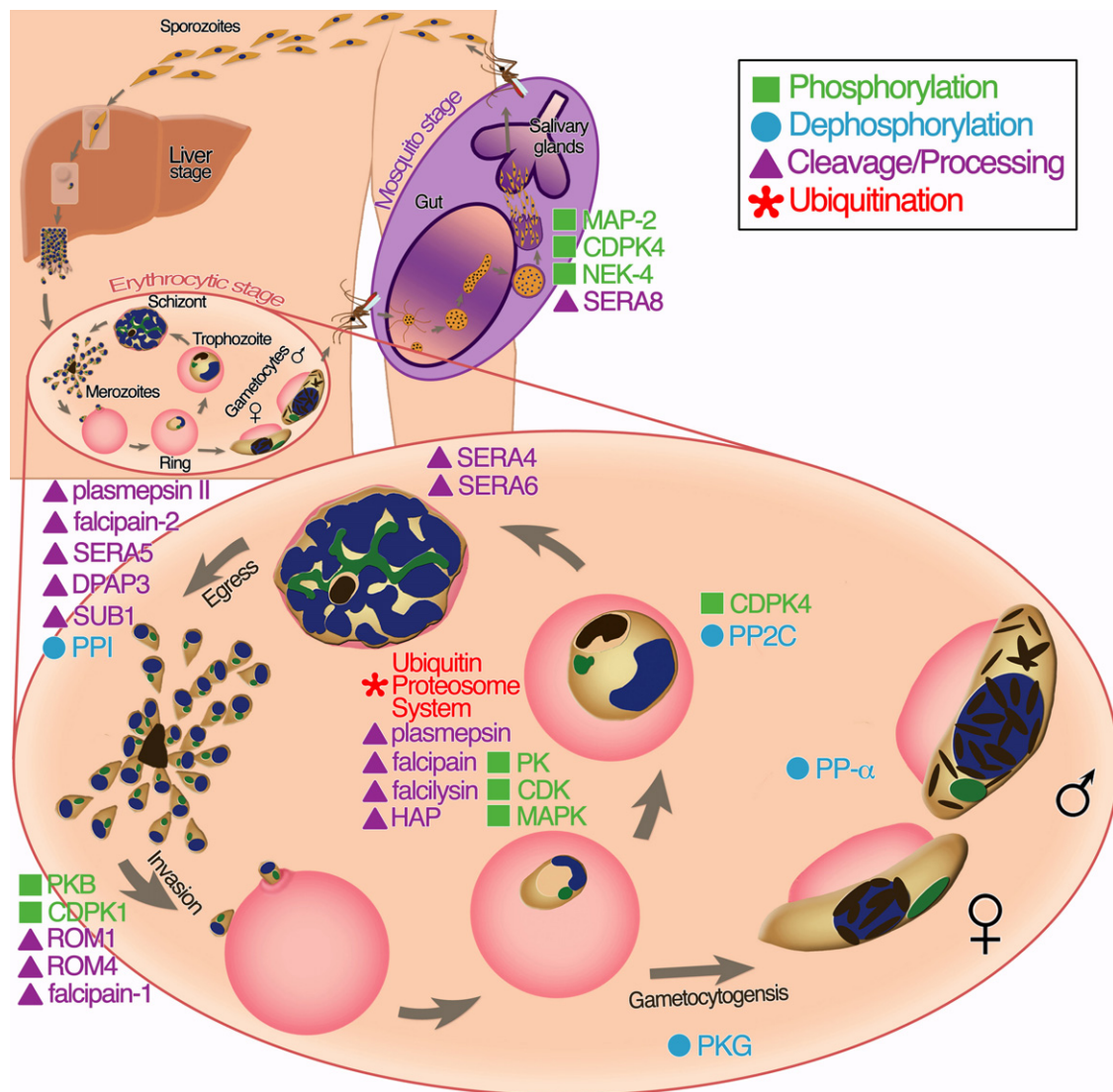
Fig. 1. General depiction of the roles of a few selected proteins involved in post-translational modifications during the life cycle of the malaria parasite.

(PFB0605w), that exhibits maximal homology to a MEKKK and a fungal PKA has been shown to regulate parasite proliferation and development [31]. Discrepancies observed between parasite and host kinases may be exploited in the search of parasite-specific kinase inhibitors as potential anti-malarial drugs but would need to be further validated *in vivo*.

2.1.2. Cell proliferation and differentiation

MAPKs are known to be key players in signal transductions, cell development and differentiation in response to a variety of stimuli [32]. Though no classical MAPK kinase has been identified in the *Plasmodium* genome, two atypical MAPK homologs have been characterized, Pfmap-1 (PF14_0294) and Pfmap-2 (PF11_0147). While the exact role of Pfmap-1 is still speculated, Pfmap-2 appears to be essential for the completion of the parasite asexual erythrocytic cycle [33]. However in *Plasmodium berghei* (*Pb*), the Pfmap-2 orthologue seems to be essential to parasite exflagellation in the mosquitos midgut [34]. Discrepancies observed between these two *Plasmodium* species will need to be further clarified. Nonetheless, it is possible to envision a complementation effect induced by species-specific *Plasmodium* kinases.

