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Plasmodium rhoptry proteins: why order is important

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Abstract

Apicomplexan parasites, including the Plasmodium species that cause malaria, contain three unusual apical secretory organelles (micronemes, rhoptries and dense granules) that are required for the infection of new host cells. Because of their specialized nature, the majority of proteins secreted from these organelles are unique to Apicomplexans and consequently not well characterized. Although rhoptry proteins of Plasmodium have been implicated in events central to invasion, there is growing evidence to suggest that proteins originating from this organelle play key roles downstream of parasite entry into the host cell. Here we discuss recent work that has advanced our knowledge of rhoptry protein trafficking and function, and highlight areas of research that require further investigation.

Plasmodium rhoptry proteins are important for multiple steps of host cell invasion

The Apicomplexan phylum comprises intracellular parasites including Plasmodium, Toxoplasma and Cryptosporidium, all of which are important human pathogens. Despite great variation in the target host cell of each parasite and subsequent disease pathology, all members of this phylum share a common feature; the presence of three specialized secretory organelles. The micronemes, rhoptries and dense granules are apically positioned structures that secrete their contents into the target host cell in a rapid and coordinated sequence of events to facilitate parasite invasion.

The invasion process of Plasmodium begins after initial contact between the merozoite form of the parasite and the host red blood cell (RBC); this adherence is of low affinity and reversible (reviewed in [1]). The parasite then orientates so that its apical end is juxtaposed to the RBC membrane to facilitate closer interactions, with invasion proceeding via the formation of a tight junction (TJ); See Glossary). The parasite enters the cell by pulling itself through this junction using an actin-myosin motor [2], simultaneously creating a vacuole (parasitophorous vacuole; PV) that separates it from the host cell cytoplasm. The vacuole membrane (PVM) fuses to surround the invaded parasite, thus providing
an environment hospitable for parasite replication [3]. Throughout these stages of invasion, the parasite sequentially discharges mediators from its three secretory organelles to facilitate entry into the host cell. Microneme proteins are the first to be released, rhoptry discharge follows, with dense granule contents secreted once the PV has been established. While the micronemes and dense granules are involved in establishing the early stages of invasion (including reorientation of the parasite and establishing the TJ [4]) and modifying the host cell, respectively, rhoptry proteins have been implicated in a diverse array of functions at various stages throughout invasion; these range from merozoite adhesion, TJ formation, establishment of the parasitophorous vacuole membrane (PVM) as well as modification of the host cell, all of which will be explored further in this review.

The rhoptries and its constituents

The rhoptries are the most prominent of the secretory organelles in Plasmodium and are synthesized de novo during parasite development. Their evolutionary origin remains unknown, being unique to Apicomplexans and bearing little morphological resemblance to other eukaryotic organelles, although studies in Toxoplasma gondii reveal rhoptries possess some characteristics typical of secretory lysosomes [5]. Rhoptries consist of two distinct regions: an apical duct known as the rhoptry neck, and a larger lipid-rich region called the rhoptry bulb, with each compartment containing distinct protein constituents (Figure 1). Some rhoptry neck proteins (RONs) are conserved between Toxoplasma, Eimeria and Plasmodium [6-8], while rhoptry bulb proteins (ROPs) are generally distinct to each species (reviewed in [7]), presumably a consequence of the different host cells (nucleated vs. non-nucleated) that each species invades. To date, more than 30 proteins have been classified as rhoptry proteins in the human malaria parasite Plasmodium falciparum (Figure 1) [9-11]. A further 27 potential rhoptry proteins with unknown functions have been identified through proteome analysis of purified rhoptries from the rodent species Plasmodium yoelii [12]. However, it is difficult to isolate rhoptries pure of other organelles and parameters such as expression profile and sequence motifs do not easily discriminate rhoptry proteins from other apical organelle proteins. As a result,
further experimental validation is required to put a more accurate figure on the number of rhoptry proteins.

