


## MICROREVIEW

# Directing traffic: Chaperone-mediated protein transport in malaria parasites

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## Abstract

The ability of eukaryotic parasites from the phylum Apicomplexa to cause devastating diseases is predicated upon their ability to maintain faithful and precise protein trafficking mechanisms. Their parasitic life cycle depends on the trafficking of effector proteins to the infected host cell, transport of proteins to several critical organelles required for survival, as well as transport of parasite and host proteins to the digestive organelles to generate the building blocks for parasite growth. Several recent studies have shed light on the molecular mechanisms parasites utilise to transform the infected host cells, transport proteins to essential metabolic organelles and for biogenesis of organelles required for continuation of their life cycle. Here, we review key pathways of protein transport originating and branching from the endoplasmic reticulum, focusing on the essential roles of chaperones in these processes. Further, we highlight key gaps in our knowledge that prevents us from building a holistic view of protein trafficking in these deadly human pathogens.

## KEYWORDS

chaperones, *Plasmodium*, protein trafficking

## 1 | INTRODUCTION

Malaria is a deadly parasitic disease that kills nearly 435,000 people every year, most of them children under the age of five in sub-Saharan Africa (World Health Organization, 2019). This disease severely impacts not only public health but also the economic wellbeing of many of the poorest countries in the world (World Health Organization, 2019). The causative agent of malaria is an obligate intracellular parasite of the genus *Plasmodium*. Several *Plasmodium* species infect humans but the majority of the death is associated with only one species, *Plasmodium falciparum* (Phillips et al., 2017). The symptoms of malaria include headache, myalgia and fevers with rigours. In severe malaria, cases can be complicated by anaemia, respiratory distress, renal failure and cerebral malaria. In

addition, placental malaria is a major cause of stillbirths, miscarriages and low birth weight in endemic regions (Fried & Duffy, 2017). These clinical manifestations are a direct result of the growth of the parasite within human red blood cells (RBC). The RBC, one of the simplest cells in the human body, lacking any organelles and nucleus, is dramatically transformed by the parasite (Cowman, Healer, Marapana, & Marsh, 2016). During invasion into the host RBC, the parasite creates the parasitophorous vacuole (PV), an enclosed space in which parasite asexual replications occurs. Cocooned within the PV, *Plasmodium* embarks on a radical transformative process that subjugates the infected RBC. The permeability, rigidity, metabolism and membrane features of the infected RBC are drastically renovated by the malaria-causing parasite to suit its needs (Spillman, Beck, & Goldberg, 2015). This is accomplished by synthesizing and transporting hundreds of

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proteins across multiple cellular membranes, including the export of over 400 parasite proteins into the host RBC (Cowman et al., 2016). Other routes serve to transfer proteins into parasite-specific organelles such as the apicoplast and rhoptries, using unique signals and mechanisms that are still under investigation. This complex network of tightly regulated trafficking is crucial to parasite survival and has therefore important clinical implications. In recent years, an accumulating body of research has revealed some of the unique molecular mechanisms that are used by the parasite to transport proteins to different cellular compartments. In this review, we will discuss the work that has been done so far and has contributed to our understanding of the parasite secretory pathway. This pathway originates in the Endoplasmic Reticulum (ER) and then diverges into multiple routes, leading to different cellular compartments and into the host cell. While describing the major findings in the field, we will primarily focus on the role of molecular chaperones as mediators of protein trafficking. The fact that these proteins typically do not rely on a strict sequence motif and are rather characterised by a broad substrate specificity, has placed them as key components in numerous cellular processes in most eukaryotic organisms, including *Plasmodium*.

## 2 | PASSPORT CONTROL: THE PARASITE ER AS A MAJOR TRAFFICKING HUB

In most eukaryotic systems, the ER-Golgi secretory pathway transports proteins to the cell membrane or for secretion. Unlike free-living organisms, *Plasmodium* uses this pathway to transport proteins not only to its own cell membrane but also to the parasitophorous vacuole, the host cell cytoplasm, the host cell membrane, and to unique parasite organelles such as the apicoplast and the rhoptries. This adds

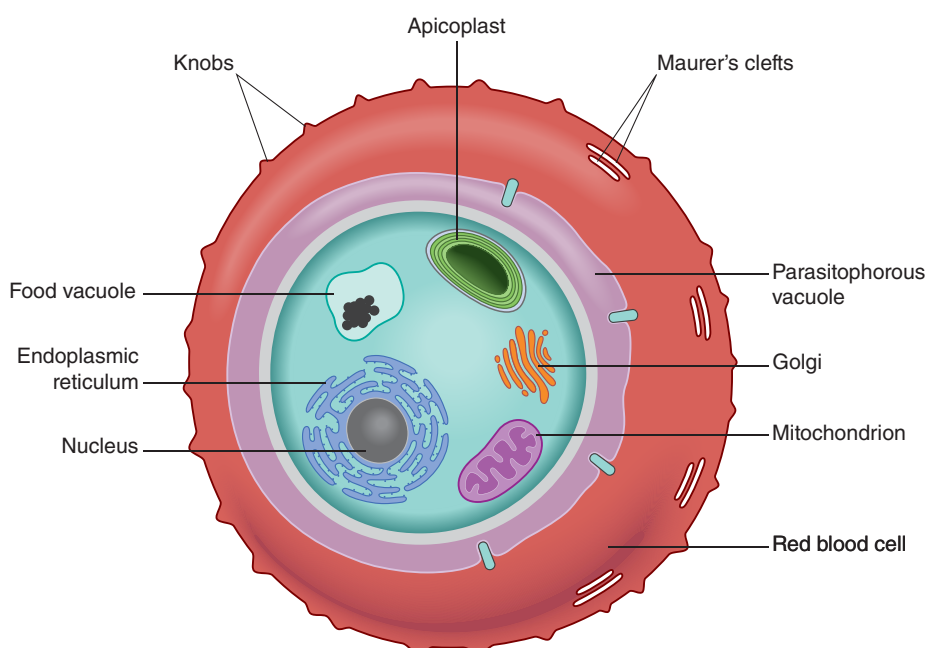
significant complexity to the parasite's secretory pathway, branching it into a highly intricate network (Figure 1).

