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1 *Plasmodium* **rhoptry proteins: why order is important**

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4 Natalie Counihan[1*], Ming Kalanon[1*], Ross L. Coppel[2] and Tania F. de Koning-

5 Ward[1]

6

7 [1]School of Medicine, Deakin University, Waurn Ponds, 3216, Australia

8 [2]Faculty of Medicine and Victorian Bioinformatics Consortium, Monash

9 University, Clayton, Victoria, 3800, Australia

10 * these authors contributed equally to this review

11 Corresponding author: de Koning-Ward, T.F. (taniad@deakin.edu.au)

12

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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

48 an environment hospitable for parasite replication [3]. Throughout these stages
49 of invasion, the parasite sequentially discharges mediators from its three
50 secretory organelles to facilitate entry into the host cell. Microneme proteins are
51 the first to be released, rhoptry discharge follows, with dense granule contents
52 secreted once the PV has been established. While the micronemes and dense
53 granules are involved in establishing the early stages of invasion (including
54 reorientation of the parasite and establishing the TJ [4]) and modifying the host
55 cell, respectively, rhoptry proteins have been implicated in a diverse array of
56 functions at various stages throughout invasion; these range from merozoite
57 adhesion, TJ formation, establishment of the parasitophorous vacuole membrane
58 (PVM) as well as modification of the host cell, all of which will be explored
59 further in this review.
60

61 **The rhoptries and its constituents**

62 The rhoptries are the most prominent of the secretory organelles in *Plasmodium*
63 and are synthesized *de novo* during parasite development. Their evolutionary
64 origin remains unknown, being unique to Apicomplexans and bearing little
65 morphological resemblance to other eukaryotic organelles, although studies in
66 *Toxoplasma gondii* reveal rhoptries possess some characteristics typical of
67 secretory lysosomes [5]. Rhoptries consist of two distinct regions: an apical duct
68 known as the rhoptry neck, and a larger lipid-rich region called the rhoptry bulb,
69 with each compartment containing distinct protein constituents (Figure 1). Some
70 rhoptry neck proteins (RONs) are conserved between *Toxoplasma*, *Eimeria* and
71 *Plasmodium* [6-8], while rhoptry bulb proteins (ROPs) are generally distinct to
72 each species (reviewed in [7]), presumably a consequence of the different host
73 cells (nucleated vs. non-nucleated) that each species invades. To date, more than
74 30 proteins have been classified as rhoptry proteins in the human malaria
75 parasite *Plasmodium falciparum* (Figure 1) [9-11]. A further 27 potential rhoptry
76 proteins with unknown functions have been identified through proteome
77 analysis of purified rhoptries from the rodent species *Plasmodium yoelii* [12].
78 However, it is difficult to isolate rhoptries pure of other organelles and
79 parameters such as expression profile and sequence motifs do not easily
80 discriminate rhoptry proteins from other apical organelle proteins. As a result,

81 further experimental validation is required to put a more accurate figure on the
82 number of rhoptry proteins.
83
84 **Biogenesis of rhoptries and trafficking of its cargo**
85 Surprisingly, there is still a limited understanding of how rhoptries are formed
86 during each cell cycle, how proteins traffic to these structures and the signals
87 that determine their segregation within the rhoptry to ensure correct secretion
88 during invasion. Transmission electron microscopy (TEM) studies performed on
89 *P. falciparum* suggest rhoptries form from the progressive fusion of Golgi-
90 derived coated vesicles, although how this is initiated is unknown [13]. Certainly,
91 rhoptry proteins pass through the conventional eukaryotic secretory pathway,
92 courtesy of a classical hydrophobic signal sequence [14], consistent with
93 rhoptries forming from Golgi-derived vesicles. As the rhoptry matures, two
94 distinctive regions separate within it, to ultimately become the rhoptry neck and
95 bulb [13]. Although *P. falciparum* possesses a stripped-down version of the
96 secretory pathway that exists in higher eukaryotes [15], it is unclear how the
97 parasite delivers proteins specifically to the rhoptries (and indeed to either the
98 neck/bulb region) given that this is not the only secretory organelle within the
99 parasite. Most likely, the sorting of cargo also occurs within the Golgi, with
100 subsequent packaging into trafficking vesicles specifically destined for the
101 rhoptries (Figure 2). In *Toxoplasma gondii,* the generation of secretory pathway
102 vesicles necessary for rhoptry biogenesis requires the presence of the large
103 dynamin-like GTPase, DrpB [16], although this is also required for the generation
104 of micronemes. Of interest, there is a DrpB orthologue in *Plasmodium* spp, but if
105 and where it functions in the rhoptry trafficking pathway is currently unknown
106 (Figure 2) [16].
107
108 The formation of intracellular transport vesicles and the selection of cargo have
109 been well studied in higher eukaryotes (Box 1) and to a limited extent in *T.*
110 *gondii.* In *Toxoplasma gondii,* depletion of AP1 using anti-sense technology
111 affects rhoptry biogenesis [17]. The recognition of motifs (tyrosine-based and di-
112 leucine) in the cytoplasmic tails of transmembrane (TM) proteins such as the
113 rhoptry protein ROP2 and the micronemal proteins MIC2 and MIC6 by the μ1

