Translation and post-translational modifications in *Plasmodium*

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Malaria is one of the most important human parasitic diseases in the world, affecting about 400 million people and killing about 1 million children every year. Our relationship with the malarial parasite has been a long one on the evolutionary scale, and the methods adopted by the parasite to thrive and colonize our bodies are truly fascinating. In order to control the disease, it is of fundamental importance to understand the biology of the parasite. In this review we discuss certain unique features in the parasite translation machinery. We also discuss the various post-translational modifications of *Plasmodium* proteins, that may result in the modulation of their function.

Keywords: *Plasmodium*, post-translational modifications, ribosomal proteins, translation.

Introduction

THE malaria parasite belonging to *Plasmodium* species is a complex eukaryote parasite that survives in different habitats in vertebrate host and mosquito vector (Figure 1). It is an obligate intracellular parasite that is transmitted by the bite of a female anopheline mosquito. Resident in the salivary glands of the mosquito, are motile forms of the parasite (sporozoites), which are injected into the human host during a blood meal. The sporozoites infect hepatocytes, undergo schizogonic divisions, and get released into circulation as merozoites, each of which can infect an erythrocyte, starting the asexual stage. After a few asexual cycles, the cells differentiate into male and female gametocytes, and when ingested into the mosquito gut, these undergo fertilization and complete the sexual stages through sporozoite production and migration to the salivary glands of the mosquito. Neither the transcriptomic nor the proteomic regulation of *Plasmodium* is well understood. Certain aspects of translational regulation of the parasite have been detailed recently[1]. In this article we shall concentrate on the ribosomal features of the translational machinery and post-translational modifications in *Plasmodium*.

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Ribosomes in *Plasmodium*

The ribosome is a macromolecular complex that synthesizes proteins from mRNAs in all cells. The structure of the ribosomes has been collated through a large number of studies worldwide, and the ribosomes constitute one of the most conserved functional units in a cell[2]. Orthologous reconstitution has shown that even crossing across prokaryotic and eukaryotic components allows protein synthesis, although the regulation of such reconstituted ribosomes is compromised. Little is known about the constituents of the *Plasmodium* ribosomes, but it has been assumed that the translation machinery is comparable to that in an eukaryotic cell. With the publication of complete *Plasmodium* genome, bioinformatic studies detected common features in the *Plasmodium* protein synthesis components[3], and subsequent annotations have been made in the database PlasmoDB (plasmodb.org).

Ribosomal RNAs

The malaria parasite *Plasmodium* is unusual in having far fewer number of structurally distinct sets of ribosomal RNA genes, although the arrangement of these rDNAs is that of a typical eukaryotic gene structure (18S–5.8S–28S, Figure 2). The unique feature of these *Plasmodium* gene sets is the stage-specific expression of the rRNAs during the parasite life cycle[4–6]. S-type rRNA is detected in sporozoites purified from the salivary glands of infected mosquitoes, the O-type occurs in the ookinete stage, whereas the A-type populates the asexual stages in the vertebrate host. The S- to A-type transition occurs in the liver and can be seen during development of sporozoites in the hepatocyte cell line[7]. Similarly, the switch from A- to S-type gene expression in rRNA can be observed during development of the parasite in the mosquito.

For most *Plasmodium* species, the overall sequence similarity between the A- and S-type rRNA genes is documented to be about 80%. Most of the differences are located in variable regions and in the internal transcribed spacer (ITS) regions[8,9]. However, in the GTPase centre of the *P. falciparum* S-type rRNA, certain critical differences exist, which suggested a functional difference between the A- and S-type rRNAs. In an attempt to define