Proteins destined for passage through the secretory pathway must begin their journey in the ER. Similar to other eukaryotes, co-translational ER import in *Plasmodium* depends on the conserved N-terminal signal peptide or a transmembrane domain (Adisa et al., 2003; Albano et al., 1999). It is during Sec61-mediated ER import that secreted proteins have their first interaction with molecular chaperones, assisting with import as well as with refolding once inside the ER (Gemmer & Förster, 2020).

Compared to other eukaryotes, the parasite ER, despite serving a complex function, lacks many components from classical ER pathways (Harbut et al., 2012). Analysis of the parasite genome reveals a reduced repertoire of conserved chaperones (Table 1). It is possible that the annotated *Plasmodium* ER chaperones play multiple essential functions or that highly diverged proteins remain to be identified.

As a key component in most eukaryotic secretory systems, a conserved ER chaperone of particular interest is the Hsp70 BiP (Binding immunoglobulin Protein) which has been shown to localise to the *Plasmodium* ER (Kumar, Koski, Harada, Aikawa, & Zheng, 1991). In other eukaryotic systems, BiP (also known as GRP78) is a multipurpose chaperone essential for ER import, proper protein folding, prevention of protein aggregation and ER stress signalling (Dudek et al., 2015; Gething, 1999; Gidalevitz, Stevens, & Argon, 2013; Vogel, Misra, & Rose, 1990). BiP is a key regulator of ER processes in eukaryotes, and likely serves equally essential and diverse functions in *Plasmodium*.

BiP function is regulated through interactions with several other ER chaperones, some of which are also encoded by the *Plasmodium* genome (Table 1). Lack of detailed studies limits our understanding of the biological roles for most of these chaperones, with the exception of PfGRP170, an atypical Hsp70 that interacts with BiP in the parasite ER, and was found to be essential for parasite replication (Kudyba



**FIGURE 1** The *Plasmodium* parasite inside the infected Red blood cell. Unlike free-living organisms, *Plasmodium* uses the secretory pathway to transport proteins not only to its own cell membrane but also to the parasitophorous vacuole, the host cell cytoplasm, the host cell membrane and to unique parasite organelles such as the apicoplast and food vacuole. AP, Apicoplast; ER, Endoplasmic reticulum; FV, Food vacuole; G, Golgi; Kn, Knobs; M, Mitochondrion; MC, Maurer's Clefts; PV, Parasitophorous vacuole; RBC, Red blood cell

**TABLE 1** Conserved ER resident chaperones in *P. falciparum*

PlasmoDB ID	Common name(s)	Classification
PF3D7_0917900	BiP; GRP78; Hsp70-2	Hsp70
PF3D7_1344200	GRP170; Hsp110; Hsp70-Y	Large Hsp70
PF3D7_1222300	Endoplasmic; GRP94	Hsp90
PF3D7_1108700	PfJ2	Hsp40
PF3D7_1318800	SEC63	Hsp40
PF3D7_0314000	HSP20-like, p23	Hsp90 co-chaperone

Note: The chaperones are often referred to using different common names. Their gene IDs are shown below.

et al., 2019). Other ER chaperones and potential BiP interactors include the Hsp90/Endoplasmic, Hsp40 co-chaperones and Protein Disulfide Isomerases (Pavithra, Kumar, & Tatu, 2007). In higher eukaryotes, the roles of some of these chaperones are well documented in regards to protein folding and secretion, but their activities in *Plasmodium* remain largely undefined. Though it is likely these ER chaperones contribute to folding and sorting of proteins preparing to leave the ER, detailed molecular studies are required to reveal their exact functions in *Plasmodium* biology.

In most eukaryotic cells, during co-translational import of proteins into the ER, asparagine residues are modified with complex oligosaccharides or glycans. Protein N-glycosylation is required not only for folding and function, but also as a signal for exit from the ER and for routing proteins to specific compartments (Andrews, 2000; Braakman & Bulleid, 2011). A specific class of chaperones bind these N-glycans, such as calnexin and calreticulin. The function of this class of lectin-chaperones is to sense the glycosylation and folding status of the substrate protein (Braakman & Bulleid, 2011). The accumulation of unfolded glycoproteins is sensed by calnexin and/or calreticulin, which activate the unfolded protein response to degrade these substrates.

However, the *Plasmodium* genome does not encode for homologs of calnexin or calreticulin or other similar lectin chaperones (Bushkin et al., 2010; Pavithra et al., 2007). Therefore, in the case of *Plasmodium* it is not enough to rely only on what we know from studies in other eukaryotes. There has been little investigation of the protein glycosylation pathway in *Plasmodium*. Previous studies have focused on glycosylphosphatidylinositol (GPI) anchors because several merozoite surface proteins are localised to the parasite membrane via this glycolipid modification (Gilson et al., 2006; Reddy et al., 2015; von Itzstein, Plebanski, Cooke, & Coppel, 2008). The available data suggest that some parasite proteins possess short N-linked glycans, such as one or two N-acetylglucosamine residues (Bushkin et al., 2010). This is in agreement with data that *Plasmodium* lacks homologs of enzymes required to synthesize complex N-glycan precursors found in other eukaryotes (Bushkin et al., 2010; Samuelson et al., 2005). Future work on the oligosaccharyltransferase complex, which transfers N-linked glycans from dolichol phosphate-linked precursors to proteins may clarify the role of this modification in parasite biology.