2.1.3. Sexual differentiation

Several identified *Plasmodium* kinases have been implicated in sexual differentiation. In *Pb*, a calcium-dependant kinase (CDPK4, PF07_0072 in *Pf*) has been shown to regulate gamete formation with the initiation of DNA replication, ookinate gliding mobilities, and mosquitos midgut invasion [35–37]. cGMP-dependant protein kinase (PFPKG, PF14_0346 in *Pf*) seems to be essential for mediating initiation of gametocytogenesis [18]. Finally, the *Pb* Pbnek-4 (MAL7P1.100 in *Pf*) has been shown to be critical for ookinate maturation [38]. The detection of an increased number of protein kinases involved in sexual differentiation demonstrates that PKs have a crucial role in regulating gametocytogenesis. These preliminary results provide a framework for identifying substrates of these *Plasmodium* enzymes to further comprehend the signalling cascade involved in sexual differentiation.

2.1.4. Parasite egress and invasion

In contrast to other pathogens, apicomplexans such as *Plasmodium* can quickly exit and enter the cell using their own machinery. Several steps involving a large collection of proteins are required in erythrocytic egress and invasion [39]. From this collection, a few protein kinases have been shown to have a role in controlling these

Table 1
Genes that are putatively involved in post-translational modifications in *Plasmodium*.

Modification	Type of protein	Protein name	Gene ID	Putative role
Phosphorylation	Casein kinase	CK1	PF11_0377	Unknown [20]
	Cyclin-dependent kinase	PK5	MAL13P1.279	Cell-cycle regulation [25–27]
	Cyclin-dependent kinase	mrk	PF10_0141	Cell-cycle regulation [25–27]
	Serine/threonine protein kinase	PK7	PFB0605w	Proliferation and development [31]
	Mitogen-activated protein kinase	map-1	PF14_0294	Unknown [33]
	Mitogen-activated protein kinase	map-2	PF11_0147	Asexual cycle regulation [33]
	Mitogen-activated protein kinase	map-2	PB000659.00.0	Exflagellation in mosquito midgut [34]
	Calcium-dependent kinase	CDPK4	PF07_0072	DNA replication, ookinete gliding mobility, mosquito midgut invasion [35–37]
	cGMP-dependant protein kinase	PKG	PF14_0346	Gametocytogenesis initiation [18]
	NIMA-related kinase	nek-4	MAL7P1.100	Ookinete maturation [38]
	Calmodulin-like protein kinase	CDPK1	PFB0815w	Secretion of microneme contents, formation of tight moving junctions [40]
	Protein kinase B	PKB	PFL2250c	Parasite gliding [41]
	Protein kinase A	PKA	PFI1685w	Mediates erythrocyte anion channels and permeability of host plasma membrane [44–45]
	FIKK orphan kinases	Multiple proteins	Multiple genes	Targets parasite proteins to host membranes [46]
Dephosphorylation	Mg^{2+}-dependent serine/threonine protein phosphatase 2C	PP2C	Multiple genes	Transcription elongation [55]
	VH1 family phosphatases	YVH1	Multiple genes	Nuclear protein activity [56]
	Protein phosphatase 1	PP1	Multiple genes	Release of infection merozoites [57]
	PPP-related protein serine/threonine phosphatase	PP-α	Multiple genes	Cell-cycle control and signal transduction in sexual stages [50]
Lipidation	Prenyl modifier	PFT α-subunit	PFL2050w	Unknown [88]
	Prenyl modifier	PFT β-subunit	PF11_0483	Unknown [88]
Ubiquitination	Ubiquitin	pUB	PFL0585w	Polyubiquitin gene that provides the ubiquitin monomers that are covalently attached to proteins. Cell-cycle regulation [106]
	Ubiquitin	UbS27a	PF13_0346	Ubiquitin moiety that provide the ubiquitin monomers for attachment to proteins. Cell-cycle regulation [108]
	Ubiquitin	UbL40	PF14_0027	Ubiquitin moiety that provide the ubiquitin monomers for attachment to proteins. Cell-cycle regulation [108]
	SUMO	PfSUMO	PFE0285c	Ubiquitin-like peptide that is covalently attached to proteins for DNA repair, cell-cycle regulation, nuclear localization and protein stability [110]
	E2 conjugating enzyme	UBC13	PFE1350c	Was found to be phosphorylated by PfPK9. Role in cell-cycle has to be validated in *Plasmodium* [111]
	Deubiquitylating/DeNeddylating enzyme	UCH54	PF14_0576	Has dual deubiquitylating and deNeddylating activity. May have roles in maintaining stable apical membrane epithelial Na+ channels [118]
Cleavage/Processing	Serine protease	Subtilisin-1 (SUB1)	PFE0370c	Activates SERA proteins. Primary regulator of egress [143]
		Subtilisin-2 (SUB2)	PF11_0381	Shedding of MSP-1 and PfAMA-1 after invasion [151]
		Subtilisin-3 (SUB3)	PFE0355c	Ubiquitous role [144]
		ROM-1	PF11_0150	Shedding of adhesins [147]
		ROM-1	PB000352.00.0	May have roles in invasion [149]
		ROM-4	PFE0340c	Shedding of adhesins [146]
		SP1	PF13_0118	Cleavage of transit peptide [123]
	Cysteine protease	Falcipain-1	PF14_0553	Putative role in invasion [153]
		Falcipain-2	PF11_0165	Hemoglobinase & role in egress [132]
		Falcipain-3	PF11_0162	Hemoglobinase [132]
		SERA-4	PFB0345c	Expressed in the PV at the late schizont stage [140]
		SERA-5	PFB0340c	Role in merozoite egress [139]
		SERA-6	PFB0335c	Expressed in the PV at the late schizont stage [140]
		SERA-8	PFB0325c	Sporozoite release from oocystes [141]
		DPAP3	PFD0230c	Primary regulator of egress [142]
		Calpain	MAL13P1.310	Unknown [132]
	Metallo protease	Falcilysin	PF13_0322	Hemoglobinase [125]
	Aspartic protease	Plasmepsin I	PF14_0076	Initiate degradation of hemoglobin [133]
		Plasmepsin II	PF14_0077	Initiate degradation of hemoglobin & role in egress [133]
		Plasmepsin IV	PF14_0075	Cleavage of denatured globin [133]
		Histo-aspartic protease (HAP)	PF14_0078	Cleavage of denatured globin [133]