**Biogenesis of rhoptries and trafficking of its cargo**

Surprisingly, there is still a limited understanding of how rhoptries are formed during each cell cycle, how proteins traffic to these structures and the signals that determine their segregation within the rhoptry to ensure correct secretion during invasion. Transmission electron microscopy (TEM) studies performed on *P. falciparum* suggest rhoptries form from the progressive fusion of Golgi-derived coated vesicles, although how this is initiated is unknown [13]. Certainly, rhoptry proteins pass through the conventional eukaryotic secretory pathway, courtesy of a classical hydrophobic signal sequence [14], consistent with rhoptries forming from Golgi-derived vesicles. As the rhoptry matures, two distinctive regions separate within it, to ultimately become the rhoptry neck and bulb [13]. Although *P. falciparum* possesses a stripped-down version of the secretory pathway that exists in higher eukaryotes [15], it is unclear how the parasite delivers proteins specifically to the rhoptries (and indeed to either the neck/bulb region) given that this is not the only secretory organelle within the parasite. Most likely, the sorting of cargo also occurs within the Golgi, with subsequent packaging into trafficking vesicles specifically destined for the rhoptries (Figure 2). In *Toxoplasma gondii*, the generation of secretory pathway vesicles necessary for rhoptry biogenesis requires the presence of the large dynamin-like GTPase, DrpB [16], although this is also required for the generation of micronemes. Of interest, there is a DrpB orthologue in *Plasmodium* spp, but if and where it functions in the rhoptry trafficking pathway is currently unknown (Figure 2) [16].

The formation of intracellular transport vesicles and the selection of cargo have been well studied in higher eukaryotes (Box 1) and to a limited extent in *T. gondii*. In *Toxoplasma gondii*, depletion of AP1 using anti-sense technology affects rhoptry biogenesis [17]. The recognition of motifs (tyrosine-based and di-leucine) in the cytoplasmic tails of transmembrane (TM) proteins such as the rhoptry protein ROP2 and the micronemal proteins MIC2 and MIC6 by the μ1
subunit of AP1 has also been implicated in their trafficking to the respective organelles [17-19]. This mechanism has been called into question for ROP2 as a more recent study revealed membrane anchoring of this protein occurs through hydrophobic and ionic interactions rather than by a classical TM domain, and thus without the protrusion of a cytoplasmic tail it is unclear how ROP2 could interact with AP1 [20]. Certainly, other modes of trafficking must exist since the presence of a TM domain or tyrosine-based motifs is not essential for the trafficking of other proteins to apical organelles [21-23].

In the case of Plasmodium, Rabs, SNAREs and AP1 have been identified in the genome [24], but the majority of known rhoptry proteins are soluble and thus the mechanism by which rhoptry cargo in Golgi-derived trafficking vesicles engage with the cytoplasmic trafficking machinery is still very much a black box. In fact, only a limited number of studies with a focus on rhoptry protein trafficking have been performed in Plasmodium. The proteins for which the most is known are members of the low molecular weight rhoptry associated protein (RAP) complex. Correct targeting of RAP2 to the rhoptries requires an interaction with RAP1 [25], with deletion of RAP1 sequence that includes the RAP2 binding site, leading to the mislocalisation of RAP2 to the endoplasmic reticulum (ER). The same is likely to be the case for RAP3 given RAP2 and RAP3 are paralogues. The high molecular weight RhopH complex (reviewed in [9]) is formed via non-covalent interactions; whether their association is required to ensure correct localization to the rhoptries remains to be investigated.

Interestingly, immunoprecipitation and fluorescence resonance energy transfer experiments have revealed that the RAP complex (and potentially the RhopH complex) interacts with the rhoptry-associated membrane antigen (RAMA) at the Golgi via the N-terminus of RAP1 [26]. RAMA is a glycoprophosphatidyl inositol (GPI) anchored protein, localizing to the ER and Golgi compartments before rhoptries are visible by TEM [27] and later present in the bulb of the mature rhoptry. It is conserved amongst Plasmodium, and its inability to be deleted in P. falciparum implies it is an essential protein [28, 29]. Taken altogether these findings led Richard et al. to propose that RAMA generates clusters of rhoptry-
destined cargo within detergent resistant membranes at the Golgi-exit face that
ultimately bud off as rhoptry-destined vesicles [26]. Intriguingly, however, no
functional equivalent of RAMA has to date been found in other Apicomplexan spp.
It should also be noted that because the C-terminus of RAMA is embedded within
the lipid bilayer [30], an unidentified TM escorter protein would have to be
present in vesicles at the Golgi-exit face, interacting with both RAMA and the
cytosolic sorting machinery to escort these protein complexes to nascent
rhoptries.