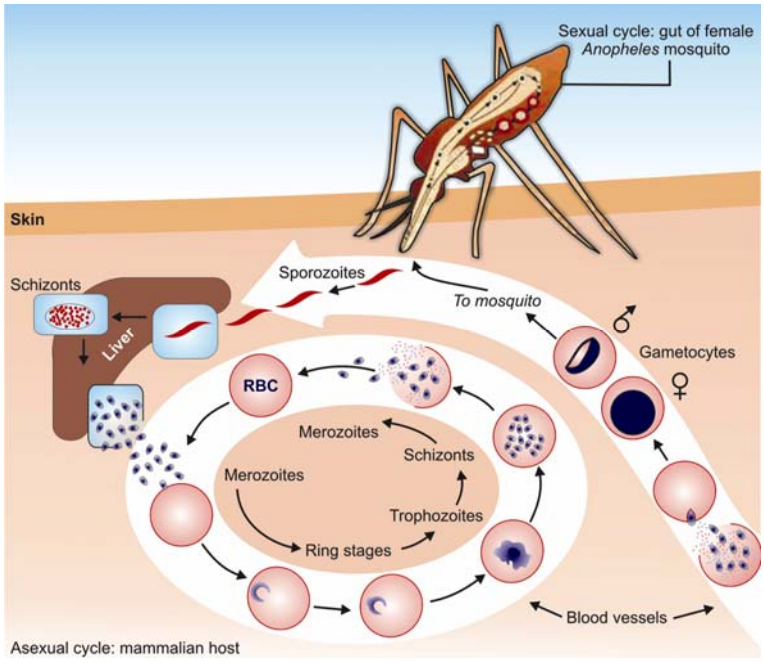

Figure 1. Life cycle of malaria parasite.

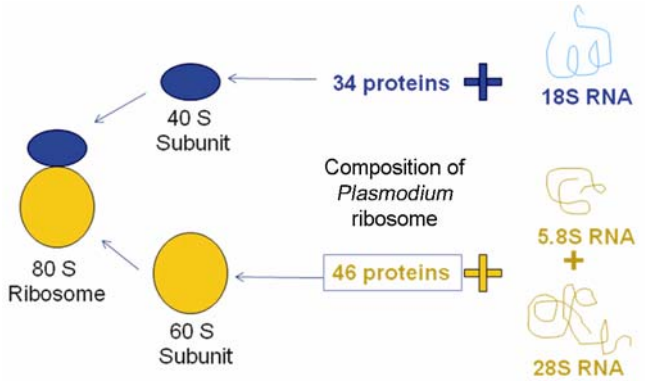

Figure 2. Composition of *Plasmodium* ribosomes as predicted by the *Plasmodium* database (PlasmoDB.org).

functional differences, the yeast wild-type GTPase centre was replaced with corresponding A- and S-type GTPase centres[10]. The results showed that the expression of either A- or S-type chimeric rRNA in yeast increased translational accuracy. Yeast containing only A-type chimeric rRNA and no wild-type yeast rRNA grew at the wild-type level, but S-type chimeric rRNA severely inhibited growth in the presence of wild-type yeast rRNA, and caused lethality in the absence of the wild-type yeast rRNA, indicating that *Plasmodium*-specific S- and A-type GTPase mutations make essential functional differences.

To further investigate the possible existence of functionally different ribosome types, the rRNA gene units of the rodent parasite *Plasmodium berghei* were studied[11,12]. On the basis of sequence differences similar to the S- and O-types of *P. vivax*[5], *P. berghei* appeared to contain four distinct copies of the rRNA units (A–D), the blood stage A-type (A and B units) and the mosquito stage S-type (C and D units). It was shown that the C-SSU gene is actually a chimera of the A-SSU and D-SSU, and that no significant differences were found in the core regions of the A-, C- and D-SSU rRNA molecules[11]. Questions were then raised regarding whether two functionally different ribosome types are indeed required for development of *P. berghei* in the mosquito. Through specific knockout studies, it was observed that functionally different ribosome types in *P. berghei* do not exist and that only one copy of the S-type genes, either the C- or the D-gene, is sufficient for complete development of the parasite in the mosquito vector[12]. The significance of the stage-specific expression of structurally distinct ribosomal RNAs is not yet clear. However, analysis of the long-term evolution of 18S genes in apicomplexans, has given rise to the postulate that these genes may evolve according to a birth-and-death model under strong purifying selection[13,14].

