Signal-mediated export of proteins from the malaria parasite to the host erythrocyte

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Intracellular parasites from the genus *Plasmodium* reside and multiply in a variety of cells during their development. After invasion of human erythrocytes, asexual stages from the most virulent malaria parasite, *P. falciparum*, drastically change their host cell and export remodelling and virulence proteins. Recent data demonstrate that a specific NH$_2$-terminal signal conserved across the genus *Plasmodium* plays a central role in this export process.

Introduction

Malaria is caused by members of the genus *Plasmodium*, which belongs to the large monophyletic assemblage of parasitic organisms that make up the phylum Apicomplexa. This phylum includes a number of other pathogens, such as *Cryptosporidium*, *Toxoplasma*, and *Theileria*, and it is distinguished in morphological terms by the presence of a specialized apical organellar complex for host cell invasion. The genus *Plasmodium* belongs to the class of the Hemosporidia and is comprised of obligate intracellular parasites switching between an arthropod vector and a vertebrate host, where they undergo cycles of asexual reproduction in blood cells. *Plasmodium falciparum* causes the most severe form of malaria in humans, with several hundred million infections each year and 1–2 million deaths (Carter and Mendis, 2002; Snow et al., 2005).

Most of the clinical symptoms and the associated mortality and morbidity of *P. falciparum* malaria are a result of the parasite undergoing continuous cycles of asexual reproduction within human RBCs. Infected RBCs (iRBCs) become rigid and develop the ability to cytoadhere to a number of cell types such as vascular endothelial cells, a mechanism that prevents the parasitized host cells from passing through to the spleen, where they would be cleared from the blood stream. These cellular modifications of iRBCs are the result of a dramatic remodelling process induced by the parasite that ultimately serves to induce cytoadherence by exposing various ligands on the iRBC surface for host cell receptors and facilitate nutrient import into the infecting parasite.

Plasmodium remodels the host RBC

Like *Toxoplasma*, *Plasmodium* parasites actively invade their host cells rather than being phagocytozed. They are transmitted to their vertebrate host when the mosquito vector takes a blood meal and releases sporozoite stages into the bloodstream. Sporozoites invade liver cells, where they undergo asexual replication to produce thousands of merozoites, which are competent to invade RBCs. The asexual cycle within the RBC takes 44–48 h, with subsequent bursting of the host cell, release of 16–32 merozoites, and reinvasion being reflected in the periodical reoccurring waves of fever during the development of the disease.

During invasion of erythrocytes (as well as hepatocytes), the parasites become enclosed within an additional membrane layer, the parasitophorous vacuole membrane (PVM), which acts as a semipermeable barrier between parasite and host, allowing for nutrient acquisition and secretion of parasite-derived factors. In early intraerythocytic stages (ring stages), the parasite initiates the development of membrane structures in the anucleated erythrocyte, which is naturally devoid of any endomembranes. This includes a tubulovesicular network, which appears to be interconnected with the PVM, and sheathlike structures underlying the iRBC membrane, termed Maurer's clefts (Fig. 1; Haldar et al., 2002). Several parasite-derived proteins such as spectrin-binding protein 1 (Blisnick et al., 2000), membrane-associated histidine-rich protein (Spycher et al., 2003), and ring-expressed antigen-1 (Hawthorne et al., 2004) have been localized to these structures.

