The parasitophorous vacuole of the blood stage malaria parasite

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During intraerythrocytic development, malaria parasites replicate within a membrane-bound parasitophorous vacuole. In this Review, Matz, Beck and Blackman explore the origin, development, molecular composition and functions of the parasitophorous vacuole during blood stage development. They also discuss the relevance of the malaria parasite's intravacuolar lifestyle for successful erythrocyte infection and provide perspectives for future research directions.

ABSTRACT

The pathology of malaria is caused by infection of red blood cells with unicellular *Plasmodium* parasites. During blood stage development, the parasite replicates within a membrane-bound parasitophorous vacuole. A central nexus for host–parasite interactions, this unique parasite shelter functions in nutrient acquisition, sub-compartmentalization and the export of virulence factors, making its functional molecules attractive targets for the development of novel intervention strategies to combat the devastating impact of malaria. In this Review, we explore the origin, development, molecular composition and functions of the parasitophorous vacuole of *Plasmodium* blood stages. We also discuss the relevance of the malaria parasite's intravacuolar lifestyle for successful erythrocyte infection and provide perspectives for future research directions in parasitophorous vacuole biology.

[H1] Introduction

Parasites of the genus *Plasmodium* are the causative agents of malaria. These unicellular organisms pose a major threat to global health and substantially impact the economies of many developing countries. In 2017, ~435,000 people died of malaria, with most deaths occurring in sub-Saharan Africa in children under five years of age[1]. Although rigorous control programs have achieved substantial reductions in the global malaria burden, eradication of this devastating disease remains one of the most testing medical challenges of the 21st century. The development of innovative, evidence-based intervention strategies strictly depends on our improved understanding of the parasite's complex biology.

The multi-stage *Plasmodium* life cycle includes transmission from an *Anopheles* mosquito to a vertebrate host[2]. Throughout its development in the vector, the parasite remains extracellular, committing to an intracellular lifestyle only upon transmission to the vertebrate host where it initially invades hepatocytes in the liver. Following a phase of substantial expansion, thousands of specialized invasive forms called merozoites **[G]** are released into the bloodstream where they invade circulating red blood cells (RBCs). The intraerythrocytic parasite transitions through ring **[G]**, trophozoite **[G]** and schizont **[G]** stages before giving rise to multiple daughter merozoites through a process referred to as schizogony. The merozoites are then released through parasite-mediated host cell rupture to establish a new round of RBC infection. These repeated infection cycles lead to the often fatal pathology of malaria, which in its most severe form features high fever, anaemia, organ failure and coma[3].

Throughout its intracellular development, the parasite resides inside a membrane-bound compartment — the so-called parasitophorous vacuole. This Review explores our knowledge of the intraerythrocytic parasitophorous vacuole with the aim of highlighting emerging frontiers in the investigation of this highly specialized host–pathogen interface.

[H1] Parasitophorous vacuole biogenesis

During invasion, the attached merozoite triggers a drastic invagination of the RBC membrane (RBCM). Through a concerted interplay of regulated protein secretion and actinomyosin-dependent force, the merozoite propels itself into this growing membrane sack, which ultimately pinches off from the RBCM to form the parasite's intraerythrocytic shelter[4] (FIG. 1).

The provenance of the parasitophorous vacuole membrane (PVM) has been extensively explored, but the unique nature of this compartment and the rapidity of its generation pose special challenges. Transmission electron microscopy (TEM) has shown that the PVM and the RBCM form a continuous phospholipid structure during invasion[5]. Accordingly, fluorescent lipophilic probes introduced into the RBCM are incorporated into the nascent vacuole at concentrations equivalent to those in the RBCM, implying that the PVM is generated entirely from the host membrane[6-8]. By contrast, metabolically incorporated fluorescent lipids from the parasite were also transferred to the vacuole, suggesting that parasite lipids contribute to PVM biogenesis[9]. Given their propensity to transfer across aqueous solutions, the appropriateness of

labelled lipid probes for these types of experiments is debatable, particularly as intermembrane transfer of such labels might be favoured during invasion owing to the close apposition of the parasite plasma membrane (PPM) and RBCM.

The surface area of the RBC prior to and post invasion was shown to remain constant, interpreted as evidence for a substantial contribution of parasite-derived phospholipids to PVM biogenesis[10]. Surprisingly, the opposite conclusion was reached in the case of *Toxoplasma gondii*, a related coccidian parasite that is able to infect almost all types of nucleated cells of warm-blooded animals. In *T. gondii*, ongoing vacuole formation did not alter host cell membrane capacitance **[G]**, suggesting that no new lipid material is inserted into the nascent vacuole[11]. Only upon pinching off of the *Toxoplasma*-containing vacuole did the host cell membrane undergo a distinct decrease in capacitance, indicating that membrane material had been depleted from the surface[11].

When invasion by *Toxoplasma* and *Plasmodium* parasites is stalled with inhibitors of the actinomyosin motor complex, vesicles and tubular extensions are observed within the host cells close to the attachment sites[12-14]. TEM studies also visualized the release of multi-lamellar membrane aggregates from the apical ends of invading *Plasmodium knowlesi* merozoites[15-17]. These membranes are most likely released from the rhoptries, twinned club-shaped apical organelles which contain tightly packed membrane whorls and which fuse and shrink upon invasion[15,16,18]. In stark contrast to the RBCM, these released membranes and the newly formed PVM do not possess intramembranous particles (IMPs), as determined by freeze fracture electron microscopy, together suggesting that the parasite secretes lipid material largely devoid of integral membrane proteins[19]. These observations concur with the exclusion of the highly abundant erythrocyte transmembrane proteins Band 3 (AE1) and glycophorin A from the vacuole[17,20]. By contrast, other host cell membrane proteins were shown to be incorporated into the *Plasmodium* PVM through a process that requires association with RBCM lipid rafts[20,21]. In *T. gondii*, host cell proteins are selectively assimilated into the PVM by passage through the differentially permeable parasite–host cell junction[22]. Whether this phenomenon is also the molecular basis for the selective recruitment of RBCM proteins into the parasitophorous vacuole of *Plasmodium* parasites remains unclear.

Upon successful vacuole formation, spherical merozoite organelles known as dense granules fuse with the PPM to release their contents into the vacuolar space[23]. Knowledge of the molecular composition of these organelles is limited, but it is clear that the dense granules supply functional molecules for the early establishment of signature pathways of the parasitophorous vacuole. Accordingly, proteins involved in RBC remodelling and nutrient acquisition (see below) are stored in dense granules prior to their vacuolar localization[24,25]. Following dense granule discharge, proteins are continuously targeted to the vacuole by means of default protein secretion, which coincides with the reappearance of IMPs in the PVM[19,26,27].

Despite the wealth of data, there is currently no consensus over the relative contributions of RBCM and parasite to PVM biogenesis[28] and in the absence of subcellular fractionation techniques that can unambiguously resolve RBCM, PVM and parasite, this issue will remain unsettled.

