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REVIEW

Family members stick together: multi-protein complexes of malaria parasites

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Abstract Malaria parasites express a broad repertoire of proteins whose expression is tightly regulated depending on the life-cycle stage of the parasite and the environment of target organs in the respective host. Transmission of malaria parasites from the human to the anopheline mosquito is mediated by intraerythrocytic sexual stages, termed gametocytes, which circulate in the peripheral blood and are essential for the spread of the tropical disease. In *Plasmodium falciparum*, gametocytes express numerous extracellular proteins with adhesive motifs, which might mediate important interactions during transmission. Among these is a family of six secreted proteins with adhesive modules, termed *Pf*CCp proteins, which are highly conserved throughout the apicomplexan clade. In *P. falciparum*, the proteins are expressed in the parasitophorous vacuole of gametocytes and are subsequently exposed on the surface of macrogametes during parasite reproduction in the mosquito midgut. One characteristic of the family is a co-dependent expression, such that loss of all six proteins occurs if expression of one member is disrupted via gene knockout. The six *Pf*CCp proteins interact by adhesion domain-mediated binding and thus form complexes on the sexual stage surface having adhesive properties. To date, the *Pf*CCp proteins represent the only protein family of the malaria parasite sexual stages that assembles to multimeric complexes, and only a small number of such protein complexes have so far been identified in other life-cycle stages of the parasite.

Keywords *Plasmodium* · Malaria · Protein family · Cell adhesion · Sexual stage · Gametocyte · Multi-protein complex

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Introduction

With an estimated 250 million cases every year and an annual death toll of one million people, the tropical disease malaria remains a major global health threat and a serious economic burden (reviewed in [1, 2]). Approximately 50% of the world's population live in affected areas within wide regions of Africa, South-East Asia, Central America, and the Eastern Mediterranean [3]. In most endemic countries, the treatment and control of malaria is undermined by the spread of drug resistant parasites, extreme poverty, and the impact of HIV infections.

Malaria is a vector-borne disease that is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding anopheline mosquitoes. There are four human malaria species, among them *P. falciparum*, the causative agent of the dangerous malaria tropica. When infecting a human host, malaria parasites initially target liver hepatocytes, causing a primary but asymptomatic infection. The parasites subsequently infect erythrocytes, and the recurrent intraerythrocytic replication cycles cause the typical symptoms of the disease, such as fever and anemia, which in severe cases are followed by respiratory distress and organ failure.

Besides three different periods of asexual replication, which take place in the human hepatocytes and erythrocytes as well as inside the mosquito vector, the malaria life

cycle also includes a phase of sexual reproduction that mediates parasite transition from the human to the mosquito and thus plays a crucial part in disease transmission. In *P. falciparum*, the malaria sexual phase begins with the differentiation of intraerythrocytic parasites to the non-replicative gametocyte lineage. Following maturation to crescent-shaped intraerythrocytic forms, the gametocytes freely circulate in the peripheral circulation in anticipation of being taken up by the mosquito during the blood meal. Environmental stimuli in the mosquito midgut during blood feeding rapidly activate the gametocytes, which within minutes egress from the host erythrocyte and undergo gametogenesis, resulting in the development of female macro- and male microgametes. The motile microgamete fertilizes the sessile macrogamete, and within 20 h, the resulting zygote transforms into a motile form, termed the ookinete. The ookinete exits the midgut lumen and crosses the gut epithelium, which marks the end of the malaria sexual phase (reviewed in [4, 5]).

It was only in the past two decades, when research focused on the sexual phase of the parasite life cycle. This interest arose upon the observation that the host's immune response to sexual stage antigens can completely inhibit the development of *Plasmodium* in the insect vector, and such antigens would therefore represent ideal candidates for the development of transmission-blocking vaccines (TBVs) (reviewed in [6]). This type of vaccine is based on human antibodies directed against select sexual stage antigens, which are taken up by the mosquito with the blood meal and which eliminate the parasite sexual stages inside the mosquito midgut, as soon as these lose protection by the enveloping erythrocyte. Hence, TBVs represent a powerful tool in malaria control efforts, particularly together with new combination-therapy drugs, and vaccines targeting morbidity and mortality due to the asexual stages (reviewed in [7, 8]).

Among the first vaccine candidates discovered in the 1980s were *Pf*s230 and *Pf*s48/45, which are expressed at the surface of the developing gametocytes and later of male and female gametes (reviewed in [4]). Both proteins share a primary structure composed of several cysteine-rich domains assigning them to the cysteine-rich motif superfamily that is comprised of ten members (Fig. 1a) [9–11]. Two additional antigens, found on the surface of zygotes and ookinetes, are *Pf*s25 and its paralog *Pf*s28, both possessing four epidermal growth factor (EGF) domains and glycosylphosphatidylinositol (GPI) anchors. The transmission-blocking potential of antisera targeting *Pf*s25 and its *vivax* malaria ortholog *Pv*s25 in humans was subject of clinical phase I trials [12, 13], further confirming the suitability of this protein for the generation of TBVs.

Additional molecules were identified in the following years, including the here discussed *Pf*CCp multi-domain

