Plasmodium species: master renovators of their host cells

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Abstract | *Plasmodium* parasites, the causative agents of malaria, have developed elaborate strategies that they use to survive and thrive within different intracellular environments. During the blood stage of infection, the parasite is a master renovator of its erythrocyte host cell, and the changes in cell morphology and function that are induced by the parasite promote survival and contribute to the pathogenesis of severe malaria. In this Review, we discuss how *Plasmodium* parasites use the protein trafficking motif *Plasmodium* export element (PEXEL), protease-mediated polypeptide processing, a novel translocon termed the *Plasmodium* translocon of exported proteins (PTEX) and exomembranous structures to export hundreds of proteins to discrete subcellular locations in the host erythrocytes, which enables the parasite to gain access to vital nutrients and to evade the immune defence mechanisms of the host.

Parasitophorous vacuole
A compartment that is formed in host cells in which apicomplexan parasites reside and develop.

New permeation pathways
(NPPs). These are induced in the membrane of infected erythrocytes to enable the passage of low-molecular-weight molecules.

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The phylum Apicomplexa encompasses various obligate intracellular parasites, including *Plasmodium* spp., which are the causative agents of malaria. Five species in the *Plasmodium* genus — namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* (with two sub-species *Plasmodium ovale curtisi* and *Plasmodium ovale walikeri*)[1] and *Plasmodium knowlesi* — can infect humans[2], with *P. falciparum* causing the most severe form of malaria and hence higher mortality rates.

An infection with *Plasmodium* spp. begins when motile forms of the parasite, which are called sporozoites, are introduced into the vertebrate host through a mosquito bite (FIG. 1). The sporozoites then travel through the bloodstream to the liver, where they infect hepatocytes[3]. Within hepatocytes, the sporozoites replicate asexually to form several thousand merozoites, which are then released into the bloodstream and infect erythrocytes, in which the parasite completes the ring, trophozoite and schizont blood stages. Rupture of the schizont causes the release of approximately 20 daughter merozoites that can invade more erythrocytes[4] (FIG. 1). Repeated cycles of erythrocytic invasion, growth and asexual reproduction rapidly expand the parasite population, which leads to malaria-associated pathologies[5]. After each cycle, a small population of the blood-stage parasites differentiates into the sexual forms of the parasite, which are known as gametocytes[6]. Over a period of approximately 10 days, male and female gametocytes develop through five distinct morphological stages. When a female *Anopheles* spp. mosquito ingests a blood meal, stage five gametocytes enter the midgut of the mosquito host, egress from the encapsulating erythrocyte and differentiate into macrogametes (female) and microgametes (male)[7]. Fertilization results in the formation of the zygote, which then transforms into a motile ookinete that can penetrate the midgut wall and develop into an oocyst (FIG. 1). Maturation of the oocysts leads to the formation and release of sporozoites, which then migrate to the salivary gland. When the mosquito takes another blood meal, the life cycle repeated.

Although most apicomplexan species infect nucleated host cells (BOX 1), *Plasmodium* spp. have evolved to survive in terminally differentiated erythrocytes, which are enucleated in mammalian hosts. Erythrocytes are small and have limited biosynthetic capacity. Within these cells, *Plasmodium* spp. are enclosed in a unique non-phagosomal compartment, which is known as the parasitophorous vacuole. To ensure rapid reproduction, intra-erythrocytic parasites require access to nutrients from the blood plasma; however, they also need to avoid detection by the surveillance mechanisms of the host immune system. To achieve this, *Plasmodium* spp. export hundreds of effector proteins into the erythrocyte compartment to subvert the functions of the host cell[8–10]. New permeation pathways (NPPs; BOX 2) facilitate nutrient uptake from the blood plasma and the disposal of toxic metabolites such as lactic acid[11,12], and protein components that have been exported from the parasite promote the insertion and display of adhesins on the erythrocyte plasma membrane. These adhesins enable the infected erythrocytes to bind to the vascular endothelium, which prevents passage through the spleen and hence clearance by splenic

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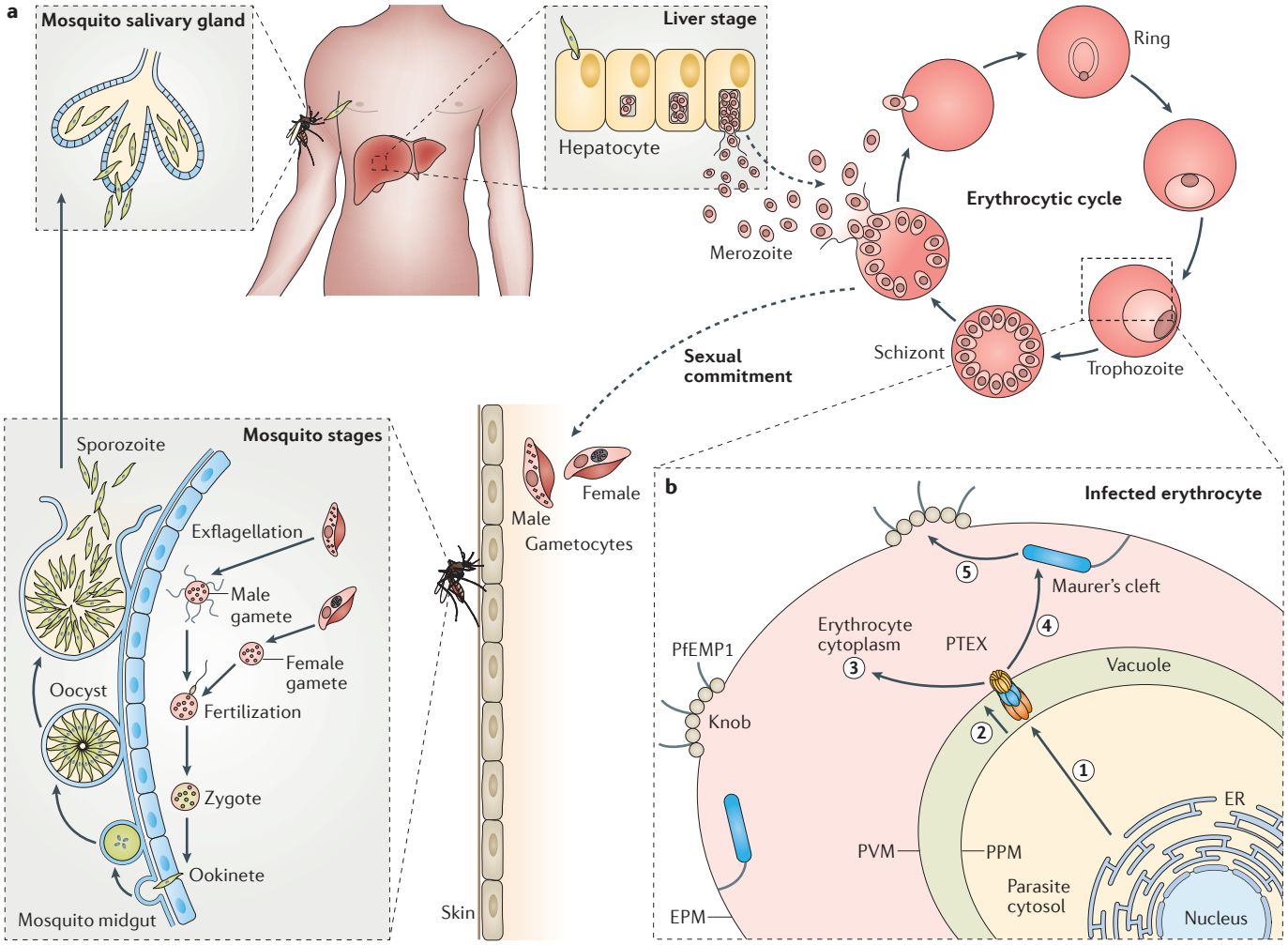