The tubovesicular network

During early blood stage development most of the PVM lies in close proximity to the PPM[18,29] and a recent study has presented evidence for functional contact zones between protein complexes of both membranes[30]. With parasite maturation the vacuole grows in size and complexity, developing large membranous loops that extend far into the RBC cytoplasm and that contain vacuolar markers such as exported protein 1 (EXP1)[31,32]. Effectively representing an exported parasite organelle, initial characterisation of this so-called tubovesicular network (TVN) was aided by visualization with fluorescent lipid dyes[33-35]. Subsequent work has shown that the *Plasmodium falciparum* TVN primarily comprises large double membrane whorls that envelop portions of RBC cytoplasm, sometimes entirely[36]. In addition, membranous tubules connect the whorls and vacuole-derived vesicular structures, together suggesting a highly convoluted and contiguous PVM system (FIG. 1b and c)[34-39]. Although the TVN is mostly associated with mature parasite stages[38], its appearance is highly variable and often it is not observed at all. Thus, time-resolved fluorescence imaging of the parasitophorous vacuole in individual infected RBCs, as previously performed for other parasite-induced host cell modifications[40], is needed to obtain a better understanding of TVN ontogeny.

Demonstrations that fluorescent probes such as BODIPY-ceramide delineate the TVN have led to claims that the extended vacuolar membranes are enriched in sphingolipids[34,35]. However, labelling commonly extends to all other membranous structures in the infected RBC, lending little support to this notion. A subcellular fractionation approach was used to demonstrate sphingomyelin synthase (SMS) activity in the 'TVN-enriched' fraction[34]; however, although those authors went to great lengths to rule out a role for contamination from other parasite compartments, the idea of sphingomyelin accumulation in the TVN remains contentious. Indeed, when over-expressed as an mCherry fusion protein, one of the parasite's two annotated SMS enzymes localized to the plasma membrane in *P. falciparum*[41]. We suggest that the physiological localization of these enzymes needs to be assessed by tagging of the endogenous genomic loci to avoid expression-related artefacts. Thus far there is no reliable and exclusive TVN marker protein known.

One possible exception is TVN junctional protein 1 (TVN-JP1). Over-expression as a GFP fusion localised this protein to specific junctional sites, bridging individual TVN sub-compartments[42]. The physiological significance of this distribution is unclear, particularly given the presence of a potential carboxyl-terminal ER retention signal (TDEL) in the TVN-JP1 sequence, which may have been obstructed by GFP. The validation of vacuolar protein localization is generally complicated by distinct sequence requirements and the unique topology of the parasitophorous vacuole (BOX 1). Artefactual or not, the observed localization pattern of GFP-tagged TVN-JP1 indicates the presence of distinct junctional TVN sub-compartments.

The significance of assembling and maintaining this complex structure remains unclear, but it is plausible to assume that the TVN serves to accommodate and organize the molecular machinery of the vacuole by providing an enlarged and compartmentalised membrane surface.

[H1] Vacuolar protein traffic

The intraerythrocytic parasite needs to overcome the paucity of nutrients and exploitable machinery in its desolate host cell environment. As a result, *Plasmodium* species have evolved specialized trafficking pathways which enable extensive remodelling of the RBC compartment by exported proteins. As a consequence of this parasite-induced host cell makeover, the infected RBC exhibits increased permeability to diverse solutes supporting rapid parasite biomass production[43,44], and displays altered mechanical properties owing to cytoskeletal modifications[45]. As the spleen removes defective RBCs from the circulation, these profound modifications of host cell architecture place the parasite at risk of destruction. Thus, to elegantly avoid splenic passage, the parasite exports adhesive molecules to the host cell surface, enabling the infected RBC to bind to the vascular endothelium, sequestering it from peripheral circulation[46]. Cytoadhesion of parasitized RBCs can severely impede the function of affected host tissues owing to vascular obstruction and the induction of localised inflammatory responses; the ensuing oedema, haemorrhage and inflammation contribute substantially to the pathophysiology of severe malaria[3,46].

Despite their functional diversity and variable subcellular distribution (FIG. 2a), all exported proteins are first secreted into the parasitophorous vacuole prior to their translocation into the RBC cytoplasm. The complicated feat of exported protein trafficking begins at the parasite ER membrane, where entry into the secretory pathway is mediated by interactions with several components of the Sec translocon **[G]**, including the Sec61 channel and the non-catalytic component of the signal peptidase complex SPC25[47]. In contrast to the classical route, the signal peptidase SPC21 does not appear to participate in the entry of exported proteins into the secretory pathway, but is rather replaced by the aspartic protease plasmepsin V[47]. Most exported proteins contain an amino-terminal *Plasmodium* export element (PEXEL) downstream of a recessed signal peptide **[G]**[48,49]. The pentameric PEXEL motif constitutes a recognition site for plasmepsin V, which cleaves the PEXEL between the third and fourth residue upon ER entry[50,51]. The newly exposed N-terminal residue is acetylated by an unknown mechanism to yield the mature protein[52].

This mode of entry into the secretory pathway is distinct from that of *bona fide* parasitophorous vacuole proteins, which usually contain an N-terminal signal peptide that is cleaved by signal peptidase, or which in exceptional cases, become N-myristoylated in order to direct them to the vacuole[26,53]. SPC21 knockdown in *P. falciparum* resulted in reduced levels of the parasitophorous vacuole-resident protein SERA5, whereas several PEXEL-containing proteins remained unaffected, highlighting the divergent entry routes of vacuolar and exported proteins into the secretory pathway[47]. Although the PEXEL is typical of most known exported proteins, PEXEL-negative exported proteins (PNEPs) also exist[54]. These typically contain a signal peptide and transmembrane domain, and do not appear to depend on SPC25, in contrast to PEXEL-containing exported proteins[47]. In spite of these differences, the N-termini of PNEPs contain information that is functionally equivalent to the mature processed PEXEL N-terminus, although this transcends a discernible linear motif[55]. It remains unclear how these early secretory events that appear to mark proteins for export are connected to cargo identification in the parasitophorous vacuole, where all types of exported protein traffic converge for translocation across the PVM[56,57].

The existence of a vacuolar protein translocon was initially indicated by experiments that revealed

protein export from the parasitophorous vacuole to be dependent on ATP and cargo unfolding[58,59]. Furthermore, chimeric cargo proteins trapped in a tightly folded state were arrested in the vacuole, where they prevented the export of other cargo, implying a blocked translocon pore[30]. The discovery of a heat shock protein 100 family member in the parasitophorous vacuole, called HSP101[60], was conspicuous given that these chaperones usually form hexameric protein unfolding machines that hydrolyse ATP to remodel cargo and thread it through the central pore of the oligomer[61]. Indeed, HSP101 was found to participate in a PVM-anchored complex subsequently named the *Plasmodium* translocon of exported proteins (PTEX; FIG. 2b)[60]. PTEX comprises at least four additional proteins: PTEX150, PTEX88, an integral membrane protein known as exported protein 2 (EXP2), and the thioredoxin TRX2. Of these five components, HSP101, PTEX150 and EXP2 comprise an essential PTEX core complex and conditional inactivation of any of these components blocks protein export across the PVM[56,57,62,63].

