Chapter 1

Introduction

The targets and role of palmitoylation in *Plasmodium* parasites

The post-translational modification (PTM) of proteins plays an essential role in modulating protein activity and function, and thus greatly influences the normal cell biology of most organisms, including *Plasmodium* parasites, the causative agents of malaria. The work described here explores palmitoylation, one of the lesser-known protein PTMs, which is used broadly to regulate protein function in other eukaryotes, but has not been well-studied in *Plasmodium*. This dissertation focuses on two broad aims: developing new approaches to identify palmitoylated cysteines in *Plasmodium falciparum* proteins, and characterising for the first time the enzymes responsible for palmitoylation in *Plasmodium* parasites. To set the background for this work, this introduction outlines what is currently known about protein palmitoylation in other eukaryotic organisms in general, and in *Plasmodium* parasites in particular.

1.1. Protein Palmitoylation: Definition

Palmitoylation is a PTM involving the addition of long-chain fatty acids to one or more internal cysteine residues via thioester (S-acyl) linkages (Figure 1.1). Proteins can be S-acylated with different fatty acid chains and thus this modification can also be referred to by the more general term, 'S-acylation'. However, the fatty acid typically used is palmitic acid, a 16-carbon saturated fatty acid, hence the term 'palmitoylation'. Critically, the thioester bond between palmitic acid and the cysteine residue is reversible, making palmitoylation a dynamically regulated protein modification, just like the much more widely studied protein PTM, phosphorylation [1].

The reversibility of the thioester bond makes palmitoylation distinct from other lipid modifications that commonly occur, such as myristoylation and prenylation. Myristoylation refers to the covalent attachment of myristate to the N-terminal glycine residue of the protein, which occurs co-translationally upon the removal of the initiator methionine residue [2]. Prenylation is the addition of a farnesyl or geranylgeranyl isoprenoid to a cysteine residue that is four amino acids from the C-terminus via a thioether linkage, which leads to subsequent proteolytic removal of the three terminal amino acids and the carboxyl-methylation of the prenylated cysteine [3]. Both myristoylation and prenylation are irreversible modifications.

While the majority of palmitoylation occurs in the form of the reversible S-acylation described above, palmitic acid can be irreversibly attached to cysteine residues if they are at the extreme N-terminus of proteins. In this case, it is thought that an intermediate thioester linkage is first formed and this is

then rearranged into a stable amide linkage (Figure 1.1). This irreversible version of palmitoylation is referred to as 'N-palmitoylation' [4].

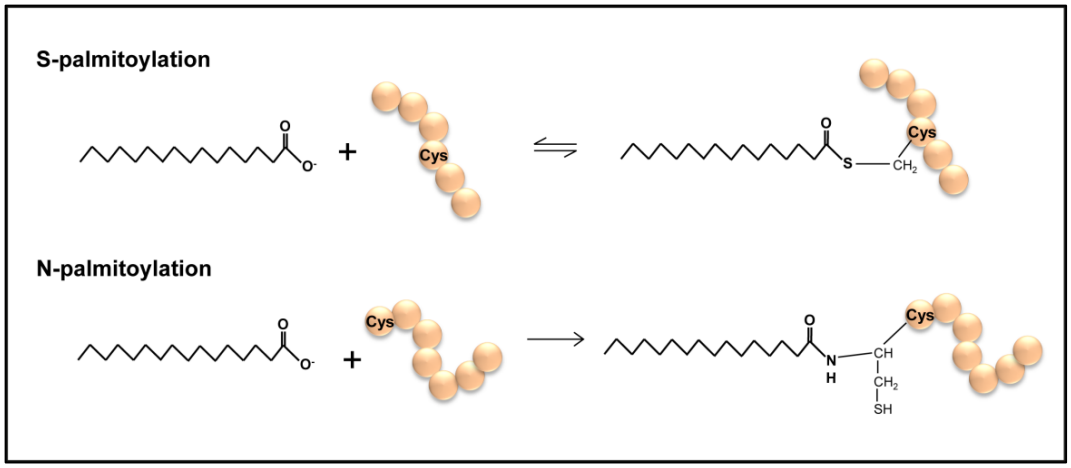

Figure 1.1: Reversible S-palmitoylation compared to irreversible N-palmitoylation. Chemical structures of palmitate attached to a protein via the reversible thioester linkage (S-palmitoylation) and the irreversible amide linkage (N-palmitoylation).

1.2. Protein acyltransferases (PATs)

The formation of the thioester bond between the palmitoyl moiety and the cysteine side chain can occur spontaneously *in vitro* in the presence of palmitoyl-CoA. It was therefore initially thought that palmitoylation took place via non-enzymatic mechanisms *in vivo* [5]. However, the recent discovery of proteins responsible for catalysing protein palmitoylation, first in *Saccharomyces cerevisiae* and later in mammalian cells, now indicates that palmitoylation is in fact an enzyme-mediated process [4, 6, 7]. These proteins are referred to as protein acyltransferases (PATs) and can be divided into two main families: DHHC-domain-containing PATs (DHHC-PATs) and MBOAT-domain-containing PATs (MBOAT-PATs) [5].

1.2.1. DHHC-PATs:

DHHC-PATs are transmembrane (TM) proteins and typically contain four to six TM-domains. The defining feature of these proteins is that they all contain a highly conserved Asp-His-His-Cys (DHHC) motif within a cysteine-rich domain [5] and this motif is commonly located on the loop between two TM-domains, and is exposed on the cytosolic side of the membrane [8, 9] (Figure 1.2A). The DHHC

signature motif, which is part of the larger approximately 50 residue cysteine-rich domain, is conserved across eukaryotes. This conserved domain allows the bioinformatics identification of DHHC-PATs, and each eukaryotic genome contains a variable number, ranging from 7 in *S. cerevisiae* to 23 in humans [5].

Besides the DHHC domain, two other regions display significant homology across the DHHC-PATs. An Asp-Pro-Gly (DPG) motif is typically found next to the second TM-domain and a Thr-Thr-x-Glu (TTxE) motif is usually found next to the fourth TM-domain, although the functional significance of these conserved areas are still unknown [5].

The DHHC-domain is thought to be directly involved in the palmitoyl transfer reaction, although not all details of the enzymatic process are currently known. All DHHC proteins so far have been found to undergo autopalmitoylation and when the cysteine residue in the DHHC motif is mutated, autopalmitoylation is abolished. This has led to the suggestion that palmitoylation of the substrate may occur via a two-step process where the PAT is first autopalmitoylated, resulting in the production of a palmitoyl-enzyme intermediate, and this is followed by transfer of the palmitoyl moiety to the substrate [5, 10]. Evidence suggests that the DHHC motif is essential for this two-step palmitoylation reaction [10]. However, this is by no means proven, and other models posit that instead of acting as an intermediate in the palmitoyl transferase reaction, the palmitoylated DHHC protein could be involved in allosteric regulation of substrate binding or in substrate specificity [5]. Indeed, other work suggests that the DHHC domain is not merely a catalytic unit and may in fact possess substrate specificity determinants [11].