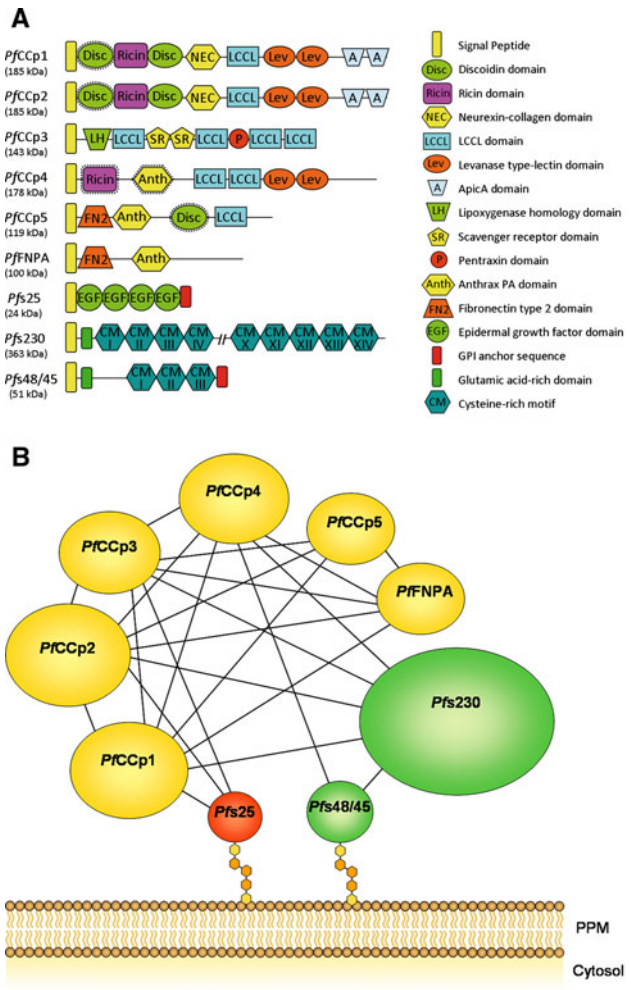

Fig. 1 **a** Schematic of the domain structure of select *Plasmodium falciparum* sexual stage proteins containing adhesive motifs, i.e., the *Pf*CCp multi-domain adhesion protein family, the EGF domain-containing protein *Pf*s25, and the cysteine-rich motif proteins *Pf*s230 and *Pf*s48/45. *Encircled black-dotted domains* indicate newly identified motifs. **b** Schematic demonstrating the proposed network of interactions between sexual stage adhesion proteins. The multimeric protein complex most likely is retained at the plasma membrane of gametocytes via binding to the GPI-anchored *Pf*s48/45. After gametocyte activation, *Pf*s25 also locates to the cell surface of the forming macrogamete, covalently linked by a GPI anchor, thereby providing an additional retention site for the multi-protein complex. *PPM* parasite plasma membrane

adhesion protein family [14]. Noteworthy is that the majority of sexual stage surface proteins have adhesive properties and can be divided into two classes. One class of sexual stage proteins, including *Pf*s230, *Pf*s48/45 and the six *Pf*CCp proteins, is expressed within the parasitophorous vacuole (PV) of the developing gametocyte and subsequently present on the gamete surface, but expression of these proteins usually ceases during fertilization. The expression of the second class of surface proteins, among them *Pf*s25 and *Pf*s28, is initiated after the parasites have

entered the mosquito midgut and persists until the ookinete has formed (reviewed in [4, 5]).

The reasons for the high number of adhesive proteins in the malaria parasite sexual stages and the coordinated expression pattern of these proteins during transmission remain elusive. A recent study from our laboratory indicated that a subset of these proteins assemble to multimeric complexes on the parasite surface. In this review, we highlight our previous findings on the *Pf*CCp protein family and provide evidence for complex formation of multiple surface-associated sexual stage proteins. We will compare these data with findings on other protein complexes of malaria parasites and speculate on the possible function of the sexual stage multimeric complexes.

Domain structure of the *Pf*CCp protein family

The *Pf*CCp protein family was identified via annotation of the *P. falciparum* genome sequence for proteins encoding multi-domain "animal-like" architectures. Six members were described exhibiting multiple adhesion domains conserved among animals and bacteria (Fig. 1a) [14], five of which share a common LCCL (*Limulus* coagulation factor C, cochlear protein Coch-5b2, and late gestation lung protein Lgl1) domain, which was eponymous for the proteins thenceforward named *Pf*CCp1 to *Pf*CCp5 (LCCL domain-containing protein). Despite the lack of a LCCL domain, a sixth protein referred to as *Pf*FNPA was assigned to the *Pf*CCp protein family due to its structural similarity to *Pf*CCp5. The predicted signal peptide sequence common to all *Pf*CCp proteins indicated an extracellular location, yet GPI-anchor signal sequences or transmembrane regions were not detected [14]. Significantly, all members of the *Pf*CCp protein family possess orthologs in other organisms of the apicomplexan clade.

*Pf*CCp1 and *Pf*CCp2 have similar architectures and are paralogs arising from gene duplication after domain accretion. *Pf*CCp1 and *Pf*CCp2 possess binding domains, corresponding to ricin, discoidin, and levanase-type lectin domains [15, 16]. C-terminal to these modules is two copies of an apicomplexan-specific cysteine-rich module, termed ApicA. Between the discoidin and levanase lectin domains are two LCCL domains [17] and a NEC module (termed after neurexins [18] and collagens).

*Pf*CCp3 contains in total four LCCL domains, a tandem repeat of a scavenger receptor (SR) domain, a lipoxygenase LH2 domain, and a region similar to pentraxin. *Pf*CCp4 possesses two center LCCL domains, followed by tandem levanase domains. *Pf*CCp5 encodes a region at the amino terminus that is similar to the type 2 domain of fibronectin (FN2), fused to a domain similar to the N-terminal region of anthrax protective antigen (PA) and a single LCCL domain near the C-terminus. As described above, *Pf*FNPA is similar to *Pf*CCp5 in overall architecture but lacks an LCCL domain. Given that *Pf*FNPA has an apparent ortholog in the distantly related apicomplexan, *Cryptosporidium parvum*, the two genes are likely to have diverged in *P. falciparum* from a common ancestor that was similar in architecture to *Pf*FNPA, followed by accretion of an LCCL domain in *Pf*CCp5.

Recent re-annotation of the *Pf*CCp protein sequences [I] revealed several additional adhesion domains, i.e., a second discoidin domain in *Pf*CCp1 and *Pf*CCp2, which is located at the N-terminus of the respective protein, and a discoidin domain in *Pf*CCp5, which is located between the anthrax PA N-terminal domain and the LCCL domain (Fig. 1a). Furthermore, two novel N-terminal adhesion domains were identified in *Pf*CCp4, which correspond to ricin domain and anthrax PA N-terminal domain. The various adhesion domains and their potential ligands are discussed as follows.

Expression of *Pf*CCp proteins in sexual stage parasites

Expression profile studies on the *Pf*CCp proteins revealed transcript and protein expression specific to gametocytes within the five developmental stages during maturation (termed stages I–V) [19, 20]. As predicted from the signal peptide sequences, the proteins are secreted and localized to the parasite plasma membrane within the PV. *Pf*CCp1, *Pf*CCp2, and *Pf*CCp3 co-localize in a punctate pattern at the parasite surface, while the surface-associated expression of *Pf*CCp4 is rather homogenous and is thus similar to the expression pattern of *Pf*s230 and *Pf*s48/45. *Pf*CCp5 and *Pf*FNPA are less abundant in gametocytes than the other four *Pf*CCp proteins and in mature gametocytes often localize to the cell poles [20].