parasitic processes. The calmodulin-like PK (CDPK1, PFB0815w in *Pf*) is required for the secretion of the parasite microneme contents and the formation of tight moving junctions [40]. Two components of the acto-myosin motor complex, the myosin A tail domain interacting protein (MTIP) and the glideosome-associated protein 45 (GAP45) have been identified to be CDPK1 substrates. In addition, PfPKB (PFL2250c) [41], an important member of the phosphatidylinositol 3-kinase-dependent signaling pathway, has been implicated to regulate parasite gliding mechanisms [42,43]. However, validating the role of PfCDPK1 and PfPKB *in vivo* remains to be established.

2.1.5. Host–parasite interaction

Recent works have begun to reveal the importance of *Plasmodium* PKs in modulating the erythrocyte membranes of infected hosts. PfPKA (PFI1685w) has been shown to alter both the activity of an erythrocyte anion channel and the permeability of the host plasma membrane [44,45]. Interestingly, the members of the *Plasmodium* FIKKs orphan kinases possess a *Plasmodium* export element (PEXEL) motif [46] that target parasite proteins to the host membrane [47,48]. Immuno-microscopy reveals that many FIKK proteins can be found in the erythrocyte cytoplasm and colocalized with Maurer's clefts protein. This finding suggests the importance of these FIKKs in the remodeling of the infected erythrocyte membrane [46].

While it is increasingly apparent that a significant number of protein kinases regulate major parasite cell cycle and differentiation events, our understanding of their targeted substrates is extremely limited. In order to comprehend the function and essentiality of each kinase, a systematic biological approach involving reverse genetics, chemical genetics and phosphoproteome studies will need to be developed.

2.2. Phosphatases

A total of twenty-seven malaria protein phosphatases (PP) have been identified in the *Pf* genome (reviewed by [49]). The identified *Plasmodium* PP clustered with the four major established eukaryotic PP families: the Metallophosphatases (PPP), the Serine/Threonine Phosphatases (PPM), the Protein Tyrosine Phosphatases (PTP) and the NLI Interacting Factor-like phosphatases (NIF). Protein sequence information as well as biochemical characterizations studies of several of these PPs in *Plasmodium* have validated their phosphatase activities and revealed significant differences with other eukaryotic organisms [49–52].

The use of phosphatase inhibitors have shown that *Plasmodium* PPs are essential and may be involved in invasion [53] and cell growth [54]. Protein–protein interaction and localization studies have highlighted a role of *Plasmodium* PPs in transcriptional elongation [55], nuclear protein activity [56] and the release of infectious merozoites [57]. Interestingly, highly conserved eukaryotic phosphatases seem to be either missing or too divergent to be detected in the *Plasmodium* genome. Among the missing phosphatases are the CDC25 homologue (known to play a major role in cell cycle control) [58], the cdc14 phosphatase (regulates mitotic events) [59] and tyrosine phosphatases [60] (though there is a report of a possible PRL tyrosine phosphatase) [61]. These findings further validate the phylogenetic distance observed between *Plasmodium spp*. and its vertebrate host. The identification of PP regulatory subunits, as well as their specific substrates, will need to be further investigated. However, it has become evident that these PPs are essential to the parasite and should be considered as potential targets for new anti-malarial strategies.

3. Acetylation

Acetylation is the addition of an acetyl functional group onto a protein substrate. Acetyltransferases transfer an acetyl group from acetyl coenzyme A onto conserved N-terminal lysine residues. The result of acetylation usually modifies DNA binding properties, protein stability, and protein–protein interactions.

Acetylation of actin at the N-terminus is a highly conserved PTM that has also been reported in *Pf* [62]. In addition to actin, *Plasmodium* histones are found to be acetylated at their N-terminal lysine residues [63]. Histone PTMs are a vital part of the 'histone code' hypothesis that proposes specific combinations of PTMs that modify chromatin structure and act as platforms for the binding of transcriptional regulators of gene expression. Consistent with other eukaryotes, *Plasmodium* histone acetylation is believed to play a major role in transcriptional regulation. In a genome-wide investigation of histone modifications and their relationship with transcriptional activation/silencing, Cui et al. (2007) [64] employed a combination of immunoprecipitation and DNA microarray detection (ChIP-chip) to show that acetylation of histone H3 (H3K9ac) was associated with active genes across the *Pf* genome. PfGCN5 was the first characterized histone acetyltransferase (HAT) subunit identified in *Plasmodium*. Recombinant *Pf*GCN5 displayed histone H3 acetylase activity *in vitro* and exists as a catalytic subunit of *P. falciparum* HAT complex. PfADA2, the yeast transcriptional coactivator homolog, has been characterized as another component of the trimeric catalytic core [65]. *In vitro* pull-down and yeast two-hybrid experiments suggest that PfADA2 and PfGCN5 are present in complex(es) and may have conserved chromatin remodeling functions. Natural compounds that inhibit HAT activity are being explored as potential antimalarials [66]. However, because HATs are conserved across species, toxicity studies will have to be further investigated.