One candidate protein that may fulfill the role of the TM escorter protein
interacting with RAMA is the *Plasmodium* orthologue of the *T. gondii* sortilin-like
receptor (TgSORTLR), which is transcribed around the same time as RAMA. A
recent study by Sloves et al. in *T. gondii* revealed TgSORTLR resides in Golgi-
endosomal compartments, and is required for the formation of rhoptries as well
as micronemes [31]. The luminal domain of TgSORTLR specifically interacts with
rhoptry protein cargo, whilst its cytoplasmic tail interacts with AP adaptins,
clathrin and other vacuolar-sorting proteins. Thus, by facilitating the association
of a sorting complex with vesicles at the Golgi exit face, TgSORTLR potentially
guides cargo through the endocytic pathway before releasing it at the rhoptries
(Figure 2). However, as TgSORTLR also interacts with cargo destined for the
micronemes, an extra layer of specificity such as temporal separation of rhoptry
and microneme protein expression must be involved to ensure proteins are
navigated from the Golgi to their correct destination. Indeed it would be
interesting to examine whether a rhoptry protein, if expressed later in the cell
cycle, would end up in the micronemes or another compartment.

In addition to a N-terminal signal sequence, rhoptry proteins in both *Plasmodium*
and *T. gondii* contain an additional pro-domain that is processed *en route* or
upon arrival at the organelle [26, 27, 32-35]. The pre-protein form of RAMA and
RAP1, for example, are cleaved to yield a smaller mature protein. In *T. gondii*, the
pro-domain plays diverse roles, mediating protein trafficking and assembly, and
in the case of the micronemal protein M2AP, it plays a critical role in the stability
and secretion of the MIC2-M2AP complex [36]. The pro-domain also potentially
plays an additional role in maintaining some rhoptry proteins as zymogens until
activation for effector function. However, there is a distinct lack of rhoptry
proteins with enzymatic activity in *Plasmodium*. Thus in *Plasmodium* the
processing event may assist protein maturation and folding, enable the RAP
complex (and other potential associated cargo) to be released from RAMA so
they are ready for secretion or be required to release RAMA from its hypothetical
TM escorter protein.

Once at the rhoptries, proteins localise to the rhoptry membrane, neck, bulb or
potentially other ‘yet to be defined’ compartments of the rhoptry [37]. The
correct localization has important implications, as neck and bulb proteins are
secreted sequentially and consequently have different roles in invasion and post-invasion events as outlined in the sections that follow. TEM studies reveal the
trafficking of vesicles from the Golgi to the rhoptry neck as the organelle is
undergoing maturation [13], suggesting that the early rhoptry proteins are
destined to become bulb proteins, while later proteins traffic to the neck
thereafter (Figure 2). RAP1 is retained in the bulb courtesy of a C-terminal motif,
with deletion of this sequence leading to its mislocalisation at the rhoptry neck
[26] and inability to localize to the PV after invasion. Whether retention motifs
are a typical feature of ROPs requires further investigation. For protein
trafficking to the rhoptry neck, perhaps Pf34 plays a similar role to RAMA in
recruiting neck proteins from within the Golgi, as hypothesized in Figure 2. Pf34
is a rhoptry neck protein that is expressed prior to the formation of the organelle
and also contains a GPI anchor [38], both features shared with RAMA. However,

Confounding the TEM observations, however, is the observation that Rh5
localises to the rhoptry bulb in late schizonts [39, 40] prior to its localization at
the apical tip of free merozoites [41]. Rh5 subsequently co-localises with Rh5
interacting partner (RIPR), which itself appears to originate from micronemes
[42]. Thus whether this pathway to the merozoite surface, via the rhoptry bulb,
is specific to Rh5, or shared with other Rh proteins is unknown but warrants
further investigation.
For the *P. falciparum* and *T. gondii* membrane-associated Armadillo Repeats-Only (ARO) protein, whose function remains to be elucidated, myristoylation and palmitoylation motifs are required for membrane attachment to the outer face of the rhoptries. Other residues within its first 20 N-terminal amino acids also ensure correct targeting to this membrane (Figure 2) [43]. Interestingly, the only putative palmitoyltransferase (PAT) that has been localized in *P. falciparum* shows Golgi distribution in late schizonts, implicating the Golgi again as the location for selective rhoptry targeting [44]. However, as there are three other PATs expressed at this stage of the parasite life cycle [43, 45], it cannot be ruled out that one of these may be bound at the rhoptry membrane to facilitate specific targeting of the *P. falciparum* ARO protein.

