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The s48/45 six-cysteine proteins: mediators of interaction throughout the *Plasmodium* life cycle

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Abstract

During their life cycle *Plasmodium* parasites rely upon an arsenal of proteins that establish key interactions with the host or vector, and between the parasite sexual stages, with the purpose of ensuring infection, reproduction and proliferation. Among these is a group of secreted or membrane-anchored proteins known as the six-cysteine (6-cys) family. This is a small but important family with only 14 members thus far identified, each stage-specifically expressed during the parasite life cycle. 6-cys proteins often localize at the parasite surface or interface with the host and are conserved in different *Plasmodium* species. The unifying feature of the family is the s48/45 domain, presumably involved in adhesion and structurally related to Ephrins, the ligands of Eph receptors. The most prominent s48/45 members are currently under functional investigation and are being pursued as vaccine candidates. In this review, we examine what is known about the 6-cys family, their structure and function, and discuss future research directions.

Graphical Abstract

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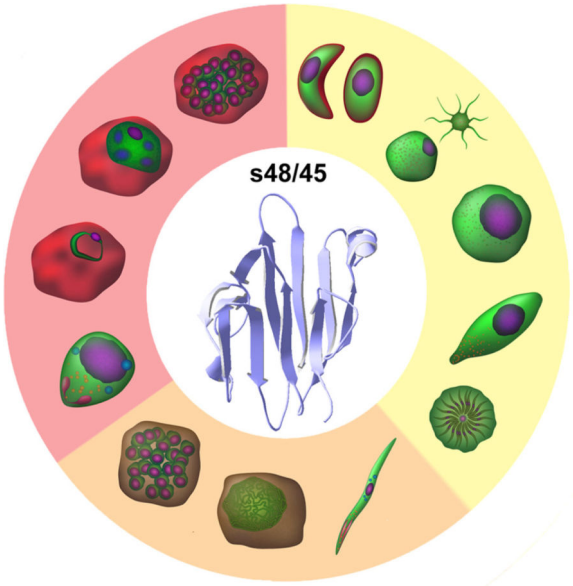

Keywords

Malaria; *Plasmodium*; s48/45; 6-cys proteins; Adhesion proteins; Host-pathogen interactions; Malaria vaccine

1. Introduction

Malaria affects millions of people throughout the world and, according to the World Health Organization (WHO), nearly half a million lives were lost to malaria in 2015 (WHO, 2015). This life-threatening disease is caused mainly by *Plasmodium falciparum* and *Plasmodium vivax* parasites. The infection cycle of *Plasmodium* is complex, with multiple stages of development occurring in a vertebrate host and an anopheline mosquito vector (Fig. 1A). *Plasmodium* parasites possess multiple families of adhesion proteins, which have evolved either with the apicomplexan parasite lineages since their descent from free living ancestors or which were acquired by the parasite from an ancient host through horizontal gene transfer. Many of these proteins mediate key interactions by the parasites' extracellular invasive stages and intracellular replicative stages either with host cells, for infection and proliferation or between sexual stages of the parasite for mating. These types of critical interactions seem to be among the functions of the members of the six-cysteine (6-cys) protein family, which in *P. falciparum* has 14 members expressed in different stages throughout the parasite cycle. These are: Pfs230, Pfs48/45, Pfs230p, Pfs47 and PfPSOP12 expressed in the sexual stages; Pf52, Pf36, PfLISP2 and PfB9 in the pre-erythrocytic stages; and Pf12, Pf12p, Pf41, Pf38, and Pf92 in the asexual erythrocytic stages (Fig. 1A, Table 1). 6-cys proteins are often expressed on the surface of the parasite and are conserved across *Plasmodium* spp. with most of the members having orthologues in human, non-human primate and rodent malaria parasites (PlamoDB (Aurrecoechea et al., 2009), attesting to the universal importance of the 6-cys family in survival and propagation of *Plasmodium*. The common feature of the family is the s48/45 domain, a structural element with six cysteines

that form disulfide bonds, which can be found as multiple modules in most of the family members (Carter et al., 1995; Gerloff et al., 2005; Arredondo et al., 2012).

2. The s48/45 domain

Plasmodium parasites have co-evolved with their vertebrate hosts for more than 150 million years (Carter and Mendis, 2002), during which time they acquired protein domains from their hosts by horizontal gene transfer (Aravind et al., 2003). In general, these domains seem to derive more often from proteins with extracellular interaction functions, implying that they were acquired by *Plasmodium* particularly due to their adhesion capabilities (Templeton et al., 2004). Some examples of adhesion domains that are known to have originated from animal hosts and transferred into apicomplexans are Sushi, Notch, Fibronectin-type II and Thrombospondin-1 (TSP-1) (Anantharaman et al., 2007); the latter are found in essential proteins such as Thrombospondin-related anonymous protein (TRAP), where the TSP-1 domain is critical for cell invasion, and circumsporozoite protein (CSP), where the domain also appears to have an important role in hepatocyte invasion (Menard, 2001; Tucker, 2004; Plassmeyer et al., 2009). More recently, the s48/45 domain has been proposed to derive from a vertebrate host protein domain involved in cell-cell interactions, an ephrin-like precursor that was probably acquired by a common ancestor of the coccidians and aconoidasidans and that eventually evolved into the SAG1-Related Sequence (SRS) and s48/45 structural domains in *Toxoplasma* and *Plasmodium*, respectively (Fig. 2A) (Arredondo et al., 2012). Initially, the s48/45 domain was believed to exist only in *Plasmodium*, however, although structural data is only currently available for *Plasmodium* proteins, the domain is reportedly present in all members of the Aconoidasida which includes haemosporidians and piroplasms (Fig. 2A) (Arredondo et al., 2012; Punta et al., 2012). In the *Plasmodium* genome the s48/45 domain expanded before speciation, resulting in the 6-cys family with distinct proteins that are conserved across species and have important roles in the different life cycle stages of the parasite, containing anywhere from one to 14 s48/45 domains (Fig. 2B). Gene duplication also played a part in the expansion of the s48/45 domain as some members of the family are found in tandem-arranged pairs (Templeton and Kaslow, 1999; Thompson et al., 2001). Nevertheless, only 17–36% overall amino acid sequence identity is observed among proteins.

The conserved cysteine motifs of the s48/45 domain that gave its name to the 6-cys family of proteins in *Plasmodium* was first identified in 1993 by Williamson et al. (1993) while studying Pfs230. The authors also noticed that the pattern was present in another protein, Pf12. As more *P. falciparum* genes were sequenced, other proteins were found to contain similar cysteine motifs and the family was established (Carter et al., 1995; Templeton and Kaslow, 1999; Thompson et al., 2001). A more detailed analysis of the arrangement of the characteristic cysteines gave rise to a prediction of disulfide bond connectivity that also proposed folding into structurally similar discrete domains (Carter et al., 1995), and eventually led to the accurate computational structural modeling of double 6-cys domains (types A and B) defined as PGSH (*Plasmodium* gamete surface homology) fragments (Gerloff et al., 2005; Gerloff, D.L., Draizen, E., 2016. 6-Cys Domain Model Database. Foundation for Applied Molecular Evolution (FfAME), UC Santa Cruz Biomolecular Engineering, USA and University of Edinburgh, Scotland. http://pgsh.soe.ucsc.edu/).

The importance of the formation of disulfide bonds for the proper folding and conformation of the 6-cys proteins was evident early on, as most of the monoclonal antibodies (mAbs) raised against native Pfs230 and Pfs48/45 only recognized the non-reduced proteins (Quakyi et al., 1987; Carter et al., 1990, 1995; Read et al., 1994). However, due to the many challenges presented by the expression of natively folded 6-cys proteins in heterologous expression systems throughout the years, the experimentally determined molecular conformation of the 6-cys domain was not obtained until recently with the solution nuclear magnetic resonance (NMR) structure of the C-terminal domain of Pf12, shown in Fig. 2A (Arredondo et al., 2012). The molecular structure of the first s48/45 domain, as termed by the Pfam Protein Families Database (Punta et al., 2012), confirmed the original predictions in terms of disulfide connectivity, and the similarity of its structure to the SRS domain from *Toxoplasma* (Fig. 2A) (He et al., 2002; Gerloff et al., 2005). The SRS superfamily comprises a group of more than 180 genes found in different stages of the life cycle of *Toxoplasma* that also tend to arrange in tandem and share the structural SRS domain (Jung et al., 2004; Wasmuth et al., 2012). The SRS proteins that have been characterized to date show that these are glycosylphosphatidylinositol (GPI)-anchored surface proteins with important functions in cell adhesion, immune evasion or virulence attenuation (Dzierszinski et al., 2000; Jacquet et al., 2001; Lekutis et al., 2001; Jung et al., 2004; Radke et al., 2004; Crawford et al., 2010; Wasmuth et al., 2012).