In contrast to N-glycosylation, research on glycosylation of serine or threonine residues in *Plasmodium* thrombospondin repeat (TSR) containing proteins show that O-fucosylation in the ER plays important roles in TSR protein function and transport (Lopaticki et al., 2017; Swearingen et al., 2016). In other model systems, this type of O-fucosylation acts as a non-canonical ER quality control pathway, where misfolded proteins do not get O-fucosylated and are targeted for ER-associated degradation (Holdener & Haltiwanger, 2019; Vasudevan, Takeuchi, Johar, Majerus, & Haltiwanger, 2015). The available data suggest that protein O-fucosylation is essential for membrane expression of several surface ligands during the vector stages of *Plasmodium*, suggesting that this non-canonical ER quality control pathway is active in these parasites (Lopaticki et al., 2017). However, the role of protein O-glycosylation, if any, during the clinically important intraerythrocytic growth of *Plasmodium* remains unknown.

### 3 | INTERNATIONAL TRANSPORT: EXPORT TARGETING IN THE ER

From the ER, the secretory pathway branches into many routes, but it is unclear how proteins are sorted and transported to their proper destinations. One major route attracts special attention and stands at the centre of several research programs: protein export. The export of proteins into the infected host cell originates in the parasite ER and ends at various locations in the Parasitophorous Vacuole (PV) and the RBC and is central to survival and virulence. The parasite exports hundreds of proteins into the host in order to change the rigidity of the RBC, modify the host cell membrane, create a membranous, Golgi-like organelle known as the Maurer's Clefts, and modify host permeability to allow favourable movement of ions and nutrients (Spillman et al., 2015). Parasite-exported proteins on the RBC membrane are required for nutrient acquisition (Nguiragool et al., 2011), immune evasion (Smith, Rowe, Higgins, & Lavstsen, 2013) and for egress from the infected RBC (Raj et al., 2014).

One subset of proteins targeted to the parasitized RBC possesses an N-terminal export signal termed *Plasmodium* Export Element (PEXEL), or the Host-Targeting motif (Hiller, 2004; Marti, 2004). The PEXEL pentameric sequence (RxLxE/Q/D) is cleaved co-translationally at the conserved Leucine by the ER-resident protease Plasmepsin V (PMV) (Boddey et al., 2010; Russo et al., 2010; Sleebs, Lopaticki, Marapana, & O'Neill, 2014). While this particular series of events has been studied in detail (Boddey et al., 2013, 2016; Tarr, Cryar, Thalassinou, Haldar, & Osborne, 2013), the significance of the PEXEL cleavage itself is not entirely clear, and the subsequent steps leading to export are largely unknown. However, a recent study showed that an early recognition of exported proteins occurs during import into the ER through interactions between PMV and a distinct subset of translocation machinery (Marapana et al., 2018). This work suggests that PMV appears to act in the place of signal peptidase for PEXEL-containing exported proteins.

A second group of proteins lack the PEXEL sequence but nonetheless make their way from the ER to the host RBC. These are called

PEXEL negative exported proteins (PNEPs) (Grüning et al., 2012; Heiber et al., 2013; Spielmann et al., 2006). Despite the lack of any known signal motif, data suggest that PNEPs share some of the export mechanisms with PEXEL proteins (Beck, Muralidharan, Oksman, & Goldberg, 2014; Elsworth et al., 2014; Grüning et al., 2012; Heiber et al., 2013).

It is not known how PNEPs and PEXEL proteins are recognised and sorted in the ER. One clue emerged from the finding that the PEXEL-cleaving protease PMV interacts with ER-resident chaperones such as BiP and Endoplasmic Reticulum chaperone (Russo et al., 2010). The same study also identified Hsp101 as another PMV interactor, but subsequent work could not substantiate this interaction (Spillman et al., 2015). Further evidence suggestive of roles for these chaperones in trafficking comes from analysis of a yeast-2-hybrid screen, which predicted interaction between BiP and the exported protein *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) (Pavithra et al., 2007). PfEMP1 is a major virulence factor inserted into the RBC membrane and allows infected cells to cytoadhere to the host's microvasculature, which has been implicated in the severity of *P. falciparum* malaria (Hsieh et al., 2016; Salanti et al., 2004; Storm et al., 2019). Another study identified interactions between PfEMP1 and ER chaperones such as Endoplasmic Reticulum chaperone and others (Batinovic et al., 2017). More work is needed to validate these predicted interactions and show direct involvement of these chaperones in the export process.

Once departing from the ER, exported proteins are thought to traverse through the Golgi, as many are sensitive to treatment with the Golgi-disturbing agent, Brefeldin A (Benting, Mattei, & Lingelbach, 1994; Grüning et al., 2012; Haase et al., 2009; Wickham et al., 2001). In mammalian and yeast systems, exit from the ER is mediated by COPII-vesicles (Barlowe, 2003; Zanetti, Pahuja, Studer, Shim, & Schekman, 2011). *Plasmodium* contains homologs for COPII vesicle components and immunofluorescence assays suggest that many of these proteins localise to the ER membrane (Lee, Moura, Miller, & Fidock, 2008). The next few steps—from Golgi to the plasma membrane and into the PV—are ill-defined and to date, no ER exit receptors or plasma membrane translocons have been identified. However, the subsequent step—in which proteins leave the PV for entry into the host—has recently been discovered as a fascinating, parasite-specific translocation mechanism.