The general question of DHHC substrate specificity –whether individual DHHC-PATs palmitoylate a specific set of targets- is a subject of particular interest for the field as a whole. For some palmitoyl-proteins, it appears that palmitoylation can be catalysed by several different PATs [11]. For example, deletion of the yeast PAT, Pfa3, reduced but did not abolish palmitoylation of the vacuolar protein, Vac8, which may indicate that the palmitoylation of Vac8 may be taken over by another PAT in the absence of Pfa3 [12, 13]. Similarly the palmitoylation of Ras in yeast was also only partially reduced in the absence of its cognate PAT, Erf [14]. Furthermore, it was also shown that multiple DHHC-PATs could be knocked-out in yeast without completely abolishing palmitoylation of substrates, suggesting possible overlap in DHHC-PAT functionality [14]. However, some palmitoyl-proteins do appear to have dedicated DHHC-PATs. For example, in yeast, palmitoylation of SNARE proteins appear to be the responsibility of the DHHC-PAT, Swf1, while another DHHC-PAT, Pfa4, appears to palmitoylate only the amino acid permeases [14], and the activities of both Swf1 and Pfa4 cannot be performed by other yeast PATs [11]. The question of whether DHHC-PATs are substrate specific therefore seems to

depend on the particular DHHC-PAT or the particular group of palmitoyl-protein substrates. However, it should be pointed out that only a handful of DHHC-PAT/substrate partnerships have been studied in detail, and there have been no studies on the global impact of DHHC-PAT deletion on palmitoylation sites, in large part because few global datasets of palmitoylation sites have so far been generated.

1.2.2. MBOAT-PATs

The membrane-bound O-acyl transferase (MBOAT) superfamily of proteins consists of membrane-bound proteins, which generally contain eight to ten TM-domains. Although they are less conserved compared to the DHHC family of proteins, MBOAT proteins possess an invariant histidine residue found within a long hydrophobic region which is thought to form part of the active site [15, 16] (Figure 1.2B). The majority of the members of the MBOAT superfamily act as enzymes for the transfer of fatty acids to the hydroxyl groups of membrane-bound target proteins [15]. However, several MBOAT proteins have also been found to catalyse N-palmitoylation, in particular, the N-palmitoylation of secreted proteins such as Hedgehog (Hh), Spitz and Wnt [17-20]. For example, in humans, the secreted morphogen Sonic hedgehog (Shh), the homologue of the Drosophila Hh protein, is N-palmitoylated, and the transfer of palmitate to the N-terminal cysteine is catalysed by Hedgehog acyltranferase (Hhat), a member of the MBOAT family [21]. Porcupine, another MBOAT protein, is also known to mediate palmitoylation of Wnt secreted proteins [20]. Thus, the MBOAT-PATs appear to be mainly responsible for the irreversible N-palmitoylation of secreted signalling proteins.

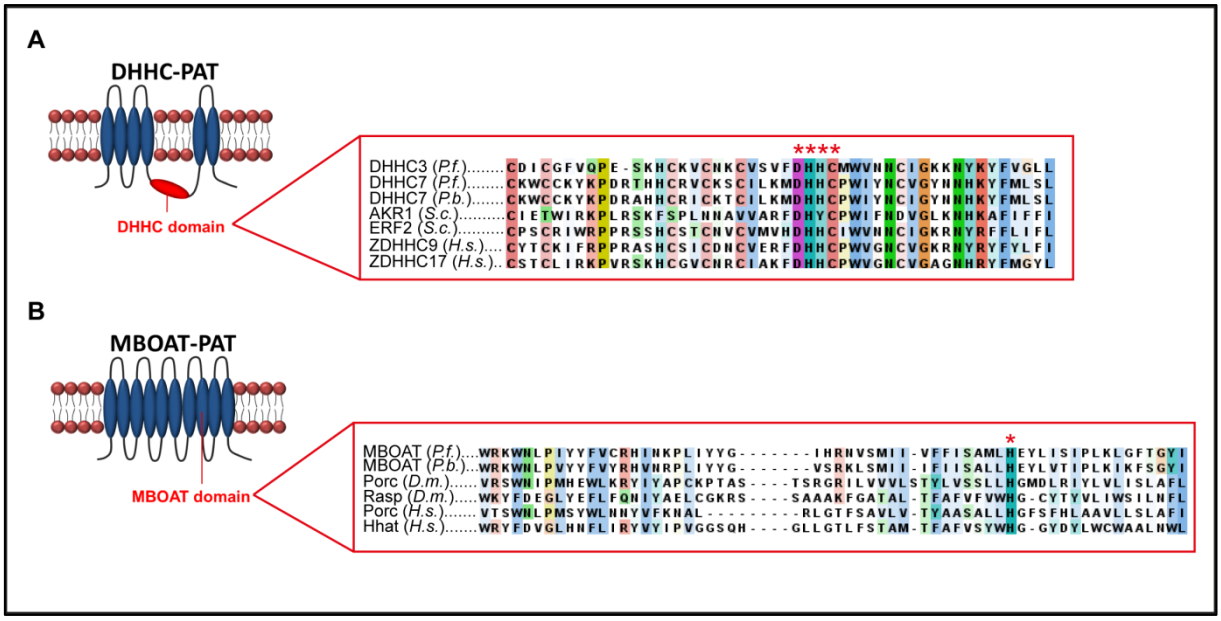

Figure 1.2: Structure of DHHC-domain and MBOAT-domain-containing PATs. (A) The generic structure of DHHC-PATs shown schematically. The conserved DHHC-domain is shown in red. Representative amino acid sequences of the DHHC domains of known DHHC-PATs from different organisms are shown aligned on the right. The characteristic DHHC sequence is highlighted with asterisks. **(B)** The generic structure of MBOAT-PATs shown schematically. The hydrophobic domain containing the conserved histidine residue is indicated as the MBOAT domain. Representative sequences of MBOAT proteins are shown aligned on the right. The conserved histidine residue which is thought to form part of the active site is highlighted with an asterisk.
P.f. - Plasmodium falciparum, *P.b. - Plasmodium berghei*, *S.c. - Saccharomyces cerevisiae*, *D.m. – Drosophila melanogaster*, *H.s. - Homo sapiens*.

1.3. Acyl-protein thioesterases

As mentioned above, S-palmitoylation is a reversible reaction as the thioester linkage can be broken, allowing the removal of the palmitate group from proteins. A class of enzymes known as acyl-protein thioesterases have been found to catalyse the depalmitoylation of proteins [22]. There are two types of acyl-protein thioesterases. The first type, known as the protein palmitoylthioesterases (PPT), of which there are two - PPT1 and PPT2, are localised in lysosomes and are involved in depalmitoylating proteins during protein degradation [23]. The second type of acyl-protein thioesterase, known as the APTs, appears to be the enzymes responsible for regulating the depalmitoylation of S-palmitoylated proteins [22]. APT1 is found in the cytosol and has been shown to remove palmitate from signalling proteins such as G protein α subunits, H-Ras and endothelial nitric oxide synthase (eNOS) [24, 25]. Although APT1 was originally found to act as a lysophospholipase, later work indicates that it in fact prefers palmitoyl-thioesters as a substrate [24, 26]. Furthermore, the inhibition of APT1 appears to

interfere with Ras localisation and signalling, which is regulated by cycles of palmitoylation and depalmitoylation [27]. This indicates that the APTs may be important players in regulating protein palmitoylation, although much is still unknown about these proteins and further study is now being performed in order to determine how and where these enzymes function *in vivo*.

1.4. Functions of protein palmitoylation

Protein palmitoylation is a widespread PTM and occurs in a large and diverse repertoire of proteins. As a lipid modification, the basic function of palmitoylation is to regulate the interaction of proteins with the lipid bilayers of cellular membranes. It is however, more than just a simple lipid anchor, and a significant role of palmitoylation appears to be in the trafficking of proteins to specific cellular membranes [28]. A well-studied example of proteins which uses palmitoylation for specific intracellular targeting is the dually-acylated Ras family of small GTPases. H-Ras and N-Ras proteins are first farnesylated, which targets the Ras proteins to the endoplasmic reticulum where they are then palmitoylated. Palmitoylated Ras proteins are trafficked to the Golgi and then to the plasma membrane via the classical secretory pathway. At the plasma membrane, Ras proteins are depalmitoylated, allowing their trafficking back to the Golgi where they can be repalmitoylated and sent back to the membrane [4, 8, 29, 30] (Figure 1.3). This dynamic cycle of palmitoylation and depalmitoylation is required for regulating the subcellular distribution of Ras proteins and in turn, may be important in regulating the locations in the cell where Ras proteins are available for signalling [3, 31].