After gametogenesis in the mosquito midgut, which involves parasite egress from the host erythrocyte, the *Pf*CCp proteins are present on the cell surface of macrogametes. For *Pf*CCp1, *Pf*CCp2, and *Pf*CCp3, partial release of the proteins was postulated [14]. Expression of the six proteins ceases within a few hours after egress; hence, the *Pf*CCp proteins belong to class I of sexual stage proteins.

To gain information on possible protein functions, we constructed gene-disruptant parasite lines for *Pf*CCp2, *Pf*CCp3, *Pf*CCp4, and *Pf*FNPA, resulting from single-crossover recombination, as well as a double-crossover knock-out mutant for *Pf*CCp1 [14, 20, 21]. Expression profile studies in the mutants (herein referred to as *Pf*CCp-KOs) led to the remarkable observation that the six *Pf*CCp proteins are expressed in a co-dependent manner and that the absence of one protein results in the complete or partial loss of all other protein family members. While the

transcript abundances of intact *PfCCp* genes in the knock-out mutants are comparable to those in wild-type parasites, the proteins are not detectable via Western blot analysis and immunofluorescence assay [21, 22]. Thus, the co-dependent expression manifests at the translational or post-translational level rather than at the transcriptional level of gene expression. The observed co-dependent expression of the *Pf*CCp family led to the assumption that the proteins interact in a way, which ensures localization to and/or retention at the correct expression site and prevents a likely degradation and loss of protein.

Intermolecular binding between *Pf*CCp proteins

Based on the co-dependent *Pf*CCp protein expression, we hypothesized that the six proteins interact in a complex and that instability in the complex due to gene ablation results in co-dependent degradation. By means of co-immunoprecipitation on wild-type gametocyte lysates followed by Western blot analysis, numerous protein–protein interactions between the distinct *Pf*CCp proteins were revealed and interplays on the molecular level were observed between all *Pf*CCp proteins (Fig. 1b) [21]. While no interactions were detectable between *Pf*CCp4 and *Pf*CCp5, the lack of signal for the respective precipitated protein by Western blot analysis is possibly due to the low efficiency of the available antisera against *Pf*CCp4 and *Pf*CCp5.

The molecular interactions between the *Pf*CCp proteins were subsequently studied via affinity chromatography co-elution binding assays, using recombinant proteins comprising selected adhesion domains. The eluted protein complexes were screened by Western blot analyses. Out of 33 combinations investigated, 18 showed adhesive interaction, while 15 recombinant protein pairs did not interact. Adhesion domains that were predominant in protein–protein binding include the LCCL domains of all *Pf*CCp proteins and the SR domains of *Pf*CCp3 (with an involvement of 32% (LCCL) and 19% (SR) of all binding events). Moreover, the ApicA, NEC, and discoidin domains of *Pf*CCp1 and *Pf*CCp2 showed involvement in interactions between *Pf*CCp proteins [21]. Subsequent latex beads-mediated cell binding assays confirmed the interaction of recombinant *Pf*CCp proteins with endogenous proteins on the macrogamete surface. Here, fluorescent latex beads, coated with recombinant proteins corresponding to *Pf*CCp1, *Pf*CCp2, or *Pf*CCp3 domains, bound significantly more often to newly emerged (*Pf*CCp protein-positive) macrogametes than to intraerythrocytic gametocytes, older (*Pf*CCp-negative) macrogametes, and *Pf*CCp3-KO (*Pf*CCp-negative) macrogametes [21].

An interaction of sexual stage adhesion proteins at the gamete plasma membrane leading to a stable protein complex has been shown before for the two TBV candidates *Pf*s48/45 and *Pf*s230 [23, 24]. Here, the GPI-anchored *Pf*s48/45 ensures the retention of *Pf*s230 to the gamete surface. While in the *Pf*s48/45-deficient gametocytes, *Pf*s230 is expressed as in wild-type parasites, it is not present on the surface of gametes after egression from the host cell [25].

New data from our laboratory indicate that the molecular interactions of sexual stage adhesion proteins are more complex than originally expected. We recently detected interactions of select *Pf*CCp proteins with *Pf*s230 and of *Pf*CCp4 with *Pf*s48/45 by co-immunoprecipitation assays on gametocyte lysates, and we further revealed the binding of these proteins with *Pf*s25 following gametocyte activation (Fig. 1b) [20] (N. Simon, A. Kuehn, S.M. Scholz, and G. Pradel, unpublished observation). *Pf*s25 belongs to class II of sexual stage proteins that are translationally repressed in gametocytes, as was shown for *Plasmodium berghei Pb*s25 [26, 27]. However, small amounts of protein can be found in vesicular structures in the maturing gametocytes, and the protein is relocated to the surface of macrogametes within minutes after activation [20].

CCp orthologous proteins

Select LCCL domain proteins, i.e., CCp1, CCp2, and CCp3, are highly conserved throughout the apicomplexan clade, including *Cryptosporidium* spp., *Toxoplasma gondii, Babesia bovis*, and *Theileria annulata*. FNPA was further described for *C. parvum* [14, 28] (reviewed in [29, 30]), and CCp5 was identified in *T. gondii* [II]. A recent genome survey study also identified the CCp family members in the early branching apicomplexan class of gregarines, *Ascogregarina taiwanensis* [31], and thus, the family is likely widely conserved in apicomplexans. The LCCL domain proteins are absent in *Tetrahymena*, for which complete genome sequence is available, and thus, it might be concluded that the acquisition and accretion of the domain architectures was relatively ancient and predates the divergence of the apicomplexan clade, but that the domains entered the apicomplexan lineage after the split of the ciliates [30].

For most of the CCp orthologs, sites of expression in the respective parasite species are yet unknown. New studies in *Babesia divergens* indicate an expression of *Bd*CCp2 in gametocytes within the tick midgut (C. Becker, S. Bonnet, UMR INRA ENVN Nantes, personal communication). In contrast, expression of *Cp*CCp1 of *C. parvum* (also referred to as Cpa135) starts in oocyst-sporozoites and increases rapidly after excystation [32]. In sporozoites, *Cp*CCp1/Cpa135 is seemingly stored in the micronemes and is excreted upon host cell invasion. Subsequently, the protein

localizes to the PV membrane. With the formation of merozoites, new transcript of *Cp*CCp1/Cpa135 is detectable [33]. It is not yet known if the protein is expressed in *Cryptosporidium* gametocytes. Notably, both *Cryptosporidium* sporozoites and *Plasmodium* gametocytes have to resist the gut environment of either mammalian host or mosquito vector.