114 subunit of AP1 has also been implicated in their trafficking to the respective
115 organelles [17-19]. This mechanism has been called into question for ROP2 as a
116 more recent study revealed membrane anchoring of this protein occurs through
117 hydrophobic and ionic interactions rather than by a classical TM domain, and
118 thus without the protrusion of a cytoplasmic tail it is unclear how ROP2 could
119 interact with AP1 [20]. Certainly, other modes of trafficking must exist since the
120 presence of a TM domain or tyrosine-based motifs is not essential for the
121 trafficking of other proteins to apical organelles [21-23].
122
123 In the case of *Plasmodium,* Rabs, SNAREs and AP1 have been identified in the
124 genome [24], but the majority of known rhoptry proteins are soluble and thus
125 the mechanism by which rhoptry cargo in Golgi-derived trafficking vesicles
126 engage with the cytoplasmic trafficking machinery is still very much a black box.
127 In fact, only a limited number of studies with a focus on rhoptry protein
128 trafficking have been performed in *Plasmodium*. The proteins for which the most
129 is known are members of the low molecular weight rhoptry associated protein
130 (RAP) complex. Correct targeting of RAP2 to the rhoptries requires an
131 interaction with RAP1 [25], with deletion of RAP1 sequence that includes the
132 RAP2 binding site, leading to the mislocalisation of RAP2 to the endoplasmic
133 reticulum (ER). The same is likely to be the case for RAP3 given RAP2 and RAP3
134 are paralogues. The high molecular weight RhopH complex (reviewed in [9]) is
135 formed via non-covalent interactions; whether their association is required to
136 ensure correct localization to the rhoptries remains to be investigated.
137
138 Interestingly, immunoprecipitation and fluorescence resonance energy transfer
139 experiments have revealed that the RAP complex (and potentially the RhopH
140 complex) interacts with the rhoptry-associated membrane antigen (RAMA) at
141 the Golgi via the N-terminus of RAP1 [26]. RAMA is a glycophosphatidyl inositol
142 (GPI) anchored protein, localizing to the ER and Golgi compartments before
143 rhoptries are visible by TEM [27] and later present in the bulb of the mature
144 rhoptry . It is conserved amongst *Plasmodium*, and its inability to be deleted in *P.*
145 *falciparum* implies it is an essential protein [28, 29]. Taken altogether these
146 findings led Richard *et al.* to propose that RAMA generates clusters of rhoptry-