Recently, single-particle cryo-electron microscopy yielded a near-atomic resolution structure of the core complex purified directly from cultured *P. falciparum*[64] (FIG. 2c). The structure revealed a hexameric HSP101 unfoldase, which is tethered to a membrane-spanning heptameric EXP2 pore through a PTEX150 heptamer. Crucially, this work enabled the structural visualisation of PTEX in two states, distinguished primarily by large-scale changes in the HSP101 hexamer between extended helical and flattened planar forms. The helical architecture has emerged as a common feature of HSP100 chaperones and is important for directional substrate feeding[65]. Remarkably, endogenous cargo was visible within the HSP101 channel in both states, suggesting a ratchet-like mechanism whereby interactions with tyrosine-bearing pore loops couple power from ATP hydrolysis to grip and pull exported cargo into the translocon channel[64].

Although not visible in the cryo-electron microscopy structure, PTEX88 has been shown to interact closely with HSP101[66]. Loss of PTEX88 does not measurably impact protein export but does reduce cytoadhesion and sequestration of infected RBCs in human and rodent malaria models[67-69]. This suggests that PTEX88 is either involved in the export of a discrete set of proteins yet to be identified, or that it may serve in some other capacity than trafficking from the vacuole, such as modification of exported proteins to support their ultimate function at the host cell surface. Additional PTEX interaction partners have been identified by proteomic analyses (FIG. 2b), but there is little evidence for a direct involvement in the translocation process[70-72].

The thioredoxin TRX2 is the only PTEX auxiliary component with a known enzymatic activity and has been postulated to reduce disulphide bridges to aid in unfolding cargo or in regulating PTEX[60]. Disruption of TRX2 in *Plasmodium berghei* causes only a mild growth defect, but does observably diminish protein export and parasite sequestration[57,68,69,73]. It has been debated whether TRX2 constitutes a *bona fide* PTEX component, owing to several reports claiming localization to either the parasitophorous vacuole[73-75], the mitochondrion[76] or to parasite organelles of unknown identity[69,73,74]. Repeated detection during proteomic analysis of PTEX suggests that TRX2 resides at least partially in the vacuole or interacts with the translocon or cargo proteins during trafficking[60,72,73]. Accordingly, the reported punctate distribution of fluorescently tagged TRX2 in the cytoplasm of both human and rodent malaria parasites might be indicative of a secretory compartment, such as the Golgi apparatus[69,74].

In the case of exported membrane proteins, which are trafficked through secretory vesicles that initially fuse with the PPM, PVM translocation is preceded by extraction from the PPM[30,55]. Recent work provides evidence for an HSP101-independent unfolding power in the parasitophorous vacuole operating on exported integral membrane proteins, suggesting a distinct PPM extraction machinery[77]. This is further supported by the observation that the PVM protein EXP1 is extracted from the PPM in a PTEX-independent manner[78].

Owing to the translocon's intravacuolar topology[64], PTEX does not appear to be involved in the refolding of exported proteins once they cross the PVM. In *P. falciparum*, an array of exported chaperones have been tentatively implicated in this process[79,80]. An additional possibility is that host machinery is co-opted for this purpose. Host HSP70 displays altered solubility in infected RBCs, which may represent recruitment to assist refolding or trafficking of exported membrane proteins[81]. More recently, the erythrocyte TCP1 ring complex was reported to interact with parasite cargo[71]. However, at present, how exported proteins are received and refolded on the RBC side of the PVM remains largely unknown.

Interestingly, both vacuolar and exported proteins are incorporated into the parasite food vacuole during the endocytic uptake of RBC cytoplasm, where some of them participate in haemoglobin digestion and haem detoxification[82,83]. There has also been a report on protein trafficking to the relic parasite plastid through the parasitophorous vacuole[84]. Together with its prominent role in protein export, these functions implicate the parasitophorous vacuole as a transitional sorting hub for effector proteins targeted to diverse subcellular locations.

[H1] Nutrient permeation across the PVM

Owing to the limited anabolic activity of the RBC, the malaria parasite struggles to obtain sufficient nutrients for rapid biomass production and so digests the available haemoglobin to support its amino acid supply[85]. However, isoleucine is completely absent from human haemoglobin whilst several other amino acids and nutrients are also underrepresented in the RBC[86]. Thus, *Plasmodium* species acquire additional metabolites from the blood plasma by exporting nutrient transporters to the RBC surface, establishing the so-called new permeability pathways (NPPs; see above)[43,44]. After crossing the RBCM, imported nutrients need to traverse the PVM. Patch clamp measurements with released PVM-enclosed asexual *P. falciparum* blood stage parasites have determined the presence of a size-selective nutrient pore, which is present at high density and is constitutively open[87]. Polyethylene glycols of different polymer lengths were used to determine a size exclusion limit of ~1.4 kDa and an effective pore diameter of 23 Å[88], through which amino acids and monosaccharides can efficiently traverse[87]. Surprisingly, the PVM pore was shown to be narrower during the parasite's liver stage development, as it only allows passage of molecules smaller than 855 Da[89]. Whether this reflects distinct pore proteins and/or different nutrient requirements during blood and liver stage development, remains to be shown.

A nutrient pore is also present in the parasitophorous vacuole of *T. gondii*-infected cells, with a size exclusion profile very similar to that of *Plasmodium* blood stage parasites (~1.3 kDa)[90]. In *T. gondii*, PVM conductance is promoted by GRA17 and GRA23, orthologs of the pore-forming PTEX component EXP2 in

Plasmodium species[91]. GRA17-deficient *T. gondii* parasites are unable to import organic compounds into the parasitophorous vacuole, a phenotype which was reverted upon complementation with *P. falciparum* EXP2[91]. Furthermore, expression of EXP2, GRA17, or GRA23 increased solute conductance in *Xenopus laevis* oocytes, consistent with channel activity[91]. EXP2 function was then examined in *P. falciparum* blood stages using regulated gene expression in combination with patch clamping[63], demonstrating a tight correlation between EXP2 protein levels and the frequency of channel detection. Truncation of EXP2 altered the channel's voltage response, further supporting the notion that EXP2 is the solute-conducting pore of the *Plasmodium* PVM[63]. The high density of the pore complex rules out the maintenance of a vacuolar transmembrane potential and suggests that the concentrations of most soluble metabolites are similar in the RBC cytosol and in the vacuolar matrix. Strikingly, no other channel activities were recorded in two independent studies with different patch clamping approaches, suggesting that no additional solute transport processes occur at the PVM[63,88].