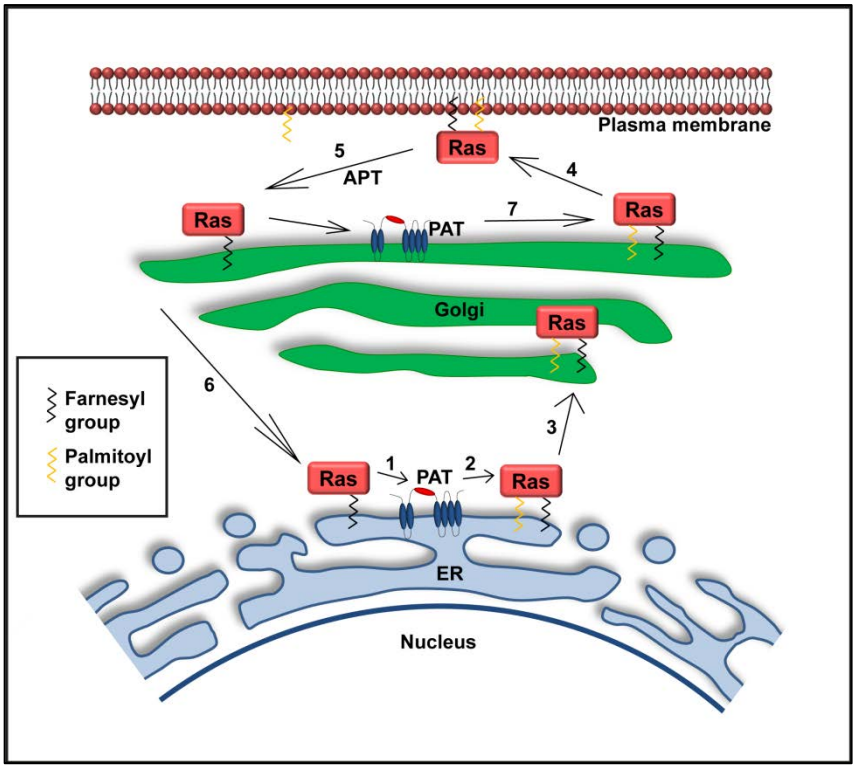

Figure 1.3: Function of palmitoylation on the subcellular trafficking of Ras proteins in mammalian cells. Ras proteins are dually-acylated proteins. The first acyl modification is the addition of a farnesyl moiety (black) and this farnesylation directs Ras proteins to the endoplasmic reticulum (ER). Farnesylation alone is not sufficient to anchor Ras proteins to the ER and a second lipid modification is required. Thus, farnesylated Ras proteins are then (1) palmitoylated with a palmitoyl group (yellow) by the putative protein acyltransferase (PAT) in the ER. This results in (2) dually-acylated Ras proteins which are either anchored at the ER membrane, or are (3) trafficked to the Golgi. From the Golgi, dually-acylated Ras can then be (4) trafficked to the plasma membrane, where they are (5) depalmitoylated by the putative acyl-protein thioesterase (APT) and trafficked back to the Golgi. At the Golgi, the farnesylated Ras proteins can either be (6) trafficked back to the ER, or (7) re-palmitoylated by a putative PAT in the Golgi, to be once again delivered to the plasma membrane. This dynamic cycle of palmitoylation and depalmitoylation regulates the subcellular distribution of Ras proteins in mammalian cells.

As well as targeting proteins to specific intracellular membranes, palmitoylation is also used to regulate protein localisation between microdomains within a specific membrane [4]. Again, Ras family proteins are the most well-studied example, where H-Ras can be palmitoylated on one or both of two cysteine residues, Cys181 and Cys184. Monopalmitoylation on Cys184 mostly retains H-Ras at the Golgi, with some inefficient delivery to the plasma membrane. For the fraction that is delivered to the plasma membrane, monopalmitoylation on Cys184, as well as palmitoylation of both cysteine residues, promotes GTP-regulated lateral diffusion of H-Ras between cholesterol-dependent and cholesterol-independent microdomains. This means that GTP-loaded Cys184 monopalmitoylated Ras,

as well as GTP-loaded dually-palmitoylated Ras segregate to cholesterol-independent microdomains, while GDP-loaded Cys184 monopalmitoylated Ras and GDP-loaded dually-palmitoylated Ras segregate to cholesterol-dependent microdomains. Monopalmitoylation of H-Ras on Cys181 meanwhile directs H-Ras to the plasma membrane, where monopalmitoylation on Cys181 dramatically reverses the lateral segregation of Ras and promotes localisation of GTP-loaded Cys181 monopalmitoylated Ras into cholesterol-dependent microdomains [32]. Palmitoylation thus appears to also influence the fine-tuning of Ras localisation on cellular membranes.

In addition to soluble proteins such as Ras, many TM-domain-containing proteins, such as multi-TM-domain spanning receptors, are also palmitoylated, despite the fact that the presence of the TM domain is sufficient for membrane binding [1]. One of the reasons for the palmitoylation of TM-domain-containing proteins could again be the fine-tuning of membrane localisation. For example, CD4 and CD8 -the co-receptors of the T cell receptor (TCR) complex- are palmitoylated, and palmitoylation is required for the localisation of these co-receptors into lipid rafts, which in turn promotes lipid raft aggregation and is important for TCR-mediated downstream signalling events [33-35]. Another example of the use of palmitoylation by receptor proteins is the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor, which is palmitoylated on different cysteines in its second TM domain and also on cysteines in its C-terminal domain. Palmitoylation of these cysteines applies multiple levels of regulation on the localisation of the receptor. For example, palmitoylation of cysteines in the second TM domain causes accumulation of the AMPA receptor at the Golgi, and palmitoylation of cysteines in the C-terminal domain promotes regulated endocytosis of the AMPA receptors, removing the AMPA receptors from the cell surface [36]. In addition to that, the dynamic palmitoylation of the post synaptic density protein, PSD-95, controls the synaptic clustering of PSD-95 and this in turn controls the recruitment of AMPA receptors to the post synaptic density. This then regulates the cycling of AMPA receptors on and off the synaptic membrane and influences synaptic plasticity and strength [37].

Finally, palmitoylation can also regulate protein-protein interactions and protein stability. An example of this function of palmitoylation is demonstrated by the yeast t-SNARE, Tlg1. This single TM-domain-containing protein is palmitoylated at a cysteine residue close to its TM-domain. Inhibition of palmitoylation results in the interaction of Tlg1 with Tul1 -an E3 ubiquitin ligase- which causes the ubiquitination of Tlg1, its targeting to the vacuole, and its subsequent degradation by the proteasome [4, 38, 39]. Another extreme and well-known example of the effect of palmitoylation on protein stability is its impact on the stability and aggregation of the huntingtin protein. The intracellular aggregation of the mutant form of huntingtin is a characteristic feature of Huntington's

disease, a debilitating neurological disorder [40]. Huntingtin is palmitoylated and the mutant form of huntingtin shows decreased palmitoylation. Inhibition of the palmitoylation of wild-type huntingtin promotes the accumulation of huntingtin protein aggregates and this is even more pronounced when palmitoylation of mutant huntingtin is inhibited. Conversely, protein aggregation is decreased when the palmitoylation of huntingtin is increased [40]. Thus, in this case, palmitoylation appears to play a protective role by regulating protein aggregation, and may play a direct role in the pathogenesis of Huntington's disease.