**Rhoptry neck proteins: from adhesins to invasins**

*Plasmodium* rhoptry proteins that localize to the neck are predominantly involved in host cell adhesion before and during invasion, with some also sharing evolutionary origins with other Apicomplexans. As there are already some excellent recent reviews on the neck proteins [1, 46] we instead focus in this section on the use of emerging technologies and methodologies that have further increased our understanding of how these proteins function.

The *Plasmodium* reticulocyte-binding homologues (Rh), comprising Rh1, Rh2a, Rh2b, Rh4 and Rh5 are adhesins, involved in establishing merozoite invasion of erythrocytes by interacting with host cell receptors. Recently, through the use of ‘avidity-based extracellular interaction screen’ (AVEXIS), the erythrocytic receptor for Rh5 was identified as basigin [47]. This high-throughput approach detects low-affinity protein interactions between bait and prey ectodomains by expressing the prey domains as pentamers, thereby increasing the avidity of any interactions [48]. Importantly, Rh5 is the only adhesin refractory to genetic disruption [40], and its interaction with basigin is required for and essential to merozoite invasion [47]. Indeed, antibodies against Rh5 can significantly inhibit the growth of all the *P. falciparum* laboratory strains tested [49]. Although *ex vivo* studies from field isolates of *P. falciparum* have shown that Rh5 is not an
abundant invasion ligand, a positive correlation was observed between the relative proportion of Rh5 expression levels in isolates relative to other Rh/erythrocyte binding antigens ligands examined and its associated parasitemia in patients [50]. If this is reflective of actual protein levels in the rhoptry, then it provides the first molecular correlate of invasion efficiency. Together these data make Rh5 a prime candidate for vaccine trials [51].

Super resolution microscopy has been another technological advance, which combined with immunoelectron microscopy and the capacity to routinely isolate merozoites [52] has provided unprecedented spatial and temporal resolution of *Plasmodium* proteins before, during and after invasion [53]. In particular, TJ formation has been studied. Localising to the TJ is a complex of secreted RON proteins comprising RON2, 4 and 5, which interact with the micronemal protein AMA1. These studies have revealed that RON4 forms an outer ring encircling the protein rings of AMA1 and a haemagglutinin epitope tagged version of RON2 during merozoite invasion [37, 53]. These nested RON4/AMA1 and RON4/RON2-HA rings clearly delineate the invaded, PVM-surrounded anterior of the parasite from its extracellular posterior, consistent with the hypothesis that the TJ is a parasite-derived protein ring that facilitates invasion into host cells (reviewed in [4, 46]). Although RON2 and its micronemal partner AMA1 have not yet been imaged together by super resolution microscopy, their localization within the RON4 ring is in keeping with previous data that indicates a direct interaction with each other. This interaction has been closely mapped by invasion inhibitory peptides and monoclonal antibodies, as well as recombinant expression of RON2 domains (reviewed in [4, 54]). Recently, co-crystallisation studies of AMA1 and RON2 peptides in both *T. gondii* [55] and *P. falciparum* [56] have provided the molecular basis for the unusually strong binding between AMA1 and RON2, as well as the structural rationale for how invasion inhibitory peptides, such as R1, and monoclonal antibodies against AMA1 inhibit its interaction with RON2.

How the interaction between RON2 and AMA1 is prevented prior to invasion is significant. Besteiro *et al.* found that, in addition to segregating RON2 and AMA1 into distinct invasion organelles, *T. gondii* also temporally separates the
components by expressing them at different developmental stages [57]. Specifically, RONs are synthesised and packaged before micronemal proteins, preventing them from interacting within the secretory system. Analogously in *P. falciparum*, the interaction between Rh5 and its interacting partner, RIPR, is observed on free merozoite apical tips, but segregated between micronemes and rhoptries in non-segregated schizonts [42]. Further, analysis of the transcriptome data of *P. falciparum* on PlasmoDB also indicates a small but discrete delay between rhoptry neck and micronemal gene transcription, with RON transcripts appearing and peaking in abundance earlier.