Pf HDAC1 and *Pf*Sir2, two of the five putative *Plasmodium* Histone Deacetylases (HDACs), have been partially characterized in *Plasmodium* [67]. *Pf*Sir2, a yeast homolog of the silent information regulator 2 (SIR2) was found to bind to parasite telomeres and cause histone de-acetylation and silencing of the *var* multi-gene family [68]. Inhibitors of HDAC activity have also been investigated for potential antimalarial drugs [69,70].

4. Methylation

Methylation is catalyzed by methyltransferases that attach a methyl group onto its substrate. Such modification increases lipophilicity and reduces the substrate solubility in water. Methylation is associated with the regulation of gene expression and protein activity. In *Plasmodium*, the most commonly methylated subtrates are the parasite histones. Genome-wide analysis of histone modification showed that H3K9me3 is associated with gene silencing [64]. Recently, chromatin immunoprecipitation of transcriptionally active *var* gene loci assessed the enrichment of histone H3 di- and trimethylation marks (H3K4me2 and H3K4me3) in the 5′ flanking region [71].

At least nine *Plasmodium* histone methyltransferases (HMT) (containing a SET-domain, characteristic of histone lysine methyltransferase), and two *Plasmodium* demethylases (containing a JumonjiC-domain, characteristic of Histone Lysine Demethylases (HDMs)) have been identified *in silico*. Phylogenetic analysis divided putative histone lysine methyltransferases (HKMTs) into five subfamilies with different putative substrate specificities. HKMTs and HDMs not only have substrate specificity but also specificity for different methyl states (mono-, di-, tri-) [72].

5. Lipidation

Lipidation is the covalent binding of a lipid group to a peptide chain and can change the activity and/or cellular localization of the modified protein. Often times, the attachment of a hydrophobic chain can help to anchor soluble proteins, or proteins with weak membrane-affinity, to the inner face of a membrane. Some examples of lipidation include N-myristoylation, prenylation, GPI-anchor addition, and palmitoylation. Discussed below are a couple of post-translational modifications *via* lipidation that have been studied so far in *Plasmodium*.

5.1. GPI-anchoring

The glycosylphosphatidylinositol (GPI) anchor is a glycolipid moiety that is added to the C-terminal of proteins after translation. Such modified proteins are attached to the outer leaflet of the cell membrane. GPI-anchored proteins are ubiquitous among eukaryotic organisms and represent a very functionally diverse group that is being extensively studied in a wide-array of organisms.

Anchoring with GPI is the major type of glycosylation that are found in *Plasmodium* [73]. It is commonly postulated that *Plasmodium* proteins are glycosylated by the erythrocytic machinery (combinatorial metabolism) during the exchanges that occur between the parasitophorous vacuole and the erythrocyte cytoplasm [74]. In the human host, the parasite's GPI anchors have been linked to the pathobiology of the disease [75] and are associated with increased levels of TNF-α leading to a systemic inflammation reaction [76,77]. It has been found that resistance to malaria in endemic areas is associated with the production of circulant antibodies directed against the parasite's GPI anchors [75] and are common targets for vaccine research. Also, most *P. falciparum* merozoite surface proteins are GPI-anchored and are involved in erythrocyte recognition and attachment to erythrocytes before invasion (reviewed by [78]). GPIs may also be involved in post-invasion processes. The *P. falciparum* GPI-anchored rhoptry protein *Pf*34 (PFD0955w) is suspected to play a role in the formation of the parasitophorous vacuole [79,80].

In the mosquito host (*Anopheles spp.*), *P. falciparum* GPIs may play a direct role in immune response [81,82]. The immune response in *Anopheles gambiae* is elicited by the parasite GPIs resulting in a significant reduction in fecundity (diminished egg production) [83]. The role of such effect on the host–parasite interactions remains open to discussion. The authors propose that the parasite may use its GPIs to trigger the mosquito immune response and "mis-direct" the specificity of the response against other pathogen-associated molecular patterns rather than ookinetes or microneme proteins essential for parasitic processes. Furthermore, the mosquito stage GPI anchored surface proteins P25 (PF10_0303) and P28 (PF10_0302) are shed during ookinete maturation and play an important role in midgut invasion [84–86]. Finally, the GPI anchor of the circumsporozoite protein (PFC0210c) plays a crucial role in sporogenesis [87]. Due to their general importance, GPIs and their biosynthesis pathway are being investigated as potential targets to antimalarial strategies.

5.2. Prenylation

Prenylation, also known as isoprenylation, is the post-translational modification of proteins by covalent attachment near the carboxyl terminal of isoprenyl lipids, a 15 carbon farnesyl or a 20 carbon geranylgeranyl group [88]. The attachment of isoprenyl lipids creates a hydrophobic tail that promotes membrane association and plays an important role in cell signal transduction, vesicle trafficking, and cell-cycle progression [89]. Unlike animals, fungi and archeabacteria, which use the classical mevalonate pathway

for isoprenoid synthesis, the *Plasmodium* synthesizes its isoprenoid precursors *via* the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway within its apicoplast, a plastid-like organelle [90].

Prenylation is mediated by three enzymes: protein farnesyltransferase (PFT), protein geranylgeranyltransferase type I and type II. PFT in *Plasmodium* has been characterized by partial purification of protein farnesyltransferase, PfPFT, and radiolabeling of prenylated proteins [88]. Prenylation precursors are incorporated into asexual synchronized parasites in a stage-specific manner with the highest amount occurring from trophozoite to schizont, and schizont to ring transitions. Dolichylation, the addition of 11 isoprene units, has also been reported in *P. falciparum* during trophozoite and schizont stages [91].