Ribosomal proteins

The possible translation factors such as ribosomal proteins required for the structure are annotated in the *P. falciparum* genome database (plasmodb.org), but most of these need to be functionally validated. A total of 212 ribosome-related proteins have been shown for the *P. falciparum* genome currently in PlasmoDB, of which about 34 and 46 are shown to be putative 40S and 60S proteins. Of these ribosomal proteins, only the 60S PfP0 protein has been validated functionally through complementation study in ScP0 conditional knockout strain of *Saccharomyces cerevisiae*[15]. It was observed that like human and other fungal P0 proteins, PfP0 could complement the ScP0-deficient yeast strain, but the kinetoplastid *Leishma-*

nia LcP0 could not, showing distinct structural differences of PfP0 compared to the other protozoan kinetoplastid orthologue[15,16]. However, PfP0 differed from ScP0 in terms of binding to the ScP0 interactor proteins, ScP1 and ScP2[15]. The *Plasmodium* P0 protein, however, does appear to bind to the *Plasmodium* P1 and P2 proteins, as the parasite extract exhibits a complex which matches the pentameric structure P0(P1)2 (P2)2 in HPLC-resolved proteins. The P1 and P2 proteins of *P. falciparum* are currently under evaluation for their various properties (Das and Sharma, unpublished results).

The P-proteins have been shown to possess non-ribosomal functions as well. In yeast, the knockout of P2 was viable in rich medium, but not under stress conditions, and changes in protein synthesis and cell division rates were noted[15,17]. Interactors of the PfP0 protein have also been documented, but these appear to contain proteins other than translation factors[18]. Ribosomal proteins are known to play varied roles besides protein synthesis[19]. Both PfP0 as well as *Leishmania* P0 protein have been implicated in protective roles[20–22]. In *Toxoplasma* transfection studies using tagged-TgP0 protein, it has been established that the cognate TgP0 protein translocates to the tachyzoite surface[23]. In *Neisseria gonorrhoeae*, the functional orthologue of P2 (L12), is shown to be surface exposed, and has been implicated in cell invasion[24].

The neutral protein P0, and the acidic proteins P1 and P2 make up a pentameric complex P0(P1)2(P2)2, which constitutes the eukaryotic stalk[25], a ribosomal protuberance of the large ribosomal subunit, functionally equivalent to the bacterial complex L10(L7/L12)[26]. The stalk interacts with the elongation factor EF-2 and plays an important role in the regulation of protein synthesis. Resistance to the antifungal sordarin has been mapped earlier to the P0 domain associated with elongation factor-binding site. Sordarin-resistant strains showed point mutations clustered in the region around 130–147 amino acid position of the P0 protein[27,28]. PfP0 conferred high sordarin resistance in *S. cerevisiae*, comparable to that of *Aspergillus fumigatus* and amino acid analysis between these species could narrow down the P0 domain of 130–147, and in particular the positions E117, R122 and V124, to play important roles in the sordarin resistance[15].

Other factors of protein synthesis

tRNAs and tRNA synthetases

A comprehensive write-up regarding tRNAs and tRNA synthetases has been documented in a recent review[1]. A total of 46 tRNA genes for 45 tRNA isoacceptors have been coded in the nuclear genome of *Plasmodium*, making it a eukaryotic organism that encodes fewest known tRNA genes[29]. These appear to have structures very similar to eukaryotic tRNAs. Most *Plasmodium* species have very low G/C content in their genomes (less than 20% G/C for *P. falciparum*)[3], while *Plasmodium* cytosolic tRNAs have a G/C content of 56%. Whether this imbalance and the fewer copies of tRNAs has any control on protein synthesis is not clear. Aminoacyl-tRNA synthetases (aaRSs) are the enzymes that confer specificity regarding charging of specific amino acids on the cognate tRNA, to form aminoacyl-tRNAs. *Plasmodium* has genes for only 37 aaRSs, and these are apparently sufficient to translate the nuclear, apicoplast and mitochondrial genomes[30].