CCp orthologs are further present in the rodent malaria parasite *P. berghei*, where they are also termed LAP proteins [34, 35]. Transcript analysis revealed a predominant expression in *P. berghei* gametocytes for the six proteins, which was confirmed by transgenic parasites expressing GFP driven by select *Pb*CCp/LAP promoters [36]. Genetic cross studies with female-deficient or male-deficient parasites reported that the functional genes are inherited from female gametocytes, indicating that the *Pb*CCp/LAP proteins are female specific [35]. Recent studies on GFP fusions of *Pb*CCp3/LAP1 (also termed *Pb*SR), *Pb*CCp1/LAP2, and *Pb*CCp5/LAP3 showed protein expression in macrogametocytes and accumulation of these proteins in the crystalloids, organelles that are formed in the ookinete and persist until the early oocyst stage [37–39].

Adhesive modules of CCp proteins

A striking feature of all CCp proteins is the presence of numerous modules, most of which were described to possess adhesive properties in other organisms and were likely acquired by the parasite via lateral transfer and converted into multi-domain adhesive molecules. With the exception of the ApicA and the levanase domains, the adhesion domains of the CCp protein family were previously identified in animal cells and bacteria and assigned to diverse adhesive functions, which are described below.

The eponymous LCCL domain was first described in the horseshoe crab *Limulus*, where it is found in the coagulation factor C, as well as identified in the vertebrate cochlear protein Coch-5b2 and in the mammalian late gestation lung protein Lgl1 [17]. For *Limulus* factor C and Lgl1, a role in antimicrobial defense via recognition of cell-surface carbohydrates of pathogens has been proposed [17]. The LCCL domain has been found in association with a number of other adhesion domains, e.g., complement B-type domains, C-type lectin domains, von-Willebrand factor A domains, or discoidin domains [17].

The prototypic discoidin domain is derived from the cell adhesion protein discoidin of the slime mold *Dictyostelium discoideum*. The domain is found in the coagulation factors V and VIII, where it promotes phospholipid binding on the surface of platelets and endothelial cells [15, 40]. Pentraxins, on the other hand, are a family of evolutionarily conserved pattern-recognition proteins that are made up of five identical subunits. Proteins of the pentraxin family are involved in acute immunological responses and often represent serum proteins. They play a role in the binding of lipoproteins and carbohydrates [41–44] and are reported to interact with other pentraxin domains as well as with complement control protein modules (also called sushi domains) [45]. Interestingly, pentraxin 3 is essential for female fertility, participating in the assembly of the cumulus oophorus extracellular matrix [46].

The LH2 lipoxygenase domain is found in a variety of membrane- or lipid-associated proteins [47], and it is suggested that the domain mediates membrane attachment via other protein binding partners. Interactions with lectin domains and other lipoxygenase domains were predicted [III]. Fibronectin, on the other hand, is a multi-domain glycoprotein found in the plasma as well as in basement membranes, where it exhibits functions in wound healing and blood coagulation, as well as in cell adhesion and migration [48]. The FN2 domain is predicted to interact with EGF domains, lectin C, and ricin domains [IV].

Ricin is a legume lectin from seeds of the castor bean plant, *Ricinus communis*. The ricin B lectin domain, present in CCp1 and CCp2, binds to carbohydrates [49]. Interactions with FN2 domains, lectins, and other ricin domains were predicted [V]. The proteins CCp1, CCp2, and CCp4 also contain a pair of levanase-type lectin domains, which are found in secreted glucosidases and levanases of *Bacillus subtilis* and proposed to be involved in sugar binding [28].

CCp4, CCp5, and FNPA share a domain termed PA (protective antigen) that is proposed to have affinity with a domain described in *Bacillus anthracis*. This bacterial pathogen produces a toxin composed of three distinct proteins, i.e., protective antigen, edema factor, and lethal factor. PA is secreted in a precursor form, which can heptamerize and form a channel in membranes, allowing the other two factors to enter the target cell. The anthrax PA N-terminal domain is reported to be involved in carbohydrate binding [50]. Perhaps the best described domain of the CCp family is the scavenger receptor (SR) domain of CCp3. SR is found in several extracellular receptors, like the macrophage scavenger receptor type I. The domain is involved in protein–protein interactions and also interacts with other SR domains [45, 51, 52].

As mentioned above, the ApicA domain is an apicomplexan-specific cysteine-rich module. The NEC domain, on the other hand, appears in a wide range of animal proteins in combination with other cysteine-rich segments [53, 54], such as neurexins [18], fibrillar collagen α globular domain [55] from vertebrates and sponges and the fibrinogen family of proteins [53]. The NEC domain is also known as fibrinogen C domain, which represents the C-termini of fibrinogen ß and γ chains and which is predicted to interact,

among others, with fibronectin type 3, laminin, discoidin, and other fibrinogen C domains [VI]. Fibrinogen is a dimeric protein involved in blood clotting and platelet aggregation [54, 56]. When the *Pf*CCp protein family was described in 2004 [14], it was the first instance where the NEC domain was reported as a stand-alone form. Several animal proteins functioning as lectins contain the NEC domain, such as ficolin and intelectin [57, 58], suggesting that it is involved in sugar binding.

Multi-protein complexes of malaria parasites

Multi-protein complexes play important roles in regulating the activity and stability of polypeptides. Moreover, protein complexes function in establishing cell–cell contacts and thus are of great importance for interactions between pathogens and host cells. For intracellular pathogens, such as the majority of apicomplexan parasites, recognition, adhesion, and invasion of host cells are essential steps during infection by zoite stages (sporozoites, merozoites, bradyzoites, tachyzoites) and are often mediated by protein–protein interactions. Examples of apicomplexan multi-protein complexes are summarized in Table 1.