Pharmaceutical companies have invested into the development of PFT inhibitors for the treatment of cancer with a few drugs in clinical trials. Interestingly, PFT inhibitors have been found to be potent antimalarials [92] and validate farnesylation as essential in the malaria parasite. Drug development for antimalarials is taking a 'piggy-back' approach since the concentrations needed to inhibit parasites are significantly lower than that of mammalian cells [89].

5.3. Palmitoylation

Palmitoylation is the covalent attachment of fatty acids to cysteine residues of proteins, often giving soluble proteins (or proteins with weak membrane avidity) a hydrophobic membrane anchor [93]. In *P. falciparum*, a 45 kDa gliding-associated protein (GAP45) was found to be both palmitoylated and N-myristoylated [94]. It is hypothesized that GAP45 may play a role in binding acto-myosin motors to the outer face of the inner membrane complex, which is implicated as the underlying force driving both gliding motility and host cell invasion in *Plasmodium* merozoites.

6. Ubiquitination

Ubiquitin is a highly conserved 76 amino acid peptide found in eukaryotic organisms. Beyond the more familiar association with protein degradation, the modification of proteins by ubiquitin conjugation is known to serve as a regulatory signal for cell proliferation, cell-stress response, transcription, cell death, DNA repair, intracellular trafficking, endocytosis and signal transduction [95]. The reversible conjugation of ubiquitin to the lysine residues in target proteins is controlled by a series of enzymes: ubiquitin activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [96]. Ubiquitin is activated by E1 and transferred to E2. Then, ubiquitin is either transferred to a monomeric E3 that catalyzes ubiquitination of the target substrate or ubiquitinated E2 forms a complex with the E3 to catalyze ubiquitination of the substrate. Deubiquitinating enzymes (DUBs) serve to reverse ubiquitin-conjugation by removing ubiquitin from substrate proteins and also help to replenish the free ubiquitin pool.

Diverse forms of ubiquitin modifications have been reported, each potentially mediating a specific function. K48-linked poly-ubiquitin chains to substrates often serve as signals for targeted protein degradation *via* the ubiquitin/proteasome system [97]. In *Plasmodium,* several studies have validated the essentiality of the proteasome and protein turnover in regulating the cell cycle progression. A collection of proteasome inhibitors has shown promising results in impeding the parasites [98–103].

Apart from protein degradation, poly-ubiquitination *via* K63-linkages seems to play important roles in DNA damage tolerances, endocytosis, ribosomal protein synthesis, and inflammatory response [104]. In addition to poly-ubiquitination, proteins can either be mono-ubiquitinated, which is the attachment of a single ubiquitin to a protein, or multi-ubiquitinated, which is the

attachment of individual ubiquitin to a substrate at multiple sites. Both mono- and multi-ubiquitination are reported to have non-proteolytic roles such as endocytosis [105] and DNA repair [106]. In the following sections, we briefly describe what has been reported so far concerning ubiquitination in *Plasmodium*.

6.1. Ubiquitin and ubiquitin-like proteins

The *P. falciparum* poly-ubiquitin gene, PfpUB (PFL0585w) is present as a single-copy on chromosome 12 with five tandem repeats of the ubiquitin open reading frame [107]. Translation of PfpUB comprises of five ubiquitin monomers that have sequence identities of no less than 94% to that of other eukaryotic species. RT-PCR and northern analysis revealed that steady-state transcript levels of PfpUB are expressed at all stages of the intraerythrocytic cycles with significant increases at the late trophozoite and schizont stages. Under heat shock, polypeptide levels of PfpUB and ubiquitinated adducts showed dramatic increases without a significant increase in steady-state transcript levels. This observation suggests that the heat shock response appears to be maintained at the level of translation [107].

Two additional ubiquitin moieties, Ub_{S27a} and Ub_{L40}, fused to the ribosomal proteins L40 (PF13_0346) and S27a (PF14_0027) have also been identified in the *P. falciparum* genome [108,109]. Expression data indicate that these ubiquitin genes are expressed throughout the *P. falciparum* life cycle [5,7]. Interestingly, though the *Pf* Ub_{L40} shows high protein sequence fidelity with other eukaryotic species, *Pf* Ub_{S27a} harbors significant divergences within the ubiquitin domain [109].

In addition to ubiquitin, ubiquitin-like proteins (UBLps) have been identified as modifiers of cellular-processes. Though a number of UBLps (ISG15, FAT10, UFM1, FUB1) that are typical in higher eukaryotes were not found in *Plasmodium*, gene expression data suggests that SUMO, NEDD8, HUB1, URM1 and ATG8 are expressed at all life-cycle stages [108,110].

Recently, Issar *et al.* (2008) [111] were the first to investigate and characterize SUMO within *P. falciparum*. Amino acid sequence comparisons revealed that *P. falciparum* gene PFE0285c (PfSUMO) has significant sequence homology to that of known SUMO orthologs from other eukaryotic organisms such as yeast, human, mouse and *Saccharomyces pombe*, while immunoblot analysis confirmed the presence of SUMO in *P. falciparum*. In addition, enzyme homology searches have identified *P. falciparum* orthologs of all the necessary members of the SUMO pathway. Using LC-MS/MS analysis, more than 20 putative SUMO substrates of *P. falciparum* proteins were identified. These protein substrates varied from histones to transcription factors to RNA helicases, which implicates a wide-ranging regulatory scope of SUMO. Immunofluorescence assays indicate that PfSUMO localizes to distinctive subcellular compartments within the *P. falciparum* and also in the host cell cytoplasm within the parasite-derived structures called Maurer's clefts.