Reversible S-palmitoylation can therefore be used as a protein regulatory tool through a variety of mechanisms, including specifying locations within or between membrane-bound organelles, and regulating protein-protein interactions and protein stability. Although N-palmitoylation is an irreversible process, this form of palmitoylation has also been found to have an important influence on protein activity, particularly that of secreted signalling ligands such as Hh and Wnt proteins [1]. The Hh and Wnt families of proteins are required for pattern formation during embryonic development. These proteins act as morphogens, forming the signalling gradients which specify growth patterning, proliferation and differentiation during the developmental process [17]. Both these families of proteins are N-palmitoylated on specific cysteine residues and the inhibition or removal of N-palmitoylation results in defective pattern formation and reduced signalling activity [1, 20, 41]. N-palmitoylation is thus thought to regulate the distribution and localisation, as well as the signalling of these secreted proteins, which in turn affect the protein gradients and pattern formation controlled by these proteins.

1.5. Types of palmitoylated proteins

From the examples described above, it is clear that palmitoylation is more than merely a membrane anchor and in fact appears to have several important regulatory functions within the cell. However, predicting which proteins might be regulated in this manner is not straightforward because there is no clear consensus sequence for palmitoylation, other than the requirement for a cysteine residue [3]. However, palmitoyl-proteins have been grouped into several classes based on the location of known palmitoylated cysteine residues and on the context of the sequences surrounding the cysteine residues [3, 8]. Examples of these classes of palmitoyl-proteins are shown in Table 1.1.

Class of palmitoyl-protein	Protein	Sequence
Type I: Palmitoylated next to or within a transmembrane domain	CD4	.....QPMALIVLGGVAGLLLFIGLGIFFCVRCRHRRRQ.....
	CD36	MGCDRNCGLIAGAVIGAV.....VAFMISYCACRSKTIK
	SNAP-25	....LGKFCGLCVCPCNKLKSS....
	TGFα	.....SALLKGRTACCHSETVV
Type II: Prenylated and palmitoylated	H-Ras	.....PPDESGPGCMSCKCVLS
	N-Ras	.....SSDDGTQGCMGLPCVVM
	Paralemmin	.....DMKKHRCKCCSIM
Type III: Palmitoylated near the N- or C-terminal end	PSD-95	MDCLCIVTTKKYRYQDEDTP.....
	$G\alpha_s$ subunit	MGCLGNSKTEDQRNE.....
	GAP43	MLCCMRRTKQVEKNDDDQKIEQDGI.....
Type IV: Myristoylated and palmitoylated	Lyn kinase	MGCIKSKGKDSLSDDGVDLKT.....
	Lck kinase	MGCGCSSHPEDDWMENIDV.....
	eNOS	MGNLKSVAQEPGPPCGLGLGLGLGLCGK.....
	$G\alpha_{i1}$ subunit	MGCTLSAEDKAAVERS....

Table 1.1: Common classes of palmitoylated proteins. Examples of different types of palmitoylated proteins found in eukaryotic systems. The amino acid sequences surrounding the palmitoylated cysteines are shown and palmitoylated cysteines are highlighted in red.

Type I palmitoyl-proteins are TM-domain-containing proteins and are commonly palmitoylated on cysteine residues located next to or just within the TM-domain, where the length of the TM-domain, as well as the length of cytoplasmic tail, influences palmitoylation of the cysteine [28]. Members of this class of palmitoyl-proteins include SNARE proteins and the above mentioned CD4 co-receptor [28] (Table 1.1).

Type II palmitoyl-proteins consist of dually-acylated proteins which are both prenylated and palmitoylated [28]. Members of this group include the Ras superfamily of proteins. These proteins possess the recognition sequence for prenylation, the C-terminal CAAX box, where 'C' is the prenylated cysteine residue, 'A' refers to an aliphatic amino acid and 'X' represents the amino acid that determines which prenyl group is added, a farnesyl or a geranylgeranyl isoprenoid [42]. Palmitoylation of the prenylated protein then usually occurs on the cysteine residue immediately upstream of the prenylated cysteine [3]. Indeed, prenylation of Ras appears to be required for its subsequent palmitoylation [28] (Table 1.1).

Type III palmitoyl-proteins are proteins which are palmitoylated on cysteines near the C-terminal or N-terminal end of the protein [28]. Members of this group of palmitoylated proteins include G_α subunit proteins and the above mentioned PSD-95 protein [3] (Table 1.1).

Lastly, Type IV palmitoyl-proteins are also dually-acylated proteins, which are modified with myristate and palmitate. These proteins possess the N-myristoylation consensus sequence, Met-Gly-X-X-X-Ser/Thr, at the N-terminal end of the protein, where the glycine residue is the N-myristoylated residue. For most of myristoylated and palmitoylated proteins, the amino acid in position three of the N-myristoylation consensus sequence is a cysteine residue, and myristoylation of glycine in position two appears to facilitate the palmitoylation of the cysteine residue [28]. However, for some proteins, palmitoylation can also occur on cysteines up to 20 amino acids away from the N-terminus [3]. Members of this class of palmitoyl-proteins include the Src family of protein tyrosine kinases [28] (Table 1.1).

While splitting palmitoylated proteins into these basic classes is helpful, it obscures the fact that there are palmitoyl-proteins that do not fall into any of these groups. The true scale at which palmitoylation is used in the cell, and the number of palmitoyl-proteins that fall outside the classical groupings has become apparent through the recent development of two independent biochemical approaches for purifying the total complement of palmitoylated proteins.

1.6. Purification of palmitoylated proteins

Early methods of identifying palmitoylated proteins involved the use of metabolic labelling with tritiated palmitic acid, which was time-consuming and mainly focused on individual proteins rather than global, whole proteome analyses [14, 43, 44]. Recently, advances in proteome-based technologies to purify and identify all palmitoylated proteins in an organism have allowed the systematic characterisation of the whole palmitoylated proteome, or 'palmitome', in different organisms. Most of these studies revealed that the size of most palmitomes numbered in the hundreds [8, 14, 45-47]. The palmitoyl-protein enrichment methods can be divided into two main techniques. The first is based on a chemical-exchange process known as acyl-biotinyl exchange (ABE) chemistry [43] and the second involves metabolic labelling followed by 'click chemistry' [45].

1.6.1 Acyl-biotinyl exchange (ABE)

Acyl-biotinyl exchange (ABE) is based on the exchange of the palmitate group with a detectable label, such as biotin, and relies on the susceptibility of the thioester linkage to cleavage by neutral hydroxylamine [43]. ABE consists of three sequential chemical steps. In the first step, the extracted and solubilised proteome sample is treated with N-ethylmaleimide (NEM), a chemical which

irreversibly binds to free cysteine thiols, causing the irreversible blockage of existing free thiols in the sample. This is followed by treatment with hydroxylamine, which specifically cleaves the thioester bonds between the palmitoyl-groups and the cysteine residues, releasing the palmitoyl-groups and exposing the previously palmitoylated thiol groups. The resultant newly-exposed free thiols are then biotinylated by a thiol-reactive biotinylation reagent, such as HPDP-biotin, and the biotinylated palmitoyl-proteins can then be affinity-purified using streptavidin agarose [14, 43, 48]. An important control in this method of palmitoyl-protein purification is the mock treatment of an equal quantity of protein sample – that is, parallel treatment of a sample under identical conditions but in the absence of hydroxylamine. In this control sample, palmitoyl-groups are not removed and thus are not biotinylated or purified, and therefore, only contaminant proteins which may occur due to inappropriate biotinylation, non-specific streptavidin binding or the non-specific purification of highly abundant proteins are expected to be purified [48]. This control sample is essential in order to assist in determining false-positive identifications, and purification of palmitoyl-proteins are measured by their enrichment in the hydroxylamine-treated 'palmitome' sample over the non-hydroxylamine-treated 'control' sample [48]. As ABE is based on the cleavage of the thioester linkage by hydroxylamine, only S-acylated proteins are purified, and proteins that are modified by the irreversible N-palmitoylation will not be purified by this method. A schematic showing the chemical exchange steps of ABE is shown in Figure 1.4.