EXP2 thus appears to form both the protein conducting channel of PTEX and the PVM nutrient pore[63]. Whereas most PTEX components are restricted to the *Plasmodium* genus, EXP2 orthologs, such as GRA17 and GRA23, are broadly conserved in vacuole-dwelling apicomplexans[91]. This remarkable double channel functionality suggests that an ancestral EXP2-family nutrient channel was adapted for protein translocation through coupling the HSP101 protein unfoldase activity to the channel through PTEX150. The discordant expression of EXP2 with other PTEX components[25,63] and incomplete co-localization of EXP2 and HSP101[38,92] suggest that the transport of small molecules occurs through translocon-independent pores (FIG. 2b). How EXP2 is installed in the PVM is unclear, although RON3, a rhoptry protein **[G]** conserved among apicomplexans that is injected into the parasitophorous vacuole during invasion, was recently reported to be crucial to both protein export and small molecule transport in *P. falciparum*, suggesting that it might serve as an EXP2 PVM insertase[93].

Despite the original demonstration of an abundant PVM pore in 1993[87], a competing model of nutrient acquisition was advanced in 1997, presenting the TVN as a specialized import structure for extracellular nutrients. Inspired by the report on sphingomyelin synthesis in the TVN[34], treatment of *P. falciparum* with a sphingolipid synthesis inhibitor was found to result in aberrant TVN assembly and reduced incorporation of fluorescent dyes, amino acids and nucleosides into the parasite[94]. The observation that inhibitor-treated parasites were still able to export proteins and remodel the host cell, was presented as evidence for ongoing parasite maturation and was thus interpreted in favour of a specific 'starvation phenotype'. The authors argued that the TVN might transiently associate with the RBC surface to import nutrients from the blood plasma[94]. However, TVN perturbation was accompanied by a much smaller parasite size, suggesting that the inhibition of sphingolipid synthesis affects parasite development from an early stage. As the TVN is predominantly formed at mature parasite stages, a lack of tubovesicular membranes upon inhibitor treatment is not surprising. Any accompanying defect in nutrient permeation can be equally well explained as a secondary effect of reduced parasite fitness or even parasite mortality. Thus, although the data provide some evidence for the importance of sphingolipid synthesis for parasite survival, no clear conclusions can be drawn about TVN function. A competing model has postulated the presence of a permanent connection or 'parasitophorous duct' between vacuole-derived extensions and the RBCM, directly supplying the

intracellular parasite with plasma-derived nutrients and macromolecules[33]. This model is not consistent with our growing knowledge about the protein export-dependent NPP and the vacuolar EXP2 pores, which together promote sequential transmembrane transport of nutrients across RBCM and PVM. Thus, the notion of a transient or permanent continuum between the blood plasma and parasitophorous vacuole remains contentious.

[H1] Vacuolar compartmentalization

Several studies have reported a 'necklace of beads' staining pattern around the parasite upon secretion of fluorescent parasitophorous vacuole reporters, suggestive of individual protein-containing pockets or foci between the PVM and PPM, particularly in ring stages[25,27,60,92,95,96]. In mature parasites, co-localization of a signal peptide-GFP fusion with BODIPY-ceramide by live fluorescence microscopy revealed protein secretion into defined regions of the vacuole and TVN[27]. Importantly, the GFP signal was absent from several vacuolar locations, all of which were prominently labelled with the lipid dye[27]. Photobleaching revealed limited protein exchange between distinct vacuole sub-compartments, including individual beads and TVN loops[27,96]. Thus, the beads and the TVN appear to fulfil equivalent functions in the spatial organization of the vacuolar proteome.

There are two possible mechanistic explanations for vacuolar sub-compartmentalization, which are not mutually exclusive: proteins are trafficked to isolated sub-compartments of the parasitophorous vacuole by site-directed secretion; and/or secreted proteins are targeted to the vacuole and subsequently redistributed. Site-directed secretion appears a plausible scenario, supported by the fact that exported proteins undergo divergent processing steps in the parasite ER[47,50,51]. The non-uniform vacuolar swelling following PTEX inactivation is also in line with discrete sites of export activity[63]. However, a split-GFP reporter system showed that non-exported vacuole-resident proteins have free access to PTEX, arguing against completely isolated sub-compartments[63]. At present, EXP1 has emerged as a key player in the spatial organization of PVM-embedded proteins. Upon genetic inactivation of EXP1, the pore protein EXP2 accumulated in few distinct PVM regions, including loops of the TVN[97]. This correlated with impaired nutrient permeation and parasite mortality, but most surprisingly did not impact protein export. Whether this sorting function of EXP1 is mediated by protein recruitment to lipid rafts within the PVM remains to be shown.

A recent study has implicated the *P. falciparum* TVN in the sequestration of export-incompetent cargo proteins[98]. Cargo that was inducibly trapped in a tightly folded state accumulated in TVN loops and also stimulated loop assembly[98]. It has thus been hypothesized that the TVN might retain cargo proteins which are not quite ready for export or which have failed to unfold, thereby preventing exhaustion of the protein translocation machinery[98]. In agreement with these observations, an exported reporter protein localized to the parasitophorous vacuole during trafficking but was specifically excluded from the loops, suggesting that this part of the TVN is not a protein export-competent site[99]. Accordingly, no translocon-exclusive PTEX components have been detected in the *P. falciparum* TVN[25,60,92,98]. Even though these observations contrast with the exclusive localization of HSP101 and PTEX88 to the TVN equivalent in *P. berghei*, it appears that spatial segregation of the protein export machinery is a shared feature among *Plasmodium* species[38,69].

Several TEM studies have revealed parasite-induced compartments within the infected RBC which appear to originate from the PVM[31,32,39]. In support of these observations, a study described detachment of vesicular compartments from the TVN that stain positive for the lipid dye BODIPY-ceramide and a soluble PV reporter protein[27]. In agreement with the double membrane morphology of TVN loops, the detached compartments exhibited a rim-like staining for the lipid dye and the secreted reporter, together implicating the TVN in the generation of autonomous double membrane vesicles and possibly supporting a role as a 'molecular garbage bin' for improperly folded export cargo[27,38,98-100].

Another study reported that EXP2-positive vesicles are predominantly generated in reticulocytes, as shown by co-localization with CD71 in *P. berghei*-, *Plasmodium yoelii*- and *P. falciparum*-infected cells[100]. Although reticulocytes generally make up only a fraction of circulating RBCs, several *Plasmodium* species, including the human pathogen *Plasmodium vivax*, show strong tropism for immature erythrocytes[101]. Residual vesicular trafficking regulators in the host cell might facilitate PVM budding, but whether these fission events represent specific adaptations towards the developmental status of the RBC remains unclear.

Fission from the parasitophorous vacuole has also been proposed for the so-called Maurer's clefts. These thin membrane lamellae are generated during the early ring stage and initially display vivid mobility inside the cell[40]. Maurer's cleft formation is completed during the early trophozoite stage, when they become tethered to the inner surface of the RBCM or cytoskeleton through proteinaceous anchors[36,40,102]. Equivalent structures with varied characteristics are generated by other *Plasmodium* species, but all appear to serve as platforms to sort exported effectors to their target sites inside the RBC compartment[103]. Although some observations point to a vacuolar origin for the Maurer's clefts, there is a lack of definitive evidence supporting this mode of genesis (BOX 2). Additional membranous compartments inside the *Plasmodium*-infected RBC include the acridine orange-stained vesicles and J-Dots, as well as coated and uncoated vesicles with an inner diameter of ~25 nm[28,36]. However, their primary origin remains unknown.