The s48/45 domain is characterized by a β-sandwich formed by a mix of parallel and antiparallel β-sheets (Fig. 2A). The domain is held together by two disulfide bonds resulting from the pairing of Cys1-Cys2 and Cys3-Cys6, and there is a third disulfide bond (Cys4-Cys5) outside the core of the domain as originally predicted (Carter et al., 1995; Gerloff et al., 2005). The Cys3-Cys6 disulfide bond is very well conserved compared with the structures of the SRS domains and it is suggested to be essential for the structural stability and proper conformation of the domain (Arredondo et al., 2012). In the early studies of the 6-cys family, some of the proteins were described to have “degenerate” domains containing only two or four cysteines in addition to the canonical 6-cys domains (Templeton and Kaslow, 1999). More recently, new members of the family were identified using a consensus 4-cys minimal motif (Ø×Ø×C×$_n$C[F,S,T] ×$_n$[F,I,L]×C×C; Ø = hydrophobic residue) which was assembled based on the MEME analysis of previously established 6-cys domains (Annoura et al., 2014). This is not unexpected since some of the SRS proteins also have only four cysteines in their domains and it is possible that, in some cases, only one disulfide bond might be enough to hold the β-sandwich in the necessary conformation to maintain function. In addition to the presence of at least one consensus motif containing the positionally conserved cysteines, the prediction to fold as an s48/45 domain and the prediction of a signal peptide were also constraints for inclusion of these proteins in the family.

To date, only the molecular structures for Pf12 and Pf41 have been solved and, as anticipated, each domain of these proteins superimposes well with the original s48/45 structure (Arredondo et al., 2012; Tonkin et al., 2013; Parker et al., 2015). However, outside the main core of the domains, interesting flexible features are observed as seen by NMR in the C-terminus of Pf12, which might also be the case for an insertion domain in Pf41 that is necessary for binding to Pf12 (Arredondo et al., 2012; Parker et al., 2015). Unfortunately, no function has been assigned to Pf12 and Pf41. Therefore, structure-function associations

relevant to parasite biology cannot be derived yet. In the meantime, molecular models based on the solved structures, are available online for a subset of the other s48/45 proteins (http://pgsh.soe.ucsc.edu/).

3. 6-cys proteins in the sexual stages

The *Plasmodium* life cycle has an obligate sexual reproduction stage that begins with the commitment to produce merozoites which, after invasion and following five stages of development (I-V), become mature male or female gametocytes (reviewed in Josling and Llinas (2015)). When stage V gametocytes are ingested by a mosquito taking a blood meal, they activate in the midgut and emerge from the erythrocyte; males exflagellate forming microgametes and fertilize female gametes to produce zygotes which then transform into motile ookinetes (Fig. 1A) (reviewed in Kuehn and Pradel (2010);Bennink et al. (2016). During traversal through the midgut epithelial cells, ookinetes activate the mosquito immune response through the JNK pathway and some are “tagged” for complement lysis and perish as they come into contact with the hemolymph (Han and Barillas-Mury, 2002; Garver et al., 2013). Surviving ookinetes, however, transform into oocysts which eventually produce thousands of sporozoites. In this section, we will discuss the 6-cys proteins found in the sexual stages of the parasite.

3.1. P230

Pfs230 is the founding member of the 6-Cys family (Williamson et al., 1993). It was originally identified by immunoprecipitation of radiolabeled gametes using transmission-blocking mouse mAbs obtained from immunizations with *P. falciparum*-purified gametes. It was named according to its molecular weight in SDS-PAGE under non-reducing conditions (Rener et al., 1983; Vermeulen et al., 1985a). Pfs230 has been extensively studied for more than 30 years due to the early recognition of its potential as a transmission-blocking vaccine antigen (Quakyi et al., 1987). For a more extensive review on earlier research on this protein alone see Williamson (2003).

There are 14 cysteine motif domains in Pfs230, some of which have four or five cysteines per domain instead of six, but they are all predicted and modeled to fold as s48/45 domains (Fig. 2B). The s48/45 domains are preceded by a glutamate-rich region and a predicted signal sequence with no GPI anchor predicted on the C-terminus (Carter et al., 1995; Gerloff et al., 2005; http://pgsh.soe.ucsc.edu/). Polymorphisms have been reported for the Pfs230 gene and it is suggested to be under positive selection (Williamson and Kaslow, 1993; Niederwieser et al., 2001; Tonkin et al., 2013). The original immunoprecipitation studies in combination with many other transcriptional, proteomic and immunofluorescence studies, agree that P230 is expressed only in male and female sexual stages starting at stage II gametocytes and continuing until fertilization is complete (Rener et al., 1983; Vermeulen et al., 1985a; Williamson et al., 1993, 1995; Florens et al., 2002; Le Roch et al., 2003; Khan et al., 2005; Eksi et al., 2006; van Schaijk et al., 2006; Pradel, 2007; Silvestrini et al., 2010; van Dijk et al., 2010). In addition, Pfs230 was confirmed to be on the plasma membrane of gametocytes and gametes by immune electron microscopy (Williamson et al., 1996). Pfs230 was also found localized on the recently described membranous protrusions on activated

gametocytes that are hypothesized to mediate their interactions (Rupp et al., 2011). As mentioned above, P230 does not have a GPI-anchoring signal, however, early studies determined that P230 forms a complex with P48/45 which is predicted to be GPI-anchored (Kumar, 1987; Kumar and Wizel, 1992); and deletion experiments showed that, in ΔPfs48/45 gametes, Pfs230 is expressed but it is not associated with the gamete surface after emergence from the red blood cell (Eksi et al., 2006) implying that P230 localizes on the membrane through its interaction with P48/45 (Fig. 1B). During gamete emergence, P230 undergoes two independent proteolytic cleavage events at the N-terminus, at sites N-terminal to the first s48/45 domain, resulting in two processed versions of the protein (300 and 307 kDa) that remain associated with the gamete (Williamson et al., 1996; Brooks and Williamson, 2000).

Disruption of P230 shed light on the crucial role of this protein in fertilization. ΔP230 parasites have a defect in male fertility that leads to significantly reduced mosquito infection and, consequently, reduced oocyst production (Eksi et al., 2006; van Dijk et al., 2010). Furthermore, in *P. falciparum*, exflagellating males are unable to bind to erythrocytes to establish exflagellation centers, which are important for fertilization (Eksi et al., 2006) and, in *Plasmodium berghei*, ΔP230 male gametes do not recognize female gametes, whereas ΔP230 female gametes have no apparent phenotype (van Dijk et al., 2010). Interestingly, the deletion of either P230 or P48/45 in *P. berghei* female gametes increased their hybridization with *Plasmodium yoelii* male gametes (Ramiro et al., 2015).

Recently, Pfs230 together with Pfs48/45 have been shown to be part of a multimeric protein complex that includes the PfCCp family members and Pfs25 (Fig. 1B), all with roles in parasite sexual reproduction and development in the mosquito (Tomas et al., 2001; Pradel et al., 2004; Scholz et al., 2008; Simon et al., 2009, 2016). Thus far, this is the only report of s48/45 proteins as part of a larger complex. A series of co-immunoprecipitation experiments showed that, in nonactivated gametocytes, Pfs230 already interacts directly with Pfs48/45 and PfCCp4 (which is in complex with other PfCCp proteins). Following gametocyte activation, Pfs230 also establishes an interaction with the other PfCCp proteins stabilizing the complex on the membrane. Absence of Pfs230 or inhibition of its proteolytic cleavage results in degradation of PfCCp proteins (Simon et al., 2016). A different study also reports the interaction of Pfs230 with PfWL1, a newly identified WD40-repeat protein that might also be part of the PfCCp complex (von Bohl et al., 2015).

It is well established that both Pfs230 and Pfs48/45 are recognized by antibodies from naturally infected individuals from malaria-endemic regions (Vermeulen et al., 1985a; Graves et al., 1988; Carter et al., 1990; Targett, 1990; Roeffen et al., 1996; Healer et al., 1999; Drakeley et al., 2004; Bousema et al., 2010; Ouedraogo et al., 2011; Stone et al., 2016;), and the response to these antigens seems to be boosted after subsequent natural infections (Skinner et al., 2015). In addition, high antibody titers are correlated with transmission reduction (Stone et al., 2016). From the beginning, Pfs230 was defined as a transmission-blocking vaccine candidate since antibodies against this protein act in a complement-dependent manner to lyse the parasite and prevent further development in the mosquito midgut (Quakyi et al., 1987; Read et al., 1994; Roeffen et al., 1995; Healer et al., 1997; Bustamante et al., 2000). The minimal effective antigenic region of Pfs230 was

originally mapped to amino acids 443–1132 referred to as region C, which contains 13 cysteines (Bustamante, 2000). These cysteines need to be properly paired for the epitopes to attain native conformation and elicit full transmission-blocking immunity, making P230 a challenging protein to produce in heterologous expression systems (Riley et al., 1995; Williamson et al., 1995; Vincent et al., 1999; Bustamante et al., 2000; Williamson, 2003; Farrance et al., 2011; Tachibana et al., 2011; Kapulu et al., 2015). However, more recently, the prodomain N-terminal to the first s48/45 domain alone was reported to be sufficient to induce transmission-blocking antibodies (Tachibana et al., 2011). As of 2015, Pfs230 has entered clinical trials in the US as an EPA (*Pseudomonas aeruginosa* exoprotein A) fusion in combination of Pfs25-EPA (Draper et al., 2015).

The *P. vivax* orthologue Pvs230 has also been evaluated as a transmission-blocking candidate. It was reported to have a similar expression and localization pattern on gametes as Pfs230, and antibody raised against a region comparable with Pfs230 region C significantly reduced oocyst numbers in the mosquito midgut (Tachibana et al., 2011). The analysis of 113 Pvs230 sequences from around the world showed that it also is under purifying selection with very low nucleotide diversity (Doi et al., 2011; Tachibana et al., 2011).