Figure 1 | **Overview of the *Plasmodium* spp. life cycle.** The *Plasmodium* spp. life cycle can be divided into two parts, asexual multiplication within the human host and sexual reproduction within the mosquito vector. **a** | The life cycle begins when female *Anopheles* spp. mosquitoes inject saliva that contains sporozoites into the human host. The sporozoites enter the bloodstream and circulate to the liver, where they actively invade hepatocytes (the liver stage). Within hepatocytes, each sporozoite replicates asexually to form hundreds of merozoites. The hepatocytes rupture, which releases the merozoites into the bloodstream, where they invade erythrocytes (the erythrocytic cycle). During the erythrocytic cycle, the parasite is encased within the parasitophorous vacuole, where it progresses through the ring (establishment), trophozoite (growing) and schizont (dividing) developmental stages. The schizont then ruptures, which releases merozoites back into the bloodstream, where they can invade new erythrocytes and continue the erythrocytic cycle. During asexual cycling a small population of the parasites commit to sexual-stage development (sexual commitment). Gametocytes develop in erythrocytes through five morphologically distinct stages, from a ring-like early stage gametocyte to a crescent-shaped mature gametocyte. After ingestion by a female mosquito, the male gametocytes undergo three rounds of DNA replication and exflagellate from the erythrocyte to release eight motile microgametes. These fertilize female gametocytes that have egressed from the erythrocyte and developed into a macrogamete. The resulting zygote differentiates into a motile ookinete, which burrows through the midgut wall to form the oocyst, the parasite undergoes asexual replication, leading to the formation of sporozoites, which rupture from the oocysts and migrate to the salivary glands of the mosquito. The sporozoites are injected into the human host during the next blood meal of the mosquito. With the exception of the zygote, *Plasmodium* spp. are haploid throughout their entire life cycle. **b** | To survive in an erythrocyte, the parasite exports proteins into the erythrocyte cytoplasm. These exported proteins remodel the erythrocyte, which enables the uptake of nutrients and waste disposal. In addition, these proteins alter the physical properties of the erythrocyte membrane to promote cytoadhesion, which is a process that is mediated by the insertion of the virulence protein *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) into knob structures at the erythrocyte membrane. Proteins that contain export motifs are recognized, processed and licensed for export within the endoplasmic reticulum (ER) and trafficked across the parasite plasma membrane (PPM) into the parasitophorous vacuole (step 1). Within the parasitophorous vacuole, proteins are recognized and exported across the parasitophorous vacuole membrane (PVM) through the *Plasmodium* translocon of exported proteins (PTEX; step 2). Once translocated, exported proteins have three final destinations: the erythrocyte cytoplasm (step 3); Maurer's clefts, which are membranous structures that act as protein sorting compartments (step 4), and the erythrocyte plasma membrane (EPM) and membrane skeleton (step 5).

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macrophages[11,13,14]. The extensive phenotypic changes in erythrocyte structure and function that are induced by *Plasmodium* spp. contribute to the pathogenesis of severe malaria.

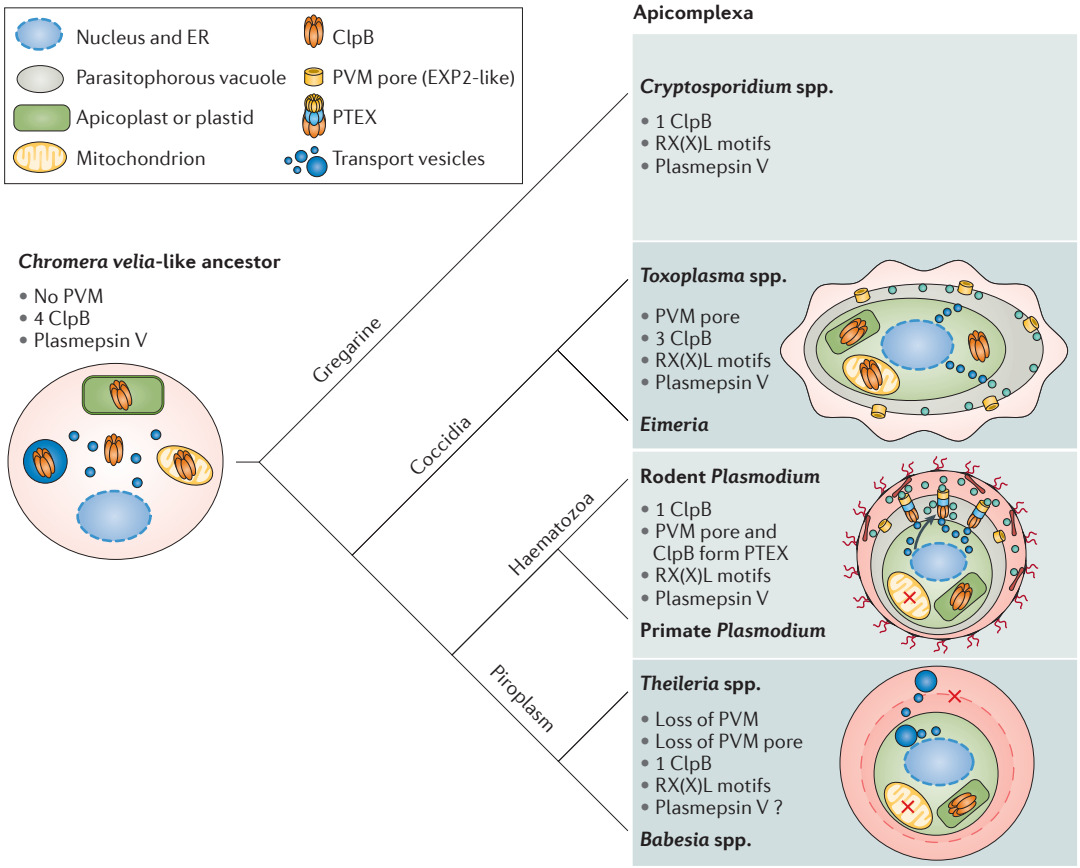
Parasite proteins that are destined for export into the host erythrocyte undergo vesicle-mediated transport from the endoplasmic reticulum (ER) to the parasitophorous vacuole before crossing the encasing

Box 1 | **The export pathway is conserved among apicomplexan parasites**

Extant apicomplexan parasites contain proteins that have signal sequences and RX(X)L *Plasmodium* export element (PEXEL)-like motifs[9,100,101], which is consistent with their capacity to export proteins beyond their plasma membrane and potentially into the host cell (see the figure). The PEXEL-like motif is a substrate for the endoplasmic reticulum (ER)-localized aspartyl protease plasmepsin V in *Plasmodium* spp.[24] and for the Golgi-resident aspartyl protease V (ASP5) in *Toxoplasma gondii*[102–104]. Other apicomplexan parasites seem to encode plasmepsin V-like proteases, which indicates that their proteins with PEXEL-like motifs may be cleaved[101].

Following cleavage, PEXEL proteins of *Plasmodium* spp. are delivered to the parasitophorous vacuole through the secretory pathway and translocated across the parasitophorous vacuole membrane (PVM) by the *Plasmodium* translocon of exported proteins (PTEX)[52,53]. In other apicomplexan parasites, the PEXEL-like motif does not necessarily license a protein for export. In *T. gondii*, PEXEL-like proteins associate with the PVM[100], the notable exception being GRA16, which is delivered to the host cell nucleus[105]. In *Babesia* spp. and *Theileria* spp., the PVM breaks down after erythrocyte invasion and proteins that have a signal sequence can be found in the host cell[101], whereas proteins that also contain a PEXEL-like motif are retained in intracellular spherical bodies[101]. The localization of PEXEL-like proteins in *Cryptosporidium* spp. remains unknown.

The PTEX seems to be specific to *Plasmodium* spp. as homologues of only two of its core proteins, heat shock protein 101 (HSP101) and exported protein 2 (EXP2), have been identified. HSP101 belongs to the ClpB family of chaperones. Although all apicomplexans contain ClpB proteins (see the figure), *T. gondii* is the only parasite that has another ClpB in its cytoplasm that is most similar to HSP101. This raises the possibility that HSP101 might be derived from the cytoplasmic version that is present in an ancestral apicomplexan parasite that may have evolved from a unicellular photosynthetic organism such as *Chromera velia*[106] (see the figure), which has a cytoplasmic-like ClpB with a signal sequence and an aspartyl protease similar to plasmepsin V[107]. Distant homologues of EXP2 are present in the apicomplexans that reside within a PVM. The homologue in *T. gondii*, GRA17, seems to function as a nutrient pore and can be functionally substituted with EXP2 (REF. 66), which suggests that EXP2 may have been a nutrient pore in the ancestor of *Plasmodium* spp. before being repurposed as a protein channel following the recruitment of HSP101. The remaining components of the PTEX may have evolved to provide a structural scaffold[54], to regulate and/or optimize the activity of the PTEX or to help export distinct subsets of protein cargo[107].