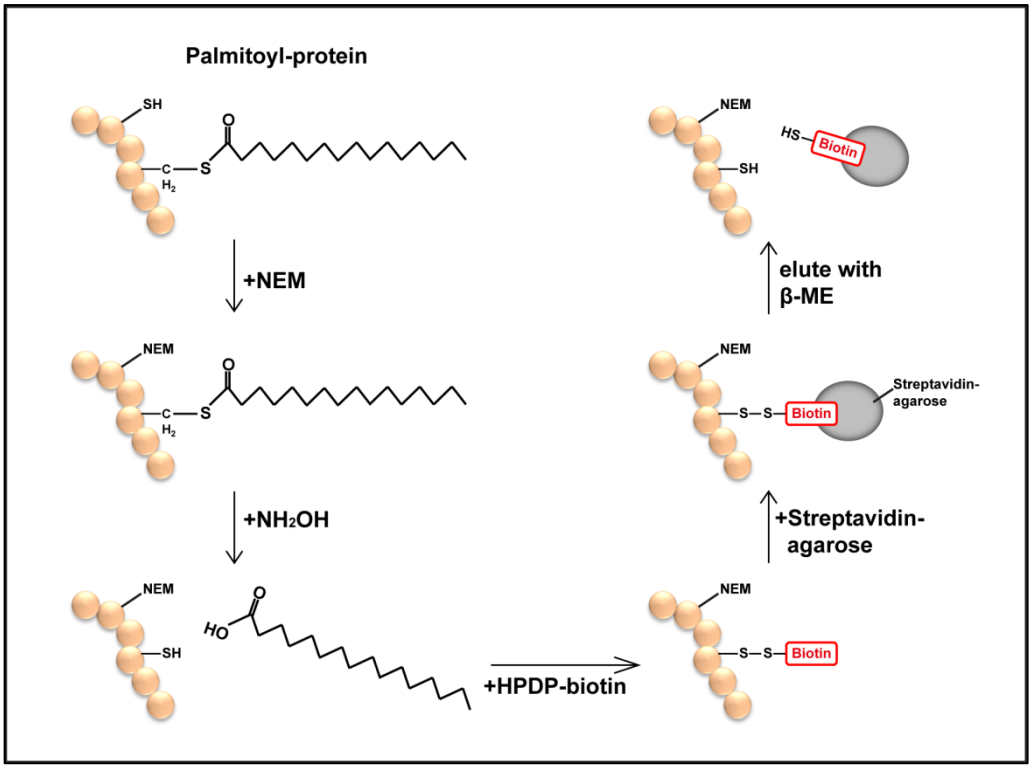

Figure 1.4: Schematic of the acyl-biotinyl exchange (ABE) method of purifying palmitoyl-proteins. This method relies on the exchange of palmitoyl groups with biotin. The extracted proteome is first treated with N-ethylmaleimide (NEM) which binds irreversibly to free thiols of unmodified cysteine residues. Palmitoyl groups are removed by treatment with hydroxylamine (NH_2OH), which cleaves the thioester linkage, resulting in the formation of newly-exposed free thiols. These free thiols can be biotinylated by a thiol-reactive biotinylation reagent, such as HPDP-biotin. The biotinylated proteins can then be purified by streptavidin-agarose affinity purification and eluted from the streptavidin-agarose beads by reduction with β-mercaptoethanol (β-ME). In the control sample, the protein extract is left untreated with hydroxylamine, the thioester linkages are thus not cleaved and palmitoyl groups are not exchanged with biotin. Proteins which are significantly enriched in the hydroxylamine-treated palmitome samples compared to the non-hydroxylamine-treated control samples can thus be classified as palmitoylated.

1.6.2 Metabolic labelling and click chemistry

This method involves the use of a palmitic acid analog that is incorporated into cellular proteins, then post protein extraction, can be conjugated to azide-linked labels using standard 'click chemistry' conditions for purification or detection [45]. Cells are first metabolically labelled with the palmitic acid analogue, 17-octadecynoic acid (17-ODYA), which contains an alkyne group. The proteome is then extracted from the metabolically labelled cells and reacted with biotin-azide under conditions conducive for the copper (I)-catalysed azide-alkyne cycloaddition (click chemistry) reaction. The click chemisty reaction essentially joins the azide and alkyne groups and thus results in the biotinylation of

the palmitoyl-proteins, which can then be affinity purified by streptavidin-agarose [8, 44, 45, 49]. As a control, cells are mock-labelled with the palmitic acid analogue and this control is important for the determination of false-positives and contaminant proteins [8, 45], just as the mock treatment control is critical in ABE purification. As this method is based on the metabolic incorporation of a palmitic acid analogue, both the thioester-linked S-palmitoylation and the irreversible N-palmitoylation can be purified by this method. A schematic showing the steps of this method of palmitoyl-protein purification is shown in Figure 1.5. Metabolic labelling with 17-ODYA followed by click chemistry methods can also be used in pulse-chase labelling experiments in order to study the dynamics of protein palmitoylation, by distinguishing between stably palmitoylated proteins and proteins which undergo rapid turnover of palmitoylation [44, 49]. Importantly, this metabolic labelling method can also be used for alternative purposes other than purification, simply by modifying the azide-linked label that is used in the click chemistry reaction. For example, replacement of biotin-azide with rhodamine-azide allows visualisation of all 17-ODYA-labelled proteins by in-gel fluorescence [45].