3.2. P48/45

Pfs48/45 was identified in a search for gametocyte antigens that are recognized by antibodies capable of blocking parasites within the mosquito midgut, and it was named Pfs48/45 because it migrated as two bands of 48 and 45 kDa in SDS-PAGE under non-reducing conditions (Rener et al., 1983; Vermeulen et al., 1985a). Pfs230 and Pfs48/45 were initially co-eluted with these transmission-blocking mAbs, and thus the two proteins were characterized in parallel in the early experiments and were soon found to interact with each other (Kumar, 1987; Kumar and Wizel, 1992). P48/45 joined the 6-cys family as the first prediction of disulfide connectivity for the s48/45 domain was made; it has three s48/45 domains, with four, five and six cysteines, respectively (Carter et al., 1995; Gerloff et al., 2005), and it is predicted to have a signal peptide and a GPI anchor (Fig. 2B) (Kocken et al., 1993). P48/45 RNA and protein expression profiles are similar to P230 with expression in gametocytes and gametes (Rener et al., 1983; Vermeulen et al., 1985a; Kocken et al., 1993; van Dijk et al., 2001, 2010; Florens et al., 2002; Le Roch et al., 2003; Khan et al., 2005; van Schaijk et al., 2006; Pradel, 2007; Silvestrini et al., 2010) and its subcellular localization mirrors Pfs230 (van Dijk et al., 2001; Eksi et al., 2006; Rupp et al., 2011). Gene deletion in *P. falciparum* and *P. berghei* and cross-fertilization experiments in rodent species showed an essential role for P48/45 in male, but not female, gametes during fertilization, with production of ookinetes drastically reduced. Motile PbΔP48/45 male gametes were able to adhere to erythrocytes to form exflagellation centers but were unable to attach to females and fertilize, as reported for PbΔ230 (van Dijk et al., 2001, 2010; Ramiro et al., 2015). It is not clear whether the observed phenotype in ΔP48/45 mutants results from the loss of a direct role of P48/45 itself or from the lack of P230 on the plasma membrane (Eksi et al., 2006). Besides P48/45 being part of the PfCCp protein complex mentioned in Section 3.1, due to its interaction with P230 (Simon et al., 2016), no other direct protein interaction has been reported for this protein to date. There is evidence that Pfs48/45 is under positive

selection, and extreme fixation of variation in this protein has been reported (Escalante et al., 1998; Conway et al., 2001; Escalante et al., 2002; Anthony et al., 2007; Tonkin et al., 2013).

Similar to Pfs230, Pfs48/45 is a transmission-blocking vaccine candidate and correct epitope conformation is also very important for eliciting effective blocking antibodies, however, protection is not complement-dependent (Vermeulen et al., 1985b; Carter et al., 1990; Targett, 1990; Roeffen et al., 1996; Healer et al., 1997; Milek et al., 1998b; Merino et al., 2016). The best epitope for blocking transmission was originally mapped to the third s48/45 domain at the C-terminus, however more recent constructs include different regions or even the full-length protein (Carter et al., 1990; Outchkourov et al., 2007, 2008; Chowdhury et al., 2009; Kapulu et al., 2015; Singh et al., 2015). Despite multiple challenges related to protein expression through the years (Milek et al., 1998a, 2000; Outchkourov et al., 2008; Chowdhury et al., 2009; Mamedov et al., 2012; Jones et al., 2013), a fusion of the C-terminus of Pfs48/45 with the N-terminus of *P. falciparum* glutamate-rich protein (PfGLURP) is currently a vaccine candidate under clinical development (Theisen et al., 2014; Draper et al., 2015; Singh et al., 2015).

Pvs48/45 is also under investigation as a transmission-blocking vaccine candidate. As for Pfs48/45, the *P. vivax* orthologue was found on the surface of gametes (Tachibana et al., 2015) and two independent groups reported significant transmission-blocking activity for antibodies raised against Pvs48/45 recombinant protein (Arevalo-Herrera et al., 2015; Tachibana et al., 2015). Population analyses report very little sequence diversity for Pvs48/45 in several parasite populations around the world (Woo et al., 2013; Feng et al., 2015; Tachibana et al., 2015; Vallejo et al., 2016).

3.3. P230p

Pfs230p, also known as PfMR5 (male-related, stage 5 antigen), was first identified as a paralog of Pfs230 when the *P. falciparum* chromosome 2 sequence became available (Gardner et al., 1998) and was predicted to have a signal peptide and five 6-cys domains in addition to some intervening “degenerate” domains with two or four cysteines (Templeton and Kaslow, 1999). Structural models indicate that P230p has 12 s48/45 domains, five of which have six cysteines with all others having two, three or four cysteines (Fig. 2B) (Gerloff et al., 2005; http://pgsh.soe.ucsc.edu/). A GPI anchor was not predicted for P230p. Transcripts were reported in *P. berghei* and *P. falciparum* in gametocytes only (Thompson et al., 2001; Le Roch et al., 2003; Hall et al., 2005; van Dijk et al., 2010; Schneider et al., 2015), particularly in stages II-V, and were absent in gametes (Eksi and Williamson, 2002). Proteomic studies, western blot analysis and a GFP reporter study indicated that P230p expresses only in male gametocytes stages IV-V (Eksi and Williamson, 2002; Khan et al., 2005; Eksi et al., 2008; Silvestrini et al., 2010). Immunofluorescence data showed Pfs230p with a cytoplasmic distribution within the gametocyte (Eksi and Williamson, 2002).

The function of P230p has not yet been described, however a role in rapid DNA replication or cytoskeletal reorganization necessary for exflagellation has been hypothesized (Williamson, 2003). Deletion mutants in *P. berghei* and *P. yoelii* have shown no effect on the parasite life cycle and in fact the P230p locus has even been used as a reporter insertion site (Janse et al., 2006; van Dijk et al., 2010; Lin et al., 2011; Hart et al., 2014). Functional

redundancy with Pfs230 has been ruled out since the expression pattern of these proteins is different and no compensating upregulation was observed for Pfs230p in a ΔPfs230 mutant (Eksi and Williamson, 2002; Eksi et al., 2006). Interestingly, P230p is the only member of the family, among those with known subcellular localization, which is not found on the surface or in an invasion-related organelle.

3.4. P47

P47, the paralog of P48/45, was originally identified in a BLAST search based on 6-cys domains from previously known members of the family and it was determined to have two domains with six cysteines separated by a linker region, as well as a predicted signal sequence and GPI anchor (Templeton and Kaslow, 1999). The linker region is actually a "degenerate" s48/45 domain with only two cysteines (Fig. 2B) (Gerloff et al., 2005; http://pgsh.soe.ucsc.edu/). Multiple transcriptional and proteomic experiments showed P47 to be expressed only in female gametocytes, starting at stage II, and on female gametes following emergence from red blood cells, both in *P. falciparum* and *P. berghei* (van Dijk et al., 2001, 2010; Florens et al., 2002; Lasonder et al., 2002; Le Roch et al., 2003; Hall et al., 2005; Khan et al., 2005; van Schaijk et al., 2006; Silvestrini et al., 2010). In addition, Pfs47 was localized to the surface of female gametocytes and gametes by immunofluorescence assays and immunoprecipitation of labeled gametes (Eksi et al., 2006; van Schaijk et al., 2006). Earlier experiments did not find a critical function for P47 in *P. falciparum* gamete fertilization and transmission-blocking activity was not observed for α-P47 antibodies (van Schaijk et al., 2006). Since Pfs47 was deemed to be dispensable, several reporter parasites were created by inserting transgenes in the P47 locus (Talman et al., 2010; Vaughan et al., 2012; Lu et al., 2016). In *P. berghei*, however, P47 was reported to have an important role in female fertility (van Dijk et al., 2010).

More recently, Pfs47 transcripts were found in ookinetes and the protein was localized to the surface by immunofluorescence. Furthermore, in this study, through a combination of genetic mapping, linkage and functional genomics applied to progeny from a *P. falciparum* strains 7G8 × GB4 genetic cross, the authors determined an essential role for Pfs47 in the evasion of the *Anopheles gambiae* innate immune response to infection (Molina-Cruz et al., 2013). Pfs47 on the surface of the ookinete mediated inactivation of JNK signaling in mosquito midgut cells that eventually resulted in inhibition of binding of the complement-like system protein TEP1 to the ookinete which in turn prevents lysis of the parasite (Ramphul et al., 2015). The identity of the mosquito receptor interacting with Pfs47 remains to be determined. Interestingly, the immune evasion function of Pfs47 is allele-dependent, and different alleles allow survival in mosquitoes from different geographical locations (Molina-Cruz and Barillas-Mury, 2014; Molina-Cruz et al., 2015), however it has been suggested that Pfs47 is not uniquely responsible for this phenomenon and that there might be other factors involved, particularly in parasite field isolates (Eldering et al., 2016). Pfs47 is very polymorphic and it is suggested to be under positive selection based on the analysis of laboratory and field isolates showing a marked geographic genetic structure (Anthony et al., 2007; Manske et al., 2012; Molina-Cruz et al., 2013; Molina-Cruz and Barillas-Mury, 2014). Most of the polymorphisms in Pfs47 and Pbs47 are found in the second s48/45

domain (van Dijk et al., 2010; Molina-Cruz et al., 2015), suggesting a role for this domain in the parasite-vector interactions required for immune evasion.