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Box 2 | **Erythrocyte remodelling leads to new permeation pathways**

Plasmodium spp. induce the formation of new permeation pathways (NPPs) in infected erythrocytes to increase membrane conductance and the uptake of small molecules, such as sugars, amino acids, nucleosides, anions and cations, to facilitate parasite growth. These pathways also provide a mechanism for the parasite to remove toxic metabolites, such as lactic acid[11,12]. To date, cytoadherence-linked asexual gene 3 (CLAG3) is the only molecular component that has been implicated in the formation of NPPs[108], although how it contributes to channel activity is not yet known. CLAG3 was assigned this misleading name on the basis that one of its paralogues (CLAG9) has been implicated in the binding of infected erythrocytes to host endothelial cells[109]. CLAG3 is produced towards the end of the parasite cell cycle and is stored in the rhoptry secretory organelles, and it enters the erythrocyte during parasite invasion[110]. It is present at the parasitophorous vacuole membrane (PVM) of the nascent ring-stage parasite but does not require the *Plasmodium* translocon of exported proteins (PTEX) to reach its final destination at the periphery of the erythrocyte[52]. Intriguingly, knockdown of the PTEX prevents the formation of the NPPs[52], which suggests that exported proteins also contribute to the activity of NPPs; however, this finding could be attributable to the arrest of parasite growth before the activation of NPPs.

parasitophorous vacuole membrane (PVM; FIG. 2). Some of these proteins, including the cytoadhesion-promoting *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), are inserted into the plasma membrane of the erythrocyte[15,16], others, such as ring-infected erythrocyte surface antigen (RESA) and PfEMP3, become associated with the membrane skeleton[16,17], whereas proteins such as glycophorin binding protein 130 (GBP130)[18] remain in the cytoplasm of the erythrocyte as soluble proteins. Furthermore, some proteins promote the formation of the exomembrane system of the parasite, which acts as a sorting depot for proteins en route to the plasma membrane of the erythrocyte[19,20]. Establishing this extra-parasitic protein-trafficking network is crucial because erythrocytes lack a membrane transport system. A large-scale gene-knockout screen in *P. falciparum* suggested that approximately 25% of the genes that encode proteins that are exported may be essential to the survival of the parasite[10].

In this Review, we describe the sequence motifs that are found in the exportomes of *Plasmodium* spp., and outline the trafficking pathways that are used to selectively transfer proteins of the exportome through the ER to the parasitophorous vacuole (FIG. 2a). We then describe the central role of a novel translocon in facilitating the passage of these proteins across the PVM and the mechanisms by which exported proteins are trafficked to different compartments within the host cell. We also discuss how exported *Plasmodium* spp. proteins remodel the structure of the host erythrocyte to promote their survival. Finally, we highlight features of the export pathway that are conserved among other medically important apicomplexans and discuss how host cell remodelling processes could be targeted to eliminate intra-erythrocytic parasites or to prevent severe disease. In this Review, we mainly focus on *P. falciparum* because host cell remodelling has mostly been studied in this species, but other species are discussed where appropriate.

Directing protein cargo for export

Plasmodium spp. need to sort protein cargo that is destined for export into the appropriate trafficking pathway. Below, we discuss the discovery of the export motif and the proteolytic cleavage events that occur in the ER, which promote export of specific proteins.

Export motifs and the predicted exportome. Early studies that used transfected *P. falciparum* parasites that expressed GFP chimaeras of the knob-associated histidine-rich protein (KAHRP; which is an exported protein that is essential for the formation of knobs) showed that a recessed amino-terminal signal sequence is sufficient to direct co-translational transport into the ER, whereas a downstream sequence is required for export across the PVM[21]. Subsequent ground-breaking studies revealed that this downstream motif contains the pentameric *Plasmodium* export element (PEXEL[22]; also known as host-targeting sequence[23]), which comprises RXLXE/Q/D (in which X denotes a charge-neutral amino acid and solidi represent alternative amino acids for that respective position). Relaxed PEXEL motifs (RXLXXE[24] and R/K/HXL/IXXE[25]) have been identified in some proteins in *P. falciparum*. The predicted PEXEL-containing exportome, which comprises more than 400 proteins in *P. falciparum*, represents 8% of the predicted proteome[9,22–24,26].

P. falciparum also exports several PEXEL-negative proteins (PNEPs)[27–29], including the PfEMP1 family, which consists of approximately 60 proteins[24] (FIG. 2). PNEPs typically contain an internal transmembrane segment that also functions as an ER entry signal[24,28,30]; although some have a standard N-terminal signal sequence[29]. PNEPs do not contain conserved elements, which makes them difficult to identify, but it has been estimated that the total *P. falciparum* exportome, including PNEPs and PEXEL proteins, comprises approximately 550 proteins (that is, 10% of the proteome)[31]. All other *Plasmodium* spp. have a much smaller number of PEXEL proteins[9]; however, they seem to have more PNEPs. Some exported proteins in *P. falciparum* and rodent malaria species exhibit variations in the ascribed PEXEL motif, and for this reason it is possible that *Plasmodium* spp. may export more proteins than currently realized[32–34].

Directing protein cargo for export. Following the discovery of the PEXEL motif, several studies investigated how this motif could license proteins for export. It was identified that this motif is proteolytically cleaved between the third and fourth positions (RXL↓XE/Q/D), co-translationally or soon thereafter, and the new N terminus is subsequently acetylated[35]; however, to date, the enzyme that is responsible for this post-translational modification is unknown. In addition, the contribution of this modification to protein export remains to be determined, as acetylation of the N terminus alone is insufficient to mediate protein export. One hypothesis is that acetylation may enhance the interaction between the new N terminus of the protein cargo and other protein components that function in the subsequent step of the export pathway. Mutation of the arginine or leucine residues in the PEXEL motif to alanine traps exported proteins in the ER, whereas mutation of the fifth position

Rhoptry
A specialized secretory organelle that is located at the apical end of apicomplexan parasites.

Parasitophorous vacuole membrane
(PVM). The membrane that surrounds the parasitophorous vacuole that, in the case of erythrocytes, is formed through the invagination of the membrane bilayer during parasite invasion.

P. falciparum erythrocyte membrane protein 1
(PfEMP1). A major virulence factor of *Plasmodium falciparum*. It is a member of a family of approximately 60 proteins of which one allele is expressed in a particular parasite.

Exomembrane system
A network of membranes that, in addition to the limiting membrane of the parasite, is present in the cytoplasm of the host cell.

Exportomes
The full complement of exported proteins in malaria parasites.

Translocon
A complex of proteins that includes a membrane-spanning channel component and provides a passage for polypeptides to cross membranes.

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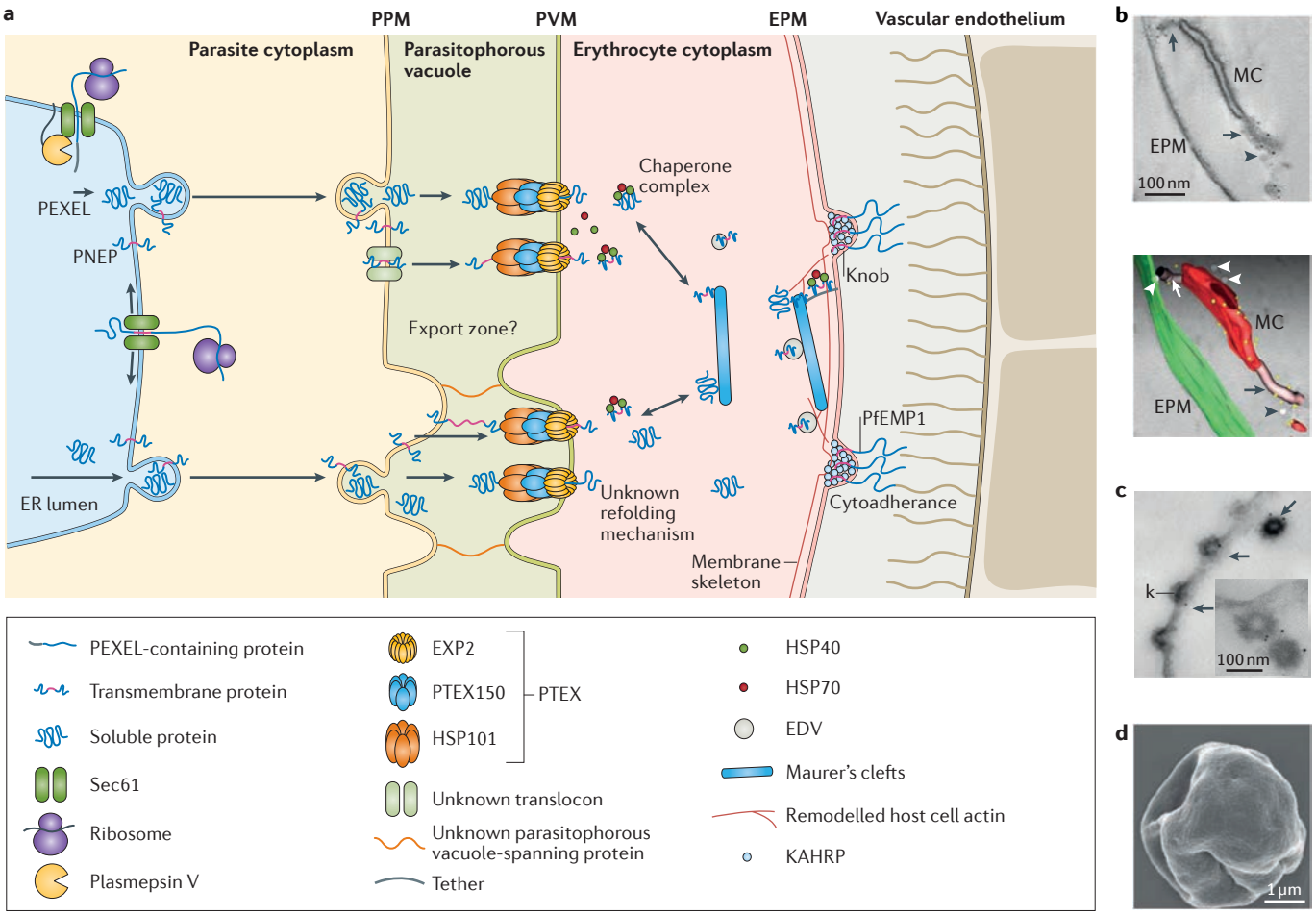