When the parasite transforms into a trophozite stage, which occupies ∼40% of the iRBC volume, the surface of the host cell becomes decorated with thousands of small punctate structures (the knobs) where knob-associated histidine-rich protein (KAHRP) and parasite-derived surface receptors such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) are concentrated (Kilejian, 1979; Pologe et al., 1987; Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). PfEMP1 mediates cytoadherence of iRBCs to various host cell receptors, and it is considered the major virulence factor of *P. falciparum*. Other exported parasite surface proteins such as members of the Rifin surface antigen family do not appear to be
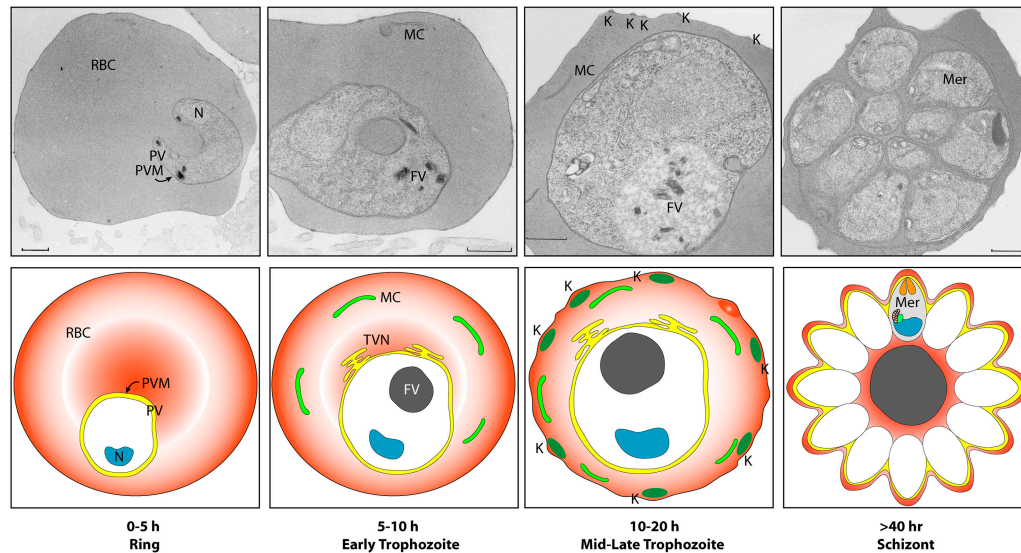
Figure 1. Asexual development of malaria parasites in the iRBC. 0–5 h: after invasion, parasites become surrounded by a parasitophorous vacuole (PV) and are visible as ring-like structures within the red blood cell (RBC; ring stage). 5–10 h: the ring-stage parasite induces membranous extensions of the parasitophorous vacuole membrane (PVM) into the host cell, which forms a tubulovesicular network (TVN) and RBC membrane–tethered Maurer's clefts (MCs). The products of hemoglobin digestion become visible with the deposition of hemozoin crystals in the food vacuole (FV). 10–20 h: as the parasite progresses to the trophozoite stage, it continues to increase its volume considerably and induces knob (K) formation on the iRBC surface. >40 h: after invasion, the parasite starts several rounds of asexual division, resulting in 16–32 daughter merozoites, which are still surrounded by the PVM until they burst out and invade uninfected RBCs. Mer, merozoite; N, nucleus. Bars, 0.5 μm.

restricted to knobs. Ring-infected erythrocyte surface antigen (RESA) and PfEMP3, on the other hand, are associated with the RBC cytoskeleton underlying the plasma membrane (Culvenor et al., 1991; Pasloske et al., 1993; Kyes et al., 1999). Although the expression of most exported parasite proteins in the host RBC peaks in early asexual stages, some are expressed even before the invasion of merozoites into an uninfected RBC. RESA, for example, is produced in the final stages of schizont development and is stored in apical organelles when the emerging merozoites are formed. After RBC invasion, the protein is released into the newly formed parasitophorous vacuole and is finally exported into the host cell, where it appears to associate with the red cell membrane skeleton (Foley et al., 1991; Rug et al., 2004).

A novel signal mediates protein export in *P. falciparum*

Toxoplasma gondii and *P. falciparum* secrete proteins into the surrounding vacuole or vacuole membrane. For example, exported protein 1 and members of a family of small membrane-bound proteins, the early transcribed membrane proteins, are localized to the PVM in *P. falciparum* (Ansorge et al., 1997; Spielmann et al., 2003). The NH$_2$-terminal hydrophobic sequence that directs these proteins into the secretory system may be located close to the NH$_2$ terminus as in other eukaryotes or recessed by up to 80 amino acids (for review see Cooke et al., 2004). In the absence of other signals, the parasitophorous vacuole appears to represent the default destination for secreted proteins in both *T. gondii* and *P. falciparum* (DeRocher et al., 2000; Waller et al., 2000; Wickham et al., 2001). Proteins are exported beyond the PVM to sites in the RBC cytoplasm or RBC membrane in a range of *Plasmodium* species. This export

process probably proceeds via a selective transporter in the PVM with the parasitophorous vacuole as an intermediate compartment. Recent studies using a GFP reporter system demonstrated that the signal sequences of KAHRP and histidine-rich protein II are necessary and sufficient for secretion into the parasitophorous vacuole and that additional targeting information for translocation through the PVM must be located within 60 amino acids downstream of the signal sequence (including a histidine-rich domain; Wickham et al., 2001; Lopez-Estrano et al., 2003).