One well-studied example of an apicomplexan multi-protein complex is represented by protein interactions comprising the so-called glideosome that has been described in *T. gondii* and several *Plasmodium* species (e.g., [59–63], reviewed in [64, 65]. This striking protein network mediates gliding motility, which is required for the movement of the parasite along host tissue barriers and for host cell invasion. A group of transmembrane proteins belonging to the TRAP family plays a key role within the glideosome due to their transmembrane linkage of the intracellular actin-myosin motor of the infective parasite stages with the host cell surface. The *P. falciparum* TRAP family includes five members, namely the sporozoite micronemal proteins *Pf*TRAP (thrombospondin-related anonymous protein; [66]), *Pf*TLP (TRAP-like protein; [62, 67]), and *Pf*TREP (TRAP-related protein; [68]) as well as the ookinete-specific micronemal protein *Pf*CTRP (CSP and TRAP-related protein; [69]) and the merozoite-specific protein *Pf*MTRAP [60]). Unifying features of *P. falciparum* TRAP proteins include a transmembrane domain, a short acidic cytoplasmic tail with one or more aromatic residues near the C-terminus, an extracellularly exposed array of adhesive domains comprised of type-1 thrombospondin-like domains and, with the exception of TLP, von-Willebrand factor A domains. When secreted by the zoite micronemes, the proteins become integrated into the apical surface of the parasite and bind to host cell receptors. For *Pf*TRAP, *Pf*MTRAP, and *Pf*TLP, it was shown that they are connected with the intracellular actin-myosin motor complex of the parasite, which is located between the parasite outer and inner membrane, by interacting with the adapter protein aldolase [59–62] (reviewed in [65]). The motor complex consists of actin and the class XIV myosin MyoA and features several additional proteins; for instance, the glideosome-associated proteins of merozoites, GAP45, and GAP50 that in concert with *Pf*MTIP (myosin tail-interacting protein) link MyoA to the inner membrane complex [59, 60, 70] (reviewed in [65]).

Similar glideosome structures were described in *T. gondii*, where the MIC (micronemal) proteins represent major key players and, indeed, much of the mechanistics were worked out using the experimental system of this pathogen. The TRAP family protein *Tg*MIC2 is responsible for linking receptors of the host cell surface with the intracellular motor complex of tachyzoites via aldolase binding [71]. *Tg*MIC2 forms a hexameric complex with *Tg*M2AP, which might be necessary for *Tg*MIC2 transport and is considered prototypical of other TRAP family members [72]. Further members of the *T. gondii* MIC family are the soluble *Tg*MIC1 and *Tg*MIC4. The two proteins interact with *Tg*MIC6 that acts as an escorter to ensure correct targeting of both proteins to the micronemes [73]. *Tg*MIC8 appears to escort the soluble adhesin *Tg*MIC3 [74]. Furthermore, *Tg*MIC6 interacts with aldolase, pointing at an involvement in host cell invasion [75]. Noteworthy, orthologs for most glideosome components were recently also identified in most other apicomplexan parasites (reviewed in [76]), including their recent discovery in *Eimeria tenella* [77]. The MIC orthologs *Et*MIC4 and *Et*MIC5 also aggregate to a multimeric protein complex [78].

Another extensive complex involved in merozoite attachment is constituted by the *P. falciparum* MSP (merozoite surface protein) group. The majority of these peripheral proteins are secreted into the PV of schizonts and subsequently binds to the surface of developing merozoites via interaction with GPI-anchored proteins such as *Pf*MSP1, which exhibits two EGF-like domains [79]. Surface-associated proteins include the MSP3/6 group, the MSP7 family as well as *Pf*41, a member of the cysteine-rich motif superfamily (reviewed in [76]). This adhesive MSP-based multi-protein complex, which consists of multiple secreted proteins assembling around a GPI-anchored EGF-domain protein, shows superficial similarities with the here discussed cell surface-associated *Pf*CCp multi-protein complex.

During invasion, a focal adhesion to the host cell, referred to as tight junction or moving junction, is established in order to enable the movement of the apicomplexan parasite toward the interior of the host cell [80]. In *T. gondii*, this attachment is mediated by a protein complex comprised of the highly conserved micronemal apical

Table 1 Constituents of multi-protein complexes in *Plasmodium falciparum*, their known or proposed functions, respective orthologs in other apicomplexan species, life-cycle expression, and gene identifier

Complex name and constituents	Properties/Features	Life-cycle stage	Function	Gene ID[a] in *P. falciparum*	Apicomplexan homologs	References
Glideosome motor complex						
TRAP family proteins	Microneme-derived, TM protein	M, O, Sp	Host cell receptor binding, binding to cytosolic aldolase	*Pf*MTRAP PF10_0281 *Pf*TRAP PF13_0201 *Pf*TLP PFF0800w	*Pssp* (*Pf*TREP, *Pf*CTRP), *Tg* (*Tg*MIC2), *Nc* (*Nc*MIC2), *Et* (*Et*p100/ *Et*MIC1), *Cp* (*Cp*TRAP-C1), *Ta*[d], *Bbo*[d]	[60, 62, 68, 127–134]
*Pf*Aldo	Cytosolic, tetrameric glycolytic enzyme, binds to cytosolic tail of *Pf*TRAP and to F-actin	ABS, O, Sp	Linkage of extracellular adhesin to actin	PF14_0425	*Pssp*, *Tg* (*Tg*ALD1), *Nc*[d], *Cp*, *Ta*, *Et*, *Bbi*	[60, 71, 135–137]
*Pf*ActI	Formation of actin filaments, regulated by actin-binding proteins (e.g., *Pf*Formin1 and *P. falciparum* profilin)	M[b], T[b], Gc[b], Sp[b]	Gliding motility and invasion	PFL2215w	All Apicomplexa	[60, 138, 139] (reviewed in [140])
Pf-MyoA	ATP-binding, binding to *Pf*MTIP	M, O, Sp	Driving force in gliding motility and invasion	PF13_0233	*Pssp*, *Tg*, *Nc*[d], *Cp*, *Ta*, *Et*, *Bbi*	[60, 141–144] (reviewed in [145])
*Pf*MTIP	Binds to *Pf*GAP45, *Pf*GAP50, and tail of *Pf*-MyoA	M, O, Sp	Linkage of motor complex to inner membrane complex	PFL2225w	*Pssp*, *Tg* (MLC1), *Nc*[d], *Cp*, *Ta*, *Et*, *Bbi*	[59, 60, 70]
*Pf*GAP45	Binds to *Pf*GAP50 and *Pf*MTIP	M, O, Sp	Linkage of motor complex to inner membrane complex	PFL1090w	*Pssp*, *Tg*, *Nc*[d], *Cp*, *Ta*, *Et*, *Bbi*	[60, 146]
*Pf*GAP50	Integral membrane protein of inner membrane complex, binds to GAP45 and *Pf*MTIP	M, O, Sp	Linkage of motor complex to inner membrane complex	PFI0880c	*Pssp*, *Tg*, *Nc*[d], *Cp*, *Ta*, *Et*, *Bbi*	[60, 146]
MSP-1 based complex						
*Pf*MSP1	GPI-anchored merozoite surface protein, fragments released upon proteolytical cleavage during invasion	M	Merozoite invasion (initial attachment[c])	PFI1475w	*Pssp*	[79, 147, 148]
*Pf*MSP3 *Pf*MSP6 *Pf*MSP7	Soluble secreted proteins, association with merozoite surface, binding to MSP-1	M	Merozoite invasion (initial attachment[c])	*Pf*MSP3 PF10_0345 *Pf*MSP6 PF10_0346 *Pf*MSP7 PF13_0197	None[d]	[149–152]
*Pf*41	Soluble secreted protein, possession of two cysteine-rich domains, binding to MSP-1	ABS[b]	Merozoite invasion (initial attachment[c])	PFD0240c	*Pssp*[d]	[153]