Figure 2 | **Key steps and compartments in the protein export pathway. a** | Diagram of protein export. Proteins are co-translationally inserted into the endoplasmic reticulum (ER) through the Sec61 protein translocon in the ER membrane. *Plasmodium* export element (PEXEL)-containing proteins are cleaved by plasmepsin V to expose a new amino terminus. Proteins that contain transmembrane domains, such as some PEXEL-negative proteins (PNEPs), are inserted into the ER membrane. These proteins are trafficked to the parasite plasma membrane (PPM) either in a soluble or membrane-bound form by secretory vesicles that fuse with the PPM. It is not known whether these vesicles are targeted towards specialized 'export zones' where the PPM is either physically connected to the parasitophorous vacuole membrane (PVM) by parasitophorous vacuole-spanning proteins or closely approaches the PVM, or whether the parasitophorous vacuole space is continuous. At the PPM, soluble proteins are directly released into the parasitophorous vacuole, where the *Plasmodium* translocon of exported proteins (PTEX) can export them. Transmembrane-containing proteins are embedded in the PPM. The mechanisms that are used to remove these proteins from the PPM and translocate them through the PTEX are not well understood. The following possibilities exist, but are not mutually exclusive. Transmembrane proteins may be translocated into the parasitophorous vacuole by an unknown translocon that is located at the PPM or targeted towards export zones that enable the proteins to be directly pulled from the PPM by the PTEX itself. To transit through the PTEX, proteins must be unfolded, potentially involving the PTEX core component heat shock protein 101 (HSP101), which is located on the *cis* side of the PVM. Once translocated, these proteins either need to be refolded immediately or after chaperone-mediated trafficking by complexes. Complexes that contain exported parasite proteins from the HSP40 and HSP70 families may be involved and have been named J-dots. The refolding machinery remains unknown. Following export, proteins can remain soluble within the erythrocyte cytoplasm or traffic to parasite-derived structures such as the Maurer's clefts or the erythrocyte membrane or membrane skeleton. Exported proteins modify the erythrocyte membrane and membrane skeleton, forming structures called knobs, the main component of which is the exported protein knob-associated histidine-rich protein (KAHRP). These knobs act as a platform for the presentation of the major virulence antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). The final step in the trafficking of surface-exposed proteins, such as PfEMP1, may involve the budding of PfEMP1-loaded electron-dense vesicles (EDVs) from the Maurer's clefts and subsequent transfer along connecting skeletal elements, such as tethers or actin structures, followed by fusion with the erythrocyte plasma membrane (EPM). Alternatively, PfEMP1 could transfer along the membrane of the tubule-like tether structures. PfEMP1 is inserted into the EPM and binds to receptors on the vascular endothelium to promote cytoadhesion. **b**–**d** | Micrographs of key structural features. **b** | Virtual section (20 nm; top panel) and rendered model (bottom panel) from an electron tomogram showing a Maurer's cleft body (rendered red), immunolabelled with ring-exported protein 1 (REX1;gold dots), flanked by two tether-like structures (arrows), one of which connects to the erythrocyte membrane. Vesicle-like structures can also be seen (arrowheads). Scale bar is 100 nm. **c** | Equinatoxin-permeabilized *P. falciparum*-infected erythrocyte showing PfEMP1 labelling associated with knobs (k) and EDVs (arrows indicate gold particles). The insert shows PfEMP1-labelled EDVs that consist of a phospholipid core and a proteinaceous outer coat. Scale bar is 100 nm. **d** | Scanning electron micrograph of an infected erythrocyte showing external protrusions (knobs). Scale bar is 1 µm. MC, Maurer's cleft. The images in part **b** are adapted with permission from REF. 111, Wiley. The image in part **c** is adapted with permission from REF. 15, Wiley. The image in part **d** is adapted with permission from REF. 112, Wiley.

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often traps proteins in the parasitophorous vacuole[36]. The ER-resident aspartyl protease plasmepsin V targets the PEXEL motif for cleavage[37,38] (FIG. 2). Plasmepsin V might function together with other proteins to direct the protein cargo into the appropriate export pathway by either shuttling the cleaved protein into a permissive protein chaperone complex or a vesicular trafficking pathway[37]. Although one study found that a reporter protein that was engineered to create an N terminus that is identical to that generated by plasmepsin V following cleavage by signal peptidase was not exported[37], other studies have shown that cleavage by signal peptidase or an exogenous protease is sufficient to target proteins for export[39,40]. This suggests that cleavage by plasmepsin V is not essential for direct coupling to the next stage of the export pathway. Moreover, a reporter protein in which the N terminus that was generated after cleavage of the PEXEL motif was that of an export-refractory sequence was found not to be exported, despite the protein being cleaved by plasmepsin V[39,40], which suggests that the sequence that is immediately downstream of the PEXEL motif is also important for export. Furthermore, the N-terminal region of the PNEP ring-exported protein 2 (REX2) is processed independently of plasmepsin V[41], but it is unclear whether other PNEPs are also proteolytically cleaved. On the basis of these findings, it was suggested that PEXEL proteins and PNEPs share cryptic trafficking elements and thus might be exported through a similar mechanism[39].

Other studies have suggested that the PEXEL motif directs export by functioning as a binding moiety for phosphatidylinositol 3-phosphate (PI3P) on the inner leaflet of the ER[42,43]. Moreover, these studies also revealed that cleavage by plasmepsin V was not necessary for the export of proteins with PEXEL-like arginine-containing motifs (RXLXE/Q/D) but instead was required to release these proteins from ER-derived membranes[42]. However, the same group subsequently reported that neither the binding of plasmepsin V nor PI3P was absolutely required for the export of GFP reporter proteins that contained a modified PEXEL motif in which the arginine was mutated to alanine[43]. Recently, a combined study that involved several research groups attempted to settle the controversy regarding the role of PI3P in protein export in *P. falciparum*. The investigators did not find any evidence of PI3P lipids residing in the ER of the parasite, but they did report that PI3P lipids were present in the membranes of the digestive vacuole and apicoplast[44]. In addition, they could only detect an interaction between the PEXEL motif and PI3P when the reporter protein was misfolded, which might explain the earlier observations. Moreover, they experimentally reiterated that a correctly positioned PEXEL motif with respect to its spacing downstream of the signal sequence, leads to efficient protein cleavage by plasmepsin V and rapid protein export[44]. However, the steps that follow cleavage by plasmepsin V still remain to be elucidated. One hypothesis is that the acetylated newly exposed N termini act as signals for recognition by receptors in the ER that link internal protein cargo to coat proteins on vesicles that are derived from the outside of the ER membrane[36].