In contrast to the numerous reported fission events, there is no conclusive evidence for the parasitophorous vacuole or any derived structures fusing with one another or with the RBCM. This is exemplified by the fact that every parasite within multiply-infected RBCs is enveloped by its own vacuole. Unpublished observations indicate that the secretion of mCherry from one *P. berghei* parasite and of GFP from another, never leads to signal overlap in dually infected murine erythrocytes (J. M. Matz, personal communication). Furthermore, the vacuole does not fuse with the RBCM during parasite egress, as previously suggested[104], which would intuitively represent a convenient mechanism for parasite release. Collectively, current evidence suggests that the malarial parasitophorous vacuole is a highly compartmentalized non-fusogenic compartment with a great capacity for membrane budding.

[H1] The vacuole during parasite egress

During the final stages of intraerythrocytic development, the parasite gives rise to several newly formed daughter merozoites. To exit the host cell and re-invade another, the parasites have to break down the PVM and RBCM in a complex choreography known as parasite egress (FIG. 3a). Inspection by electron and video

microscopy revealed cases of merozoites free within the RBC cytoplasm late during schizogony, suggesting that parasitophorous vacuole disruption precedes RBCM rupture[105-107]. This was later supported by immuno-electron microscopy showing re-localization of S-antigen from the vacuole to the cytoplasm of the intact RBC upon PVM disintegration[108,109], as well as by imaging transgenic *P. falciparum* parasites expressing vacuole-localized or exported GFP fusions[109]. In early schizonts, the fluorescent proteins exhibited the expected localization to the parasitophorous vacuole or to the RBC cytoplasm, respectively. Upon initiation of egress, both signals equilibrated across the PVM while the RBCM was still intact, elegantly proving that vacuole disruption occurs first[109]. Time-resolved TEM and fluorescence microscopy of gametocyte egress indicate that sexual and asexual blood stages share the inside-out dynamics of host cell exit[110,111].

These observations are supported by numerous more recent egress inhibitor studies. Parasite egress is controlled by internal signalling pathways and the action of secreted and vacuole-resident proteolytic enzymes[112-114]. Accordingly, certain kinase and protease inhibitors block egress at different steps in the process, a fact that has been extensively exploited in the dissection of the molecular pathways involved. Selective inhibitors of the parasite cGMP-dependent protein kinase (PKG) prevent discharge of a subtilisin-like serine protease called SUB1 into the vacuolar lumen[115]. PKG acts in concert with another, calcium-dependent parasite protein kinase called CDPK5 which is also required for egress, although its precise role is unclear[116,117]. Substrates of SUB1 include components of the merozoite surface protein 1 complex and members of the serine-rich antigen family of papain-like proteins (SERA), which regulate merozoite liberation upon processing[118-124]. Maturation of SUB1 is in turn mediated by an aspartic protease called plasmepsin X. Accordingly, pharmacological inhibition or genetic disruption of plasmepsin X results in egress arrest[125,126]. Similarly, preventing SUB1 discharge with PKG inhibitors or by genetic ablation causes the parasites to arrest within an intact vacuole[127-129]. By contrast, the cysteine protease inhibitor E64 which abates activity of the protease SERA6 in *P. falciparum* blocks parasite egress at a later stage, allowing efficient lysis of the PVM but trapping merozoites within the RBC[107,127,129].

PVM disruption is preceded by a brief stage of vacuolar rounding in which the daughter merozoites arrange around the central food vacuole in an orderly fashion, a process also referred to as ‘flower formation’ or rounding up[130]. One study used fluorescent markers of the parasitophorous vacuole matrix and membrane to demonstrate that the PVM is in close contact with the daughter merozoites during late schizogony, sometimes even folding into the gaps between multiple merozoites, a phenomenon described previously[131,132]. Upon flower formation, the PVM marker assumed a circular staining pattern, whereas the luminal marker remained distributed around the merozoites, indicating that the PVM loses its close association with the parasites[131]. The vacuolar volume remained constant during rounding, refuting earlier assertions of an osmotic swelling mechanism[106,131]. It is conceivable that physical connections between the PPM and PVM become disconnected during this phase to allow subsequent merozoite release. Under such conditions, the parasitophorous vacuole would assume its most energy-efficient shape, becoming close to spherical. Alternatively, the re-orientation of the merozoites during flower formation might simply unfurl the PVM.

One to two minutes following rounding up, the parasitophorous vacuole is breached by soluble vacuolar

contents (FIG. 3b) and haemoglobin[127,131]. The PVM still appears intact at this point until it is ultimately degraded to multi-lamellar vesicles shortly before host cell egressdeparture[107,127] (FIG. 3c). Live microscopy of transgenic parasites expressing fluorescently tagged EXP2 revealed that the PVM ruptures at discrete foci and progressively decomposes from the sites of breakage[131]. PVM permeation and PVM breakdown appear to be discrete steps during egress, because the permeable PVM does not rupture in parasites arrested with PKG inhibitors[127]. This intermediate state of high permeability but structural integrity was observed to last up to 30 minutes in untreated *P. falciparum* schizonts[127] and is reminiscent of the egress mode of *Plasmodium* liver stages, which also features a period of vacuolar leakage prior to PVM disruption[133-135]. By contrast, another study observed a tight temporal correlation between vacuolar leakage and vacuole rupture proper during asexual blood stage egress[131]. This latter scenario is similar to the reported egress phenotype of *P. falciparum* gametocytes, which were shown to degrade the parasitophorous vacuole within less than a minute following activation of the egress pathway[111].

Final rupture of the PVM coincides with the poration and collapse of the RBCM around the parasites only moments before merozoite release[107,115,124,127,129-131]. As well as perhaps indicating that PVM rupture and RBCM poration may be mediated by the same effector protein or proteins, this signifies the presence of a physical barrier around the parasites for as long as an intact host cell is present. Disruption of the RBCM is a rapid process, but the parasite clearly mediates a step-wise dismantling of its protective niche, further highlighting the significance of the *Plasmodium*-containing vacuole until the very end of RBC infection.

The molecular mechanisms underlying the dismantling of the *Plasmodium* blood stage PVM remain elusive. Observations in the related coccidian parasite *T. gondii* suggest the involvement of both perforating and membrane lytic proteins[136,137]. Although the genome of the malaria parasite encodes five perforin-like proteins, all are individually dispensable in asexual blood stages and have been implicated in other life cycle stages, including gametocytes, ookinetes and sporozoites[138,139]. Interestingly, a vacuole-localized parasite phospholipase has been demonstrated to promote *P. berghei* liver stage egress[133]. However, its genetic ablation did not obviously impair blood stage proliferation[133]. The parasite appears to express additional enzymes with the potential for lipolytic activity[140], yet their possible roles in blood stage egress remain to be determined.