Table 1 continued

Complex name and constituents	Properties/Features	Life-cycle stage	Function	Gene ID[a] in *P. falciparum*	Apicomplexan homologs	References
Moving junction complex						
*Pf*AMA1	Microneme-derived, TM protein	M, Sp	Mediating rhoptry secretion during RBC invasion[c]	PF11_0344	*Pssp*, *Tg*, *Nc*, *Tspec*[d], *Et*, *Bbo*[d]	[77, 81, 154–156]
*Pf*RON2 *Pf*RON4 *Pf*RON5	Intermolecular binding, binding to *Pf*AMA1	M	Mediating rhoptry secretion during RBC invasion[c]	PfRON2 PF14_0495 PfRON4 PF11_0168_v5.5 PfRON5 MALBP1.73	*Pssp*, *Tg*, *Nc*[d,e], *Tspec*[d], *Et*, *Bbo*[d], *Cm*[d,f]	[77, 82, 83, 88, 89]
Rhop complex						
*Pf*RhopH1 *Pf*RhopH2	Intermolecular binding of *Pf*RhopH1/2/3, RhopH complex locates to newly formed PV upon invasion, transported to RBC cytosol	ABS	RBC invasion, cytoadherence	*Pf*RhopH1 PFB0935w (clag2) MAL7P1.229 (clag8) PFC0120w (clag3.1) PFC0110w (clag3.2) PFI1730w (clag9) *Pf*RhopH2 PFI1445w	*Pssp*	[157–159]
*Pf*RhopH3	Binding to *Pf*RAMA, RBC membrane, see RhopH1	ABS	RBC invasion, cytoadherence	PFI0265c	*Pssp*	[90, 158]
*Pf*RAMA	TM protein in rhoptry membrane, peptide p60 binds to RBC membrane upon discharge	ABS	Trafficking/retention of RhopH proteins and RAP proteins to rhoptries[c], rhoptry biogenis[c], RBC invasion	MAL7P1.208	*Pssp*	[90, 101, 160]
*Pf*RAP1	Binding to *Pf*RAMA and *Pf*RAP2, *Pf*RAP3, binding to RBC surface	M, Sch	Merozoite invasion	PF14_0102	*Pssp*, *Tr*[d]	[99, 161]
*Pf*RAP2 *Pf*RAP3	Binding to *Pf*RAP1, binding to RBC surface, homologous proteins	M, Sch	Merozoite invasion	*Pf*RAP2 PFE0080c *Pf*RAP3 PFE0075c	*Pssp* (here, only one RAP2/RAP3 protein present)	[99, 100, 161, 162]
Knob complex						
*Pf*EMP1	TM protein, binding to host cell receptors (e.g., CD36, ICAM-1, CSA)	ABS	Cytoadherence, immune evasion, immunomodulation	~60 *var* genes	*Pk*	[163–166]

Table 1 continued

Complex name and constituents	Properties/Features	Life-cycle stage	Function	Gene ID[a] in *P. falciparum*	Apicomplexan homologs	References
KAHRP	Binding to cytosolic tail of *Pf*EMP1, actin, spectrin, ankyrin, self-association	T, Sch	Knob formation, linkage to host cell cytoskeleton	PFB0100c	*Pv*[d], *Pk* (fragment)[d]	[109, 110, 113, 114, 167]
*Pf*EMP3	Binding to spectrin	T	Alteration of RBC membrane deformability[c] and Maurer's clefts morphology[c]	PF07_0026	*Pssp*, *Tg*, *Nc*[d], *Tspec*[d], *Bbo*[d], *Tt*[d]	[168–171]
MESA/*Pf*EMP2	Binding to protein 4.1R	T, Sch	Alteration of RBC membrane deformability[c]	PFE0040c	All apicomplexa[d]	[167, 172, 173]
*Pf*322	Association with Maurer's clefts, possession of DBL domain, TM protein	T, Sch	*Pf*EMP1 trafficking[c], Maurer's clefts morphology[c], RBC membrane deformability[c]	PF11_0506	All apicomplexa[d]	[174–177]
Sexual stage surface complex						
*Pf*CCp protein family	Association with plasma membrane, partial release upon gametogenesis, possession of multiple adhesion domains, intermolecular binding between all *Pf*CCp proteins	Gc, Gm	Adhesive properties	*Pf*CCp1 PF14_0723 *Pf*CCp2 PF14_0532 *Pf*CCp3 PF14_0067 *Pf*CCp4 PFI0185w *Pf*CCp5 PFA0445w *Pf*FNPA PF14_0491	*Pssp*, *Tg*, *Nc*[d], *Ta*, *Bbo*, *Cssp, At*	[14, 31, 178]
*Pf*s48/45	Surface expression, linkage to plasma membrane via GPI anchor, possession of cysteine-rich domains, binding to *Pf*s230, *Pf*CCp4	Gc, Gm	Fertilization of macrogametes by microgametes	PF13_0247	*Pssp*	[20, 23, 24, 179]
*Pf*s230	Association with plasma membrane, possession of multiple cysteine-rich domains, fragments released upon proteolytical cleavage during invasion, binding to *Pf*s48/45, *Pf*CCp1, *Pf*CCp2, *Pf*CCp3, *Pf*CCp4	Gc, Gm	Binding of exflagellating microgametes to RBCs, oocyst formation[c]	PFB0405w	*Pssp*	[20, 23–25][g]

Table 1 continued

Complex name and constituents	Properties/Features	Life-cycle stage	Function	Gene ID[a] in *P. falciparum*	Apicomplexan homologs	References
*Pf*s25	Surface expression, linkage to plasma membrane via GPI anchor, possession of EGF domains, binding to *Pf*CCp1, *Pf*CCp2, *Pf*CCp3	Gm, Z, O	Binding to laminin (*Pb*), calreticulin (*Pv*), protection during ookinete development[c], traversal of midgut epithelium[c] (*Pb*)	PF10_0303	*Pssp*	[180–182][g]