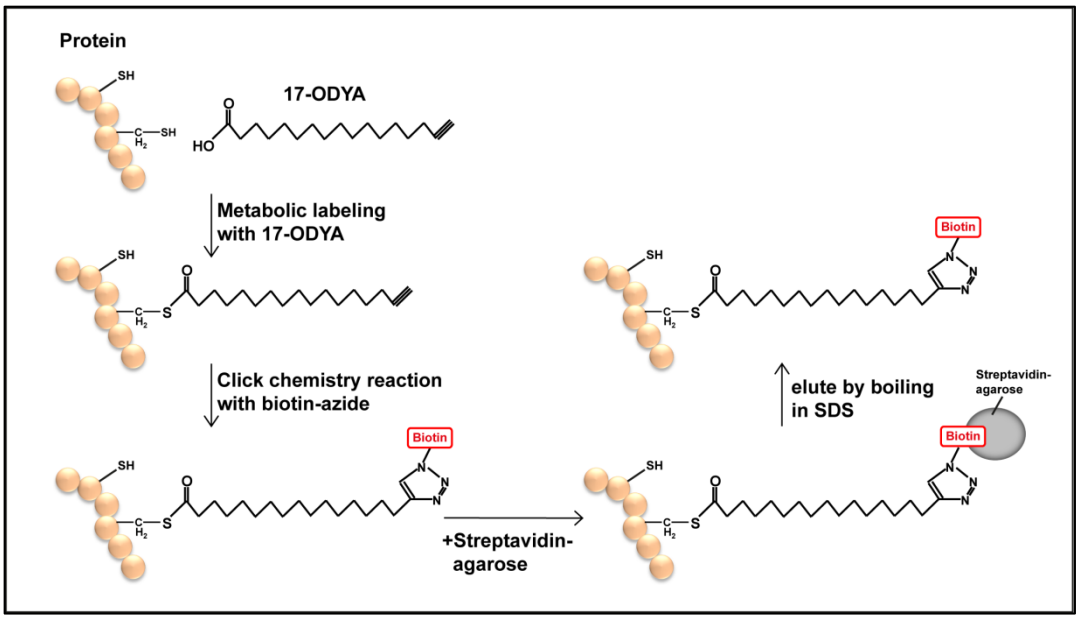

Figure 1.5: Schematic of the metabolic labelling and click chemistry method of purifying palmitoyl-proteins. This method relies on the *in vitro* metabolic labelling of proteins with a palmitic acid analog. Cells are metabolically labelled with the alkyne-containing palmitic acid analog, 17-octadecynoic acid (17-ODYA). Proteins are extracted from the cells and proteins labelled with 17-ODYA are reacted with biotin-azide under copper (I)-catalysed azide-alkyne cycloaddition (click chemistry) reaction conditions, resulting in the biotinylation of 17-ODYA-labelled proteins. The biotinylated proteins are purified by streptavidin-agarose affinity purification and eluted from the streptavidin-agarose beads by boiling in SDS. In the control sample, cells are mock-labelled by treatment with DMSO instead of 17-ODYA, and subjected to the same conditions as described above. Proteins which are significantly enriched in the 17-ODYA-treated samples compared to the mock DMSO-treated control samples can thus be classified as palmitoylated.

1.6.3. Comparison of palmitome purification methods

Both methods of palmitome purification described above bring with them their own set of false-positive identifications. ABE is based on the complete and irreversible blockage of free thiols with NEM in order to prevent the purification of non-palmitoylated free thiols. Insufficient blockage with NEM can thus give rise to false identifications. This can also result in the enrichment of highly abundant proteins whose free thiols may not have been completely blocked. Additionally, ABE will also enrich all other proteins which use thioester linkages, such as enzymes which use thioester intermediates as part of their reaction mechanisms [44, 50]. Meanwhile, metabolic labelling and click chemistry methods can result in the enrichment of proteins which have incorporated the 17-ODYA metabolic label, but are not in fact S-acylated. For example, glycosylphophotidylinositol (GPI)-anchored proteins can incorporate the 17-ODYA label although they are not palmitoylated [50].

Although false-positive identifications are reduced in click chemistry purification methods, this method relies on the ability of the proteome of interest to be labelled with 17-ODYA, as well as on the efficiency of the metabolic labelling. Despite the larger background found with ABE, all the chemical exchange reactions of ABE take place post extraction of the proteome and thus does not have to rely on the *in vitro* or *in vivo* metabolic incorporation of an exogenous label.

Thus, both methods of palmitome purification have their strengths and weaknesses, and datasets from both methods, as with any large-scale profiling method, will require careful quantitation, analysis and validation. Nevertheless, these methods have still resulted in the identification of several hundreds of potential palmitoylated proteins in multiple organisms and can provide new insight into the extent of protein palmitoylation in biology.

1.7. Malaria: A major global parasitic disease

Malaria, a disease caused by the mosquito-borne Apicomplexan parasite, *Plasmodium*, still remains a wide-spread global health problem. According to recent reports from the World Health Organisation (WHO), in 2010, there were about 219 million cases of malaria and an estimated 660,000 deaths due to malaria, with approximately 90% of all malaria deaths occurring in the African continent [51]. Although this disease has been eradicated in some parts of the world, more than two billion of the world's population still live in malarial regions and thus are at risk of contracting malaria [52]. Across the years, there have been several combined efforts working towards the global eradication of malaria. These campaigns, which include the use of more efficacious drugs and anti-vector measures such as insecticide-treated bed nets, along with economic development and improved health

infrastructure in some countries, have helped to reduce the burden of malaria, but whether these methods alone will be able to completely eradicate the disease is contentious [53, 54], and the on-going problem of *Plasmodium* drug resistance means that any gains that are being made currently are liable to erosion unless new drugs are continually developed.

1.8. The life cycle of *Plasmodium* parasites

Plasmodium parasites are members of the phylum Apicomplexa, which is characterised by the apical complex, a group of specialised organelles found at apical end of the invasive parasite stages, and the apicoplast, a rudimentary plastid descended from alga through secondary endosymbiosis [55]. Four species of *Plasmodium*, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, are found to cause malaria in humans and are transmitted by female *Anopheles* mosquitoes. Of the four, *P. falciparum* is the most virulent and deadly, causing the most severe forms of malaria and responsible for most of the disease morbidity and mortality [53, 54]. More recently, another species of *Plasmodium*, *P. knowlesi*, which normally infects macaque monkeys, has also been found to infect humans in some areas [56].

Plasmodium parasites are obligate intracellular parasites and thus spend most of their life cycle within the cells of their host, evading the immune system and feeding on the host cell. The life cycle of these parasites in both the mosquito vector and the vertebrate host is complex, involving the invasion of a variety of cell types and many transitions from one developmental stage to another at different points in the life cycle.

The life cycle begins when the human host is bitten by an infected *Anopheles* mosquito, resulting in the transmission of sporozoites, a motile form of the parasite, into the bloodsteam. The sporozoites migrate to the liver and invade hepatocytes, where they then develop into tens of thousands of merozoites. The merozoites then enter the bloodstream and rapidly invade erythrocytes, thus beginning the asexual intraerythrocytic stages. During the intraerythrocytic cycle, the parasites develop and replicate within the host erythrocytes, firstly from ring stages, to the highly metabolically active trophozoite stages, and lastly to the mature schizont stages where replication occurs to form daughter merozoites. Eventually, the infected erythrocytes burst, releasing the daughter merozoites into the bloodstream, where they are free to reinvade new erythroctyes. Each intraerythrocytic cycle takes approximately 48 hours and it is the synchronous rupture of infected erythrocytes every 48 hours which induces the periodic symptoms of malaria such as fever, chills and anaemia. Furthermore, a distinctive feature of *P. falciparum* is the ability of the intraerythrocytic

stages of the parasite to bind to endothelium and sequester in organs such as the brain, which can lead to the symptoms of cerebral malaria [53].

During the intraerythrocytic cycle, some parasites can differentiate into male and female sexual forms, known as gametocytes, in response to as yet unknown factors. These forms remain in the bloodstream until they are taken up by a mosquito during a blood meal. Once in the mosquito midgut, they transform into the activated male and female gametes and fertilization occurs, resulting in the production of the diploid ookinete. The motile ookinete crosses the mosquito midgut wall and forms an oocyst on the midgut basal lamina, where hundreds of haploid, genetically distinct sporozoites are formed. Rupture of the oocyst occurs, releasing the sporozoites, which migrate to the salivary glands and are transmitted to the human host during the next blood meal [53, 54]. A diagram adapted from [8], showing all the steps of the *Plasmodium* life cycle, in both the mosquito vector and the human host, is shown in Figure 1.6.

All of the steps taken by the parasite in order to complete its life cycle, even during just the intraerythrocytic cycle, from the invasion of erythrocytes [57, 58], to the remodelling of the infected erythrocyte to promote the survival of the parasite [59], and to the egress of the parasite in order to invade new erythrocytes [60], involve the use of many different proteins and multiple signalling cascades. This provides a potential source of targets which can be used for therapeutic interventions.