#### 4 | PROTEINS WITHOUT BORDERS: EXPORT INTO THE HOST CELL

The final barrier between parasite and host is the parasitophorous vacuole membrane (PVM): parasite proteins destined for the RBC must find passage through this membrane to exit the vacuole. Entry-way to the host cell is provided by the *Plasmodium* translocon of exported proteins (PTEX) (de Koning-Ward et al., 2009; Riglar et al., 2013). The core of the translocon is composed of the integral membrane protein EXP2, PTEX150 and the AAA+ ATPase Hsp101 (Bullen et al., 2012; Charnaud, Kumarasingha, Bullen, Crabb, & Gilson, 2018; Ho et al., 2018). Conditional knockdown of PTEX

components block export of proteins into the RBC and lead to their accumulation in the PV (Beck et al., 2014; Elsworth et al., 2014; Garten et al., 2018). One of these essential PTEX components is the chaperone Hsp101. As a AAA+ ATPase, Hsp101 functions to couple ATP hydrolysis with protein unfolding as the client protein is fed through the translocon (Ho et al., 2018). Indeed, exported proteins that are locked into a tightly folded state are trapped within the PV and jam the PTEX translocon (Gehde et al., 2009; Mesén-Ramírez et al., 2016; Riglar et al., 2013). These data demonstrate that PTEX is an exit through which parasite proteins enter the host cell, and that chaperone-mediated unfolding is a key event in the export process.

How exported proteins are refolded and trafficked inside of the host cell largely remains a mystery. *P. falciparum* encodes a number of exported chaperones that may be involved in these processes. These chaperones include a single Hsp70—PfHsp70x—and numerous Hsp40 co-chaperones (Külzer et al., 2012; Maier et al., 2008). PfHsp70x is found in the PV as well as in mobile puncta within the host cytoplasm called J-dots, and has been found associated with PTEX (Elsworth et al., 2016; Külzer et al., 2010, 2012). Subsequent research into PfHsp70x function has shown that while it may have a role in supporting parasite virulence, the chaperone is dispensable for asexual growth in culture (Charnaud et al., 2017; Cobb et al., 2017). The non-essential nature of PfHsp70x stands in contrast with the Hsp40 co-chaperones exported by *P. falciparum*, some of which appear to be indispensable for host cell remodelling and are refractory to genetic disruption (Maier et al., 2008). At least two of these Hsp40s associate with PfHsp70x in J-dots (Külzer et al., 2010, 2012; Petersen et al., 2016; Zhang et al., 2017). The function of J-dots remains unknown, but it has been hypothesized that they play a key function in trafficking after proteins cross PTEX, though the dispensability of PfHsp70x complicates this theory. RBCs contain human Hsp70 proteins, and therefore one intriguing possibility is that the parasite Hsp70 may have redundant function with its human orthologue (Pasini et al., 2006). In fact, infection with *P. falciparum* affects the normal localization of the human Hsp70, as the protein is soluble in non-parasitized RBCs but is found in detergent-resistant fractions following infection (Banumathy, Singh, & Tatu, 2002). Together, these works suggest a model wherein export is aided by parasite chaperones as well as by human chaperones present in the host RBCs.

PfEMP1 is an exported transmembrane protein that plays a key role in *P. falciparum* virulence (Hsieh et al., 2016; Salanti et al., 2004; Storm et al., 2019). Therefore, research into PfEMP1 provides a relevant case study into the roles chaperones may play in trafficking within the RBC. Knockdown of PTEX components blocks export of PfEMP1, suggesting the transmembrane protein uses PTEX to enter the host cell (Beck et al., 2014; Elsworth et al., 2014). Moreover, evidence shows that the protein is not inserted into a membrane during trafficking from the PVM to Maurer's clefts (MCs), and that it is trafficked as part of a soluble protein complex in the RBC cytoplasm (Knuepfer, Rug, Klonis, Tilley, & Cowman, 2005; Papakrivovs, Newbold, & Lingelbach, 2005). This complex is likely to include chaperones, as PfEMP1 is found associated with PfHsp70x in the RBC cytoplasm during trafficking (Külzer et al., 2012), although knockdown

or knockout of PfHsp70x had little-to-no effect on PfEMP1 trafficking (Charnaud et al., 2017; Cobb et al., 2017).

Like PfEMP1, other proteins whose final destination is the RBC membrane and/or the knob structures that surround membrane-inserted PfEMP1 are first trafficked to the MCs (Kaviratne, Khan, Jarra, & Preiser, 2002; Petter et al., 2007; Wickham et al., 2001). Evidence thus far support vesicle-independent trafficking from the PV to Maurer's clefts, and suggests that proteins other than PfEMP1 are also trafficked in soluble complexes (Hanssen et al., 2008; Knuepfer et al., 2005; Spycher et al., 2006). The transport of proteins across the RBC to the MCs is merely the beginning of the intriguing biological problem of protein trafficking to Maurer's clefts. The mechanism allowing proteins to enter the MC membrane and lumen, perhaps through an undiscovered translocon, remain to be defined. And once proteins have reached the organelle, how do they refold to achieve correct conformations? How are transmembrane proteins accurately inserted in the correct orientation? Proteomic analysis of Maurer's clefts detected several different heat shock proteins (Vincensini et al., 2005). These MC-associated chaperones may play similar functions to the roles other chaperones play in the ER, PTEX and the host cell, assisting in protein translocation and movement to the RBC membrane.

## 5 | DOMESTIC TRAVEL: TRANSPORT TO THE APICOPLAST

The parasite contains a unique organelle called the apicoplast, an ancient endosymbiont that carries its own genome (Gould, Waller, & McFadden, 2008; Keeling, 2009; McFadden & van Dooren, 2004). The apicoplast harbours essential metabolic pathways, including synthesis of isoprenoids, fatty acids and iron-sulfur clusters and many enzymes involved in these pathways were proposed or found to be localised to the apicoplast (Ralph et al., 2004; Seeber & Soldati-Favre, 2010; van Dooren & Striepen, 2013). With the exception of the very few genes that are encoded by the apicoplast genome, most of the apicoplast proteins are encoded by the cell nucleus and need to be transferred to maintain a functional organelle (Boucher et al., 2018; Wilson et al., 1996).