ABS Asexual blood stages, *At Ascogregarina taiwanensis*, *Bbo Babesia bovis*, *Bbi Babesia bigemina*, *Cp Cryptosporidium parvum*, *Cm Cryptosporidium muris*, *Cssp Cryptosporidium subspecies*, *Et Eimeria tenella*, *Gc* Gametocyte, *Gm* Gamete, *M* Merozoite, *Nc Neospora caninum*, *O* Ookinete, *Sch* Schizont, *Pb Plasmodium berghei*, *Pk Plasmodium knowlesi*, *Pr Plasmodium reichenowi*, *Pssp Plasmodium subspecies*, *Pv Plasmodium vivax*, *Sp* Sporozoite, *T* Trophozoite, *Ta Theileria annulata*, *Tt Tetrahymena thermophila*, *Tg Toxoplasma gondii*, *TM* Transmembrane, *Tspec Theileria species*, *Z* Zygote

[a] Gene identifier of PlasmoDB [I]

[b] According to mass spec data from PlasmoDB [I]

[c] Proposed function

[d] According to OrthoMCL DB [VII]

[e] Only ortholog of *Pf*RON2

[f] Only ortholog of *Pf*RON4

[g] Unpublished observations of the authors

membrane antigen 1 (AMA1) [81], which is inserted into the parasite plasma membrane, and of the four rhoptry neck proteins RON2/4/5/8 [82, 83]. Recent data led to the provocative hypothesis that the receptor for *Tg*AMA1 located at the host cell surface is provided by *Tg*RON2, which upon secretion becomes inserted into the host cell plasma membrane, whereas *Tg*RON4, *Tg*RON5, and *Tg*RON8 locate to the cytosolic face of the host cell [83, 84]. Although the intracellular motor complex had been proposed to associate with the site of the tight junction, direct interaction partners have not been identified [85]. An additional role of the moving junction complex might be the exclusion of certain host cell plasma membrane proteins from the newly formed PV [86, 87]. AMA1 and RON orthologs are also present in other apicomplexan parasites [77, 84, VII]. In *P. falciparum*, the *Pf*RON2, *Pf*RON4, *Pf*RON5, and *Pf*AMA1 have been proposed to form a multimeric complex [88, 89]. *Pf*AMA1 is translocated to the merozoite surface before invasion (reviewed in [76]). A recent study suggests a role after reorientation and establishment of the tight junction, which succeed the initial weak contact between merozoite and red blood cell (RBC). Therefore, *Pf*AMA1 might be involved in the secretion of rhoptry contents which presumably form the nascent PV [89].

Another rhoptry-derived protein complex crucial for parasite survival is the Rhop complex of *P. falciparum*, which features two subgroups of protein associations, referred to as *Pf*RhopL and *Pf*RhopH complexes (low and high molecular weight rhoptry protein complex, respectively, [90]). *Pf*RhopH complex is formed by the non-covalent-associated polypeptides *Pf*RhopH1 (155 kDa), *Pf*RhopH2 (140 kDa), and *Pf*RhopH3 (110 kDa) [91, 92]. *Pf*RhopH1 is encoded by one gene of the *rhoph1/clag* gene family (named after the cytoadherence-linked asexual gene, *clag9*, [93]) comprised of five polymorphic paralogous genes, while *Pf*RhopH2 and *Pf*RhopH3 are encoded by a single gene [94, 95]. The binding of the *Pf*RhopH complex to the erythrocyte membrane is essential for merozoite invasion as in vitro studies showed, employing inhibitory antibodies raised against *Pf*RhopH3 as well as *Pf*RhopH3 peptides masking the putative receptor at the RBC surface [90, 96, 97]. In addition to cytoadherence, the *Pf*RhopH complex is also proposed to participate in PV formation (reviewed in [98]). The low molecular weight *Pf*RhopL complex comprises rhoptry-associated proteins such as *Pf*RAP1 (83 kDa), *Pf*RAP2 (40 kDa), and *Pf*RAP3 (37 kDa) [99], and likewise *Pf*RhopH complex binds to the erythrocyte cell surface [100]. Both *Pf*RhopH and *Pf*RhopL complexes bind non-covalently to the rhoptry-associated membrane antigen (RAMA), which is anchored to the inner face of the rhoptry membrane via a GPI tail, thereby mediating the retention of the protein complexes prior to

rhoptry discharge [90, 101]. Both RhopH and RhopL complex constituents have been shown to possess homologs in several *Plasmodium* species [102–104], but not in other apicomplexan parasites [88]. However, RhopH1/Clag exhibits a region homologous to RON2, suggesting a common function of this region within the respective protein complex [88].

A prominent multimeric protein association modifying a host cell membrane is responsible for the formation of knob-like protuberances of the erythrocyte membrane after infection of malaria parasites (reviewed in [105, 106]). Knob formation in erythrocytes is limited to infections by *P. falciparum* and underpins virulence mechanisms causing the symptoms of severe malaria. Erythrocyte knobs mediate cytoadherence of these cells to a variety of host cell receptors, among them CD36 and ICAM-1 of endothelial cells, leading to clotting and capillary damage and subsequently resulting in organ failure (reviewed in [107]). Fundamental for knob formation is the knob-associated histidine-rich protein KAHRP, which is deposited at the erythrocyte plasma membrane facing the cytoplasm [108, 109]. KAHRP seems to assist the presentation of *Pf*EMP1 (erythrocyte membrane protein 1) via binding to the *Pf*EMP1 cytosolic region [110]. *Pf*EMP1 is an antigenically highly diverse transmembrane protein comprised of multiple adhesion modules that enable the protein to bind to the different types of host cell receptors (reviewed in [111, 112]). KAHRP further interacts with actin and spectrin, thereby establishing a linkage of the knobs with the erythrocyte cytoskeleton [113, 114]. Components of the erythrocyte cytoskeleton in turn interact with further parasite-derived proteins such as *Pf*EMP3, mature-parasite-infected erythrocyte surface antigen (MESA), and *Pf*322, which presumably alter the deformability of the cytoskeleton (reviewed in [106]).

Multimeric complexes of sexual stage parasites: which role do they play?