In *P. vivax*, P47 is also expressed in gametocytes but reportedly localized to the cytoplasm. Nevertheless, antibodies against Pvs47 significantly reduced oocyst numbers in mosquito feeding assays with *P. vivax*-infected blood. Population analysis of Pvs47 isolates form Thailand, Vanuatu and Colombia reported very few polymorphisms (Tachibana et al., 2015) but multiple polymorphisms were reported for Pvs47 in Korean isolates (Woo et al., 2013). In rodent malaria species, P47 is one of the fastest evolving proteins and it is under strong positive selection in *P. berghei* and *P. yoelii* (van Dijk et al., 2010; Ramiro et al., 2015).

3.5. PSOP12

Putative secreted ookinete protein 12 (PSOP12) was identified in a reverse genetics screen of *P. berghei* proteins expressed by the ookinete (Ecker et al., 2008). This protein has one C-terminal 4-cys s48/45 domain and a predicted signal sequence but no GPI anchor (Fig. 2B) (Ecker et al., 2008; Annoura et al., 2014). Mass spectrometry data indicates it is expressed in gametocytes, ookinetes and oocyts in *P. berghei* and in *P. falciparum* (Florens et al., 2002; Hall et al., 2005; Ecker et al., 2008; Silvestrini et al., 2010). Live and immunofluorescence microscopy of PSOP12-GFPtagged parasites detected the protein on the surface of male and female gametocytes, gametes and ookinetes (Sala et al., 2015). *Plasmodium berghei* ΔPSOP12 parasites showed only a mild, non-significant reduction in oocyst production, resulting in normal numbers of sporozoites and normal transmission to mice (Ecker et al., 2008). Nevertheless, a recent vaccine study using baculovirus-expressed PbPSOP12 reported a small but significant transmission-blocking activity for antibodies to this protein both in vitro and in mice (Sala et al., 2015). Further characterization of SOP12 in *P. falciparum* and *P. yoelii* is required to determine whether robust blocking transmission activity can be achieved by targeting this protein.

4. 6-cys proteins in the pre-erythrocytic stages

After traversal of the mosquito midgut epithelium, the ookinete attaches to the basal lamina of the midgut and transforms into an oocyst which grows, undergoes sporogony and produces sporozoites. Escape from the oocyst allows the sporozoites to disperse in the hemolymph and eventually reach and recognize specific receptors on the salivary gland epithelium, prompting them to actively invade and take up residence within the gland ducts. The next time the infected mosquito takes a blood meal, sporozoites are deposited in the host skin where, by means of gliding motility, they will make their way to a blood vessel and enter it. They are then transported to the liver, where they cross the liver sinusoidal endothelium to reach the parenchyma. Following the traversal of multiple hepatocytes, and the successful identification of key receptors on a suitable host hepatocyte, the sporozoite invades by establishing a parasitophorous vacuole. Within this protective compartment, the sporozoite transforms into a trophozoite, enters schizogony and eventually differentiates into thousands of exo-erythrocytic merozoites ready to egress and start the asexual cycle of red blood cell infection (reviewed in Vaughan et al., 2008; Aly et al., 2009). It is evident that during the pre-erythrocytic phase of infection there are multiple points at which parasite-host

cell interactions are necessary for effective life cycle progression. In this section we discuss the members of the 6-cys family which fulfill important roles in these interactions.

4.1. P36

P36 was identified in the above-mentioned BLAST search (Section 3.4) as having two 6-cys domains and it is predicted to have a secretory signal peptide but not a GPI anchor sequence (Fig. 2B) (Templeton and Kaslow, 1999). This protein was reported to be expressed in *P. berghei* gametocytes by northern blot analyses (van Dijk et al., 2010; Thompson et al., 2001) and by proteomic studies (Hall et al., 2005; Khan et al., 2005); however, deletion of P36 in *P. berghei* did not have detrimental effects on fertilization (van Dijk et al., 2010). In addition, P36 expression in *P. falciparum* and *P. yoelii* sporozoites was demonstrated by proteomic experiments (Lasonder et al., 2008; Lindner et al., 2013) and reverse transcription (RT) - PCR (Labaied et al., 2007; VanBuskirk et al., 2009). Western blot analysis suggested that P36 is expressed in salivary gland sporozoites and not in oocyst sporozoites (Ishino et al., 2005). The actual subcellular localization of this protein has not been reported to date. Pf36 does not seem to be very polymorphic, as population studies of 11 *P. falciparum* isolates showed non-significant results for polymorphisms in this protein (Anthony et al., 2007).

Deletion of P36 in *P. falciparum* or rodent malaria parasites did not show any apparent defect in blood stage replication, development in the mosquito or sporozoite infection of the salivary glands (Ishino et al., 2005; Labaied et al., 2007; VanBuskirk et al., 2009). PbΔP36 sporozoites demonstrated an important role for this protein in hepatocyte invasion as these mutants were not able to productively invade hepatocytes and could only infrequently establish a productive infection in mice (Ishino et al., 2005). Similar results were reported for PfΔP36 parasites in hepatocyte invasion assays in vitro, where parasite developmental arrest occurred early inside the host cell (VanBuskirk et al., 2009). Moreover, rapid clearance of PyΔP36 parasites from mouse livers has been recently described (Kaushansky et al., 2015). P36 (together with P52, Section 4.2) is critical for the establishment and/or maintenance of the parasitophorous vacuole during invasion as PyΔP36 parasites can enter hepatocytes but then are observed free in the cytoplasm of the host cell (Labaied et al., 2007; Ploemen et al., 2012). Although there was abundant evidence that P36 is important for successful invasion of hepatocytes by sporozoites, the exact role of the protein had not been determined. However, a recent study points towards the interaction of P36 with host EphA2, a hepatocyte-expressed receptor tyrosine kinase that naturally binds GPI-anchored EphrinA1 to mediate cell-cell interactions (Kaushansky et al., 2015). This is an intriguing finding in light of the structural similarity of s48/45 domains with the Ephrin fold. Although binding of P36 to EphA2 was not directly demonstrated, the work showed that recombinant P36 interfered with EphrinA1 binding to EphA2, indicating an interaction (Kaushansky et al., 2015). The importance of EphA2 in pre17 erythrocytic infection was corroborated by the observation that EphA2 knockout mice were dramatically less susceptible to sporozoite infection compared with wildtype mice and that sporozoites require hepatocytes with high expression of EphA2 to support invasion with parasitophorous vacuole formation (Kaushansky et al., 2015). It is tempting to hypothesize that the structural similarities between the host Ephrin fold and parasite s48/45 domains have been exploited by the parasite to allow interaction with Eph receptors for host cell engagement.

4.2. P52

P52 (named according to molecular mass) was identified in the first comprehensive sporozoite transcriptome study (Kappe et al., 2001) and in a comparative genomics study as a paralog of P36 and therefore named P36p (Thompson et al., 2001). It has two 6-cys domains and it is predicted to have a signal sequence and a GPI anchor (Fig. 2B) (Kappe et al., 2001; Thompson et al., 2001). Sequence analysis of 13 *P. falciparum* isolates suggested that Pf52 is under positive selection (Tonkin et al., 2013). Transcriptional and proteomic analyses in *P. berghei, P. yoelii* and *P. falciparum* agree and show that P52 is only expressed in sporozoites (Kappe et al., 2001; Le Roch et al., 2003; Ishino et al., 2005; van Dijk et al., 2005, 2010; Labaied et al., 2007; Lasonder et al., 2008; VanBuskirk et al., 2009; Lindner et al., 2013). While P52 transcripts were reported in both oocyst sporozoites and salivary gland sporozoites (Kappe et al., 2001), western blot analysis showed expression only in salivary gland sporozoites of *P. berghei* (Ishino et al., 2005). Immunofluorescence and immune electron microscopy (EM) tentatively localized P52 to the sporozoite micronemes (Ishino et al., 2005; VanBuskirk et al., 2009). In addition, the protein was reported to be translocated to the surface of the sporozoite during gliding motility (Ishino et al., 2005).

Deletion of P52 resulted in a similar phenotype as observed for ΔP36 mutants in vitro and in vivo, where sporozoites arrest soon after entering hepatocytes and are not able to achieve productive invasion (Ishino et al., 2005; van Dijk et al., 2005; van Schaijk et al., 2008; VanBuskirk et al., 2009). Simultaneous deletion of P36 and P52 had a more profound infection phenotype than the single deletion mutants (Labaied et al., 2007; VanBuskirk et al., 2009). The strong phenotype is caused by the mutant's inability to form and/or maintain a parasitophorous vacuole very early in/after invasion (Labaied et al., 2007; Ploemen et al., 2012). Because ΔP36ΔP52 parasites are severely attenuated in vitro and in vivo, they can be used for immunization and this conferred sterile immunity against wildtype sporozoite challenge in mice (Labaied et al., 2007). PfΔP36ΔP52 parasites were also shown to be severely attenuated in a controlled human malaria infection study (Spring et al., 2013) and thus deletions of Pf36 and Pf52 alongside a third gene deletion are now part of a Genetically Attenuated Parasite (GAP) vaccine candidate that is undergoing clinical testing (Mikolajczak et al., 2014).