Almost all apicoplast-targeted proteins contain an N-terminal bipartite sequence that is cleaved upon successful import into the apicoplast. The first part is a signal peptide, reflecting the fact that the route to the apicoplast begins at the ER and goes through the secretory pathway (Waller, Reed, Cowman, & McFadden, 2000). The second part is exposed in the ER upon signal peptide cleavage and quite resembles the transit peptide (TP) that leads plant proteins to the chloroplast (Foth et al., 2003). The apicoplast TP varies in length and has no consensus sequence or conserved motif. It is usually 150-200 amino acids long, overall positively charged, unstructured, and enriched with hydrophilic and basic amino acids (Foth et al., 2003). These somewhat relaxed requirements make *in silico* predictions challenging; however, a few groups have developed different algorithms to identify apicoplast-targeted proteins (Boucher et al., 2018; Cilingir,

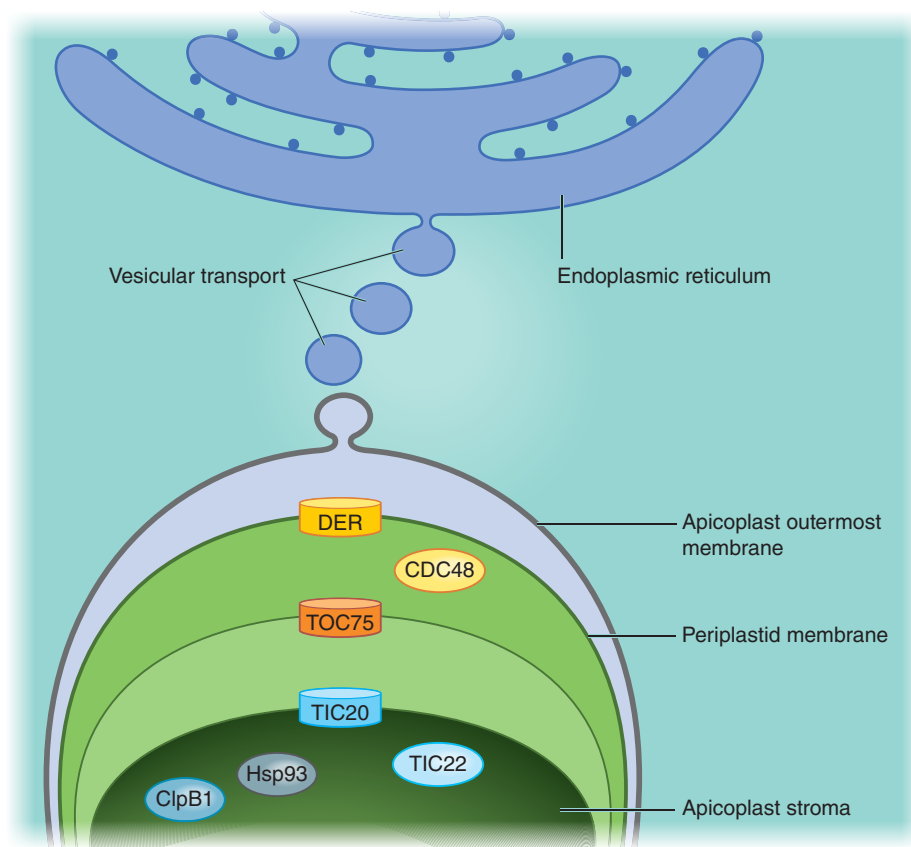
Broschat, & Lau, 2012; Zuegge, Ralph, Schmuker, McFadden, & Schneider, 2001). How is the apicoplast proteome (estimated to include around 500 proteins) sorted and discriminated from hundreds of other transported proteins? Here too, ER chaperones may be involved due to their abundance and broad substrate specificity. Interestingly, apicoplast TPs are predicted to bind Hsp70, and when these predicted binding sites were mutated, targeting to the apicoplast was disrupted (Foth et al., 2003). Moreover, an Hsp70 inhibitor with an antimalarial activity was shown to inhibit apicoplast targeting (Ramya, Karmodiya, Suroliya, & Suroliya, 2007; Ramya, Suroliya, & Suroliya, 2006). It is known that apicoplast TPs are structurally disordered, suggesting that chaperones are involved in their transport (Gallagher, Matthews, & Prigge, 2011).

Not much is known about the journey from the ER to the apicoplast, and how proteins reach, recognise and cross the outermost of the four apicoplast membranes (Figure 2). Several studies reported the appearance of vesicle-like structures containing apicoplast-targeted proteins upon disruption of apicoplast integrity (Bowman et al., 2014; Florentin et al., 2017; Gisselberg, Dellibovi-Ragheb, Matthews, Bosch, & Prigge, 2013; Yeh & DeRisi, 2011). This was interpreted as the accumulation of transport vesicles that under normal conditions travel from the ER to the apicoplast; however, upon apicoplast damage they are forced to diffuse in the cytoplasm without being able to dock at their destination. Such vesicle transport will most probably require the presence of v-SNAREs and t-SNAREs in the ER and apicoplast, respectively, as well as other vesicle-mediated transport components, none of them yet identified.