Two independent studies have now shown that a short peptide is necessary for protein export into the host RBC in *Plasmodium* (Hiller et al., 2004; Marti et al., 2004). This motif, termed PEXEL (*Plasmodium* export element; Marti et al., 2004) or HT (host targeting) **s**ignal (Hiller et al., 2004), with the consensus R/KxLxE/Q, is located 15–20 amino acids downstream of the NH$_2$-terminal hydrophobic signal sequence and mediates export of proteins across the PVM. Site-directed mutagenesis demonstrated that positions 1, 3, and 5 of the motif were equally important for export of a reporter. However, the involvement of surrounding amino acids could not be ruled out because the motif has not been embedded into a completely heterologous context (Marti et al., 2004). Intriguingly, proteins embedded in RBC membranes, including the major virulence factor PfEMP1 as well as Rifin and Stevor antigens, show high conservation of the export motif as well, indicating that they utilize the same export pathway as soluble proteins. However, recent data have suggested that PfEMP1 is not exported by vesicular trafficking through the RBC cytosol (Knuepfer et al., 2005b; Papakrivos et al., 2005), raising the possibility that the protein already achieves a soluble state before translocation through the PVM.

Surprisingly, the second study defined a rather different tryptophan-rich export motif for PfEMP1, which is not NH_2 terminal but lies within a conserved region of duffy binding-like (DBL) domains (Hiller et al., 2004). Because each PfEMP1 has multiple DBL domains, each PfEMP1 molecule would have several of these targeting motifs. In addition, DBL domains are present in a range of other characterized ligands, most notable of which are proteins involved in merozoite invasion that are trafficked to organelles within the invasive merozoite form. Therefore, the contribution of this internal motif for PfEMP1 export remains unclear.

Toward defining the *Plasmodium* "exportome"

The identification of this signal allowed the prediction of the *P. falciparum* "exportome" and a broad classification of exported proteins; although the term "secretome" has been used as well (Hiller et al., 2004), we prefer exportome because it describes a defined subset of secreted proteins. Initial motif searches indicated that the exportome includes the known surface exposed antigens (e.g., PfEMP1 and Rifin; Kyes et al., 1999), other membrane-associated proteins (e.g., Stevor and the two transmembrane Maurer's cleft protein families; Cheng et al., 1998; Sam-Yellowe et al., 2004), and proteins that interact with host cytosol components, such as KAHRP, PfEMP3, and RESA. Exported proteins include virulence factors on the surface of the RBC (i.e., PfEMP1 and Rifin) and potential remodelling factors facilitating the generation of protein and nutrient transport pathways in the iRBC.

We have recently developed an algorithm, ExportPred, to predict the exportome of malaria parasites (unpublished data). A similar approach has previously been used to predict proteins targeted to the *Plasmodium* chloroplast remnant (i.e., the plastid; Foth et al., 2003). A more accurate prediction of the *P. falciparum* as well as rodent and other primate malaria parasite exportomes combined with paralogue and orthologue clustering provides, for the first time, a genus-wide picture of the nature and extent of exported proteins. This study confirms the strong bias toward two-exon genes encoding exported proteins and identifies a large number of novel gene families in *P. falciparum*. Of particular interest are two families of DnaJ proteins (co-chaperones to HSP70 with an NH_2-terminal J domain) as well as 11 novel families with multiple helical domains that cluster into three groups with either two, one, or no transmembrane domains. One of these novel families, termed PHIST (*Plasmodium* helical interspersed subtelomeric family), consists of 72 paralogues in *P. falciparum*. This family can be divided into three subgroups: PHISTa is entirely *P. falciparum* specific; PHISTb shares one orthologue with *Plasmodium vivax* and encodes a subset containing DnaJ domains (including RESA); and PHISTc is entirely shared between the two primate malaria lineages. Interestingly, cross-comparison of the ExportPred output with recent microarray data that specifically analyzed gametocyte stages indicates that a number of exported proteins, including PHISTa and PHISTb paralogues, are specifically up-regulated in early sexual stages. This supports the idea that infected erythrocytes are subject to a similar re-