6.2. Ubiquitin enzymes

Computational studies have identified over a hundred proteins predicted to be involved in the reversible conjugation of ubiquitin or ubiquitin-like proteins in *P. falciparum* alone [108,110].

6.2.1. Ubiquitin activating enzymes (E1)

Eight putative E1 have been identified in the parasite genome [108]. While primary sequence identity has been observed in the core ubiquitin activating enzyme domain, sequences outside of this core diverge rapidly as the functional requirements for these E1 enzymes change to specifically interact with their respective E2 conjugating enzymes. Sequence analysis indicates the existence of *Plasmodium* E1 paralogs for UBA1 and UBA1-like proteins, and UBA2, UBA3, UBA4, ATG7 proteins, which mediate the activation of ubiquitin-like proteins SUMO, NEDD8, URM1, and ATG8, respectively.

6.2.2. Ubiquitin conjugating enzymes (E2)

Fourteen putative E2 paralogs were found in *P. falciparum* [108]. They exhibited extensive conservation with other eukaryotic E2 proteins. Gene expression data for nine of the fourteen *Plasmodium* E2s reveal a diverse pattern of steady-state mRNA at different stages of the intraerythrocytic cycle, suggesting the existence of a temporal profile of delivering ubiquitin or UbLps to different E3s, indicating a potential additional level of temporal control in ubiquitination during the parasite's life cycle.

Recently, a *Pf* homolog (PfUBC13) of the E2 ubiquitin-conjugating enzyme 13 (UBC13) was characterized and found to be a substrate of the *Pf* protein kinase PfPK9 [112]. Reverse-phase HPLC and *in vitro* ubiquitination assay show that PfPK9 phosphorylates PfUBC13 at S106 and suppresses ubiquitin conjugating activity. Though the physiological role of PfUBC13 is unknown, the highly-conserved UBC13 (coupled with an ubiquitin E2-variant protein) assembles K63-linked ubiquitin chains [113], which mediate non-proteolytic pathways [114]. UBC13's conjugating activity regulates various cellular processes such as DNA repair [115], tumor suppressor p53 activity [116], and mitotic progression [117].

6.2.3. Ubiquitin ligating enzymes (E3)

Though *Plasmodium* E1 and E2 enzymes exhibited strong conserved homology with other E1 and E2 enzymes from other eukaryotes, *Plasmodium* E3 ligases were found to be highly divergent and the most abundant. Within the 54 putative E3 ligases identified, all superfamilies (HECT, RING, U-box, and cullin) of E3 ligases are represented within *P. falciparum* with E3 RING finger proteins making up the majority of the *Plasmodium* E3 ligases [108].

Functional annotation analysis reveals that these *Plasmodium* ligases have a wide array of potential roles including cell cycle regulation, trafficking, DNA repair, chromatin structure, and mRNA transport. However several of these proteins seem to be specific to the Apicomplexa phylum. While the functional analysis of these parasite-specific putative E3s will need to be further validated *in vitro* and *in vivo*, two selected E3 ligases seem to be essential to the parasite erythrocytic cycle and have already shown *in vitro* ubiquitination activity validating further the importance of this pathway in parasite development (Chung and Le Roch, unpublished data).

6.2.4. Deubiquitinating enzymes

While ubiquitin ligases catalyze the attachment of ubiquitin and UbLs, DUBs hydrolyze ubiquitin and UbLps [118]. Depending on the computational tools employed, 18 or 29 *Plasmodium* DUBs were found [108,110]. Five distinct gene families were identified: the ubiquitin C-terminal hydrolases (UCHs); the ubiquitin-specific peptidases (USPs/UBPs); the ovarian tumor (OTU) domain proteins; the Josephin or Machado-Joseph disease (MJD) proteins and the JAMM (Jab1/MPN domain-associated metalloisopeptidase) domain proteins. In addition, DUBs for UbLps including SUMO, ATG8, and NEDD8 have also been identified.

PfUCH54 was the first DUB characterized in *P. falciparum* [119]. Using electrophilic probes that detect enzymes capable of removing ubiquitin and other ubiquitin-like proteins, PfUCH54 was found to possess both deubiquitination and deneddylation activity. Using known active site residues and crystal structure of homologous DUBs, PfUCH54 was found to have high homology to UCHL3 [119], an enzyme that has been reported to also have dual deubiquitinating and deneddylating activity [120]. Though the function of *Pf*UCH54 is not known, mouse UCHL3 is required to maintain a stable apical membrane epithelial sodium channel, facilitating the dynamic recycling of sodium channels at the apical surface [121].

Though functional analysis studies will need to further validate the roles of ubiquitination pathways in *Plasmodium*, E3 ligases and DUBs may represent a good target for new therapeutic interventions due to their diversity and potential involvement in parasite-specific pathways.

7. Protein cleavage and processing

Proteases are major virulence factors in parasitic diseases as largely reviewed by [122] and [123]. Five main classes of proteases have been identified: (1) cysteine proteases, (2) serine proteases, (3) threonine proteases, (4) aspartic proteases, and (5) metallo-proteases. A computational analysis identified 92 proteases in the genome of *P. falciparum*, 83 of them being transcribed during the erythrocytic cell cycle and 67 being translated during the parasite's life cycle [124]. Proteases are commonly involved in a wide array of biological processes such as lysosomal proteolysis, precursor protein processing or trafficking.