After reaching the apicoplast and traversing through the first membrane, the next membrane to cross is the periplastid membrane, which was originated during the secondary endosymbiosis event that created the organelle. Several lines of evidence suggest the involvement of a system derived from the ER-associated degradation (ERAD) machinery, which was adapted to assist in translocating proteins across the periplastid membrane (Figure 2). It appears that *Plasmodium* and other related apicomplexan parasites have a second set of putative ERAD proteins with predicted apicoplast localization. Specifically, the *Plasmodium* homolog of Der1, the potential ERAD translocon, was reported to localise to the periphery of the plastid (Kalanon, Tonkin, & McFadden, 2009; Spork et al., 2009). In the closely related apicomplexan parasite *Toxoplasma gondii*, knockout of *der1* completely blocked apicoplast transport and killed the parasites (Agrawal, van Dooren, Beatty, & Striepen, 2009). In support of this model, a subsequent study in *T. gondii*, has identified two other components in this pathway, both homologous to ERAD components (Fellows, Cipriano, Agrawal, & Striepen, 2017). One of these is a AAA+ ATPase on the periplastid membrane, homologous to the Cdc48 chaperone that extracts ER proteins into the cytosol for degradation (Fellows et al., 2017). This suggests that similar to the PTEX component Hsp101 on the PV membrane, a different AAA+ ATPase is involved in crossing the apicoplast periplastid membrane (see below).

In line with the evolutionary origin of the apicoplast, its two inner most membranes are homologous to chloroplast membranes. Subsequently, it was hypothesized that plant homolog complexes





**FIGURE 2** Trafficking from the ER to the apicoplast organelle. Protein trafficking to the apicoplast starts at the ER and is thought to involve vesicular transport. The mechanism of transportation across the outermost apicoplast membrane is unclear. Translocation across the second outermost barrier, the periplastid membrane, takes advantage of an ERAD-like machinery involving the potential ERAD translocon DER1 and the assisting AAA+ ATPase CDC48. Consistent with the evolutionary origin of the apicoplast, the two innermost membranes are homologs to chloroplast membranes and presumed to involve similar translocation mechanisms, such as Toc (Translocon of the Outer Chloroplast membrane) and Tic (Translocon of the Inner Chloroplast membrane) that facilitate translocation into the stroma. For the outermost membrane, only the Toc pore forming component, Toc75, is encoded by apicomplexan genomes. For the innermost membrane, two central components of the Tic complex, Tic20, a potential pore forming protein, and Tic22, a chaperone with an unclear function, have been identified in the closely related parasite *T. gondii*

such as Toc (Translocon of the Outer Chloroplast membrane) and Tic (Translocon of the Inner Chloroplast membrane) facilitate translocation into the stroma (McFadden & van Dooren, 2004). A homolog of the Toc pore forming component, Toc75, is encoded by apicomplexan genomes; however, no other homolog of Toc proteins has been identified (Bullmann et al., 2010; McFadden & van Dooren, 2004).

As for the innermost membrane, one of the central components of the Tic complex—Tic20, a potential pore forming protein—has been identified in *T. gondii* and mutants were shown to be non-viable and defective in apicoplast import (van Dooren, Tomova, Agrawal, Humbel, & Striepen, 2008). However, all other central Tic components seem to be missing from the genomes of different apicomplexan parasites (McFadden & van Dooren, 2004). The only other Tic homolog identified in *Plasmodium* and *Toxoplasma* is Tic22 with a proposed yet unclear chaperone function (Glaser et al., 2012; Kalanon et al., 2009). It is possible that chaperones and receptors with diverse sequences but conserved functions play the roles of the missing components.

Homologs of Hsp93 (Clp chaperones) are predicted to localise to the apicoplast (van Dooren & Striepen, 2013), and may, together with other molecular chaperones facilitate the translocation through the innermost membrane. For example, several chaperones from the prokaryotic Clp family have been localised to the apicoplast, and may assist in protein transport to the organelle (El Bakkouri et al., 2010). A possible candidate is PfClpB1, a homolog of bacterial and plant chaperones that unfold proteins to cross membranes. The highly related PfClpB2, also referred to as Hsp101, localizes to the PVM and is part of the PTEX translocon as was discussed above (Beck et al., 2014; Elsworth et al., 2014; Ho et al., 2018). The two *Plasmodium* ClpB genes share a high degree of homology, and most probably resulted from a gene duplication event, suggesting similar functions in different membranes (Florentin et al., 2017). Alternatively, given the homology of PfClpB1 to bacterial and plant ClpB disaggregases (Ngansop, Li, Zolkiewska, & Zolkiewski, 2013), it may serve a different role in the apicoplast lumen, refolding proteins. If that is the case, other chaperones, such as the apicoplast-encoded PfClpM chaperone (El Bakkouri et al., 2010), may be involved in unfolding proteins during import.

It is still unclear how peripheral proteins such as Tic20, Toc75 or Der1 are separated from other TP-cleaved stromal proteins and find their way into their specific localization on each one of the four membranes.

## 6 | EXPORT/IMPORT: TRAFFICKING TO THE FOOD VACUOLE

The food vacuole (FV; also called digestive vacuole) is a unique compartment that is crucial to the parasitic life style. This is an acidic lysosomal-like organelle that functions as a catabolic centre that primarily breaks down host haemoglobin to produce essential nutrients, and to free space in the host cytosol to allow for parasite expansion (Francis, Sullivan, & Goldberg, 1997). The flux into the food vacuole, therefore, comes from the outside, in the form of endocytic vesicles engulfing host cytoplasm and haemoglobin, although the exact molecular mechanisms are still debated (Dluzewski et al., 2008; Elliott et al., 2008; Jonscher et al., 2019; Lazarus, Schneider, & Taraschi, 2008). Haemoglobin breakdown is a multistep process, mediated by diverse parasite proteases. It starts with Plasmepsins and Falcipains, two groups of aspartic and cysteine proteases (Goldberg, 2005; Rosenthal, 2011) and the resulting peptides are further digested by designated metalloproteases and aminopeptidases (Dalal & Klemba, 2007; Eggleston, Duffin, & Goldberg, 1999; Klemba, Gluzman, & Goldberg, 2004). The haemoglobin breakdown generates toxic free heme, which is detoxified in the FV through conversion into inert hemozoin crystals (Jani et al., 2008). This process is targeted by antimalarial drugs such as chloroquine and, as a consequence, led to the emergence of drug resistance markers in the FV such as the P-glycoprotein PfMDR1 and the chloroquine resistance transporter PfCRT (Valderramos & Fidock, 2006).