modelling process in both asexual and sexual stages, including the capability to cytoadhere in order to avoid clearance of the host cell during spleen passage (Day et al., 1998; Rogers et al., 2000). Moreover, this study identified a small subset of exported proteins that are conserved across the genus but absent from other Apicomplexan lineages such as *Coccidia* (that includes *Cryptosporidium* and *Toxoplasma*). The conserved proteins mostly belong to these novel families and are clustered in a few subtelomeric loci syntenic between *P. falciparum* and *P. vivax*. This strongly suggests the presence of a core exportome in a common *Plasmodium* ancestor (unpublished data). Finally, the radiation of DnaJ proteins in *P. falciparum* may be explained by the emergence of PfEMP1, which contains multiple DBL domains. Although the highly folded DBL domains are widespread in the genus *Plasmodium*, they are usually restricted to the invasive merozoite stages, whereas PfEMP1 is exported to the host cell, where it may recruit a specific refolding machinery before exposition on the RBC surface.

What is the nature of the corresponding translocation machinery?

The identification of a small complement of exported proteins conserved across the genus and the functional expression of the motif derived from *P. vivax* and *Plasmodium gallinaceum* proteins (Marti et al., 2004) indicates a general export mechanism in the genus *Plasmodium* and raises questions with respect to the nature of the corresponding translocation machinery. The presence of the motif on both soluble and membrane-anchored proteins implies that there is common machinery responsible for protein export in the PVM. There are an increasing number of known systems for signal-mediated protein transport through biological membranes. Translocation systems in prokaryotic and eukaryotic cells can be divided into structural or functional terms: the pore can either be formed by a single protein or by an oligomeric complex, which forms a translocon. Examples of the former include bacterial outer membrane secretion pathways via β-barrel pore structures, and examples of the latter include the Sec pathway in prokaryotes and eukaryotes, the mitochondrial and chloroplast import machineries, and bacterial outer membrane secretion pathways.

Although the Sec pathways translocate unfolded proteins, others may also mediate translocation of folded proteins or even complexes (e.g., secretion pathways in bacterial outer membranes and the twin arginine translocation system for peroxisomal import; for review see Thanassi and Hultgren, 2000; Schnell and Hebert, 2003). Preliminary bioinformatic analysis of the *Plasmodium* genome indicates that it has genes encoding the ER Sec61 translocation, mitochondrial (translocases of the outer and inner mitochondrial membranes), and possibly chloroplast machineries but no known complex translocation system for folded proteins (such as the twin arginine translocation system; unpublished data). However, simple primary structure-based algorithms (such as basic local alignment search tool) may not be appropriate in this context. For example, β-barrel proteins from outer bacterial and organelle membranes are highly divergent in their primary sequences. Therefore, experimental approaches similar to those used to identify the mitochondrial and chloro-