After passage through the ER, cleaved PEXEL-containing proteins and PNEPs are loaded into secretory vesicles and trafficked to the plasma membrane of the parasite (FIG. 2). Soluble protein cargo is released directly into the parasitophorous vacuole for subsequent transport into the erythrocyte. By contrast, membrane proteins are either transported through the secretory pathway as soluble chaperoned complexes[45] or, in the case of PNEPs, are likely to be inserted into the ER membrane for vesicular delivery to the plasma membrane of the parasite, into which they are subsequently incorporated. PNEPs may be directly extracted from the plasma membrane and then transported across the PVM into the erythrocyte, or they are first extracted into the parasitophorous vacuole before transport into the erythrocyte[39] (FIG. 2).

Crossing the PVM

To enter the host cell, cleaved PEXEL proteins and PNEPs must cross the PVM. The PVM contains pores that enable the free passage of metabolites up to 1,400 Da (REF. 46). However, parasite proteins do not passively diffuse across the membrane; this led to the hypothesis that a translocon exists at the PVM. Indeed, the *Plasmodium* translocon of exported proteins (PTEX; FIG. 2) was identified at the PVM[47], which is consistent with the observations that proteins need to be in an unfolded state to cross the PVM[48] and that ATP is required[49].

Composition and localization of the PTEX. The PTEX comprises five components in *P. falciparum*[47] and in the rodent malaria species *Plasmodium berghei*[50] (FIG. 3). The three core components are the AAA+ ATPase heat shock protein 101 (HSP101), exported protein 2 (EXP2) and PTEX150. Two additional components of the PTEX — thioredoxin 2 (TRX2) and PTEX88 — are non-essential proteins during the blood stages of the parasite life cycle[50,51]. By contrast, disruption of the genes that encode the three core proteins has not been successful, presumably because these proteins are essential for the survival of the parasite[47,50,51]. Conditional knockdown of the expression of HSP101 (REF. 52) and PTEX150 (REF. 53) provided the first functional evidence that the PTEX has a crucial role in the export of diverse types of soluble and transmembrane domain-containing protein cargo, including PEXEL proteins and PNEPs.

Several studies have investigated the composition of the native intact PTEX and its constituents using blue-native polyacrylamide gel electrophoresis (BN–PAGE) and immunoprecipitation under non-reducing conditions and under increasing stringency. EXP2, PTEX150 and HSP101 were found to assemble in that order relative to the PVM to form a stable complex that is ~1,230 kDa in size[54,55] (FIG. 3). Mass spectrometry and western blotting of the ~1230 kDa BN–PAGE species confirmed that all components of the PTEX, except TRX2 (but including PTEX88), are present in this complex, with HSP101 and PTEX150 at a stoichiometry of 1:1 (REF. 55). However, the stoichiometry of the remaining components in the PTEX remains unclear owing to the lack of experimental tools, such as antibodies, that are specific for PTEX88

Knobs
Electron-dense protrusions on the membrane of erythrocytes that are infected with *Plasmodium falciparum* and that increase the stiffness and adhesiveness of the erythrocyte.

Plasmodium export element (PEXEL). A pentameric amino acid motif in the amino-terminal region of proteins that guides their export.

PEXEL-negative proteins (PNEPs). *Plasmodium* export element (PEXEL)-negative exported proteins lack a PEXEL motif and contain a hydrophobic region that mediates entry into the secretory pathway.

Plasmepsin V
A parasite aspartyl protease that cleaves the *Plasmodium* export element (PEXEL) motif.

Plasmodium translocon of exported proteins (PTEX). The *Plasmodium* translocon of exported proteins provides a selective gateway for parasite proteins to traverse the parasitophorous vacuole membrane and access the host erythrocyte.

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and TRX2. The absence of TRX2 from the ~1,230 kDa species may be due to its small size and low expression levels that fall below the detection limit. In addition to the full-size multimeric complex of ~1,230 kDa, EXP2, HSP101 and PTEX150 are found in protein species that are ~600 kDa, ~500 kDa and ~250 kDa in size, respectively[54,55]. These represent either homo-oligomers or smaller complexes that comprise other proteins (such as TRX2 or PTEX88), as each subcomplex only reacted with antibodies to EXP2, HSP101 or PTEX150 rather than with several antibodies. This suggests that cycles of assembly and disassembly of several components of the PTEX may be integral to its function.

Immunofluorescence microscopy studies revealed that the PTEX is localized in discrete regions of the parasitophorous vacuole and PVM. Similarly, exported cargo proteins exhibit a 'necklace of beads' pattern at the parasitophorous vacuole[21,56]. Super-resolution light microscopy and immunoelectron microscopy of GFP-tagged PfEMP1 indicated that this protein localizes to bulging regions of the parasitophorous vacuole that have a diameter of ~150 nm (REF. 15). These structures may represent export zones where the PTEX machinery resides and where protein cargo accumulates before export (and conversely where non-exported proteins are excluded; FIG. 2); however, experimental support for this notion is still lacking. It is intriguing to consider how this compartmentalization might be achieved and whether protein cargo is specifically directed from the ER to the sub-compartments of the parasitophorous vacuole that are enriched for the PTEX or whether some type of 'barcoding' is required to direct protein cargo that arrives in the parasitophorous vacuole to the PTEX (FIG. 2).

Extensions and whorls that emanate from the parasitophorous vacuole form the tubulovesicular network[57–60]. Proteins that are resident in the parasitophorous vacuole and exported proteins seem to be excluded from these structures in *P. falciparum*[15,56,61], but evidence suggests that related structures in *P. berghei* may contain components of the PTEX[60]. EXP2 has also been observed in membranous vesicles in reticulocytes that are infected with *P. falciparum* and the rodent parasites, *Plasmodium yoelii* and *P. berghei*[62]. These vesicles are usually absent from infected mature erythrocytes, which indicates that the localization of EXP2 may be determined by the environment of the host cell[62]. Whether these vesicles represent membranous structures that have budded from the PVM and have a role in protein export remains to be determined.

Contribution of components of the PTEX to protein export. Protein translocons exhibit structural features that are intrinsic to their function[63]. Accordingly, it is expected that the PTEX comprises a protein-conducting channel (also known as the pore), a molecular motor to power translocation, adaptors to facilitate the direct or indirect docking of protein cargo to the target membrane, and chaperones to keep proteins in a translocation-competent state and to help re-fold the proteins once inside the erythrocyte compartment (FIGS 2,3). As translocons are often modular to accommodate diverse types of protein cargo under different environmental conditions, the PTEX may recruit additional adaptor components to help translocate the exportome.

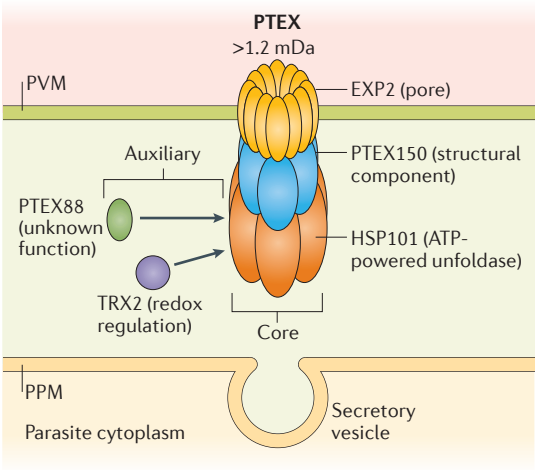

Figure 3 | **A putative model of the translocon.** To enter into the host cell, cleaved *Plasmodium* export element (PEXEL) proteins and PEXEL-negative proteins (PNEPs) must cross the parasitophorous vacuole membrane (PVM) through the *Plasmodium* translocon of exported proteins (PTEX). This ~1.2 mDa complex is localized in discrete regions of the parasitophorous vacuole and PVM. The PTEX comprises homo-oligomers of three components: an AAA+ ATPase heat shock protein 101 (HSP101), exported protein 2 (EXP2) and PTEX150, which are essential for the survival of *Plasmodium* spp. Two additional components, PTEX88 and thioredoxin 2 (TRX2), are part of the PTEX, but are not essential for the survival of the parasite. They are classified as auxiliary components that are presumably recruited to the PTEX to optimize the translocation of protein cargo. EXP2 has been proposed to function as the protein-conducting channel (the pore) in the PVM that facilitates protein transport. PTEX150 has been suggested to have a structural role and to regulate the stability of the PTEX. HSP101, which is located on the *cis* side of the PVM, is possibly the molecular motor of the PTEX. TRX2 might contribute to the unfolding of cargo proteins to promote their export, or it could regulate the activity and/or assembly of components of the PTEX through a redox-based mechanism. The function of PTEX88 remains unknown. PPM, parasite plasma membrane.