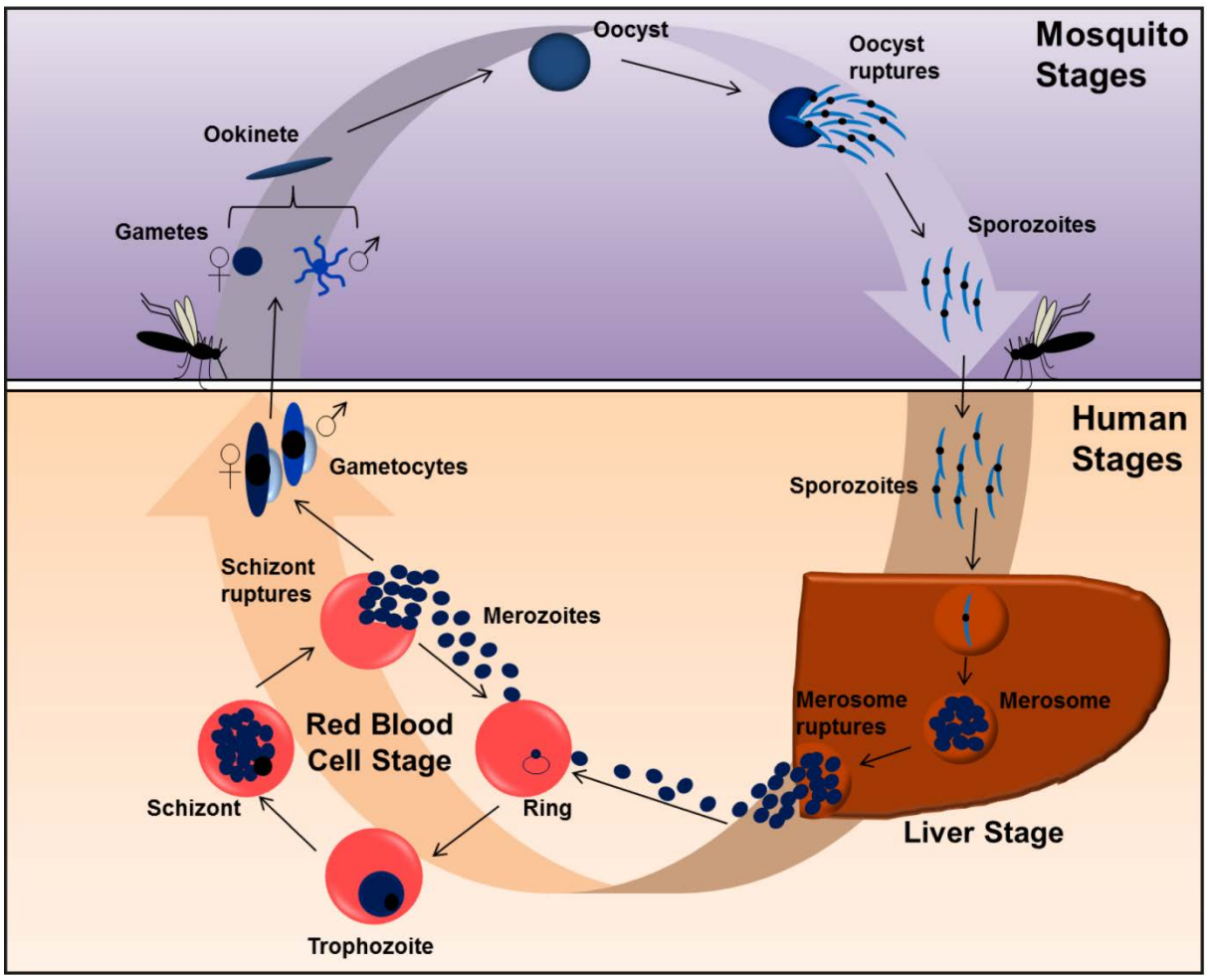

Figure 1.6: Schematic of the *Plasmodium* parasite's life cycle. The bite of an infected *Anopheles* mosquito transmits the motile sporozoite forms of the parasite into the host bloodstream. The sporozoites invade the hepatocyte cells of the liver, where they develop into tens of thousands of merozoites. The merozoites enter the bloodstream and invade erythrocytes. This is the beginning of the asexual intraerythrocytic stages of *Plasmodium*, where the parasites replicate and develop within host erythrocytes. The cycle begins at the ring stages, moves on to the highly metabolically active trophozoite stages, and ends at the mature schizont stages, where replication occurs to form daughter merozoites. The merozoites burst from the erythrocytes and re-enter the bloodstream, where they are free to invade new erythrocytes. During the intraerythrocytic stages, some parasites differentiate to form male and female gametocytes, the sexual forms of the parasites. The gametocytes are taken up by the mosquito during a blood-meal, and fertilisation occurs in the mosquito, producing the diploid ookinete. The ookinete crosses the mosquito midgut wall and forms the oocyst, where hundreds of haploid, genetically distinct sporozoites are formed. The sporozoites are released when the oocyst ruptures, and migrate to the mosquito salivary glands, where they can be transmitted to the human host during the next bloodmeal. This schematic was adapted from [8].

1.9. The need for new drug targets

There is currently no effective vaccine for any *Plasmodium* species, and all past efforts to control malaria have been based on the use of anti-malarial drugs for the prevention and treatment of infection, and insecticides for the elimination of the mosquito vector. However, these efforts have been repeatedly thwarted by the development of resistance of the parasite against the available anti-malarial drugs as well as resistance of the mosquito against insecticides [54, 61]. Drug resistance is one of the major problems facing malaria control. Over the past several decades, parasites have become resistant to most of the commonly used anti-malarial drugs, such as chloroquine, and the antifolate drugs such as sulfadoxine-pyrimethamine, both of which were once frontline therapies for malaria but are now largely ineffective due to widespread resistance [62]. Artemisinin derivatives and artemisinin-based combination therapies are now the standard frontline therapeutic interventions for malaria [51, 61]. However, sobering reports have now revealed that parasites with increased tolerance to these new drugs have emerged in South East Asia [51, 63] and with it, the fear that the continued widespread use of the artemisinin-based drugs could potentially result in the global spread of artemisinin resistance [63].

Furthermore, the lack of a vaccine against the malaria-causing parasites hinders efforts to completely eradicate malaria. Currently, the most promising candidate vaccine is RTS,S, which is based on the *P. falciparum* circumsporozoite protein and targets the pre-erythrocytic stages of infection. However, although the RTS,S vaccine has been shown to prevent infection and reduce the occurrence of severe malaria, this vaccine is still only approximately 50% efficacious [53, 54, 64]. There is therefore an urgent and on-going need for the identification of new drug targets, and the signalling pathways and proteins involved in regulating parasite growth and development through PTMs are one potential source of novel targets. Given that palmitoylation plays a role in regulating cellular processes in other eukaryotes, palmitoylation is a process that warrants investigating as a potential drug target in *Plasmodium* parasites.

1.10. Palmitoylation in *Plasmodium*

As detailed above, protein palmitoylation appears to play diverse and important roles in the regulation of protein localisation and function in many organisms, and is widely used in many cellular processes, as indicated by the large sizes of the palmitomes of the various organisms studied. It is gradually being discovered that this is also the case in *Plasmodium* parasites. Previously, only three proteins were experimentally shown to be palmitoylated in *P. falciparum*. Glideosome-associated

protein 45 (GAP45) and calcium-dependent protein kinase 1 (CDPK1) are components of the invasion motor complex, a multi-protein complex found at the inner membrane complex (IMC) of the parasite invasive stages, and thought to be required for the generation of the force required to actively propel the parasite into the host cell during invasion [57, 58, 65]. Calpains are cysteine proteases with well-conserved catalytic domains that are thought to be involved in various cellular processes such as cell signalling and migration, cell differentiation and development, and cell-cycle regulation, although the exact role of the calpains is still poorly characterised [66]. These three proteins -GAP45, CDPK1 and calpain- are dually-acylated in *P. falciparum*, modified by both a myristate and a palmitate group, and these acyl modifications appear to be important for the membrane attachment, targeting and localisation of these proteins [67-69].