[H1] Why live in a parasitophorous vacuole?

Plasmodium parasites devote considerable resources to the assembly and maintenance of their intraerythrocytic niche by providing lipids for PVM expansion as well as energy and functional molecules for vacuolar pathways. Yet, the primary role of the parasitophorous vacuole during blood stage development remains elusive. Vacuolar compartments are often of great benefit for intracellular pathogens, because they provide a safe hiding place whilst still allowing for host–cell interactions[141]. Exemplifying this protective function, host-induced degradation of the *T. gondii*-containing PVM results in rapid parasite killing within the host cell cytoplasm[142]. By contrast, *Plasmodium* parasites have no obvious reason for hiding in a vacuole during blood stage development. After all, they replicate inside a terminally differentiated cell which is devoid of organelles, pathogen recognition pathways or cell-autonomous defence mechanisms. Therefore, one is

left to wonder what the benefit of the intraerythrocytic parasitophorous vacuole might be.

Maintenance of a parasitophorous vacuole is not imperative for successful parasitism of the erythrocyte. As an example of this, the closely related piroplasm parasites *Babesia* spp. and *Theileria* spp. initially form a vacuole during RBC invasion but degrade this temporary envelope only a few minutes later[143-145]. Despite this, *Babesia* spp. replicate many aspects of *Plasmodium*-induced host cell makeover, including the trafficking of virulence factors, rigidification of the RBC, induction of intraerythrocytic membranous compartments and knob-like protrusions of the RBCM, as well as cytoadhesion and parasite sequestration[146-148]. Together, these observations demonstrate that efficient host cell remodelling does not require a parasitophorous vacuole. By contrast, the mechanisms that have evolved to achieve protein translocation across the *Plasmodium* PVM appear extraordinarily complex in the light of the alternative strategy of a cytosolic lifestyle. Similarly, the vacuolar adaptations required to enable nutrient permeation and egress are fundamentally molecular coping mechanisms to overcome the hurdle imposed by the surrounding PVM. Novel proteomic approaches targeting the parasitophorous vacuole hold promise for the identification of factors necessitating the parasite's intraerythrocytic niche (BOX 3).

Despite the lack of cell-autonomous defence mechanisms in the erythrocyte, the parasitophorous vacuole of the malaria parasite may fulfil a protective function. As a result of their role in oxygen transport, both infected and uninfected erythrocytes are continuously exposed to reactive oxygen species, which threaten the integrity of cellular membranes and proteins[149]. One prominent source of radical-induced cell damage is free haem, concentrations of which can reach ~20 μM in uninfected RBCs, possibly owing to spontaneous haemoglobin degradation[150]. Enzymatic and non-enzymatic redox regulators protect the RBC from haem and radical-induced damage[151], but as a fast-growing organism the malaria parasite likely requires additional adaptations to withstand these harsh conditions. As such, the parasitophorous vacuole could provide an important layer of protection. Perhaps relevant to this, the abundant PVM protein EXP1 was shown to act as a glutathione transferase with the capacity to detoxify haem *in vitro*, providing some support for a potential function of the parasitophorous vacuole in redox protection[152]. However, this enzymatic activity of EXP1 does not contribute to parasite blood stage development *in vivo*[97].

The maintenance of the parasitophorous vacuole during blood stage development could also be rooted in the parasite's preceding expansion phase in the mammalian liver, because the capacity of the hepatocyte to initiate innate and adaptive defence mechanisms might necessitate an intravacuolar niche during exoerythrocytic schizogony. Indeed, transgenic rodent malaria parasites which lack expression of 6-Cys family-related proteins and consequently lose their PVM, arrest development within the hepatocyte and rapidly trigger host cell apoptosis and adaptive immune responses[153-155]. As the machinery for maintaining and operating a parasitophorous vacuole is encoded in the *Plasmodium* genome anyway, the parasite might simply adhere to this strategy during blood infection. In this context, it may be notable that the life cycle of *Babesia* parasites lacks a preceding tissue stage in the mammalian host prior to RBC infection. It is, however, questionable whether the strategy of simply adhering to a liver stage-specific adaptation outweighs the costs of vacuole construction, maintenance and operation during RBC infection.

Surprisingly, *P. falciparum* blood stages were shown to be capable of undergoing schizogony in an

axenic culture system in which parasites are maintained in a gelatinous matrix of RBC homogenate and nutrient supplements[156]. Despite the absence of both PVM and intact host cells, the parasites remained viable as indicated by parasite segmentation, the incorporation of rhodamine 123 **[G]** and the generation of haemozoin **[G]**[156]. These observations raise the question as to whether *Plasmodium* parasites can also survive outside their vacuole in the context of RBC infection. As of yet, the ultimate purpose of the parasite's intraerythrocytic vacuole remains a mystery.

[H1] Conclusions and future perspectives

Over 60 years since the first description of the parasitophorous vacuole in *Plasmodium*-infected RBCs[29], extensive research has only really begun to unravel the functions of the parasite's intraerythrocytic niche. Although our understanding of vacuolar protein export, nutrient acquisition and compartmentalization continues to improve, many fundamental questions remain unanswered. How exactly is the parasitophorous vacuole generated? How does it grow? By what means is it degraded at egress? What evolutionary pressures underlie its development and what is its ultimate physiological purpose? Another largely unexplored area is the composition and spatial organization of PVM lipids. How does the parasite traffic lipids from and to the PVM? Do specific lipids form functional microdomains within the PVM and does this relate to differential protein targeting? Given the obvious importance of the parasitophorous vacuole as a central host–pathogen interface, we cannot afford to neglect these questions. Parasitophorous vacuole biology remains controversial and challenging to dissect, and we should not be discouraged by the wealth of often conflicting data. Despite decades of research, many characteristics of the vacuole remain disputed and are in desperate need of revisiting. The ongoing development of new molecular tools and their implementation in the field of malaria research will hopefully enable us to settle some of the long-standing controversy and solidify our understanding of this intriguing parasite habitat.

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All authors contributed substantially to the conceptualization and writing of the manuscript. J.M.M. conceived the manuscript. The first draft was written by J.M.M. and J.R.B. with input from M.J.B. All authors edited and reviewed the manuscript before submission.