147 destined cargo within detergent resistant membranes at the Golgi-exit face that
148 ultimately bud off as rhoptry-destined vesicles [26]. Intriguingly, however, no
149 functional equivalent of RAMA has to date been found in other Apicomplexan spp.
150 It should also be noted that because the C-terminus of RAMA is embedded within
151 the lipid bilayer [30], an unidentified TM escorter protein would have to be
152 present in vesicles at the Golgi-exit face, interacting with both RAMA and the
153 cytosolic sorting machinery to escort these protein complexes to nascent
154 rhoptries.
155
156 One candidate protein that may fulfill the role of the TM escorter protein
157 interacting with RAMA is the *Plasmodium* orthologue of the *T. gondii* sortilin-like
158 receptor (TgSORTLR), which is transcribed around the same time as RAMA. A
159 recent study by Sloves *et al.* in *T. gondii* revealed TgSORTLR resides in Golgi-
160 endosomal compartments, and is required for the formation of rhoptries as well
161 as micronemes [31]. The luminal domain of TgSORTLR specifically interacts with
162 rhoptry protein cargo, whilst its cytoplasmic tail interacts with AP adaptins,
163 clathrin and other vacuolar-sorting proteins. Thus, by facilitating the association
164 of a sorting complex with vesicles at the Golgi exit face, TgSORTLR potentially
165 guides cargo through the endocytic pathway before releasing it at the rhoptries
166 (Figure 2). However, as TgSORTLR also interacts with cargo destined for the
167 micronemes, an extra layer of specificity such as temporal separation of rhoptry
168 and microneme protein expression must be involved to ensure proteins are
169 navigated from the Golgi to their correct destination. Indeed it would be
170 interesting to examine whether a rhoptry protein, if expressed later in the cell
171 cycle, would end up in the micronemes or another compartment.
172
173 In addition to a N-terminal signal sequence, rhoptry proteins in both *Plasmodium*
174 and *T. gondii* contain an additional pro-domain that is processed *en route* or
175 upon arrival at the organelle [26, 27, 32-35]. The pre-protein form of RAMA and
176 RAP1, for example, are cleaved to yield a smaller mature protein. In *T. gondii,* the
177 pro-domain plays diverse roles, mediating protein trafficking and assembly, and
178 in the case of the micronemal protein M2AP, it plays a critical role in the stability
179 and secretion of the MIC2-M2AP complex [36]. The pro-domain also potentially

180 plays an additional role in maintaining some rhoptry proteins as zymogens until
181 activation for effector function. However, there is a distinct lack of rhoptry
182 proteins with enzymatic activity in *Plasmodium*. Thus in *Plasmodium* the
183 processing event may assist protein maturation and folding, enable the RAP
184 complex (and other potential associated cargo) to be released from RAMA so
185 they are ready for secretion or be required to release RAMA from its hypothetical
186 TM escorter protein.
187
188 Once at the rhoptries, proteins localise to the rhoptry membrane, neck, bulb or
189 potentially other 'yet to be defined' compartments of the rhoptry [37]. The
190 correct localization has important implications, as neck and bulb proteins are
191 secreted sequentially and consequently have different roles in invasion and post-
192 invasion events as outlined in the sections that follow. TEM studies reveal the
193 trafficking of vesicles from the Golgi to the rhoptry neck as the organelle is
194 undergoing maturation [13], suggesting that the early rhoptry proteins are
195 destined to become bulb proteins, while later proteins traffic to the neck
196 thereafter (Figure 2). RAP1 is retained in the bulb courtesy of a C-terminal motif,
197 with deletion of this sequence leading to its mislocalisation at the rhoptry neck
198 [26] and inability to localize to the PV after invasion. Whether retention motifs
199 are a typical feature of ROPs requires further investigation. For protein
200 trafficking to the rhoptry neck, perhaps Pf34 plays a similar role to RAMA in
201 recruiting neck proteins from within the Golgi, as hypothesized in Figure 2. Pf34
202 is a rhoptry neck protein that is expressed prior to the formation of the organelle
203 and also contains a GPI anchor [38], both features shared with RAMA. However,
204 further work is required to test this theory.
205
206 Confounding the TEM observations, however, is the observation that Rh5
207 localises to the rhoptry bulb in late schizonts [39, 40] prior to its localization at
208 the apical tip of free merozoites [41]. Rh5 subsequently co-localises with Rh5
209 interacting partner (RIPR), which itself appears to originate from micronemes
210 [42]. Thus whether this pathway to the merozoite surface, via the rhoptry bulb,
211 is specific to Rh5, or shared with other Rh proteins is unknown but warrants
212 further investigation.