Initiation, elongation and release proteins

Since *P. falciparum* has an A/T-rich genome, and also contains generally long 5′-untranslated regions (~350 bases)[31], initiation at the appropriate AUG sequence is important. Recently, an algorithm to predict TIS in the mRNA of asexual stages of *P. falciparum* was employed and experimental validations of sequence features have been carried out[32,33].

The factors required for initiation, elongation and release of the polypeptide chain are currently annotated in the *P. falciparum* genome database (PlasmoDB) and in a recent review[1]. However, once again, validation of most of these is yet to be carried out. Among the factors specifically characterized are initiation factors in the eIF-4F complex (eIF-4E, eIF-4G and eIF-4A)[34–37]. The mRNA cap-binding activity by eIF-4E has also been demonstrated[35]. The interactions of eIF-4E with eIF-4G and eIF-4A (PfH45) have also been documented. The translation initiation factor eIF-5A from *P. falciparum* contains the unique amino acid, hypusine, which has been shown to be post-translationally modified by parasite deoxyhypusine synthase and hydroxylase[38].

Eukaryotic elongation factor 1A (eEF-1A) plays a central role in protein synthesis, cell growth and morphology. In *Plasmodium* species, the two copies of eef-1a (eef-1aa and eef-1ab) are identical and genetically linked, implying that they produce eEF-1A with identical functions, and the duplication might therefore be explained as a gene dosage phenomenon in order to produce sufficient protein[39]. Through generation of a *P. berghei* knockout that lacked an eEF-1a gene, it was shown that the level of eEF-1A production affected the proliferation of blood stages and parasite fitness. These parasites could complete the vertebrate and mosquito phases of the life cycle, but the growth phase was distinctly retarded[40]. Protein interactions have been seen amongst several elongation factors of the parasite, such as the phosphoprotein eEF-1b and the G protein eEF-1a (the functional homologues of prokaryotic EF-Ts and EF-Tu respectively) with eEF-1d and eEF-1g[41,42].

Organellar protein synthesis in Plasmodium

In addition to the nuclear genome, there are cellular organelles, the apicoplast and mitochondria, and these

organellar genomes contain rDNA sequences[43]. The later appear to be polycistronically transcribed and developmentally regulated[44]. Translational activity has been definitively documented only in the apicoplast[45–47]. However, while apicoplast seems to code for some of the translation factors, mitochondrial DNA, a unit of 6 kb codes for very little. Thus, all the remaining factors are presumably acquired from the parasite cytosol. Putative prokaryotic-like initiation factors can be identified in the nuclear genome, and some of these possess apicoplast or mitochondria targeting pre-sequences[1]. Definitive information exists for the encoding of apicoplast specific EF-Tu, and recent data show that peptide chain elongation in the apicoplast may be facilitated by a complex of plastid-encoded EF-Tu and nuclear-encoded EF-Ts, presumably in conjunction with an apicoplast-targeted EF-G[48]. The apicoplast genome also contains genes encoding 26 tRNA isoacceptors[29]. An analysis of the database indicates that the apicoplast genes, which contain mono- or polycistronic structures, appear to lack the purine-rich prokaryotic ribosome-binding Shine–Dalgarno (SD) sequence upstream of their AUG codons and are A/T-rich in nature. Correspondingly, the complementary pyrimidine-rich, anti-SD sequence at the 3′-end of the bacterial small ribosomal subunit 16S rRNA, is also an A/T-rich sequence in the apicoplast-encoded ssu16S rRNA[1].