In *Plasmodium*, trafficking is a vital biological process, especially with regards to targeting proteins to the apicoplast. Trafficking pathways leading to the apicoplast have not yet been elucidated but are known to involve the presence of both a signal peptide and a transit peptide that are processed along the pathway [125]. Wu *et al.* identified a serine protease Signal Peptidase 1 (SP1, PF13_0118) that could be responsible for the cleavage of signal peptides [124]. In addition, metalloprotease falcilysin (PF13_0322 in *P. falciparum*) [126] has been recently implicated in transit peptide cleavage [127].

Another important parasite-specific pathway is the PEXEL-mediated pathway that targets parasite proteins to the surface of infected erythrocytes. This pathway is thought to promote parasite evasion from the host immune system [128]. Chang *et al.* (2008) demonstrated that exported proteins are processed at their N-terminal end [129]. This processing involves acetylation and cleavage of the PEXEL motif in the endoplasmic reticulum. The authors suggest that this N-terminal processing may be more generally utilized for many exported soluble proteins. However, the protease responsible for such cleavage remains to be identified.

In *Plasmodium*, various proteases play key roles in hemoglobin degradation, egress and invasion during the parasite erythrocytic cycle. The roles of such proteases were usually determined from inhibitor studies, which emphasize the potential to target such enzymes by anti-malarial drugs.

7.1. Hemoglobin degradation

The degradation of hemoglobin involves various proteases called hemoglobinases [130] and provides the essential amino acids that are taken up by the parasite [122,131,132].

The cysteine proteases falcipain-2 (PF11_0165) and falcipain-3 (PF11_0162) are known to play a role in the early steps of hemoglobin degradation within the parasite food vacuole (see [133] for a review). In addition to these cysteine proteases, degradation of hemoglobin seems to involve metallo (falcilysin) and aspartic proteases such as plasmepsins [131]. Four plasmepsins are present and active in the food vacuole of *P. falciparum*, plasmepsin I (PF14_0076), II (PF14_0077), IV (PF14_0075) and a histo-aspartic protease (HAP, PF14_0078) [134–136]. It was postulated that plasmepsins I and II are matured and released in the food vacuole by protein cleavage [134,137]. A recent study demonstrated that falcipain-2 and falcipain-3 are responsible for plasmepsins preprocessing and that auto-processing can occur when falcipain activities are inhibited, providing an alternative pathway to activate plasmepsins [138].

7.2. Parasite egress

Parasite proteases are also known to trigger the degradation of parasite and host membranes, leading to the egress of infectious parasites. The role of these proteases in cell egress has been recently reviewed [139]. Briefly, the proteases implicated in parasite egress are falcipain II, plasmepsin II and putative papain-like SERA proteases. SERA proteins are a family of nine members, which are activated by a subtilisin-like serine protease SUB1 (PFE0370c). Among them are SERA-4 (PFB0345c), SERA-5 (PFB0340c, [140]) and SERA-6 (PFB0335c), which are essential proteins that are expressed in the parasitophorous vacuole of the late trophozoite and schizont stage (erythrocytic cycle) [141]. Additionally, SERA-8 (PFB0325c), another member of the SERA protein family, is essential for sporozoite release from oocytes (mosquito stage) [142].

The involvement of the subtilisin-family serine protease *Pf*SUB1 and the cysteine protease dipeptidyl peptidase 3 (DPAP3, PFD0230c) as primary regulators of parasite egress have been recently evidenced using serine and cysteine protease inhibitors [143]. Just prior to egress, the essential serine protease *Pf*SUB1 is discharged from the exonemes into the parasitophorous vacuole space [144]. Inhibition of both DPAP3 and *Pf*SUB1 blocks the processing of the serine repeat antigen protein SERA-5, which correlates with the inhibition of membrane rupture. In *Plasmodium*, two other subtilisin serine proteases of unknown function can be found: *Pf*SUB2 (PF11_0381) and *Pf*SUB3 (PFE0355c) (reviewed in [145]). *Pf*SUB2 is believed to play an important role in the erythrocytic cycle while *Pf*SUB3 appears to be more ubiquitous as it is expressed during the asexual blood stage, in gametocytes and in sporozoites [6].

7.3. Parasite invasion

The critical role of proteases in merozoite invasion of erythrocytes has been largely studied and reviewed [39,146]. Briefly, invasion involves contact, interaction and junction between the merozoite and the red blood cell surface *via* adhesins and various GPI-anchored proteins, such as *Pf*AMA-1 and MSPs followed by the active entry of the parasite within the host. In order for invasion to be completed, these interactions are interrupted by the shedding of the protein coat covering the merozoite surface made of various MSPs and adhesins. The proteases involved in such removal are called sheddases. For example, the adhesins Duffy binding ligand erythrocyte-binding antigen (DBL-EBP) 175 (EBA-175, MAL7P1.176) is shed from the merozoite at around the point of invasion by the rhomboid protease *Pf*ROM4 (PFE0340c) [147]. Rhomboid proteases are ubiquitous intramembrane serine proteases (see [148–150] for an extensive description of the different types of rhomboid proteases). Also, sporozoite invasion of hepatocytes is reported to be mediated by the shedding of *Pf*AMA-1 and the thrombospondin-related adhesive protein (TRAP) by a serine protease [151].

Double cleavage of MSPs is required for invasion and probably involves serine proteases 9 See Harris, Yeoh *et al.* (2005) [152] for further details 0. Very recently, it was suggested that MSP-1, MSP-6 and MSP-7 undergo proteolytic maturation catalyzed by *Pf*SUB1 before egress, maturation that is essential for further processing of MSPs involved in invasion [153].