Initially phenotypic studies on *Pf*CCp-KO lines were performed in order to reveal protein function. It was anticipated that a knock-out phenotype would manifest at the gametocyte stages, thus mirroring the stage specificity of *PfCCp* transcript and protein expression. Surprisingly, despite the loss of all *Pf*CCp proteins in the respective *Pf*CCp-KO lines, all lines exflagellated upon gametocyte activation and apparently normal ookinete and oocyst formation was observed [14, 20, 21] (A. Kuehn, G. Pradel, unpublished observations). In *Pf*CCp2- and *Pf*CCp3-KO mutants, however, no sporozoites were detected in the salivary glands. *Pf*CCp4-KO parasites, on the other hand, did not reveal any differences in the mosquito-specific development compared to wild-type parasite [20], and no phenotype analyses on mosquito-specific parasites stages have so far been performed for *Pf*CCp1-KO and *Pf*FNPA-KO. Hence, *Pf*CCp2-KO and *Pf*CCp3-KO parasites appeared to be blocked in the transition of midgut sporozoites to the salivary glands [14], and thus, the respective knock-out phenotype for these mutants manifested in the blockage of sporogonic mosquito-specific parasite stages, in which *Pf*CCp proteins were not present.

Similar phenotypes were observed, when the orthologous genes were knocked out in *P. berghei*. Loss of function mutants were generated for *Pb*CCp1/LAP2, *Pb*CCp2/LAP4, *Pb*CCp3/LAP1, and *Pb*CCp4/LAP6 [35] as well as for two double knock-out mutants, *Pb*CCp1/CCp3 and *Pb*CCp1/CCp4 [36]. For all mutants, the formation of sporozoites in the midgut oocysts of parasite-infected mosquitoes was aborted [35, 36]. Interestingly, sporulation of *Pb*CCp3/LAP1 (termed *Pb*SR in this study) appeared to be normal in in vitro assays, pointing to the involvement of mosquito factors in the loss-of-function phenotype of this protein [38].

Up to date, there is no explanation for the obvious difference in the stage of expression and stage of knock-out phenotype for the *Pf*CCp proteins. It might represent a delayed phenotypic effect, in which reduced fertilization events result in a lower number of ookinetes and subsequently oocysts, in turn leading to a diminishing number of salivary gland sporozoites. An alternative explanation is offered by the observation that select *Pb*CCp/LAP proteins of *P. berghei* associate with the crystalloid, a cytoplasmic aggregation in the developing ookinete [37–39, 115], which has been suggested to serve as storage for protein required during oocyst maturation [116, 117]. *Pb*CCp3/LAP1 (*Pb*SR) seems to play a role in crystalloid formation leading to the assumption that the impaired sporulation of *Pb*CCp-KO mutants results from the absence of crystalloids [38]. Notably in this context, both the LCCL domain protein family and the ookinete crystalloid are inventions specific to the apicomplexan clade.

In view of our current data and despite the observed delayed knock-out phenotypic effect, we presume that the *Pf*CCp functions lie in mediating molecular interplay during sexual reproduction. We hypothesize that the *Pf*CCp proteins assemble to surface-associated multimeric protein complexes via intermolecular binding between select adhesion motifs. The secreted *Pf*CCp proteins, together with *Pf*s230, are kept to the gametocyte surface by interacting with the GPI-anchored *Pf*s48/45 and, after activation, with *Pf*s25 to the macrogamete surface (Fig. 1b). Such assembly of secreted adhesion proteins around a GPI-anchored EGF-domain protein would be analog to the MSP-based multimeric complexes on the surface of merozoites.

The *Pf*CCp complexes might be involved in important adhesive processes of macrogametes during sexual reproduction and transmission. On the one hand, the complexes might play a role in promoting contact between the emerging gametes within the blood meal. The physical contact between mating gametes is a requirement to initiate fertilization but can represent a major challenge for midgut parasites under natural conditions due to the low number of gametocytes taken up by the mosquito [118]. For example, the complexes might support the binding of macrogametes to microgametes due to interaction of select *Pf*CCp proteins with *Pf*s230 or *Pf*s48/45 on the microgamete surface. In this context, recent studies described the identification of the microgamete protein GCS1/HAP2 in *P. berghei* that enables gamete fusion, but not initial binding between the two mating partners, which appears to involve other adhesion proteins [119, 120].

On the other hand, the complexes might mediate the protection of the exposed macrogametes from the aggressive environment of the mosquito midgut by forming a barrier between parasite and midgut content. During egress from the host erythrocyte, the emerging gametes become vulnerable to the factors of the mosquito midgut, resulting in an approximately 300-fold loss in parasite number between gametocytes and ookinetes [121]. Harmful factors of the midgut content include digestive enzymes, midgut bacteria as well as potential immune defense molecules of the mosquito. It was recently shown that a subset of immune genes become upregulated by the mosquito microbial flora, which then reduce the infection level of malaria parasites [122]. Of particular interest is an immune gene family of fibrinogen-related proteins (termed FREP), which is upregulated after challenge with bacteria or malaria parasites. One family member, FBN 9, interacts with gut bacteria as well as with rodent and human malaria parasites in the mosquito midgut epithelium [123]. Further harmful components of the blood meal include human antibodies and complement proteins. Noteworthy, complement of the alternative pathway can be active for several hours post-feeding, as was shown in rodent and avian malaria models [124–126]. The predicted binding of selected sexual stage protein-specific adhesion domains with complement control protein modules might provide a key to identifying potential protection mechanisms of the sexual stage parasite.

Concluding remarks

Apicomplexan parasites exhibit a variety of life-cycle stage-dependent protein complexes, all of which are inventions specific to the clade. Proteins of these complexes either show lineage specificity, like the Rhop proteins of the malaria parasite erythrocytic stages, or possess adhesion motifs of wider conservation, as found in the TRAP and the CCp families, which were acquired by the pathogen via lateral gene transfer. The multi-protein complexes are utilized by the parasite for the different types of intercellular contact, ranging from attachment to and gliding on target cells to host cell invasion and cyto-adherence of infected erythrocytes. While the majority of multimeric complexes are so far described for the infective parasite stages, a new type of complex involving the *Pf*CCp protein family was recently identified in the sexual stages of the malaria parasite *P. falciparum*. The complex-forming proteins support the adhesiveness of macrogametes, but their detailed functions remain elusive. Follow-up studies will reveal if these complexes mediate intercellular contact, the way it was described for the complexes of other life-cycle stages, and which receptors they would use for these binding events. Another aspect worth investigating is the possible connection of these adhesion proteins to intracellular complexes by yet unknown linker proteins, thereby either participating in motility or in protein trafficking and/or re-cycling. Whichever role the complex-forming *Pf*CCp proteins play for the malaria parasite sexual stages, the evolutionary conservation of the numerous adhesion modules and architectures of those proteins strongly argues that the proteins exhibit similar functions in other apicomplexan species.

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