Considering the very similar phenotypes observed in deletion mutants of P36 and P52, and the precedence set by the P230-P48/45 complex in gametocytes, it is possible that P36 and P52 are forming a complex where the GPI-anchored P52 would attach to the parasite plasma membrane and bind secreted P36. The P52/P36 complex could establish contact with the EphA2 receptor on the hepatocyte surface and this interaction would initiate the formation of the parasitophorous vacuole and productive invasion (Fig. 1B). However, a direct interaction between P36 and P52 has not yet been reported. It is also possible that the two proteins function in separate, but closely related, pathways or are part of a larger complex that fully disassembles only when both proteins are absent, resulting in the stronger phenotype of the double gene knockout.

4.3. LISP2 (Sequestrin)

Plasmodium falciparum Liver stage-Specific Protein 2 (LISP2) was first identified by immunoprecipitation from parasite-infected erythrocytes and was originally named Sequestrin (Ockenhouse et al., 1991); however, it was only recently recognized as a 6-cys protein (Arredondo et al., 2012; Annoura et al., 2014). LISP2 is predicted to have a signal sequence but not a GPI anchor. Two independent groups reported two s48/45 domains for this protein, one domain with four cysteines and the other with six (Fig. 2B) (Arredondo et al., 2012; http://pgsh.soe.ucsc.edu/), and two other groups report that LISP2 only has the one 6-cys domain (Orito et al., 2013; Annoura et al., 2014). Across *Plasmodium* spp., the most conserved region contains the 6-cys domain (Orito et al., 2013), implying the essentiality of this domain for the function of the protein.

The original study reported LISP2 on the surface of cytoadherent *P. falciparum*-infected erythrocytes as observed by immunofluorescence assay. In addition, it was reported to bind to CD36 (Ockenhouse et al., 1991), however no other studies characterizing this interaction have been reported to date and its role in blood stages remains unknown. On the other hand, transcripts of LISP2, as well as the protein, were identified in abundance in a proteomics study of late *P. yoelii* liver stages (Tarun et al., 2008) and more recently in a expressed sequence tag analysis of *P. berghei* liver stages (Orito et al., 2013). These studies also determined that LISP2, named after its expression pattern comparable with LISP1 (Tarun et al., 2008), is exclusively expressed in the mid-to-late liver stages stages. Another group has independently confirmed these results by characterizing stage-specific expression using the promoter region of *P. berghei* LISP2, in conjunction with a dual luminescence system (Helm et al., 2010; De Niz et al., 2015). Immunofluorescence assays of liver stages showed that LISP2 is transported to the vacuolar space and then, after processing, exported into the hepatocyte cytoplasm (Orito et al., 2013; Itani et al., 2014).

Targeted disruption of LISP2 in *P. berghei* demonstrated an important role for this protein at the time of liver stage schizogony since the majority of the ΔLISP2 parasites formed aberrant exo-erythrocytic merozoites with a defect in their release, delaying onset of blood stage patency (Orito et al., 2013). A second ΔLISP2 parasite, created by a different group, was characterized and shown to have lower levels of parasite liver stage burden in mice at late developmental time points. Further analysis in vitro showed drastically reduced merozoite surface protein 1 (MSP-1) expression, a merozoite surface marker that is normally expressed late in liver stage schizogony (Annoura et al., 2014), confirming an important role for LISP2 in late liver stages, particularly in the formation of merozoites. Furthermore, a very recent study reported another *P. berghei* ΔLISP2 parasite to be more severely attenuated in mouse infections than previously reported, attributing the difference to the full deletion of the gene including the N-terminus (Kumar et al., 2016). It is possible that this protein has a dual role; first in exo-erythrocytic schizogony and then, after processing and translocation to the hepatocyte cytoplasm, a second function perhaps related to parasite egress or immune evasion.

4.4. B9

B9 was recently identified as a 6-cys family member through a genome search for proteins containing a minimal 4-cysteine motif derived from the already known s48/45 domains (Annoura et al., 2014). B9 has a single 4-cys s48/45 domain at its C-terminus, and it is predicted to have a signal sequence and a GPI anchor (Fig. 2B). Immunofluorescence assays suggested that B9 localizes to the plasma membrane of liver stages. In sporozoites, however, the protein seems to be transcriptionally repressed as RT-PCR data shows the presence of transcripts but protein expression was not observed until after hepatocyte infection, peaking at 5 h p.i. in *P. berghei* and starting at 24 h p.i. in in vitro *P. falciparum* liver stages (Annoura et al., 2014). In contrast, peptides corresponding to B9 were identified in a proteomic study of *P. falciparum* sporozoites (Lindner et al., 2013). The analysis of *P. berghei, P. yoelii* and *P. falciparum* ΔB9 parasites showed developmental arrest early after invasion of hepatocytes with a very small number of parasites able to continue development. Neither the arrested nor the developing ΔB9 parasites were positive for parasitophorous vacuole membrane markers, indicating a critical role for B9 in its integrity (Annoura et al., 2014). Deletion of B9 is part of a second GAP vaccine candidate that was shown to induce sterile protective immunity in the *P. berghei* model (van Schaijk et al., 2014) and the *P. falciparum* version will likely undergo clinical testing in the near future.

5. 6-cys proteins in asexual erythrocytic stages

The clinically symptomatic asexual cycle of replication of *Plasmodium* in red blood cells begins with a loose association of the merozoite with the erythrocyte surface, followed by reorientation to establish apical contact with the cell, followed by irreversible attachment of the merozoite and creation of a tight junction that allows the progressive entrance into the cell, and ultimately the establishment of the parasitophorous vacuole; each one of these steps requiring multiple protein-protein interactions of the parasite with the erythrocyte (reviewed in Cowman et al., 2012; Koch and Baum, 2016). Once the merozoite has sealed itself inside the erythrocyte, protein export and remodeling of the cell begins. The parasite progresses through the ring and trophozoite stages, finally differentiating into schizonts that produce a new generation of merozoites, ready to invade erythrocytes and start another asexual replication cycle. In the trophozoite stages of *P. falciparum*, knob-like structures containing parasite cytoadhesion molecules allow the parasites to bind to the vascular endothelium and sequester, causing severe malaria (reviewed in Maier et al., 2009). In this section we will review the 6-cys proteins expressed in the asexual erythrocytic stages.

5.1. P12

P12 is the smallest member of the 6-cys family and was first identified in a screen of a *P. falciparum* genomic library expressed on the surface of mammalian cells and tested for reactivity against human immune sera from *P. falciparum*-exposed African adults (Elliott et al., 1990). P12 has two 6-cysteine motifs similar to those detected in Pfs230 and it was deemed to be the structural archetype of the family (Williamson et al., 1993; Carter et al., 1995; Gerloff et al., 2005); its NMR analysis provided the first experimentally determined molecular structure for the 6-cys family (Fig. 2B) (Arredondo et al., 2012; Tonkin et al., 2013). Pf12 was shown to be a GPI-anchored protein by the proteomic analysis of

radioactive glucosamine-labeled late schizont membranes (Gilson et al., 2006). Although transcripts have been detected in late schizonts, sporozoites and gametocytes in *P. falciparum* and *P. berghei* (Bozdech et al., 2003; Le Roch et al., 2003; Hall et al., 2005; Gilson et al., 2006; van Dijk et al., 2010), protein expression has only been confirmed for schizonts/merozoites by western blot (Taechalertpaisarn et al., 2012; Tonkin et al., 2013) and membrane proteome analyses (Sanders et al., 2005; Gilson et al., 2006). Immunofluorescence microscopy indicated that Pf12 is localized on the surface of the merozoite with a more prominent signal on the apical end that colocalizes significantly with the rhoptry neck protein RON4 (Taechalertpaisarn et al., 2012; Tonkin et al., 2013) and electron microscopy studies localized Pf12 to the rhoptry organelles of the merozoite (Silvia Arredondo, unpublished data). However, it has been suggested that P12 is shed from the merozoite following proteolytic cleavage (Taechalertpaisarn et al., 2012).

Based on its expression profile and localization, Pf12 was anticipated to be involved in erythrocyte invasion, however ΔP12 *P. falciparum* parasites did not show any deficiency in invasion or other apparent phenotypes during the asexual replication cycle (Taechalertpaisarn et al., 2012). In addition, neither native Pf12 from culture supernatants nor recombinant protein showed direct binding to red blood cells (Taechalertpaisarn et al., 2012), in contrast to earlier reports of Pf12 synthetic peptides binding and displaying modest invasion inhibition activity (Garcia et al., 2009). Antibodies raised against recombinant Pf12 did not show any invasion inhibition activity either (Taechalertpaisarn et al., 2012). Co-immunoprecipitation experiments established an interaction of Pf12 with Pf41 and this has been confirmed by biochemical and structural experiments using recombinant protein (Fig. 1B) (Taechalertpaisarn et al., 2012; Crosnier et al., 2013; Tonkin et al., 2013; Parker et al., 2015). Although the molecular structures for Pf12 and Pf41 have been solved (discussed in Sections 2 and 5.3), it has not been possible to derive functional insights from these structures.

Pf12 appears to be a strong natural immunogen; in addition to its initial identification by immune sera from African populations (Elliott et al., 1990) and immunoprecipitation by immune sera from Papua New Guinea (Sanders et al., 2005), studies have reported high seroprevalence against Pf12 in Kenyan and Papua New Guinean children with a weak association with protection from symptomatic malaria in the latter (Richards et al., 2013; Osier et al., 2014). However, P12 is remarkably conserved and believed to be under purifying selection (Tetteh et al., 2009; Tonkin et al., 2013), an indication of a crucial function for this protein that has yet to be determined.