DNA from *Plasmodium* mitochondria consists of a head-to-tail tandemly repeated array with a unit length of about 6 kb, contains three open reading frames (Cox1, Cox3 and Cytb) and fragments of large and small subunits of rRNA[49]. Although unusual translational machinery has been implicated in mitochondria of *Plasmodium*[50], direct evidence is lacking and very little is known regarding mitochondrial translation in *Plasmodium.*

Post-translational modifications in Plasmodium spp: In response to developmental cues and environmental stimuli, proteins undergo a wide variety of chemical modifications that are collectively referred as post-translational modifications (PTMs)[51,52]. In a protein, such modifications can occur at a single site or at multiple sites, leading to the generation of a large variety of variants. The potential of PTMs to generate diversity in the functional proteome of a cell seems limitless. For example, there are approximately 30,000 open reading frames in the human genome, but it is predicted to give rise to ~ 1–2 million different protein variants[53]. Structurally, such modifications must 'add' (e.g. phosphorylation adds a phosphate group) or 'subtract' mass from the native protein (e.g. proteolysis) molecule. Apart from changing the mass, the diversity generated offers new chemistry, new recognition pattern for partner molecules, turning 'on' and 'off' of enzyme activity, alter the lifetime and location of the protein, etc. by altering the protein charge, conformation and hydrophobicity. Increasingly, attention of investigators is being drawn to detailed analysis of PTMs with the realization that they are important for the smooth physiological functioning of cellular processes by increasing the inventory of side chains available to proteins.

P. falciparum, the causative agent for the most virulent forms of human malaria has ~ 5300 genes (open reading frames)[3]. The parasite has a complex life cycle (Figure 1) involving several different developmental stages and two different hosts. During the course of its life cycle, the parasite is exposed to wide variation in environmental conditions, e.g. temperature (may vary from 25°C in mosquito to ~ 40°C in human patients), pH, etc. that necessitates high degree of flexibility and adaptability in its functional proteome. Regulatory processes can come into play at the level of expressed transcriptome, translation and/or modification of the functional proteome (PTMs). Recent studies based on microarray analysis of parasite transcriptome indicate that steady-state level changes in mRNA occur during parasite development. But, there are very few regulatory motifs and transcription regulators that could be identified leading to the perception that transcription in *Plasmodium* is hard-wired and protein levels are primarily determined by post-translational mechanisms[54,55]. It is likely that regulation of post-transcriptional processes (repression/activation of transcriptional and PTMs) may be the main mechanism to the control life cycle and adapt/respond to the changing environment. Such considerations have led to suggestions that PTMs may be rampant in *Plasmodium* and has generated immense interest in analysing such modifications[56]. Here we review the approaches being used for analysis of PTMs and current status of such studies on the malaria parasite. Given the limitation on size and scope of this review, we have chosen a few examples and approaches to introduce the reader to the complex field of PTMs, and how it is adding to our understanding of parasite biology.

Approaches for analysis of PTMs: Traditionally, PTM analysis was mostly restricted to the one 'protein at a time' paradigm. Use of specific radioactive precursors allowed the determination of stochiometry of incorporation and isolation of the labelled peptides after proteolytic digestion of the protein. The labelled pepides could be sequenced to determine the specific residues in the protein that are modified. Such approaches were very slow and were not suitable for global PTM analysis. Use of specific antibodies and modifying group-specific fluorescence dyes/antibodies (e.g. Pro-Q Diamond, a dye specific for phospho-proteins or anti-Y-P, S-P/T-P antibodies) allowed simultaneous observation of several modified proteins on a two-dimensional gel. However, such analysis provides information about a very limited set of chemical groups that have modified the protein, and also does not provide information about the residues that are modified. Recent developments in protein separa-

tion by two-dimensional gel electrophoresis (2DE)[57], comparative analysis using dye multiplexing (fluorescence dyes, Cy2, Cy3 and Cy5) technologies[58] and protein sequencing by mass spectrometry[59] have allowed determination of PTMs and changes in variant profiles in response to environmental or developmental signals. Such analysis usually does not lead to exact identification of the nature of the modifying group or site of modification, but provides a quick comparative profile of different variants or isoforms[58,60,61]. More recently, global approaches involving different combinations of separation methods (PAGE, HPLC, IMAC, etc.) coupled with mass spectrometry-based peptide sequencing for the identification of proteins as well as modifying groups are yielding comprehensive information about the proteome-wide PTMs. A host of experimental work-flows can be used to fulfil the specific purpose of the experimenter. However, there is no single protocol to provide complete analysis of all PTMs in a given cellular proteome[62–64].