The key interaction between AMA1 and RON2 has supported the hypothesis that invading Apicomplexans provide both the ligand and its cognate receptor for TJ formation [7]. However, a conditional knockdown of AMA1 in *Plasmodium berghei* using the flippase recombinase-flippase recognition target FLP-FRT inducible knockdown system found that sporozoite invasion of hepatocytes was not impaired *in vivo* or *in vitro*, although the functional loss of AMA1 subsequently prevented merozoite invasion of erythrocytes [58]. By contrast, similar loss of RON4 significantly decreased sporozoite invasion of hepatocytes. These unexpected findings suggest that AMA1 is not universally required for zoite invasion and is not a prerequisite for TJ formation, although it appears to be required for merozoite invasion of erythrocytes.

In addition to adhesins that initiate invasion after host recognition, Zuccala et al. applied a bioinformatics approach to identify rhoptry proteins putatively involved in TJ-mediated invasion, which they have termed ‘invasins’ [37]. Amongst several candidate invasins, this *in silico* approach identified components of the TJ-forming RON complex as well as the Apical Sushi Protein (ASP). ASP is a rhoptry neck protein that may be orthologous to *T. gondii* RON1 [59] or *Cryptosporidium parvum* PRP1 [60], although the overall sequence similarity is poor, and only *Plasmodium* ASP is GPI-anchored. Intriguingly, the host cell receptor for Rh4, complement receptor 1 (CR1), also contains tandem sushi domains that may interact with Rh4 to facilitate invasion [61]. However, the function of ASP and its sushi domains remain unknown.
Several other proteins that localize to the Plasmodium rhoptry neck remain to be functionally characterized, including Pf34, AARP and RON6 (Figure 1, reviewed in [46]). Whether these proteins are involved in adhesion or invasion requires further investigation, but at the moment leaves open the possibility that rhoptry neck proteins may engage in even more diverse functions.

**Rhoptry bulb proteins function predominantly downstream of invasion**

In contrast to the rhoptry neck proteins, *Plasmodium* rhoptry bulb proteins (Figure 1) share no homology with rhoptry proteins from other Apicomplexans, suggesting they have evolved to suit their target host cell. It should be noted that some of the designated *Plasmodium* ROP proteins will require further characterization to confirm their localization (Box 2). In *T. gondii*, many of the identified ROPs are kinases and their biological functions include interacting with host signaling pathways (reviewed in [7, 62]) and interfering with host immunity [63-67]; both roles are consistent with enhancing parasite survival within host cells. On the other hand, no *Plasmodium* ROPs are predicted to have kinase activity, and to date they have been implicated in diverse roles ranging from rhoptry biogenesis, host cell invasion, PV formation and host cell modification, although functional data supporting these is limited.

The rhoptry bulb contains two well-characterized protein complexes identified through immunoprecipitation experiments on rhoptry extracts: the RAP and RhopH complexes. Members of these complexes are all highly abundant proteins and are conserved amongst the malaria species, although in *P. falciparum*, the number of ROP genes has expanded due to gene duplication events. For example, RAP2 and RAP3 are paralogues in *P. falciparum*, whereas other *Plasmodium* species harbor only a single RAP2/3 gene. Gene duplication has also occurred in the RhopH complex; RhopH1 is a product of a multigene family of which there are five known variants in *P. falciparum* (clag 2, 3.1, 3.2, 8 and 9) [68, 69]. Interestingly, other malaria species have only two RhopH1 genes: a clag9 orthologue and a clagx gene that shares similarities with other clag genes.
Previously, the RAP complex has been implicated in invasion, as antibodies and peptides specific for RAP1 can partially block \textit{in vitro} invasion of RBCs. RAP1 is also immunogenic, as anti-RAP1 can be detected in the sera of naturally infected hosts, although vaccines directed against this protein do not completely protect against challenge infection (reviewed in [9]). This lack of protection is consistent with what we now know about the sequence of invasion, which has revealed that ROPs are actually secreted into the PV/PVM after formation of the TJ [53]. Certainly, the immunogenicity of RAP1 can be accounted for by abortive invasive events, whereby attached merozoites release rhoptry contents without invasion of a host cell, or phagocytosis of erythrocytes infected by early stage parasites. The timing of ROP release now also fails to explain the role of these proteins during invasion as suggested by studies that have demonstrated the binding of various bulb proteins such as RhopH3 to the RBC surface. These may reflect potential interactions that would occur between ROPs and RBC proteins transferred to the PVM during invasion, or are the result of non-specific binding of sequences to lipid membranes, which constitute both the RBC surface and the PVM.