Recently however, using both ABE and metabolic labelling followed by click chemistry methods, coupled with quantitative mass spectrometry, it was discovered that protein palmitoylation in *Plasmodium* is more extensive than previously known, with more than 400 putative palmitoyl-proteins found in the asexual intraerythrocytic schizont stages of *P. falciparum* [50]. Even more intriguing was the discovery that treatment of schizont-stage parasites with 2-bromopalmitate (2-BMP), a small molecule compound which inhibits palmitoylation [70], for 6 hours appears to have different effects on different palmitoyl-proteins. Some proteins are unaffected and remain palmitoylated, while other proteins appear to be either moderately or severely affected by 2-BMP treatment. These different effects induced by the inhibition of palmitoylation point to the existence of both stable as well as dynamic palmitoylation in *Plasmodium* [50].

The proteins identified in the *P. falciparum* palmitome include those which fall into the general types of palmitoyl-proteins described above, as well as proteins which are part of processes known to involve protein palmitoylation in other organisms [8, 50]. However, more interestingly, a significant number of proteins identified as palmitoylated are proteins which are involved in parasite specific processes such as invasion, drug resistance, cytoadherance and parasite development [50].

Parasite invasion of the host erythrocyte is one of the parasite specific processes which appear to involve palmitoylation. Invasion is a complicated process involving the recognition and binding of surface receptors on the erythrocyte, and an actin-myosin invasion motor complex, which generates the force required to push the parasite into the host erythrocyte [57, 58]. All the steps of the invasion process involve many different proteins, and bioinformatics analysis suggest that more than 400 proteins play a role in the invasion of parasites into erythrocytes [71]. Of these invasion-associated proteins, 74 are identified as palmitoylated [50]. The fact that so many of the invasion-linked proteins appear to be palmitoylated suggests that palmitoylation may be critically involved in

regulating the invasion process of the parasite. This suggestion is supported by the fact that inhibition of palmitoylation with 2-BMP significantly inhibits erythrocyte invasion [50]. In fact, palmitoylation appears to be directly involved in maintaining the stability of components of the invasion motor complex such as GAP45 and myosin A tail domain-interacting protein (MTIP), as treatment of parasites with 2-BMP results in the degradation of both GAP45 and MTIP [50]. Although exactly how protein palmitoylation regulates invasion is still unknown, it is clear that this PTM appears to be required for the normal function of some of the proteins responsible for parasite invasion.

Perhaps more surprising was the discovery that a fraction of schizont proteins identified as palmitoylated are parasite exported proteins [50]. Following invasion, several hundred parasite proteins are exported across the parasite plasma membrane to specific sites in the erythrocyte where they modify and remodel the erythrocyte in order to support parasite survival and development. This remodelling results in structural and morphological changes within the erythrocyte, such as increased rigidity and adhesiveness, and decreased deformability of the plasma membrane, which in turn leads to cytoadherence and sequestering of infected erythrocytes, a pathological characteristic of *P. falciparum* infection [59].

Some of these parasite exported proteins, including the major virulence factor, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), the protein responsible for anchoring PfEMP1 to the surface of the erythrocyte, knob-associated histidine-rich protein (KAHRP), and mature-parasite-infected erythrocyte surface antigen (MESA) were identified as palmitoylated [50]. All of the parasite exported proteins require trafficking across several membranes in order to move from the parasite to its site in the host erythrocyte. Even within the erythrocyte, the exported proteins are either retained in the Maurer's clefts, parasite organelles which are tethered to the erythrocyte membrane, or transported to the erythrocyte membrane itself. The fact that some of these exported proteins are palmitoylated raises the question as to whether palmitoylation is involved in regulating the trafficking and localisation of these proteins from the parasite to the erythrocyte.

This initial work suggests for the first time that palmitoylation plays an important role in regulating multiple aspects of *Plasmodium* biology. To further our understanding of *Plasmodium* palmitoylation, two critical questions need to be addressed. Firstly, where and when the *Plasmodium* PATs are expressed, as well as the substrates for individual PATs, are still unknown. Secondly, although a list of palmitoylated proteins in *Plasmodium* now exists, the exact sites which are palmitoylated are still not known. The work described in this dissertation was performed in an attempt to answer these questions.

1.11. Aims and objectives

The work described in this dissertation can be divided into two main aims as detailed below:

1.11.1. Aim 1: To determine the sites of palmitoylation in Plasmodium.

The *P. falciparum* palmitome has now been purified from intraerythrocytic schizont stages, resulting in the identification of over 400 putative palmitoyl-proteins [50]. However, the specific cysteines which are palmitoylated are still unknown for most *P. falciparum* palmitoyl-proteins and due to the lack of a consensus sequence for palmitoylation, are impossible to predict in the majority of cases. As is the case for all eukaryotes, while some proteins identified in the *P. falciparum* palmitome fall into the defined classes of palmitoyl-proteins described above where palmitoylation sites are predictable (for example, 44% of the palmitome are TM-domain-containing proteins, and 8 proteins identified in the total palmitome contain the N-myristoylation motif followed by a cysteine residue within 10 amino acids of the motif [50]), in the majority of cases, palmitoylation sites are impossible to predict. Information on the sites of palmitoylation could be used in the generation of testable hypotheses about the function of palmitoylation for a particular protein of interest, and would also provide critical information for subsequent studies of DHHC-PAT substrate specificity.

The first goal of this project was therefore to expand further on the analysis of the *P. falciparum* palmitome by developing methods to allow the identification of specific palmitoylated cysteines on individual palmitoyl-proteins. This was achieved by adapting the ABE methodology to purify palmitoylated peptides, rather than intact palmitoyl-proteins. As such peptides should by definition contain a palmitoylated cysteine residue, a palmitome-wide list of palmitoylated cysteines could be generated.

1.11.2. Aim 2: To investigate the molecular basis of palmitoylation by characterising the Plasmodium PATs.

Little is currently known about PATs in *Plasmodium*. A protein-protein BLAST search of the *P. falciparum* genome using the 50 amino acid DHHC cysteine-rich domain [5] reveals 12 potential DHHC-PATs, all of which share some homology with DHHC-PATs from other eukaryotes. These DHHC-PATs also have homologues in other *Plasmodium* species, although one of these proteins, PFB0140w, has no homologue in rodent *Plasmodium* species, which only have 11 DHHC-PATs. Some *P. falciparum* DHHC-PATs appear to be expressed constitutively throughout the life cycle stages, while other DHHC-PATs appear to be more regulated, with higher expression in particular life cycle stages

[8]. Only one of the *P. falciparum* DHHC-PATs (PFC0160w) has been characterised, and is found to be localised to the Golgi [72], one of the common localisations of DHHC proteins in other organisms. The localisation of the other DHHC-domain-containing proteins in *Plasmodium* is currently unknown, and whether they actually act as PATs in *Plasmodium* has never been formally tested.

In contrast to the DHHC proteins, only one protein is identified by protein-protein BLAST search as a potential MBOAT-protein in *P. falciparum.* However, this protein (PFC0995c) has been characterised to act as a diacylglycerol O-acyltransferase [73], an activity common to MBOAT family proteins in other organisms [15]. It is unknown whether this protein also acts as an MBOAT-PAT in *Plasmodium*.

Given this significant gap in our understanding of palmitoylation in *Plasmodium*, the second aim of this project was to characterise for the first time the *Plasmodium* DHHC and MBOAT proteins. This was achieved by determining the different localisations of as many of these proteins as possible in the parasite, as well as by determining the effect of deleting DHHC-PAT encoding genes on parasite growth and development. In addition, in order to formally prove that *Plasmodium* DHHC and MBOAT proteins have palmitoyl transferase activity, a novel PAT activity assay was developed using a mammalian cell expression system. This assay was used to establish enzymatic activity, and to carry out investigations into the substrate-specificity of individual *P. falciparum* DHHC-PATs.

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