Identification of molecular components required for PTMs in Plasmodium: For any PTM to occur in a parasite, it is imperative that either the parasite has the enzymatic machinery needed to catalyse such reactions, or it imports the relevant host proteins. Extensive studies have been done on the enzymatic machinery involved in a number of PTMs in eukaryotic cells. Several investigators have used the approach based on homology searches to determine whether the relevant genes needed to code the enzymes required for specific PTMs are present in the parasite genome. Further, validation is needed to confirm that these genes express, and active forms of proteins are produced in the cell. Several enzymes involved in phosphorylation/dephosphorylation, acetylation/deacetylation, methylation/demethylation, ubiquitination/deubiquitination/lipidation, etc. have been identified and in some cases their role in PTMs has also been validated[56]. Table 1 presents a summary of molecules involved in some of the most commonly observed PTMs in *Plasmodium*.

Multiple isoforms of Plasmodium proteins indicate abundance of PTMs: Many PTMs are known to alter the pI of a protein without affecting the molecular mass significantly. Such variants of a protein appear as a series of horizontal spots on a 2D gel and allow one to determine the lower limit of the number of isoforms that a protein has in a given sample. Identity of such proteins is established either using the mass spectrometry or immunological (Western) methods[61]. Bozdech's group in Singapore used this approach and undertook a comparative analysis of the proteome as the *P. falciparum* undergoes intraerythrocytic multiplication cycle[65]. Several protein spots from 2D gels were identified using MS methods. The results showed that many proteins, viz. actin-1, enolase, glyceraldehyde-3-phospahte dehydrogenase, phosphoglycerate kinase, lactate dehydrogenase eukaryotic initiation factor (elF)4A, elF5A, HSP70-1, HSP70-2, HSP86, EF2, etc. are extensively modified in *Plasmodium*. Several truncated isoforms (proteolytically cleaved forms) were also detected[60,65]. Proteolytic processing is known to play an important role in hemoglobin degradation, merozoite egress and host-cell invasion processes. Truncated forms of merozoite surface proteins (MSP1, MSP7), rhoptry-associated proteins (RAP1, RAP2, Rap3), proteins from the CLAG gene family (RHopH1, RHopH2, RHopH3), and glideosome-associated protein (GAP50) could be detected[60]. Some proteins were extensively modified and different isoforms localized in different subcellular compartments. For instance, *Plasmodium* enolase showed about 18 different variants, several of which are truncated forms (eight) and some are multiply phosphorylated, whereas others are conjugated to other proteins to give rise to higher molecular mass isoforms[60,61,65,66]. *In situ* localization[67] and subcellular fractionation showed the presence of enolase in cytosol, nucleus, cell membrane, cytoskeletal elements and food vacuole. Further, different isoforms are associated with different organells, suggesting moonlighting functions for this protein. It is likely that specific PTMs regulate recruitment of enolase to different organelles in the cell[61]. Enolase also showed stage-specific variation in isoform profiles and relative abundance[68].

Although existence of multiple isoforms is evident from these studies, our understanding of their physiological significance is rather rudimentary. Increasingly, efforts are being made to understand the role of PTMs in functional control of cellular processes. MSP1 that is attached to the surface membrane through a GPI anchor is proteolytically cleaved during invasion of the red blood cell. A C-terminal ~ 19 kDa fragment of MSP1 is rapidly endocytosed into small vacuoles that coalesce to form the food vacuole[69]. Such observations point to the importance of understanding the role of PTMs in regulation of cellular events as well as host–parasite interaction.