A role of cysteine proteases (falcipain) in erythrocyte invasion has also been proposed but remains elusive. Inhibitors of falcipain-1 (PF14_0553) block invasion of host red blood cells [154] whereas parasites with a disrupted falcipain-1 gene were not affected in terms of asexual growth [155]. More recently, the presence of an endogenous cysteine protease inhibitor in *Pf*, falstatin, has been identified [156]. The presence of such an endogenous inhibitor may

be another level of proteolytic control of certain parasite/host proteases in order to facilitate erythrocyte invasion.

Proteolytic processing is finally involved in the maturation of proteins secreted by the merozoite-specific organelles, rhoptries. Rhoptry-associated proteins (RAP) are believed to play a role in invasion and are activated upon proteolytic cleavage. For example, the rhoptry-associated protein RAP-1 is matured *in vivo* by multiple processing steps [157–159].

8. Concluding remarks

In addition to the PTMs presented above, there are several other PTMs in *Plasmodium* that are being investigated but, due to space-constraints, are unable to be fully expanded upon in this review. However, we would like to briefly mention a few. For example, a *Plasmodium* cathepsin-C-like protein is reported to be modified *via* O-sulfonation [160]. Also, polyglutamylation of tubulin was found in the microtubule organizing centers and post-mitotic microtubular structures of *Plasmodium* [161]. Lastly, several chaperone proteins, involved in the proper folding of proteins, have been described in *Plasmodium* and are reported to be involved in roles such as trafficking [162–165].

Today, transcriptional studies make up a significant amount of publications that are dedicated to understanding mechanisms regulating the *Plasmodium* developmental cycle. However, recent evidences suggest that *Plasmodium* may not follow the classical transcriptional model and may have rigid transcription machinery, which may indicate the need to reallocate investigative efforts into other fields in order to better understand the parasite's life-cycle regulation. With the exception of phosphorylation/dephosphorylation, post-translational modifications in *Plasmodium* have been understudied and are largely overshadowed by the classical view that proteins are mainly regulated at the transcriptional level. With further investigation, post-translational regulation may reveal to be a bigger factor in parasite development than previously thought.

For example, though it is apparent that proteases are necessary to destabilize host cell membranes during invasion and egress, the mechanisms of how these proteases are activated and tightly controlled temporally are yet unclear. However, there are increasing reports that PTMs may play a major role in both malarial invasion and egress. As already described above, it is suggested that additional proteases, such as SUB proteases, may act as regulators of egress by processing, and thereby activating, distinct effector proteins or signal transduction pathways [139,144]. In addition, it is reported that *Pf* protein kinases, such as CDPK, may also be involved in regulating invasion and egress [19].

Besides having a major regulatory role within the parasite, PTMs may also play key roles outside the parasite by manipulating the host's signaling pathways thereby usurping normal cellular processes for survival and escape from immune responses. Currently, there are an increasing number of reports that show PTMs to play an integral role in host–pathogen interactions, or cross-talk, in bacteria, viruses and protozoa [166–168]. For example, it has been shown that host organisms employ a wide range of post-translational modifications to initiate their immune responses in order to avoid invasion by pathogens. For instance, host cells use ubiquitination in defense strategies as a way to degrade parasitic proteins and also to activate inflammatory and anti-apoptotic genes in a non-degradative fashion [169]. As a countermeasure, it has been shown that pathogens are able to avoid and exploit these ubiquitination defense strategies by secreting proteins of their own that either deubiquitinate or inhibit the host ubiquitinating enzymes [169,170]. It is also reported that bacterial pathogens are able to provoke histone modifications (*via* acetylation and

phosphorylation) and chromatin remodeling in host cells, thereby manipulating the host's transcriptional programming and diminish the host innate immune response [167]. The utilization of PTMs within host–pathogen interactions for the purposes of pathogenic growth and immune evasion is an emerging field, which may prove to be both widespread and diverse in the mechanisms at work.

The utilization of PTMs to manipulate host cell signaling and immune response may also be true in *Plasmodium*. In *P. falciparum*, it is reported that kinases and phosphatases were found among the 320+ proteins predicted to be secreted from the parasite [48]. More recently, over 30 proteins have been validated to be secreted from the *Pf*, of which 27 proteins are novel extracellular proteins [171]. Several post-translational modifiers, such as kinases, phosphatases, and proteases, were confirmed to be among the secreted proteins. Furthermore, preliminary functional analysis suggests that these secreted proteins are possibly involved in immune evasion and signaling [171].

Our current understanding of the roles and extent of PTMs within *Plasmodium* is still greatly limited due to the relative novelty and inherent challenges of this particular field. More genome-wide approaches, along with their proper biological validation, will provide significant advances in filling the gaps of our present understanding of the regulatory mechanisms driving this deadly parasite. Though there is still much more to uncover, current reports collectively show that *Plasmodium* PTMs have vital roles in all aspects of the parasites' life cycle, including host–pathogen interactions. Because of the numerous types and virtually incalculable combinations of PTMs, the possibilities of regulation at the post-translational level are vast. In addition to being vitally essential, many *Plasmodium* PTM proteins are both highly divergent and specific to the parasite, making *Plasmodium* PTM proteins excellent candidates for drug targeting. Thus, *Plasmodium* PTMs may open new venues for drug discovery and may prove to be more significant in the regulation of this deadly parasite than previously acknowledged.

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