Interestingly, FV-localized proteins start their journey with the secretory pathway in the ER and Golgi (Klemba et al., 2004). But then, instead of being directly trafficked to their destination, they are shipped to the parasite membrane to join the endocytic vesicles containing host cytoplasm and haemoglobin, and are transferred together back to the FV (Abu Bakar, Klonis, Hanssen, Chan, & Tilley, 2010; Dasaradhi et al., 2007; Subramanian, Sijwali, & Rosenthal, 2007). Studies of trafficking signals for Plasmepsin and Falcipain proteases revealed at least two essential stretches of amino acids required for trafficking to the FV (Dasaradhi et al., 2007; Subramanian et al., 2007). The mechanism behind these two signals require further study, but current evidence suggests that multiple protein-protein interactions may be necessary for FV localization (Deponete et al., 2012). It is entirely unclear how these proteolytic enzymes are clustered in the plasma membrane and directed to the endocytic foci that are routed back into the FV. There is no current evidence for the involvement of molecular chaperones, however their abundance, localization and previous reported functions, place them as potential mediators of this unusual trafficking route.

## 7 | AN EPIC JOURNEY: FROM THE ER TO THE APICAL ORGANELLES

To perpetuate the asexual lifecycle, *P. falciparum* utilizes special secretory, apical organelles that are required for egress from the current host cell and invasion into the new host cell. Present in most genera of apicomplexans, these organelles have raised much interest for therapeutic interventions. These specialised organelles include the large club-shaped rhoptries, the electron-dense micronemes and exonemes and the vesicular-like dense granules. While the fascinating roles that these organelles play in parasite egress and invasion are well studied (and reviewed in Singh & Chitnis, 2017), it is still not entirely clear how proteins get to these organelles to begin with. Studies with the related parasite *Toxoplasma gondii* have identified some of the complex signals that are important for trafficking (Venugopal & Marion, 2018), but these may or may not be similar in *Plasmodium* (Kats, Black, Proellocks, & Coppel, 2006).

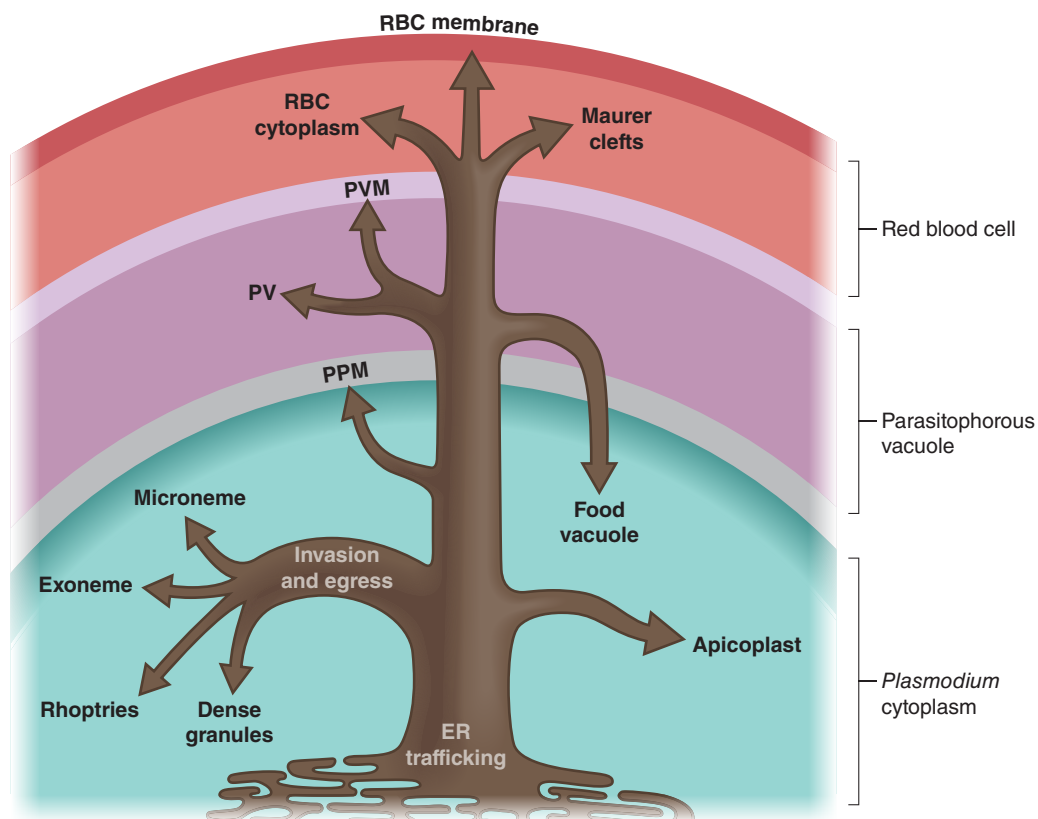
The conserved *de novo* biogenesis of this class of apical organelles is highly coordinated and occurs during the late stages of the intraerythrocytic lifecycle (Margos et al., 2004). They all seem to originate from Golgi-derived transport vesicles that are translocated to the apical tip via microtubules and motor proteins (Bannister, Hopkins, Fowler, Krishna, & Mitchell, 2000; Fowler et al., 2001; Schrevel et al., 2008). Thus, from the earliest point in their formation, the apical organelles rely on ER and Golgi trafficking. Later on, protein trafficking to the newly formed organelles also begins in the ER, but then an unknown set of signals direct them to the rhoptries, micronemes or dense granules. The trafficking of many of these proteins involve proteolytic maturation, which was shown to play a critical role in microneme and rhoptry localization in *Toxoplasma* (Bradley & Boothroyd, 2001; Dubois & Soldati-Favre, 2019; El Hajj et al., 2008; Striepen, Soldati, Garcia-Reguet, Dubremetz, & Roos, 2001). In this related parasite, certain modifications such as GPI-anchors and transmembrane domains with conserved motifs seem to play a role in microneme localization (Binder, Lagal, & Kim, 2008; Di Cristina, Spaccapelo, Soldati, Bistoni, & Crisanti, 2000; Gaji, Flammer, & Carruthers, 2011; Reiss et al., 2001). In *Plasmodium*, however, it is unclear whether cleavable pro-domains play any role in targeting of microneme proteins, although some of them appeared to be GPI-anchored (Hackett, Sajid, Withers-Martinez, Grainger, & Blackman, 1999; Harris et al., 2005; Healer, Crawford, Ralph, McFadden, & Cowman, 2002; Topolska, Lidgett, Truman, Fujioka, & Coppel, 2004).