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Fig. 1 | **The parasitophorous vacuole of the malaria parasite. a** | Biogenesis of the parasitophorous vacuole. The merozoite induces invagination of the red blood cell membrane (RBCM) during invasion. Active merozoite motility and subsequent membrane scission result in the formation of the parasitophorous vacuole (blue). **b** | Remodelling and trafficking of the parasitophorous vacuole membrane (PVM). The vacuole can form a continuous space around the parasite (1). The frequently observed 'necklace of beads' morphology is indicative of constriction zones between parasite and PVM (2). Membrane whorls emerge from the PVM (3) and envelop host cell cytoplasm, resulting in formation of the tubovesicular network (TVN) (4) and the release of double membrane vesicles (5). The TVN also features distinct junctional sites (green), as well as partially interconnected tubular (6) and vesicular compartments (7). Maurer's clefts (MCs) are believed to bud off from the parasitophorous vacuole and become tethered below the RBCM through proteinaceous anchors (dark blue) (8). There is debate as to whether the MCs remain connected to the vacuole or not. Endocytosis of host cell cytoplasm leads to the formation of intraparasitic endosomes lined by the PVM and parasite plasma membrane (PPM) (9). The PVM is ultimately degraded during haemoglobin digestion and concomitant haemozoin formation (dark brown rectangles) (10). **c** | Morphology of the parasitophorous vacuole in maturing parasites. Shown are live fluorescence micrographs of transgenic *Plasmodium berghei* parasites expressing mCherry-tagged PV1 (Ca and Cb) or EXP2 (Cc and Cd) Shown are the 'necklace of beads' (Ca), tubular extensions (Cb), TVN whorls (Cc) and budding from the PVM (Cd). Red, tagged protein; blue, nucleus. Scale bar, 5 μm.

Fig. 2 | **Molecular transport across the parasitophorous vacuole membrane. a** | Various locations of exported *Plasmodium* proteins. Shown are live fluorescence micrographs of transgenic *Plasmodium berghei* parasites expressing mCherry-tagged versions of a PEXEL reporter exported into the RBC cytoplasm (top), intra-erythrocytic *P. berghei*-induced structures 1 (IBIS1) localizing to the Maurer's cleft equivalent (middle), or the erythrocyte membrane-associated protein 1 (EMAP1) (bottom). Red, tagged protein; blue, nucleus. Scale bar, 5 µm. **b** | Nutrient and protein traffic across the parasitophorous vacuole membrane (PVM). EXP2 (purple) forms a heptameric pore facilitating free nutrient diffusion across the PVM. The pore complex also translocates exported virulence factors (grey) upon assembly with PTEX150 (yellow) and HSP101 (green). Auxiliary factors of the *Plasmodium* translocon of exported proteins (PTEX), including PTEX88, thioredoxin 2 and surface protein P113 (brown), are thought to assist protein unfolding and delivery. The exported protein-interacting complex (EPIC), consisting of PV1, PV2 and the transmembrane protein EXP3 (orange), is thought to deliver protein cargo to PTEX. Upon translocation, red blood cell (RBC) chaperones, such as the TCP1 ring complex (red) might receive and refold the cargo. **c** | Structure of PTEX in distinct 'engaged' and 'resetting' states determined by cryo-electron microscopy (PDB IDs 6E10 and 6E11)[64]. ATPγS molecules are highlighted in red. View from the RBC cytosol shows cargo trapped in the engaged HSP101 channel (grey, bottom).

Fig. 3 | **The parasitophorous vacuole during egress. a** | The parasitophorous vacuole membrane (PVM) (blue) undergoes distinct morphological phases during parasite egress. Upon completion of schizogony, the merozoites align around the central food vacuole (dark grey) and the parasitophorous vacuole rounds up. The PVM becomes perforated and is then broken down into multilamellar vesicles, which coincides with red blood cell membrane (RBCM) poration and collapse. Subsequent RBCM rupture releases the merozoites and the cycle starts anew. **b** | Equilibration of soluble protein contents across the PVM prior to merozoite exit. Shown are live fluorescence micrographs of mCherry-tagged PV2 (orange) in transgenic *Plasmodium berghei* schizonts before (left), during (middle) and following parasite egress (right). Scale bar, 5 µm. Initially, fluorescence is closely associated with the merozoite periphery, then spreads throughout the red blood cell cytoplasm upon PVM disruption and ultimately diffuses into the extracellular medium, leaving only a brightly stained fraction within the parasite's digestive vacuole upon completed egress. **c** | Fragments of ruptured PVM (black boxes) are observed within the RBCM (white arrowhead) of E64-treated *Plasmodium falciparum* schizonts by transmission electron microscopy of high-pressure frozen, freeze-substituted thin sections (left). M, merozoite; Scale bar, 1 µm. PVM fragments frequently form multi-lamellar vesicles (right), within which PVM-associated protein complexes can be observed (black arrowheads). Shown is an average of ten central slices from a tomogram reconstructed from a -60^{o} → +60^{o} dual-axis tilt series collected on a 120 kV electron microscope. Scale bar, 100 nm. Electron microscopy images courtesy of Claudine Bisson (Birkbeck, University of London).

Box 1 | **Validation of parasitophorous vacuole localization**

Vacuolar residency is most commonly validated by genetic incorporation of protein tags, as opposed to the laborious and time-consuming generation of specific antibodies for immunofluorescence. Proper validation requires tagging of the endogenous gene, as over-expression of an additional copy can lead to localization artefacts. Furthermore, introduced tags may affect protein function owing to steric hindrance and interference with protein interactions. Thus, successful tagging of an essential single-copy genomic locus lends support to the physiological localization and functionality of the expressed fusion protein.

Before tagging, carboxyl-terminal targeting information such as ER retention signals (RS) must be considered. The functionality of such motifs can be assessed by appending them to the carboxy-terminus of a secreted reporter, such as a signal peptide (SP) GFP fusion[70] (see the figure). Alternatively, the tagging sequence can be incorporated directly upstream of the putative ER retention signal. Upon ER retrieval, the fusion protein will be retained inside the parasite instead of localizing to the parasitophorous vacuole. The amino-terminus of vacuolar proteins is less accessible to tagging owing to the presence of important sequence information that promotes entry into the secretory pathway.

The confirmation of vacuolar targeting by microscopy is hampered by the close apposition of the parasite plasma membrane (PPM) and parasitophorous vacuole membrane (PVM). Thus, co-localization with a parasitophorous vacuole marker protein to the periphery of the parasite is generally considered insufficient. There are, however, additional indicators of vacuole residency using conventional fluorescence microscopy. If the circumferential pattern is accompanied by protrusions, such as tubules or loops, a vacuolar localization can be assumed, because these structures are core features of the tubovesicular network (TVN), whereas the parasite surface is relatively smooth. Previous studies have also examined egressing merozoites to validate vacuolar localization. Soluble proteins of the parasitophorous vacuole matrix should be released or may be restricted to the central food vacuole, owing to the internalization of parasitophorous vacuole material during haemoglobin uptake. By contrast, the parasite surface should usually be devoid of any signal due to free protein dispersion upon merozoite release[70,157] (FIG. 3b). An additional localization to the disintegrating PVM is indicative of membrane association[158]. It should be noted that maturation events may in some cases lead to changes in the solubility profile of vacuolar proteins, as exemplified by SERA5 where the full-length protein is soluble in the vacuole but processing by SUB1 during egress leads to generation of amino- and carboxy-terminal fragments that bind to the merozoite surface[159].