Future developments and challenges

Availability of the genome sequence of an organism provides the required framework for the identification of a protein in a gel spot using mass spectrometry. This is routinely carried out in laboratories equipped with mass spectrometers. However, structural characterization to determine the nature of chemical modification and the specific aminoacyl residue that has undergone the modification in an electrophoretic variant, is technically demanding and requires advanced mass spectrometry-driven proteomics. Recent attempts by Stunnenberg's group to analyse acetylation/methylation and other modifications in *P. falciparum* histones have provided interesting insights[70]. In several species, strong correlation between active promoters and H3K4me3 and H3K9ac modifications has been observed. In *P. falciparum* (ring

Table 1. *In silico* identification of the number of genes involved in some of the common post-translational modifications in *Plasmodium* proteome[56]

Modifying enzyme	No. of putative genes	Putative role/remarks
Protein kinases	86–99 (refs 76, 77)	TyrK and STE family of protein kinases not found. However, tyrosine phosphorylation of proteins in *Plasmodium* has been reported[74]. Phosphorylations are implicated in cell-cycle regulation, cell proliferation, sexual differentiation, parasite egress and invasion, etc.
Protein phosphatases	27 (ref. 78)	Involved in invasion, cell growth, merozoite release, etc.
Acetylases	21 (ref. 79)	Acetyltransferases (source: http://plasmodb.org/)
Deacetylases	5 (ref. 80)	
Methylases	9 (ref. 81)	Histone methylation
Demethylases	2	Histone demethylation
Protein farnesyl transferase (PFT)	1 (ref. 82)	PFT inhibitors are antimalarials. Highest prenylation of proteins observed in transition from trophozoite to schizont.
Ubiquitination	E1 ≥ 8; E2 ≥ 14; E3 ≥ 54 (ref. 83)	Plasmodia have polyubiquitin gene. Conjugating enzymes E1, E2 and E3 have been reported. Numbers mentioned here are based on *in silico* search of *P. falciparum* genome sequence data.
Deubiquitination	18 or 29 (ref. 83)	Iso-peptidases required for proteosomal degradation of proteins.

stage), such histone modifications are homogenous across the genes, marking active or inactive genes equally. However, in schizont stage they are enriched at the 5′-end of the active genes, thus revealing an unforeseen and unique plasticity in the use of the epigenetic marks and implying the presence of distinct epigenetic pathways in gene silencing/activation throughout the erythrocytic cycle[71].

For large-scale, proteome-level PTM analysis, multiple strategies are emerging depending on the specific modification that one would like to study[72]. Phosphorylation is one of the most abundant and important PTM that several proteins undergo. It is also highly dynamic. Methods involving selective enrichment of phosphopeptides and then sequencing them using LC-MS/MS type of protocols have proved quite rewarding[64,73]. In one such effort, we studied soluble fraction of *P. falciparum* cellular extract, enriched phosphopeptides (after trypsin digestion) using TiO_2 beads and analysed the sample in a MS/MS experiments. Several phosphorylation sites in a variety of proteins could be identified. Using similar approach, in a recent study on extracts of *P. falciparum* schizont stage cells, about 5000 different phosphopeptides could be identified[74]. Such affinity-based approaches for the selective separation of sub-proteome of interest coupled with high accuracy, high-resolution mass spectrometer can provide global PTM profile for a proteome. Mapping the modified sites on 3D structure of the protein may provide insights into their functional significance.

A more challenging task will be to understand the physiological significance of such PTMs. One approach can be to mutate the residue that is getting modified and develop *in vivo* (transfection) or *in vitro* approaches to determine the function of the PTM[75]. Antibody-based pull-down assays can be used, where a multi-protein complex can be isolated and PTMs in the protein of interest can be determined. Similarly, an isoform associated with any specific sub-cellular fraction can be analysed for the PTMs, thereby establishing the molecular basis for such recruitment. Although the advent of high-resolution, multi-dimensional separation technologies and a host of mass spectrometers are allowing near-complete determination of proteome-level PTMs, the task of assigning physiological functions to specific PTMs is going to be a daunting one, requiring a wide variety of experimental strategies. Accumulating knowledge about the PTMs in *Plasmodium* and their role in regulating parasite development cycle as well as host–parasite interaction may offer novel opportunities for the design of new interventional strategies.

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