As for transport to *Plasmodium* rhoptries, no specific signals have yet been identified. However, certain regions within rhoptry proteins have been shown to be essential for localization (Ghoneim, Kaneko, Tsuboi, & Torii, 2007; Sherling et al., 2017) as well as interactions with other rhoptry proteins (Baldi et al., 2000; Hallée, Boddey, Cowman, & Richard, 2018; Morse, Webster, Kalanon, Langsley, & McFadden, 2016; Richard et al., 2009). The rhoptry associated protein 1 (RAP1) was shown to contain a bipartite signal necessary and sufficient for trafficking to the rhoptries (Richard et al., 2009).

Further, the RAP1 rhopty targeting signal was shown to interact with another rhopty protein, the rhopty associated membrane antigen, suggesting that this interaction may be required for rhopty targeting (Richard et al., 2009). This possibility has not been explored further and the rhopty targeting signals for other rhopty proteins remain to be identified. More recent work, has suggested that certain members of the golgi-endosomal protein trafficking pathway, such as homologs of sortilin or VPS10p, are repurposed for targeting proteins to rhopties (Hallée et al., 2018). The *Plasmodium* sortilin homolog has been shown to interact with a rhopty protein (Hallée et al., 2018), however, more work is required to show a direct involvement of the vacuolar protein sorting pathway in directing proteins to the rhopties. Trafficking to the dense granules and exonemes is even less studied and current models rely on similarities to the other apical organelles as well as to findings in *Toxoplasma* (Karsten et al., 1998). Distinct and conserved motifs that can be used to predict any of these subcellular localizations have not been identified yet, and the specific mechanisms that will facilitate the sorting of these signals are yet to be found.

## 8 | CONCLUDING REMARKS AND THE ROAD AHEAD

Protein trafficking in malaria parasites has been the focus of many research programs in recent years. As a result, our understanding of basic cellular processes in the parasites has increased dramatically, shedding light on new virulence factors and potential drug targets. The finding and characterisation of the PTEX complex and the accompanying export machinery revealed parasite-specific mechanisms that are molecularly distinct from its human host. Similarly, the pathway to the apicoplast relies on bacterial and plant-like machinery that is equally divergent from higher eukaryotes. In a eukaryotic pathogen that shares a high degree of molecular similarity with its host, the potential for components in these pathways to serve as drug targets is relatively high. Nevertheless, our understating of this huge trafficking network is still limited and many of the components are still unidentified. Specifically, the parasite's ER—the origin of the secretory pathway and the master regulator of almost all trafficking routes—is still poorly understood (Figure 3). How are proteins sorted in the ER?



**FIGURE 3** The parasite ER is a master regulator of trafficking routes. The parasite's ER is the origin of the secretory pathway and a major sorting and branching hub. It is where hundreds or even thousands of proteins are sorted and shipped via different routes towards distinct organelles and cellular compartments. These include intracellular parasite-specific organelles such as the apicoplast and a specific subset of secretory organelles which are involved in invasion/egress, including micronemes, exonemes, rhopties and dense granules. The ER route also leads to the parasite plasma membrane (PPM), where some proteins are shipped back into the cell towards the food vacuole to assist with haemoglobin digestion. Other secreted proteins may find themselves in an extracellular confined space called the parasitophorous vacuole (PV) or to the membrane that surrounds it (PVM). Finally, proteins that cross the PVM will be shipped to different compartments in the host Red Blood Cell (RBC) including the RBC cytoplasm, RBC membrane and parasite-generated organelles in the RBC such as the Maurer's clefts



What mechanisms and signals are used? Do protein post-translational modifications, such as addition of glycans, function in protein trafficking? How is protein trafficking regulated? How does it maintain normal protein transport in the face of severe environmental assaults such as oxidative stress and rapid temperature changes? With a reduced repertoire of conserved chaperones (Table 1) and thus a limited stress response machinery, the parasite ER may reveal itself as *Plasmodium's* Achilles heel. However, most of the key factors in the ER and outside, have not been found yet. This can be attributed to the challenging genetics of *P. falciparum* that hamper large unbiased screens, and to the fact that a large proportion of its genes have no homologs in other organisms. Moving forward becomes possible with the use of emerging new genetic technologies and by carefully building on existing knowledge. The latter includes data of parasite-specific mechanisms but cannot ignore players with conserved or predicted functions. Such is the case of molecular chaperones that are the cornerstone of protein trafficking in almost every cell. Studying their biological functions and significance in a parasitical context has led to important discoveries, and pursuing this path will continue to reveal novel and complex networks that are central for the parasitic lifestyle.

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#### CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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