EXP2 is thought to function as the membrane-spanning component of the PTEX (FIG. 3). Although EXP2 does not have canonical transmembrane segments, it is tightly associated with the PVM[47,54,64] and is predicted to adopt a similar conformation to haemolysin E (HlyE), a toxin in *Escherichia coli* that forms dodecameric pores in host cell membranes[47]. Although protein substrates that were stuck in the process of translocation across the PVM were present in a complex with EXP2 (REF. 65), functional evidence that EXP2 acts as the PTEX pore is still lacking. Interestingly, all apicomplexans that reside in a parasitophorous vacuole encode an orthologue of EXP2 (BOX 1). In *Toxoplasma gondii,* this orthologue is known as GRA17, which, similarly to EXP2, is secreted from the dense granules and localizes to the PVM[66]. The

Dense granules
Small vesicular bodies that are located at the apical end of the cytoplasm in the invasive form (merozoite) of the malaria parasite.

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EXP2 protein of *P. falciparum* can complement the function of GRA17 in *T. gondii*. However, knockdown of the expression of GRA17 in *T. gondii* did not affect the export of GRA24 and the PEXEL-containing protein GRA16. Instead it led to a decrease in the movement of small molecules across the PVM[66]. Furthermore, membrane conductance was enhanced in *Xenopus laevis* oocytes in which GRA17 and EXP2 were overexpressed[66], but the time that was required to alter the conductance was much longer than would be expected for proteins that have functions in pore formation.

These findings suggest the intriguing possibility that EXP2 may have a dual function in the transport of proteins and small molecules. The inability to knockout *exp2* during the blood stages of the parasite life cycle[50,51], presumably because this is lethal to the parasite, and the decreased patency of conditional EXP2 mutants[67] are consistent with this notion. Indeed, the transcriptional expression profile of EXP2 is different to that of other components of the PTEX, and although EXP2 colocalizes with components of the PTEX, it is also found in other compartments of the PVM[47,60]. Thus, in the presence of other components of the PTEX, EXP2 is likely to have a role in protein export, but when the other components of the PTEX are not available for complex formation EXP2 might contribute to the transport of small molecules (BOX 1; FIGS 2,3).

HSP101, which is located on the *cis* side of the PVM, is most likely to be the molecular motor of the PTEX (FIGS 2,3). The inhibition of HSP101 function in *P. falciparum*[52] and knockdown of HSP101 expression in *P. berghei*[53] traps protein cargo in the parasitophorous vacuole in a soluble state and arrests parasites at the transition between the ring stage and the trophozoite stage. Arrested *P. falciparum* parasites, in which the function of HSP101 was blocked, remained viable for some time and could grow again when the function of HSP101 was restored. These results are consistent with the hypothesis that HSP101 provides the energy that is required for protein translocation and/or promotes the unfolding of protein cargo. Loss of viability by inhibiting the function of HSP101 may develop from the failure to export essential cargo, or the failure to pass a cell cycle check-point due to the accumulation of unfolded protein.

In bacteria and archaea, the translocation of secretory proteins through the SecY pore triggers the SecA ATPase on the *cis* side of the membrane to 'push' proteins from the cytoplasm into the extracellular space[68]. In the organelles of eukaryotic cells, soluble molecular chaperones of the HSP70, HSP90 or HSP100 protein families power protein translocation by 'pulling' from the lumenal (*trans*) side of the membrane[68–70]. *P. falciparum* exports HSP70-x into the cytoplasm of erythrocytes[30]; however, HSP70-x is only present in *P. falciparum* and the closely related *Plasmodium reichenowi,* but not other *Plasmodium* spp. HSP70-x associates with exported HSP40 proteins in J-dots within the cytoplasm of erythrocytes[30] (FIG. 2). This has led to the suggestion that HSP70-x is involved in the trafficking of *P. falciparum*-specific proteins, such as PfEMP1, rather than driving protein translocation per se. Human HSP70 is another potential motor component that drives the PTEX. Following the infection of erythrocytes with *P. falciparum*[71], HSP70 associates with the PVM and is highly enriched in PTEX150 pull-down assays[47]. Therefore, although pushing by HSP101 represents the most likely energy source that drives protein translocation, a push–pull mechanism that involves chaperones on both sides of the membrane is possible (FIG. 2).

PTEX150 is also essential for the survival of *Plasmodium* spp. in erythrocytes[47,53]. PTEX150 exhibits no homology to proteins that are expressed outside the *Plasmodium* genus and has no domains of known function. PTEX150 binds tightly to both HSP101 and EXP2 (REF. 54). Truncation of 125 amino acids from the carboxy-terminal end of the protein leads to a decrease in its association with other PTEX subunits, which indicates that this region regulates the stability of the PTEX and that PTEX150 has a structural role, linking HSP101 to EXP2 (REF. 55) (FIGS 2,3). Consistent with this, knockdown of PTEX150 gene expression results in decreased parasite growth and protein translocation across the PVM[53].

The remaining two validated components of the PTEX, TRX2 and PTEX88, are not essential for the survival of *P. berghei* in mice[50,51] and can therefore be considered accessory proteins of the PTEX. However, disruption of *trx2* increased the length of the *P. berghei* cell cycle[50] and significantly decreased the global expression of proteins on the surface of the infected erythrocyte[53]. Thus, TRX2 may contribute to the unfolding of protein cargo to promote their export, or it could regulate the activity and/or assembly of components of the PTEX through the reduction and oxidation of disulfide bonds within respective components. Mice that were infected with *P. berghei* parasites in which *ptex88* was knocked-out showed a moderate growth phenotype, which may develop from the decreased ability of the parasite to sequester in organs and their increased clearance from the circulation. Consequently, infected mice do not succumb to cerebral malaria[72,73]. Although knockdown of the expression of PTEX88 in *P. falciparum* did not affect parasite growth *in vitro,* it decreased the cytoadherence capacity of the infected erythrocyte[73]. However, loss of PTEX88 expression in *P. berghei* and *P. falciparum* does not lead to an obvious defect in the export of numerous proteins[72,73] and thus one possibility is that PTEX88 functions as an adaptor for HSP101 to translocate a specific subset of protein cargo.

Protein transport in the erythrocyte

After proteins of the parasite gain access to the erythrocyte cytoplasm through translocation across the PVM by PTEX, they need to traffic to their final cellular location where they exert their function. Exported proteins have three possible destinations within the erythrocyte: the erythrocyte membrane and membrane skeleton, membranous structures called Maurer's clefts, or the erythrocyte cytoplasm (as soluble proteins; FIGS 1,2).

After exiting the PTEX, proteins that are destined for the erythrocyte plasma membrane seem to transit through the erythrocyte cytoplasm through a vesicle-independent

Patency
The time point during an infection when parasites are detectable.

J-dots
Proteinaceous chaperone complexes that are present in the cytoplasm of erythrocytes that are infected with *Plasmodium falciparum*.

Maurer's clefts
Flattened, single-membrane-bound structures that are constructed by *Plasmodium falciparum* in the cytoplasm of infected erythrocytes. These clefts receive exported protein cargo and deliver it to the surface of the erythrocyte.

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Lamellae
Thin, plate-like membranous structures.

Cisterna
Flattened membrane disc-like structures.

Dynamins
Large GTPases that are implicated in the budding and scission of nascent vesicles from their parent membranes.

route as chaperone-associated transport complexes[17,74] (FIG. 2). Consistent with this, the deletion of membrane-targeting domains from exported proteins leads to the accumulation of soluble, exported proteins within the erythrocyte cytoplasm[39,75]. Similarly, fluorescence photobleaching of PfEMP1–GFP and PfEMP3–GFP chimaeras reveals rapid protein diffusion, which is consistent with protein complex-mediated trafficking processes. In addition, it was shown that a population of PfEMP1 is associated with HSP70-x in J-dots[30], which supports the notion that chaperones are involved in the trafficking of PfEMP1 to Maurer's clefts (FIG. 2).

Maurer's clefts function as a sorting depot. The trafficking of parasite proteins to the erythrocyte membrane is dependent on the formation of an exomembrane system and prominent features of this system are Maurer's clefts[76,77] (FIG. 2). These closed cisternal compartments are thought to function as a sorting depot for proteins en route to the erythrocyte membrane[19,20]. A full complement of Maurer's cleft-like structures are formed soon after invasion of the erythrocyte[78], and resident Maurer's cleft proteins are presumably transported to pre-formed structures through the chaperoned delivery system that is described above (FIG. 2).