Pv12, the Pf12 orthologue in *P. vivax*, was shown to be transcribed in schizonts and localized to the surface and rhoptries of merozoites by immunofluorescence (Li et al., 2012; Cheng et al., 2013; Moreno-Perez et al., 2013). The interaction of Pv12 and Pv41 has recently been reported in addition to a novel interaction with PVX_110945 (Hostetler et al., 2015). Low genetic diversity was observed for Pv12 in a Colombian parasite population (Forero-Rodriguez et al., 2014) but a higher number of polymorphisms was found in parasite populations from the China-Myanmar border (Wang et al., 2014). Pv12 is recognized by sera of naturally infected patients (Chen et al., 2010; Moreno-Perez et al., 2013; Franca et al.,

2016) and a strong association in reduced risk of clinical disease and antibody levels was recently reported (Franca et al., 2016).

5.2. P12p

P12p is a paralog of P12 found in tandem with p12 on chromosome 6, and it was first reported to be a member of the family by Gerloff and co-workers (Gerloff et al., 2005). Similar to P12, there are two 6-cys domains in P12p, and a signal sequence and GPI anchor are predicted (Fig. 2B) (Gilson et al., 2006). P12p was reported to be transcribed in the blood stages in *P. falciparum* by microarray and in *P. berghei* by northern blot analysis (Gilson et al., 2006; van Dijk et al., 2010). However, mass spectrometry data indicates P12p is also present in *P. falciparum* and *P. yoelii* sporozoites (Lasonder et al., 2008; Lindner et al., 2013). Deletion of P12p in *P. yoelii* did not show any detectable phenotype at any stage of the infection cycle (our unpublished results). Thus, the function of P12p remains unknown and requires further investigation.

5.3. P41

P41 was identified in the same BLAST search as Pf36 and Pfs47 and it was described to have a secretory signal peptide, two 6-cysteine domains and an “intervening region” that was recently shown to be an important element within the first domain (Fig. 2B) (Templeton and Kaslow, 1999; Parker et al., 2015). P41 lacks a GPI anchor but, as mentioned in Section 5.1, it is part of the second confirmed heterodimer within the family, interacting with P12 (Fig. 1B) (Taechalertpaisarn et al., 2012; Crosnier et al., 2013; Tonkin et al., 2013; Hostetler et al., 2015; Parker et al., 2015). Correspondingly, the expression and localization profiles for Pf41 are similar to Pf12 (Hall et al., 2005; Sanders et al., 2005; van Dijk et al., 2010). Pf41 was found in the proteome of merozoite surface membranes and antibody against the inter-domain region localized it to the surface of merozoites within mature schizonts and in the apical organelles in free merozoites by immunofluorescence microscopy (Sanders et al., 2005). Biochemical studies and targeted deletion for Pf41, carried out in parallel with Pf12, showed no critical function for this protein in erythrocyte binding and invasion (Taechalertpaisarn et al., 2012), contrary to the reported inhibitory activity of P41 peptides (Garcia et al., 2009). Pf41 is the second 6-cys protein for which the molecular structure has been solved (Parker et al., 2015). Initial biochemical studies on the Pf12/Pf41 complex suggested the proteins to have an anti-parallel arrangement (Tonkin et al., 2013). The actual structure of Pf41 revealed a more interesting feature: what was originally described as an interdomain region is actually an intra-domain insertion that is essential for binding to Pf12 and is protected from proteolytic cleavage upon interaction of the two proteins (Parker et al., 2015).

Pf41, seemingly under positive selection (Tonkin et al., 2013), is recognized by sera from naturally infected individuals with reported seroprevalences of 32–88% (Sanders et al., 2005; Richards et al., 2013; Osier et al., 2014). In addition, high correlation with malaria disease protection was reported in a study of a cohort of Kenyan children (Osier et al., 2014), while only weak association with protection from symptomatic malaria was observed in a population of Papua New Guinean children (Richards et al., 2013).

The expression and subcellular localization profiles for Pv41 are similar to the *P. falciparum* orthologue, however the apical localization in the merozoite was not reported (Angel et al., 2008; Chen et al., 2010; Cheng et al., 2013). Positive balancing selection was suggested for Pv41, particularly in the C-terminus of parasites from the China-Myanmar border (Wang et al., 2014). Alternatively, low genetic diversity was found for Pv41 in parasites from Colombia with a few substitutions in the C-terminus under positive selection (Forero-Rodriguez et al., 2014). Pv41 is also recognized by sera from naturally infected individuals and a strong association for protection is reported for Pv41 antibodies in a Papua New Guinean cohort (Chen et al., 2010; Cheng et al., 2013; Franca et al., 2016).

5.4. P92

Pf92 was initially identified in a proteomic study of detergent-resistant membranes of mature asexual blood stages (Sanders et al., 2005). Interestingly, P92 is the only protein in the family that does not have an orthologue in rodent *Plasmodium* spp. The 6-cys model database indicates it has a double s48/45 domain, however it has also been reported to have a single C-terminal s48/45 domain with only four cysteines (Arredondo et al., 2012; Annoura et al., 2014; http://pgsh.soe.ucsc.edu/). It is predicted to have a signal sequence and it has been validated as a GPI-anchored protein in the same proteomics study as Pf12 (Fig. 2B) (Gilson et al., 2006). Transcriptional analysis indicated that Pf92 is expressed in blood stage schizonts (Bozdech et al., 2003; Le Roch et al., 2003; Sanders et al., 2005; Gilson et al., 2006). Expression of Pf92 in late schizonts and merozoites has also been confirmed by proteomic experiments (Sanders et al., 2005, 2007; Gilson et al., 2006), western blot analysis (Obando-Martinez et al., 2010; Kennedy et al., 2016) and immunofluorescence microscopy, which showed localization on the surface of the merozoites using a GFP-tagged version of the protein (Sanders et al., 2005). In addition, Pf92 peptides were reported in oocyst-derived sporozoites (Lasonder et al., 2008).

Among the most abundant proteins found in schizont-derived membranes (Sanders et al., 2007), Pf92 was recently described to have an important role in preventing complement-dependent lysis of merozoites. Kennedy et al. (2016) showed that Factor H, a complement regulator that normally binds to cell receptors to prevent “self” destruction by the complement cascade, is recruited by merozoites, representing yet another immune evasion strategy of the *Plasmodium* parasite. Immunoprecipitation experiments identified Pf92 as the main protein recruiting Factor H to the surface of merozoites (Fig. 1B). Deletion mutants of Pf92 were not able to bind to Factor H and showed significant growth reduction after four in vitro replication cycles. Although the binding domains in Factor H were successfully mapped, the corresponding interacting regions in Pf92 remain to be determined (Kennedy et al., 2016). As mentioned above, Pf92 is not found in rodent malaria species, however Factor H is part of the mouse complement system and human Factor H is capable of regulating mouse complement (Pouw et al., 2015), raising questions regarding the identity of the rodent *Plasmodium* protein or proteins that may be functionally substituting for Pf92.

Pf92 has been reported to be under positive selection in the analysis of 13 isolates from PlasmoDB sequences (Tonkin et al., 2013) and under weak negative selection in the *P. falciparum* Gambian parasite population (Tetteh et al., 2009). While Pf92 elicits an immune

response in individuals from endemic regions, with ~80% seropositivity observed in a cohort of Kenyan children, there was no significant correlation with protection from clinical disease episodes (Osier et al., 2014).

5.5. P38

Pf38 was identified as a member of the 6-cys family in a comparative genomics study (Thompson et al., 2001). This protein has two s48/45 domains with four and six cysteines, respectively, with a predicted signal peptide, and it has been determined to have a GPI anchor (Fig. 2B) (Thompson et al., 2001; Gilson et al., 2006; http://pgsh.soe.ucsc.edu/). P38 is reported to be expressed in schizonts, sexual stages and sporozoites by several transcriptomic and proteomic studies (Bozdech et al., 2003; Le Roch et al., 2003; Gilson et al., 2006; Silvestrini et al., 2010; Lindner et al., 2013). In addition, northern blot analysis showed transcripts for P38 in gametocytes and blood stages of *P. berghei* (Thompson et al., 2001; van Dijk et al., 2010). Pf38 was found in detergent-resistant membranes from late schizont/merozoites (Sanders et al., 2005, 2007; Gilson et al., 2006) and confirmed to localize on the surface of merozoites by immunofluorescence microscopy in both *P. yoelii* and *P. falciparum* blood stages (Feller et al., 2013; Harupa, A., 2015. Identification and functional analysis of novel sporozoite surface proteins in the rodent malaria parasite *Plasmodium yoelii,* Doctoral thesis, Freie Univeritat Berlin, Germany). However, live fluorescence microscopy using a Pf38-GFP fusion showed this protein mainly in the apical end of merozoites (Sanders et al., 2005). P38 has also been reported to localize to sporozoite micronemes, using an hemagglutinin- (HA) tagged P38 in *P. yoelii* (Harupa, A., 2015. Doctoral thesis, cited earlier), and on gametocytes, macrogametes and zygotes (Feller, 2013).

Deletion mutants of P38 have shown no obvious phenotypic differences compared with wildtypes both in *P. berghei* and *P. yoelii* (van Dijk et al., 2010; Harupa, A., 2015. Doctoral thesis, cited earlier). However, Pf38-derived peptides were reported to show a 20–33% inhibition of erythrocyte invasion in vitro (Garcia et al., 2009); and anti-Pf38 antibody significantly inhibited the growth of *P. falciparum* blood stages, in addition to inhibiting zygote development (Feller et al., 2013). P38 is the only member of the family that has been shown to have a phosphorylated serine residue in proteomic studies (Aurrecoechea et al., 2009; Treeck et al., 2011; Pease et al., 2013; Lasonder et al., 2015) but whether this residue modification is important for function remains to be determined.