To better understand the function of the ROPs, loss-of-function studies have been performed via gene disruption in \textit{P. falciparum} (Table 1) and \textit{P. berghei}. Disruption of RAP1 was only possible in one particular \textit{P. falciparum} strain that utilizes alternative parasite ligands for invasion [25]. Interestingly, although no \textit{in vitro} phenotype was observed, RAP2 targeting to the rhoptry was disturbed in the RAP1 knockout. In \textit{P. falciparum}, RAP3 is not essential for merozoite invasion, most likely because RAP2 can complement the loss of RAP3 [70]. In \textit{P. berghei} which only has a single RAP2/3 gene, a successful knockout of RAP2/3 has not been achieved [71]. This suggests that the RAP complex may indeed be crucial for parasite survival in an \textit{in vivo} setting.

For the RhopH complex, which is transferred to the host cell cytoplasm and plasma membrane post-invasion, several functions have been assigned (Figure 1) [72-76]. The \textit{P. falciparum} RhopH1/clag9 has been implicated in the cytoadherence of infected RBCs to the microvasculature, preventing clearance.
through the spleen, factors that contribute to severe malaria [72]. Recently RhopH1 has been associated with establishing new permeation pathways in the RBC; the clag3 variants, which are inserted into the RBC membrane, alter host cell permeability to a diverse range of solutes and thus potentially aid in nutrient acquisition [76]. Additionally, clag3 null mutants display a significant growth defect \textit{in vitro}, with no invasion phenotype observed [77]. The function of clag2 and clag8 in \textit{P. falciparum} is unknown, although it is likely they have some degree of functional redundancy with clag3. In keeping with this, the single orthologues of \textit{P. falciparum} clag9 and clag 2/3/8 in \textit{P. berghei} are refractory to gene deletion [78], which implies they have a crucial functional role \textit{in vivo}.

While these studies have highlighted novel roles for \textit{P. falciparum} ROPs, several questions remain unanswered, especially concerning the timing of secretion and trafficking of RhopH proteins. Although RhopH1 proteins (clag3 and clag 9 variants) are implicated in functions downstream of invasion, their expression peaks in schizonts. Another RBC-membrane interacting protein (ring-infected erythrocyte surface antigen; RESA) that is also maximally expressed in schizonts, is instead found in the dense granules. By contrast, the knob-associated histidine rich protein (KAHRP), which is also implicated in cytoadherence, demonstrates a peak level of expression after invasion during ring stages. Whilst RESA and KAHRP are most likely translocated across the PVM by a multimeric protein machinery termed the \textit{Plasmodium} translocon for exported proteins (PTEX) [79, 80], PTEX is unlikely to transport RhopH as a complex. As the RhopH complex initially localizes to the PVM after invasion, and antibodies to RhopH2 later label the Maurer’s Clefts [73], it remains to be examined whether the RhopH complex is exported into the RBC via initial cleft formation or via another mechanism.