Several proteins in Maurer's clefts have been shown to have important roles in shaping these organelles and in the efficient delivery of protein cargo to the erythrocyte surface. For example, inducible knockdown or deletion of REX1 in the *P. falciparum* parasite line 3D7 results in the formation of stacked Maurer's clefts. Furthermore[79], when a highly charged repeat-sequence domain in REX1 is deleted, giant stacks of clefts form and in some cases remain interconnected and attached to the PVM[79], which indicates that REX1 has a crucial role in maintaining the structure of Maurer's clefts. In addition, these results suggest that Maurer's cleft lamellae are formed from a single focus at the PVM and are then severed into individual cisterna and released. There is no sequence homology between REX1 or other Maurer's cleft-associated proteins and the vesicle coat and severing proteins that mediate canonical vesicle-dependent trafficking in other organisms. However, many of the proteins that reside in Maurer's clefts have domains that are predicted to form extended α-helices and it is possible that these α-helices form helical 'coats' that constrict membranes and promote fission in a manner similar to dynamins[80,81].

The transmembrane Maurer's cleft-associated protein PfEMP1 trafficking protein 1 (PTP1) has an important role in the trafficking of two surface-exposed antigens — PfEMP1 and subtelomeric variant open reading frame (STEVOR). PTP1 is associated with a subpopulation of J-dots, and its genetic deletion results in the fragmentation of the Maurer's clefts and arrest of the export of PfEMP1 and STEVOR at the PVM[82]. This suggests roles in chaperoning proteins to, and maintaining the structure of, Maurer's clefts. Several other proteins that are associated with Maurer's clefts also have roles in maintaining the morphology of Maurer's clefts or in promoting the efficient delivery of PfEMP1 to the surface of erythrocytes (TABLE 1).

Trafficking to the erythrocyte membrane. Exported proteins are either trafficked directly to the erythrocyte membrane and membrane skeleton by chaperone-mediated complexes, or indirectly through Maurer's clefts. Forward trafficking of proteins from the Maurer's clefts to the erythrocyte membrane may use chaperone-mediated complexes or a vesicle-mediated transport mechanism. Associated with the Maurer's clefts are numerous 25 nm uncoated vesicles[20,83–85] and a population of 80–100 nm electron-dense vesicles (EDVs) that have a thick coat[15,85] (FIG. 2). EDVs appear in the early trophozoite stage of parasite development when PfEMP1 is first delivered to the erythrocyte surface[85]. PTP2 is a marker for these vesicles[82,86], and they are also labelled by antibodies against PfEMP1 and PfEMP3 (REFS 15,87). Distinct tether-like structures (tubes approximately 25 nm by 200 nm)[85,88] are also closely associated with the Maurer's clefts. These structures often connect to the erythrocyte membrane skeleton and may help to physically tether the Maurer's clefts to the membrane skeleton, but do not provide a membrane continuum[85,88]. Membrane-associated histidine-rich protein 2 (MAHRP2) is a marker of tethers, and these structures are also labelled by antibodies against PfEMP1 (REF. 15). The roles that these vesicular and tether-like structures have in the trafficking of proteins, such as PfEMP1, to the erythrocyte membrane, are poorly understood. The process may involve the budding of PfEMP1-loaded EDVs from the Maurer's clefts and subsequent transfer along connecting skeletal elements (see below), followed by fusion with the erythrocyte membrane. Alternatively, PfEMP1 could transfer along the membrane of the tethers and then bud onto the erythrocyte surface (FIG. 2).

Remodelling the host erythrocyte membrane. During the trophozoite stage of an infection with *P. falciparum*, the host erythrocyte has a markedly altered surface morphology, under going substantial changes in permeability through the establishment of the NPPs (BOX 2) and in deformability. The roles that exported proteins have in driving these modifications are starting to be resolved.

Approximately 20 h post-invasion, the host membrane becomes distorted with knob-like protrusions, which are caused by the assembly of KAHRP into a spiral cone-like structure that is linked to the erythrocyte membrane cytoskeleton[89]. PfEMP1 is inserted into the membrane and concentrated at these knob structures[19,83] (FIG. 2). Knob-deficient *P. falciparum*-infected erythrocytes have decreased rigidity and decreased levels of cytoadhesion[90,91].

Additional structural modifications of the infected erythrocyte membrane have been identified by ultrastructural studies. Atomic force microscopy has shown that the erythrocyte spectrin–actin skeletal meshwork expands as the parasite matures[92,93]. Cryo-electron tomography revealed branched actin filaments (each ~500 nm in length) that connect the Maurer's clefts to the erythrocyte membrane skeleton in the vicinity of the knobs[94] (FIG. 2). These filaments seem to be produced by parasite-mediated mining of erythrocyte actin from cytoskeletal junction points[92,93], which potentially leads

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Table 1 | **Exported proteins of *Plasmodium falciparum* that have roles in PfEMP1 trafficking**

Protein name [PlasmoDB ID]	Location	Export class	Function	Interacting partners	Refs
REX1 [PF3D7_0935900]	Maurer's cleft	PNEP	• Decreased cytoadhesion following gene deletion, as PfEMP1 is retained at Maurer's clefts • Deletion causes the 'stacking' of Maurer's cleft into Golgi-like bundles	SEMP1*	27,75,79, 111–113
MAHRP1 [PF3D7_1370300]	Maurer's cleft	PNEP	• Gene deletion decreases PfEMP1 trafficking to the erythrocyte surface, which leads to impaired cytoadhesion • Important for Maurer's cleft stability	Unknown	114–116
SBP1 [PF3D7_0501300]	Maurer's cleft	PNEP	• Deletion affects the morphology of Maurer's cleft, PfEMP1 trafficking, cellular rigidity and cytoadhesion • Interacts with the erythrocyte membrane skeleton	• Spectrin • Protein 4.1R • PTP1	117–120
PTP1 [PF3D7_0202200]	• Maurer's cleft • Erythrocyte cytoplasm	PEXEL	• Involved in PfEMP1 trafficking • Deletion of this protein affects Maurer's cleft architecture, causing vesiculation	• PfEMP1 • SBP1 • PIESP2‡ • GEXP10§ • PTP6 • PF3D7_0532300	10,121
PTP2 [PF3D7_0731100]	• Maurer's cleft • EDV	PEXEL	• Involved in PfEMP1 trafficking • May have a role in the formation of EDVs from Maurer's clefts	Unknown	10,86
PTP3 [PF3D7_1478600]	Erythrocyte cytoplasm	PEXEL	• Involved in PfEMP1 trafficking • Deletion increases erythrocyte rigidity	Unknown	10
PTP4 [PF3D7_0730900]	Unknown	PEXEL	• Involved in PfEMP1 trafficking • Deletion decreases erythrocyte rigidity	Unknown	10
PTP5 Exoantigen PF70 [PF3D7_1002100]	Unknown	PEXEL	• Involved in PfEMP1 trafficking • Deletion decreases erythrocyte rigidity	Unknown	10,122
PTP6 [PF3D7_1302000]	Unknown	PEXEL	• Involved in PfEMP1 trafficking	PTP1	10

EDV, electron-dense vesicle; MAHRP1, membrane-associated histidine-rich protein 1; PEXEL, *Plasmodium* export element; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; PNEP, PEXEL-negative protein; PTP, PfEMP1 trafficking protein; REX1, ring-exported protein 1; SBP1, skeleton-binding protein 1; SEMP1, senescence-associated epithelial membrane protein 1. *REX1 was co-precipitated with SEMP1 (REF. 123); ‡ The PlasmoDB ID for PIESP2 is PF3D7_0501200. §The PlasmoDB ID for GEXP10 is PF3D7_0113900 (hyp8).

to the observed reorganization of the membrane skeleton of the host cell[95]. The differential phosphorylation of skeletal components that is induced by exported parasite proteins could also lead to changes in the molecular organization of erythrocyte membrane proteins[96,97]. Several exported parasite proteins have been implicated in actin mining, membrane skeleton reorganization and modulation of cellular deformability (TABLE 2).