Population analyses showed that, in the Gambian and Papua New Guinean parasite populations, Pf38 is a polymorphic gene and it seems to be under modest balancing selection with a stronger signature of balancing selection for Domain I of the protein in New Guinea (Tetteh et al., 2009; Reeder et al., 2011). Analysis of laboratory isolates, however, did not yield significant results regarding polymorphisms for this gene (Anthony et al., 2007). Pf38 is recognized by antibodies of naturally infected individuals from different populations with high seroprevalence (Sanders et al., 2005; Richards et al., 2013; Osier et al., 2014). The study of a cohort of Papua New Guinean children showed that antibodies against Pf38 were correlated with intermediate protection from symptomatic malaria (Richards et al., 2013),

and in a different cohort of infected Kenyan children, Pf38 also ranked highly in the association of protection from clinical episodes (Osier et al., 2014).

In *P. vivax*, Pv38 has two s48/45 domains, however the N-terminal domain has five cysteines rather than four (Wang et al., 2014; http://pgsh.soe.ucsc.edu/). Pv38 was reported to be present in membranes from asexual blood stages by western blot analysis and localized on schizonts with a speckled pattern (Mongui et al., 2008). Low genetic diversity has been reported for Pv38 in parasites from Colombia and, similarly to the *P. falciparum* orthologue, balancing selection was suggested for the N-terminal domain of the protein (Forero-Rodriguez et al., 2014). Parasites from the China-Myanmar border were reported to have significant genetic differentiation in comparison to the Colombian population, and in this case positive balancing selection was suggested for Domain II of Pv38 (Wang et al., 2014).

6. Concluding remarks

It appears as if the suggested acquisition of the s48/45 domain by the malaria parasite from its host, its expansion in the 6-cys family and its preservation through millions of years of evolution, have conferred multiple and significant advantages to *Plasmodium* parasites. To date, there are three main categories in which the adhesive functions of the s48/45 proteins cluster to ensure parasite survival and life cycle progression: fertilization in the sexual stages (P230, P48/45 and PSOP12), establishment or maintenance of the parasitophorous vacuole membrane to sustain infection of hepatocytes (P36, P52, B9) and immune evasion (P47, P92 and perhaps LISP2, P12, P41, P38, P12p). Another common theme is becoming apparent: a GPI-anchored family member attaches to the parasite plasma membrane and binds a secreted member by forming a heterodimer, raising the question as to whether some members serve only as scaffolds while others perform effector/binding functions. The non-conserved regions outside the main s48/45 domains of the proteins deserve closer analysis as well. Could the flexible loops and extensions away from the main domain core hold the key to the adhesive properties of these proteins, as may be the case of the intra-domain region in Pf41 or the N-terminus to the first s48/45 domain in Pfs230Δ Is the intrinsic value of the s48/45 domain in its versatility in allowing extensive peripheral flexibility while maintaining robust stability within the core? The solution of the molecular structures of the 6-cys family members for which functions and host partners are known, for example the co-crystal of Pf92 and Factor H, or P36 and EphA2, will be very useful in answering these questions. It is reasonable to ask whether P38 is a scaffold throughout different parasite stages, stabilizing unrelated or other s48/45 proteins. Is there redundancy among “scaffold” members? Proteolytic cleavage to create functional protein might also be a more general feature of 6-cys proteins, as proteolysis has been reported for P230, LISP2, P12 and perhaps P41 which upon cleavage loses the ability to bind to P12. Is the host-interaction functionality only activated after proteolysis? To better understand the interactions of these proteins we must also look into loss/gain of function for the same protein across species, for example for P47 and P230 in *P. berghei* and *P. falciparum.* It would be very informative to define the specific protein regions involved in female fertilization or male exflagellation, respectively. P230 remains a very interesting protein; with its 14 domains it is tempting to imagine the opportunities for multiple functions and to date it appears to have at least three separate functions: fertilization and exflagellation in males and mating barriers in females. Is this

multiplicity of function what makes P230 a strong transmission-blocking vaccine candidate? If so, we may have to look at the s48/45 family from a more global perspective and perhaps through a combination of in silico and in vitro approaches define minimal conserved conformational epitopes that may elicit strong cross-reactive antibody responses that can interfere with the function of multiple s48/45 proteins at once, attacking parasite progression at multiple points in the life cycle. Finally, the advent of new and more robust parasite transgenesis techniques such as the CRISPR/Cas9 system will allow for precise genetic manipulations to introduce mutations at specific residues, delete full domains or even swap domains between members of the family or across species. This will accelerate the functional characterization of the s48/45 proteins and the identification of essential epitopes for more effective vaccines, addressing the many questions we still have about this intriguing family in the near future.

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Highlights

- The *Plasmodium* 6-cys protein family has 14 members that share the s48/45 domain.
- s48/45 proteins are found in all parasite stages and are conserved across species.
- s48/45 proteins have functions in fertilization, parasitophorous vacuole membrane fitness and immune evasion.
- Some of the s48/45 proteins are under investigation as vaccine candidates.

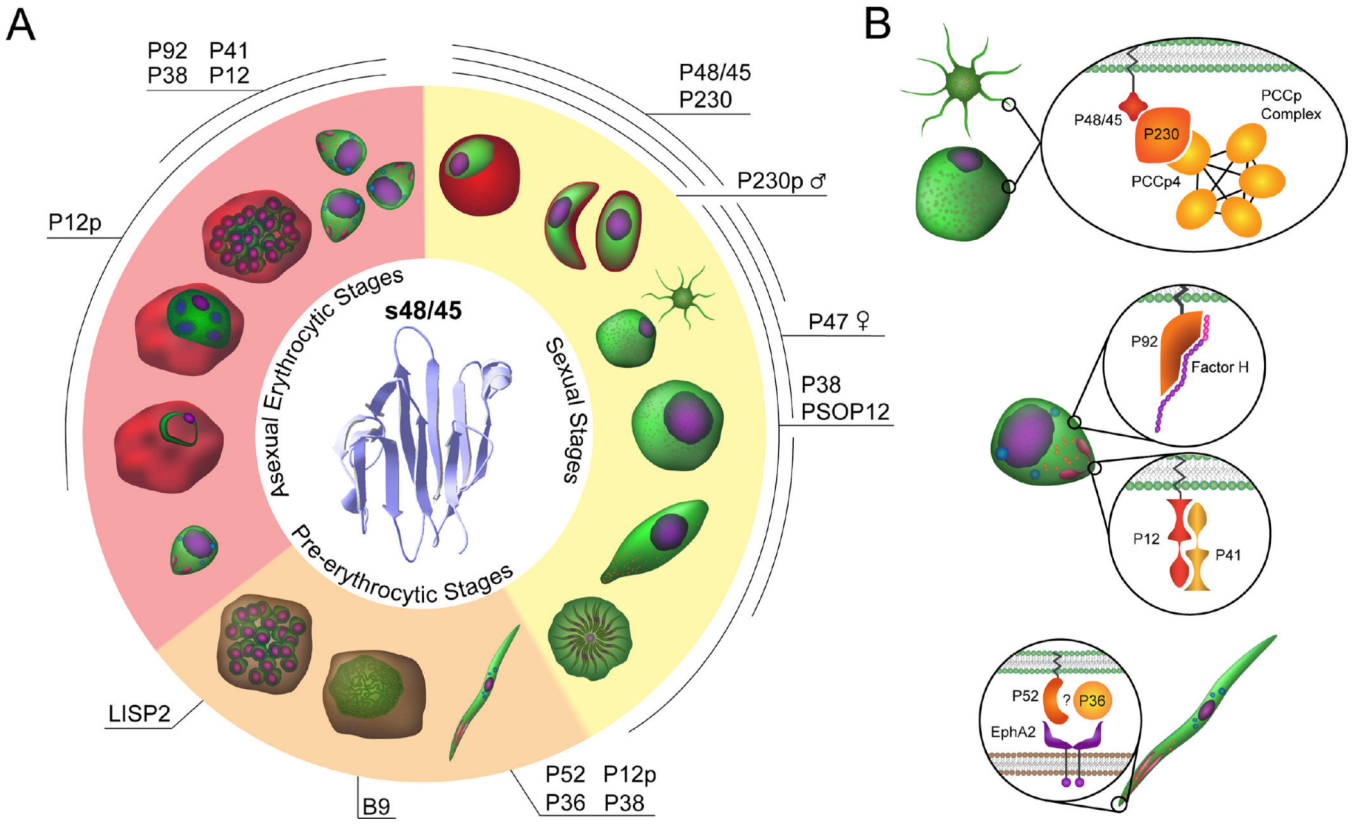

Fig. 1.