\textbf{Concluding remarks}

The biological functions of many \textit{Plasmodium} rhoptry proteins remain largely unknown due to the lack of appropriate and/or robust methodologies to study products of genes essential to parasite survival within RBCs [81]. The ordered and sequential release of proteins from the three apical organelles was thought to reflect the function of each organelle’s contents; micronemes secreted
proteins required for invasion and the establishment of the TJ, rhoptry proteins were implicated in PV and PVM formation, and dense granules proteins were required to modify the host cell after invasion had been completed. However, this model is now under challenge, as Rh5 is suggested to be part of the attachment process, RON2/RON4 are implicated in TJ formation with the micronemal protein AMA1, and ROPs are implicated downstream of PVM formation, in processes that include altering host cell permeability to enhance nutrient acquisition.
Table 1. Gene deletion studies in *P. falciparum*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Findings</th>
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<tr>
<td>RAMA</td>
<td>Refractory to deletion</td>
<td>[29]</td>
</tr>
<tr>
<td>RAP1</td>
<td>Gene disruption, no <em>in vitro</em> phenotype in D10 parasite line</td>
<td>[25]</td>
</tr>
<tr>
<td>RAP3</td>
<td>Gene disruption, no <em>in vitro</em> phenotype</td>
<td>[70]</td>
</tr>
<tr>
<td>RhopH1/Clag3</td>
<td>Reduced <em>in vitro</em> growth</td>
<td>[77]</td>
</tr>
<tr>
<td>RhopH3</td>
<td>Refractory to deletion</td>
<td>[82]</td>
</tr>
<tr>
<td>RALP</td>
<td>Refractory to deletion</td>
<td>[10]</td>
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Box 1. Sorting of protein cargo in eukaryotes

Eukaryotic proteins that enter the secretory system are translocated across the membrane of the endoplasmic reticulum into the lumen, before they are selectively packaged into transport vesicles bound for the Golgi complex. Protein cargo then passes through the Golgi to the trans-Golgi network, where proteins are sorted further depending on their final destination (e.g., lysosome, vacuole, cell surface). The selection and packaging of cargo at the Golgi-exit face is reliant on coat proteins assembling at the surface of the donor membrane to form transport vehicles. The formation of intracellular transport vesicles and the selection of cargo in higher eukaryotes involve subunits of adapter protein complexes (AP) that form part of the protein coat on the cytoplasmic face of clathrin-coated vesicles, and these interact with specific sequence motifs within the cytoplasmic tails of transmembrane (TM) proteins (reviewed in [83]). For Apicomplexans, other modes of trafficking to the apical organelles must exist as Plasmodium rhoptry proteins do not possess TMs with cytoplasmic tails, and the TM of some micronemal proteins (which possess tyrosine motifs in their cytoplasmic tails) are not required for proper targeting. For example, pro-domains of rhoptry and microneme proteins and the endosomal cargo receptor TgSORTLR are implicated in trafficking to these organelles. Moreover, unlike higher eukaryotes, where secretory granules are derived from the trans-Golgi network, Toxoplasma gondii proteins and potentially those from other Apicomplexans are routed through the early and late endosomes of the endolysosomal system before delivery to the rhoptry and micronemes. This may provide a mechanism by which secretory proteins can be processed by proteases. Also involved in the trafficking pathway are Rab GTPases and SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) that promote tethering of the transport vesicles to the target membrane and fusion to the target membrane, respectively.

Box 2. Poorly characterised bulb proteins

Several proteins are thought to localize to the rhoptry bulb but require further characterization to confirm this positioning. Repetitive organellar protein (ROPE) has characteristics similar to mammalian spectrin and is thought to
function in invasion by interacting with the RBC cytoskeleton [84]. Several
*Plasmodium* spp. encode an integral stomatin-like protein (band 7-related
protein) [85]. This stomatin orthologue, which partially co-localises with
RhopH2, is delivered to the cytoplasmic face of the PVM and is proposed to play a
role in PVM formation. Rhoptry-associated leucine-zipper like protein (RALP1)
contains four heptad repeats of leucines, resembling a leucine zipper [10]. RALP1
colocalises with RAP1 in merozoites, but as it is not observed in newly invaded
cells, its function is unknown.
Outstanding questions

- What is the identity of the TM escorter that engages with *Plasmodium* rhoptry cargo and the cytosolic trafficking machinery to facilitate trafficking to the rhoptry?

- Does the rhoptry organelle consist of more compartments than just the bulb and neck in order to accommodate proteins with diverse functions that act at different stages of host cell attachment and invasion?

- How do proteins localize to distinct compartments of the rhoptry? Is this via temporal expression of bulb and neck proteins concurrent with maturation of the organelle? What stops the neck and bulb proteins mixing and how are some proteins retained in the rhoptry whilst other constituents are being secreted?

- If Rh5-basigin interaction occurs upstream of TJ formation, are there multiple triggers for rhoptry release, with the first one triggered by the EBA/Rh ligand interaction with cognate receptors on the host cell [86] and the second after the AMA1 and RON2 interaction?