The physical roles of knobs and membrane skeleton remodelling in increasing the stiffness of the infected erythrocyte and enhancing its adhesiveness are becoming clearer (FIG. 2). A coarse-grained molecular dynamics model of the *P. falciparum*-infected erythrocyte membrane indicates that skeletal remodelling combined with the deposition of knobs (which both stiffens the membrane and leads to enhanced vertical interactions with the membrane skeleton) can account for the increase in rigidity[95]. The high stiffness that results from the deposition of knobs and skeletal reorganization facilitates the adhesion of the infected erythrocyte to endothelial cell ligands by distributing the tensional forces that are imposed on individual PfEMP1 molecules across the entire knob region and through to the cytoskeleton. This increases the maximum force that is sustainable by PfEMP1-mediated linkages[95]. Therefore, increases in the density of knobs are predicted to not only enable better presentation of PfEMP1, but also to increase rigidity and facilitate the adhesion of infected erythrocytes to low-copy-number ligands in regions of the microvasculature that exhibit increased blood flow (FIG. 2). The ability of *P. falciparum* to adhere to the microvasculature is important as it enables the parasite to evade clearance through the spleen. However, it is also a contributing factor to organ dysfunction in patients with severe disease, as sequestered parasites can block blood vessels and cause ischaemia, and induce the production of pro-inflammatory cytokines that can damage endothelial cells and increase vascular permeability[5].

Outlook

Since the discovery of the trafficking signals that mediate protein export in *Plasmodium* spp. more than a decade ago, research on host cell remodelling by malaria parasites has proceeded rapidly, mainly owing to advances in genetic engineering and imaging technologies[98]. Molecular insights have revealed how the exportome of *Plasmodium* parasites facilitates survival in erythrocytes — host cells that are both metabolically limited and under constant surveillance by the spleen. However, the function of many exported proteins still remains to be determined.

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Table 2 | ***Plasmodium falciparum*** **exported proteins that function at the erythrocyte skeleton**

Protein name [PlasmoDB ID]	Location	Export class	Function	Interacting partners	Refs
KAHRP [PF3D7_0202000]	Erythrocyte skeleton	PEXEL	• Main component of knobs • Deletion leads to parasites that lack knobs and that have reduced rigidity and adhesion under flow	Spectrin, actin, PfEMP1	21,90, 124,125
RESA (formerly known as Pf155) [PF3D7_0102200]	Erythrocyte skeleton	PEXEL	• Deletion leads to a decrease in erythrocyte rigidity • May function to stabilize the modified erythrocyte membrane	Spectrin	16, 126–128
MESA (also known as PfEMP2) [PF3D7_0500800]	Erythrocyte skeleton	PEXEL	• Natural genetic deletion leads to reduced rigidity	Protein 4.1R	129,130
PfEMP3 [PF3D7_0201900]	• Maurer's cleft • Erythrocyte skeleton	PEXEL	• Deletion leads to decreased rigidity of the erythrocyte by disruption of the spectrin–actin–protein 4.1R interaction	Spectrin	127,131
LyMP [PF3D7_0532400]	Erythrocyte skeleton	PEXEL	• Gene deletion leads to a decrease in cytoadherence • Contains a PHISTb domain	PfEMP1	132–134
FIKK4.2 [PF3D7_0424700]	K-dots	PEXEL	• Deletion leads to decreased rigidity and decreased cytoadhesion • No direct effect on PfEMP1 trafficking	Unknown	97,135
SURFIN 4.2 [PF3D7_0424400]	• Maurer's cleft • Erythrocyte membrane	PNEP	• Deletion decreases erythrocyte rigidity	Unknown	10,136
STEVOR* [Gene family]	• Maurer's cleft • Erythrocyte membrane	PEXEL	• Increased expression of STEVOR decreases deformability • Mediates resetting to uninfected erythrocytes	Unknown	137,138
Conserved *Plasmodium* membrane protein [PF3D7_0404600]	Unknown	PNEP	• Deletion decreases erythrocyte rigidity	Unknown	10
Conserved *Plasmodium* protein [PF3D7_0801900]	Unknown	PNEP	• Deletion decreases erythrocyte rigidity	Unknown	10
Exported protein [PF3D7_1401600]	Unknown	PEXEL	• Deletion decreases erythrocyte rigidity • *P. falciparum* conserved region (TIGR FAM 01639) • PHISTb protein	Unknown	10
Pf332 [PF3D7_1149000]	• Maurer's cleft • Erythrocyte skeleton	PNEP	• Deletion increases erythrocyte rigidity and decreases cytoadhesion • Binds to erythrocyte skeleton through actin • Involved in Maurer's cleft morphology and PfEMP1 trafficking	Actin SEMP1‡	139–141
GBP130 [PF3D7_1016300]	• PVM • Erythrocyte cytoplasm	PEXEL	• Infected erythrocyte rigidity increased following gene deletion	SEMP1‡	10,18
Exported protein [PF3D7_0220100]	Unknown	PEXEL	• Deletion increases erythrocyte rigidity • May promote host erythrocyte survival	Unknown	10
Exported protein (hyp12) [PF3D7_1301400]	Unknown	PEXEL	• Deletion increases erythrocyte rigidity	Unknown	10
Exported protein [PF3D7_0424600]	Unknown	PEXEL	• Deletion affects knob formation • RESA-like molecule • PHISTb protein	Unknown	10,142
Exported protein [PF3D7_1039100]	Unknown	PEXEL	• Deletion affects knob formation • Contains a DnaJ domain	Unknown	10

GBP130, glycophorin binding protein 130; KAHRP, knob-associated histidine-rich protein; LyMP, lysine rich membrane-associated PHIST protein; MESA, mature parasite-infected erythrocyte surface antigen; PEXEL, *Plasmodium* export element; Pf332, *Plasmodium falciparum* antigen 332; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; PHISTb, poly-helical interspersed subtelomeric subclass b; PNEP, PEXEL-negative protein; PVM, parasitophorous vacuole membrane; RESA, ring-infected erythrocyte surface antigen; SEMP1, senescence-associated epithelial membrane protein 1; STEVOR, subtelomeric variant open reading frame; SURFIN4.2, surface-associated interspersed gene 4.2. *See REF. 143 for a complete list of STEVOR proteins. ‡Pf332 and GBP130 were co-precipitated with SEMP1 (REF. 123).

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Although *Plasmodium* spp. share some common trafficking pathways with other apicomplexans and higher organisms, they have also evolved crucial new components (BOX 1) — the functions of which are mostly unknown.

Several key gaps remain in our understanding of how processing events that occur in the ER translate to recognition by the protein export machinery at the PVM. It is unclear why *Plasmodium* spp. have two classes of exported protein (those that contain PEXELs and PNEPs) and why they require both plasmepsin V-dependent and plasmepsin V-independent pathways to traffic proteins from the ER to the parasite membrane. Could this be related to differences in the topology and subcellular localization of proteins or do they have independent evolutionary origins? In addition, the events that occur between PEXEL processing in the ER and the recognition of cargo by the PTEX remain to be determined. For example, it is not known whether proteins that are destined for export have a sequence barcode that specifically shuttles them to the PTEX or whether this specificity is achieved by the secretory pathway, whereby exported proteins are delivered to export compartments in the parasitophorous vacuole in which the PTEX resides. Similarly, aspects of the mechanisms by which proteins exit the PTEX, are refolded and delivered to their final location in the host erythrocyte require further investigation.

Finally, the unique components of the protein export pathway provide potential new drug targets, and inhibitors of plasmepsin V have been produced[99]. A more detailed understanding of the steps in the export pathway may reveal new strategies to target the parasite, such as blocking the assembly of the PTEX or the delivery of PfEMP1 to the surface of infected erythrocytes. The latter could lead to the generation of parasites with low virulence that could be used in vaccine trials. Future studies are also required to clarify whether some of the features of the export pathway in the asexual erythrocytic stages are also conserved among the gametocyte and liver-stage parasites and hence whether drugs that target host cell remodelling may be effective across the malaria life cycle.

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Competing interests statement
The authors declare no competing interests.

DATABASES
PlasmoDB: http://plasmodb.org/plasmo
PF3D7_0102200 | PF3D7_0113900 | PF3D7_0201900 | PF3D7_0202000 | PF3D7_0202200 | PF3D7_0220100 | PF3D7_0404600 | PF3D7_0424400 | PF3D7_0424600 | PF3D7_0424700 | PF3D7_0500800 | PF3D7_0501200 | PF3D7_0501300 | PF3D7_0532300 | PF3D7_0532400 | PF3D7_0730900 | PF3D7_0731100 | PF3D7_0801900 | PF3D7_0935900 | PF3D7_1002100 | PF3D7_1016300 | PF3D7_1039100 | PF3D7_1149000 | PF3D7_1301400 | PF3D7_1302000 | PF3D7_1370300 | PF3D7_1401600 | PF3D7_1478600 |

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