Box 2 | **Origin of the Maurer's clefts**

In a host cell lacking an endomembrane system and its associated machinery, trafficking of exported membrane proteins to the host cell periphery presents a particular challenge which is met through the *de novo* formation of the Maurer's clefts in *Plasmodium falciparum*-infected red blood cells[103]. However, their mode of genesis remains unknown. Several electron microscopy studies have demonstrated the emergence of lamellar bodies from the parasitophorous vacuole, interpreted as Maurer's cleft biogenesis[160]. However, the presence of EXP1 suggests that these lamellae actually represent projections of the tubovesicular network (TVN)[31,35]. It is striking that neither luminal nor membrane-associated parasitophorous vacuole marker proteins are detected in the Maurer's clefts, despite their initial secretion from dense granules and expression during the early ring stage[25,40]. This may indicate that either: all vacuolar proteins are retained in the lumen and membrane of the vacuole during Maurer's cleft budding; or that they are degraded inside the Maurer's clefts; or that the origin of the Maurer's clefts is in fact not the parasitophorous vacuole.

A fluorescence microscopy study has reported the transient localization of a GFP-tagged Maurer's cleft marker to the parasite periphery. However, fluorescence spread to the parasitophorous vacuole only in more mature stages[161], which does not agree with the reported timing of Maurer's cleft biogenesis[40] and is likely caused by overexpression of the construct. Immuno-gold staining revealed focal labelling of the parasitophorous vacuole membrane (PVM) with an endogenous Maurer's cleft protein in electron micrographs, but labelled regions did not show any signs of membrane budding[161]. Since the export of Maurer's cleft proteins continues upon completion of Maurer's cleft formation and is thus largely independent of vacuolar budding, the mere presence of parasitophorous vacuole-associated Maurer's cleft proteins cannot be regarded as evidence for ongoing Maurer's cleft biogenesis because all exported proteins are expected to localize to the vacuole prior to their PTEX-dependent translocation[40].

3D reconstruction of the parasitophorous vacuole and Maurer's clefts has yielded conflicting evidence as to whether they form a lipid continuum in mature stages, which might be partially explained by the use of different laboratory strains[36,37,162,163]. The presence of parasitophorous vacuole–Maurer's cleft junctions in mature parasites, however controversial, does not necessitate a vacuolar Maurer's cleft origin as such junctions might be established after complete Maurer's cleft formation. Reliable proof could be provided by multi-labelling immuno-electron microscopy-mediated detection of protruding but still vacuole-attached lamellar compartments containing an early expressed Maurer's cleft marker. At the same time, antibodies against a signature parasitophorous vacuole protein should denote the vacuole, but not the nascent Maurer's cleft. In the absence of such evidence, the origin of the MCs continues to be a gaping hole in our knowledge of *Plasmodium*-induced host cell make-over.

Box 3 | **Proteomics of the parasitophorous vacuole**

A full understanding of the functional and evolutionary relevance of the *Plasmodium* parasitophorous vacuole requires detailed knowledge of its molecular makeup. Although the inability to isolate the parasitophorous vacuole membrane (PVM) has thus far prohibited lipidomic analysis, several studies have begun to shed light on the vacuolar protein composition. The first systematic proteomic investigation of the parasitophorous vacuole matrix exploited a combination of differential permeabilization, biotin labelling and mass spectrometry[164]. Although several established vacuolar constituents were identified, the repertoire of labelled proteins also contained cytoplasmic and ER-resident parasite factors. Conversely, many known highly expressed parasitophorous vacuole proteins evaded detection using this approach[164].

The sequential use of pore-forming toxins and the non-ionic detergent saponin should theoretically permit the isolation of vacuolar matrix proteins[165]. The pore-forming toxins facilitate selective red blood cell membrane lysis and removal of host cell cytoplasm, while saponin is thought to lyse the parasitophorous vacuole membrane (PVM) without affecting the integrity of the parasite plasma membrane (PPM). A recent study describes the mapping of the *Plasmodium yoelii* proteome using a comparable strategy[166]. Unfortunately, confident assignment of subcellular localization is hampered by the frequent leakage of parasite cytoplasm across the PPM upon saponin treatment[53,167].

With the advent of proximity-dependent biotinylation technology (BioID), the catalogue of known parasitophorous vacuole proteins continued to grow. In two recent studies, secreted reporter proteins were fused to the promiscuous biotin ligase BirA* in order to label the vacuolar compartment, thereby recovering several previously unknown proteins of the parasitophorous vacuole[157,158]. Owing to the vacuole's interposed topology, proteomic analysis is complicated by the frequent off-target identification of proteins from the ER, Golgi apparatus, PPM and host cell compartment, necessitating independent validation[157,158] (BOX 1). An alternative approach set out to bypass these limitations by exploiting refined prediction algorithms to detect signatures of vacuolar residency, including possession of a signal peptide and the absence of apicoplast transit peptides or export motifs, leading to the identification of three novel parasitophorous vacuole proteins[70].

All published attempts to unravel the vacuole's proteome have resulted in the discovery of previously unknown vacuole molecules. Owing to the inherent methodological limitations discussed above, however, many secreted parasite factors probably remain undetected. Interestingly, genome scale experimental genetics data suggest dispensable functions during asexual blood stage development for most of the newly identified proteins[168,169]. We have previously hypothesized that the presence of highly abundant yet dispensable proteins might provide a favourable molecular crowding effect for important vacuolar pathways[70].

The overwhelming majority of the identified proteins harbour no functional annotations, pointing towards a plethora of tailor-made adaptations at this central host–pathogen interface. One prominent example of this phenomenon is the *Plasmodium*-specific early transcribed membrane protein family (ETRAMP) which

comprises a set of small and highly charged transmembrane proteins of unknown function that oligomerize in the PVM[95,170]. The *Plasmodium*-specific nature of most vacuolar proteins offers invaluable perspectives for the development of evidence-based intervention strategies that specifically target the parasite's intraerythrocytic niche without harming the host.

Glossary

Merozoite: Invasive parasite stage, which is released during host cell egress to infect erythrocytes.

Ring stage: Young parasite stages, which mark the onset of intraerythrocytic development after invasion.

Trophozoite: Intraerythrocytic parasite stage characterised by rapid growth and biomass production.

Schizont: Intracellular parasite stage that undergoes multiple rounds of nuclear division and concerted cytokinesis to form new daughter merozoites.

Membrane capacitance: The ability of a biological membrane to store energy in the form of an electrical charge, the magnitude of which is directly proportional to the membrane surface area.

Sec translocon: ER-resident protein complex which translocates secretory proteins from the cytoplasm across or into the ER membrane.

Signal peptide: Short amino-terminal peptide of 16-30 amino acids which directs secretory proteins to the Sec translocon and which is cleaved upon translocation.

Rhodamine 123: A fluorescent potentiometric dye which accumulates in mitochondria in a manner which is dependent on membrane polarization.

Haemozoin: Crystals of haemoglobin-derived haem in the parasite's food vacuole.

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