The s48/45 six-cysteine protein family in the *Plasmodium falciparum* life cycle. (A) Development of *Plasmodium* in the mosquito vector and vertebrate host through the three main stages of infection (clockwise: sexual stages shaded in yellow (gametocyte stage II, gametocytes stage V, gametes, zygote, ookinete, oocyst), pre-erythrocytic stages shaded in orange (sporozoite, mid-liver stage, late liver stage), and asexual erythrocytic stages shaded in pink (merozoite, ring, trophozoite, late schizont, merozoites). Proteins that contain at least one s48/45 domain (the molecular structure is shown in ribbon mode (Protein Data Bank (PDB) ID: 2LOE)) are expressed in the different stages of the parasite life cycle as labeled. PSOP12, putative secreted ookinete protein 12; LISP2, liver stage-specific protein 2. (B) Protein interactions of the s48/45 family. The interactions that have been confirmed or proposed based on the literature are depicted in the insets on the corresponding parasite stages. From top to bottom: the confirmed interaction of the PfCCp-Pfs230 (14 s48/45 domains) complex with Pfs48/45 (three domains) on the surface of gametes; the confirmed interactions of Pf92 (one domain) with Factor H, and of Pf12 with Pf41 (two domains each) on the merozoite; and the suspected interactions of P52 and P36 (two domains each) with EphA2 in the sporozoite. Figure reproduced with permission, ©Adriana Lippy.

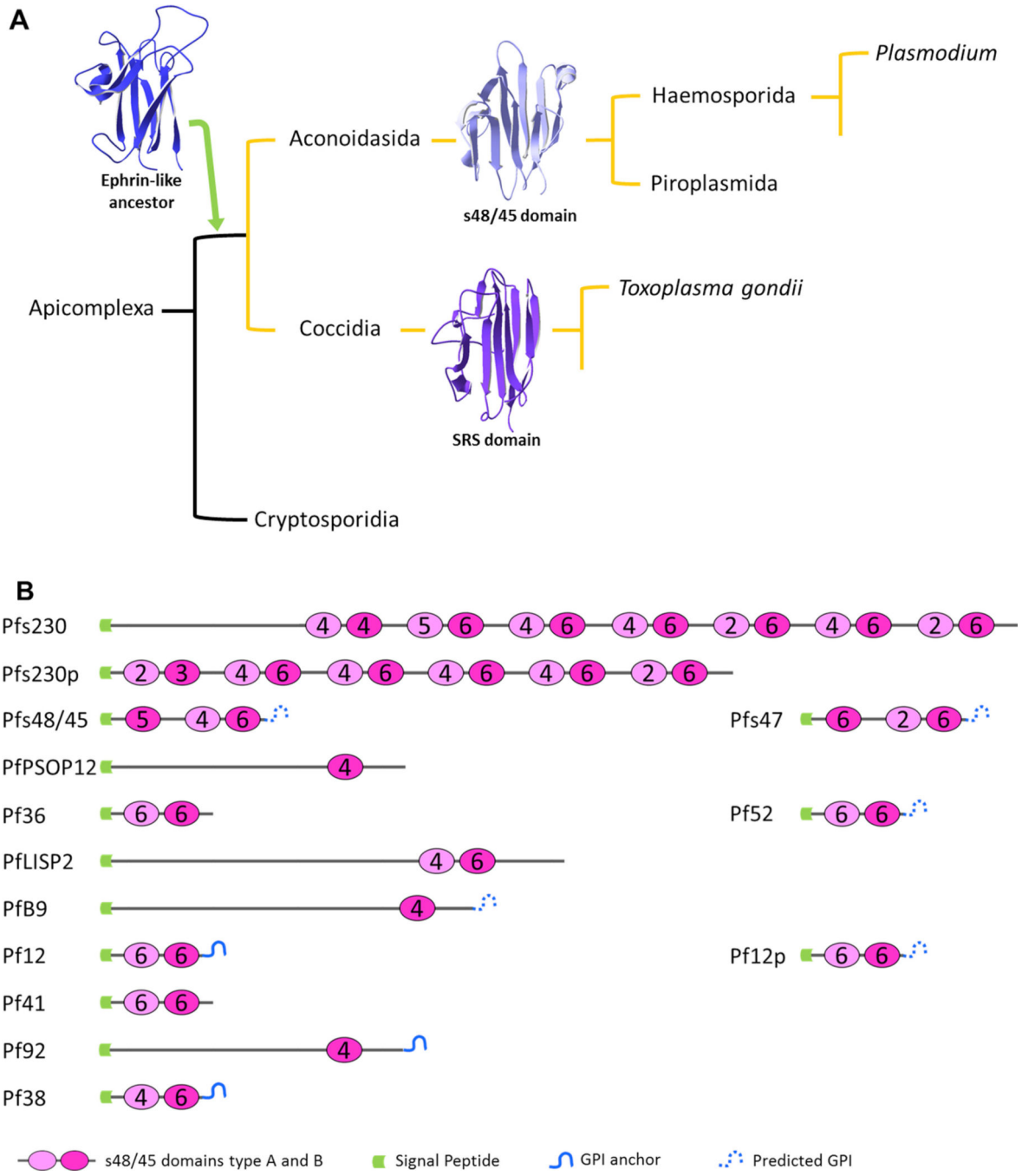

Fig. 2.

The evolution and distribution of the s48/45 six-cysteine protein domain. (A) The s48/45 domain is suggested to derive from an ancient Ephrin-like domain that was acquired by an ancestor of the aconoidasidans and coccidians that eventually evolved into the current s48/45 domain in *Plasmodium* and the SAG1-Related Sequence (SRS) domain in *Toxoplasma* (ribbon structures shown: Protein Data Bank (PDB) ID: 3WO3-b for Ephrin-like; 2LOE for s48/45; 1KZQ-a D1 for SRS), (figure adapted from Arredondo et al. (2012). (B) Illustration of the *Plasmodium falciparum* s48/45 family members showing the arrangement of the

domains in each protein, the number of cysteines present in each domain and the predicted signal sequences and glycosylphosphatidylinositol (GPI) anchors.

Table 1

Features of the known members of the *Plasmodium* six-cysteine (6-cys) s48/45 protein family. Data were extracted from PlasmoDB (Aurrecoechea et al., 2009), except for columns listing the number of s48/45 domains, Stage, Subcellular Localization and Function which were summarized from the reviewed literature.

Name	PlasmoDB Accession No.	Chromosome	No. of amino acids[a]	MW (kDa)[a]	s48/45 Domains	Orthologues	Stage	Subcellular Localization	Function
Pfs230	PF3D7_0209000	2	3135	363.2	14	Pv, Pk, Pc, Pr, Pb, Py, Pch	M and F gametocyte and gametes	Plasma membrane of gametocytes and gametes	Male fertility, exflagellation center formation, cross fertilization.
Pfs230p	PF3D7_0208900	2	2508	293.7	12	Pv, Pk, Pc, Pr, Pb, Py, Pch	M stage IV-V gametocytes	Gametocyte cytoplasm	ND
Pfs48/45	PF3D7_1346700	13	448	51.5	3	Pv, Pk, Pc, Pr, Pb, Py, Pch	M and F gametocyte and gametes	Plasma membrane of gametocytes and gametes	Male fertility, cross fertilization.
Pfs47	PF3D7_1346800	13	439	50.8	3	Pv, Pk, Pc, Pr, Pb, Py, Pch	F gametocytes and gametes; ookinete	Surface of gametocytes, gametes and ookinetes	Evasion of the mosquito immune system
PfPSOP12	PF3D7_0513700	5	735	87.3	1	Pv, Pk, Pc, Pr, Pb, Py, Pch	M and F gametocytes, ookinetes, oocyts	Surface gametocytes, gametes and ookinetes	Possibly related to transmission
Pf36	PF3D7_0404400	4	379	44.6	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Sporozoite	ND	Hepatocyte invasion
Pf52	PF3D7_0404500	4	478	56.3	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Sporozoite	Micronemes	Hepatocyte invasion, PVM formation
PfLISP2	PF3D7_0405300	4	1964	233.7	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Mid-late liver	Vacuolar space/ exported to host cytoplasm	Schizogony in the Liver
PfB9	PF3D7_0317100	3	969	114.7	1	Pv, Pk, Pc, Pr, Pb, Py, Pch	Early liver	Plasma membrane	Maintenance of PVM
Pf12	PF3D7_0612700	6	347	39.4	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Late schizont/ merozoite	Rhoptries, merozoite surface	ND

Name	PlasmoDB Accession No.	Chromosome	No. of amino acids[a]	MW (kDa)[a]	s48/45 Domains	Orthologues	Stage	Subcellular Localization	Function
Pf12p	PF3D7_0612800	6	371	43.5	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Blood stage and sporozoite	ND	ND
Pf41	PF3D7_0404900	4	378	43.1	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Late schizont/ merozoite	Merozoite surface and apical organelles	ND
Pf92	PF3D7_1364100	13	796	92.7	1	Pv, Pk, Pc, Pr	Late schizont/ merozoite	Merozoite surface	Recruitment of Factor H for complement lysis protection
Pf38	PF3D7_0508000	5	349	40.6	2	Pv, Pk, Pc, Pr, Pb, Py, Pch	Schizont, merozoites, sporozoites, gametocytes	Merozoite surface and apical organelles. Sporozoite micronemes. Gametocyte surface.	Suggested erythrocyte invasion.

[a]Includes signal peptide

Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pk, *Plasmodium knowlesi*; Pc, *Plasmodium cynomolgi*; Pr, *Plasmodium reichenowi*; Pb, *Plasmodium berghei*; Py, *Plasmodium yoelii*; Pch, *Plasmodium chabaudi*; M, male; F, female; ND, not determined; PVM, parasitophorous vacuole membrane.