- How does RhopH1/clag3 contribute to anion channel formation at the host erythrocyte membrane and/or solute transport? How does the RhopH complex get exported into the host RBC and traffic to the RBC membrane?

Glossary

Adhesins: merozoite surface proteins involved in host cell interactions and induction of invasion. Adhesins include the micronemal erythrocyte binding antigens (EBA) family, EBA175, EBA140, EBA181, and the rhoptry-localised reticulocyte-binding homologues (Rh) family, Rh1, Rh2a, Rh2b, Rh4 and Rh5.

Invasins: a new term describing parasite ligands involved in establishing tight-junction mediated invasion.

Basigin (Ok blood group antigen, CD147, EMMPRIN, M6): an erythrocyte receptor that interacts with the rhoptry adhesin Rh5.
Parasitophorous vacuole (PV): vacuole found in infected host cells where most Apicomplexan parasites reside and develop.

Tight junction (TJ): a dynamic junction formed between the apical end of the invading merozoite and the host erythrocyte and hence is often also referred to as the moving junction. The *Plasmodium* merozoite TJ is predicted to be composed of the RON complex (RON2, RON4, RON5) and the micronemal protein AMA1.

RAP complex: a low molecular weight protein complex localized to the rhoptry bulb, comprised of heterodimers of rhoptry associated protein 1 (RAP1) and RAP2, or RAP1 and RAP3.

RhopH complex: a high molecular weight protein complex comprising RhopH1, RhopH2 and RhopH3.

Rhoptry-associated membrane antigen (RAMA): an essential and abundant glycophaspatidyl inositol (GPI)-anchored protein that accumulates in the ER and Golgi compartments in lipid rafts prior to rhoptry development, implicated in rhoptry biogenesis.

R1 inhibitory peptide: a 20-residue peptide that is able to inhibit invasion of *P. falciparum* merozoites by binding to AMA1 and preventing TJ formation.

Sushi domain: cysteine-rich domains that typically regulate complement ligands, also known as short complement repeats (SCRs). These domains are found in the rhoptry neck protein ASP, as well as CR1, the erythrocyte receptor for Rh4.
**Figure 1. Rhoptry neck and bulb proteins during and after invasion**

RONs predominantly localise to the merozoite surface adhesion site or tight junction during attachment and invasion, although the function and final localisation of several RONs remain unknown. ROP proteins are secreted after the parasite has fully entered the erythrocyte, and may be involved in forming the PVM. Some ROPs have been detected at the Maurer’s clefts (MC) and even the erythrocyte plasma membrane (ePM) after invasion. Again, the final localisation of some ROPs remain unknown. A, adhesion site; D, dense granule; M, microneme; R, rhoptry; N, nucleus; TJ, tight junction; nPVM, nascent parasitophorous vacuole membrane; PVM, parasitophorous vacuole membrane; MC, Maurer’s clefts; ePM, erythrocyte plasma membrane.

**Figure 2. Model of protein sorting to the rhoptries in Plasmodium.**

Proteins destined for the rhoptries are co-translationally inserted through Sec61 at the endoplasmic reticulum (ER) via their signal sequence and trafficked to the Golgi in COPII-coated vesicles. At the Golgi exit face, the GPI-anchored protein RAMA aggregates with other rhoptry bulb cargo such as the RAP complex within detergent resistant membranes, and these regions ultimately bud off as rhoptry-destined vesicles (CCV; clathrin coat vesicles). The Sortilin-like receptor (SORTLR) potentially acts as a rhoptry escorter, facilitating an interaction with adapter proteins (AP) and the cytosolic trafficking machinery. The CCVs fuse at the target membrane to release cargo into nascent rhoptries. Here, proteolytic processing of the rhoptry cargo facilitates their release from the trafficking complex, with a bulb-retention motif in the RAP complex preventing dispersal throughout the entire rhoptry as it matures. The Armadillo Repeats-Only (ARO) protein is targeted to the rhoptry membrane; whether this is predetermined at the Golgi or once it has trafficked at the rhoptry remains to be elucidated. The GPI-anchored rhoptry neck protein Pf34 may perform a similar role to RAMA. Whilst DrpB is also involved in rhoptry biogenesis, its localization and function in rhoptry biogenesis and trafficking is yet to be revealed.