Figure 2. **A model for signal-mediated protein export during the asexual cycle.** Approximately 400 parasite proteins have a predicted PEXEL motif that is expected to direct export to the RBC cytosol. Many of these may play roles in trafficking of PfEMP1 to the RBC surface or modifying the RBC membrane in other ways to suit the parasite's needs. *Plasmodium* prepares for the invasion and remodelling process of a new host cell in late asexual stages (schizont stage) by transporting crucial molecules to the three prototypic apicomplexan organelles (the microneme [Mn], the rhoptry [Rh], and the dense granule [Dg]) located close to the apical end of the parasite. While microneme proteins are mainly involved in initiating invasion (stages I–III), rhoptry and dense granule proteins are implicated in establishment of the parasite in the newly invaded host cell (stages III–VI). RESA (arrows), for example, is targeted to the dense granules in late schizogony, released into the parasitophorous vacuole (PV) after invasion, and subsequently exported into the red blood cell (RBC), where it associates with the red cell cytoskeleton to stabilize the newly invaded RBC membrane. The presence of a PEXEL motif in RESA implies the establishment of the corresponding translocation machinery very early after formation of the PVM. This allows the virulence factor PfEMP1 to be translocated already in early ring stages. It then appears to be transported as a soluble complex and inserted into the Maurer's cleft (MC) membrane. In trophozoite stages, knob components such as KAHRP and PfEMP3 are exported and travel as large complexes through the RBC cytosol until they transiently associate with components on the MC. Further transport of KAHRP, PfEMP3, and PfEMP1 leads to knob formation and surface exposition of PfEMP1. It is not known whether Rifin and Stevor proteins are also trafficked as soluble complexes in the RBC cytosol or whether there is vesicular transport between the PVM, MCs, and possibly the RBC membrane. Mi, mitochondrion; A, apicoplast. Other abbreviations are the same as those in Fig. 1.
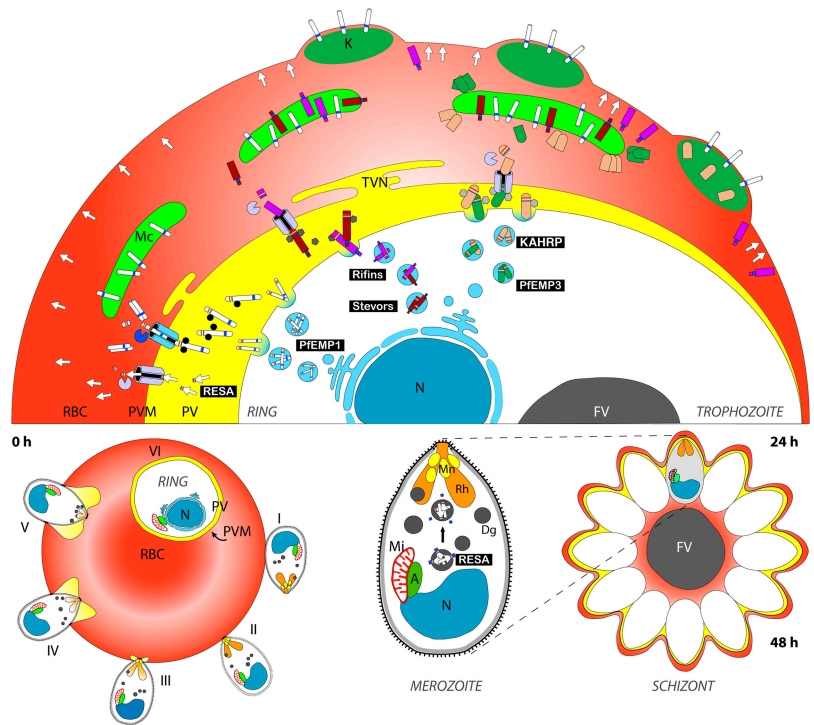
plast import machineries (i.e., the generation of a translocation intermediate and subsequent pull-down of the translocation intermediate–translocon complex; Eilers and Schatz, 1986; Schulke et al., 1999) are likely to be the most promising approaches for identifying such complexes in *Plasmodium*.

Surprisingly, an NH_2-terminal motif similar to the PEXEL motif has recently been identified in a number of virulence factors, which are secreted from plant pathogens into their host cells (Rehmany et al., 2005). These parasites (such as *Phytophthora infestans*) belong to the phylum Heterokonts, which, together with the dinoflagellates, Apicomplexa, and ciliates, form a large monophyletic subassemblage of the chromalveolates (Harper et al., 2005). Therefore, it is possible that 1) both plant and blood parasites have recruited the same machinery to translocate factors into the respective host cells and 2) that similar signals may also be identified in other chromalveolates.

Transport mechanisms in the host cell

Until recently, it was assumed that soluble exported proteins like KAHRP are secreted into the RBC cytoplasm, whereas membrane-anchored proteins such as PfEMP1 or Rifin are incorporated into trafficking vesicles. This view was supported by ultrastructural studies showing strings of vesicles budding off the PVM and PfEMP1-labeled vesicles in the RBC cytoplasm (Trelka et al., 2000; Taraschi et al., 2003). However, these structures have mainly been observed upon treatment of iRBCs with the trafficking inhibitor aluminium tetrafluoride. In contrast, recent studies investigating the mode of PfEMP1 secretion suggest that this surface antigen is transferred as a protein complex to the Maurer's clefts, where it becomes asso-

ciated with these membranes (Knuepfer et al., 2005b; Papakrivos et al., 2005). This data favors a nonvesicular mode of protein transport between the PVM and Maurer's clefts, at least for soluble proteins and PfEMP1. PfEMP3 and KAHRP are also present as large complexes in the RBC cytosol until they bind to the Maurer's clefts (Wickham et al., 2001; Kriek et al., 2003; Lopez-Estrano et al., 2003; Knuepfer et al., 2005b; Papakrivos et al., 2005), where they may be assembled into the cytoadhesion complex or participate in its assembly before the transfer of PfEMP1 to the RBC membrane (Waterkeyn et al., 2000; Wickham et al., 2001). Sequences involved in binding transient and permanent resident proteins onto the Maurer's clefts are not completely defined. However, protein domains that confer association with Maurer's clefts are located in the charged region between amino acids 69 and 120 of KAHRP (Wickham et al., 2001) and between amino acids 120 and 500 of PfEMP3 (Knuepfer et al., 2005a). These same regions could tether PfEMP1 to Maurer's clefts and initiate its insertion into the membrane. Indeed, the positively charged histidine-rich region of KAHRP has been shown to bind to the negatively charged acidic terminal segment region of PfEMP1 in vitro (Waller et al., 1999). How are other exported proteins, such as the Rifin and Stevor antigens containing two predicted transmembrane domains, transported to the Maurer's clefts and RBC surface? A recent study demonstrated that 1) Stevor is transported through the parasite's secretory pathway as a membrane-anchored protein and 2) that the majority of the protein is membrane bound in the Maurer's clefts (Przyborski et al., 2005). In addition, it has been shown that the presence of at least one transmembrane domain is sufficient to target both Rifin and Stevor

to the Maurer's clefts once the protein is translocated into the RBC cytosol (Marti et al., 2004; Przyborski et al., 2005). This suggests that for Rifin and Stevor, but also likely for all of the more recently identified exported proteins with one or two putative transmembrane domains (Sam-Yellowe et al., 2004; unpublished data), the default pathway is the transport to the Maurer's clefts. However, how these proteins are trafficked through the parasitophorous vacuole, the putative translocon, and to the Maurer's clefts remains enigmatic.

Maurer's clefts are characterized by a translucent lumen and an electron-dense coat (Atkinson et al., 1988; Elford et al., 1997; Kriek et al., 2003). Initially, these compartments appear as twisted and branched structures located near the parasitophorous vacuole, but they gradually relocate closer to the RBC membrane and appear to be tethered by fibrous connections to the RBC membrane skeleton (Atkinson and Aikawa, 1990; Waterkeyn et al., 2000; Kriek et al., 2003). There is currently some debate as to whether the Maurer's clefts are independent structures or subdomains of the tubulovesicular network. A recent study used fluorescence microscopy of cells labeled with lipid probes and electron microscopy of serial sections to examine the structures in the *P. falciparum*–iRBC cytosol (Wickert et al., 2003). These authors postulated that Maurer's clefts and the tubulovesicular network form part of a continuous meshwork. Other reports using reconstructions from serial electron micrograph sections (Bannister et al., 2004) and analysis of the dynamics of GFP chimeras of Maurer's cleft–associated cargo (Wickham et al., 2001; Knuepfer et al., 2005b) suggest that Maurer's clefts are distinct entities. However, nonvesicular transport between the PVM and the Maurer's clefts does not exclude the possibility that these two structures and the tubulovesicular network are structurally linked. A general model on the different steps of protein export into the host RBC during the asexual cycle is presented in Fig. 2.

Outlook

Apicomplexa comprise a phylum of highly specialized organisms that successfully adapted to survive in numerous host cell types. The malaria parasites invade and replicate within the quiescent erythrocytes of its host as a strategy for persistence and transmission. This requires drastic remodelling of the host cell during the development of the intraerythrocytic parasite. The presence of a conserved motif mediating protein export in different plasmodial species suggests that the corresponding translocation machinery must also be conserved. The discovery of a signal-mediated mechanism for protein transport into the host erythrocyte by parasites of the genus *Plasmodium* and the subsequent prediction of the exportome now provides the foundation for a better understanding of the factors involved in the successful intracellular lifestyle of this parasite family. The exported proteins unique to certain *Plasmodium* species will provide important insights into the different functions required for survival within their respective host cells. Additionally, the identification of a set of exported proteins that is conserved across different *Plasmodium* will point to a subset that is important for survival of the parasite in this environment. This is something that may be critical in developing novel antimalarial drugs to this infectious disease, potentially targeting multiple species and life stages.

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