213

214 For the *P. falciparum* and *T. gondii* membrane-associated Armadillo Repeats-
215 Only (ARO) protein, whose function remains to be elucidated, myristolylation
216 and palmitoylation motifs are required for membrane attachment to the outer
217 face of the rhoptries. Other residues within its first 20 N-terminal amino acids
218 also ensure correct targeting to this membrane (Figure 2) [43]. Interestingly, the
219 only putative palmitoyltransferase (PAT) that has been localized in *P. falciparum*
220 shows Golgi distribution in late schizonts, implicating the Golgi again as the
221 location for selective rhoptry targeting [44]. However, as there are three other
222 PATs expressed at this stage of the parasite life cycle [43, 45], it cannot be ruled
223 out that one of these may be bound at the rhoptry membrane to facilitate specific
224 targeting of the *P. falciparum* ARO protein.

225

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

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

233

234 The *Plasmodium* reticulocyte-binding homologues (Rh), comprising Rh1, Rh2a,
235 Rh2b, Rh4 and Rh5 are adhesins, involved in establishing merozoite invasion of
236 erythrocytes by interacting with host cell receptors. Recently, through the use of
237 'avidity-based extracellular interaction screen' (AVEXIS), the erythrocytic
238 receptor for Rh5 was identified as basigin [47]. This high-throughput approach
239 detects low-affinity protein interactions between bait and prey ectodomains by
240 expressing the prey domains as pentamers, thereby increasing the avidity of any
241 interactions [48]. Importantly, Rh5 is the only adhesin refractory to genetic
242 disruption [40], and its interaction with basigin is required for and essential to
243 merozoite invasion [47]. Indeed, antibodies against Rh5 can significantly inhibit
244 the growth of all the *P. falciparum* laboratory strains tested [49]. Although *ex vivo*
245 studies from field isolates of *P. falciparum* have shown that Rh5 is not an

246 abundant invasion ligand, a positive correlation was observed between the
247 relative proportion of Rh5 expression levels in isolates relative to other
248 Rh/erythrocyte binding antigens ligands examined and its associated
249 parasitemia in patients [50]. If this is reflective of actual protein levels in the
250 rhoptry, then it provides the first molecular correlate of invasion efficiency.
251 Together these data make Rh5 a prime candidate for vaccine trials [51].
252
253 Super resolution microscopy has been another technological advance, which
254 combined with immunoelectron microscopy and the capacity to routinely isolate
255 merozoites [52] has provided unprecedented spatial and temporal resolution of
256 *Plasmodium* proteins before, during and after invasion [53]. In particular, TJ
257 formation has been studied. Localising to the TJ is a complex of secreted RON
258 proteins comprising RON2, 4 and 5, which interact with the micronemal protein
259 AMA1. These studies have revealed that RON4 forms an outer ring encircling the
260 protein rings of AMA1 and a haemaglutinin epitope tagged version of RON2
261 during merozoite invasion [37, 53]. These nested RON4/AMA1 and RON4/RON2-
262 HA rings clearly delineate the invaded, PVM-surrounded anterior of the parasite
263 from its extracellular posterior, consistent with the hypothesis that the TJ is a
264 parasite-derived protein ring that facilitates invasion into host cells (reviewed in
265 [4, 46]). Although RON2 and its micronemal partner AMA1 have not yet been
266 imaged together by super resolution microscopy, their localization within the
267 RON4 ring is in keeping with previous data that indicates a direct interaction
268 with each other. This interaction has been closely mapped by invasion inhibitory
269 peptides and monoclonal antibodies, as well as recombinant expression of RON2
270 domains (reviewed in [4, 54]). Recently, co-crystallisation studies of AMA1 and
271 RON2 peptides in both *T. gondii* [55] and *P. falciparum* [56] have provided the
272 molecular basis for the unusually strong binding between AMA1 and RON2, as
273 well as the structural rationale for how invasion inhibitory peptides, such as R1,
274 and monoclonal antibodies against AMA1 inhibit its interaction with RON2.
275
276 How the interaction between RON2 and AMA1 is prevented prior to invasion is
277 significant. Besteiro *et al.* found that, in addition to segregating RON2 and AMA1
278 into distinct invasion organelles, *T. gondii* also temporally separates the

279 components by expressing them at different developmental stages [57].
280 Specifically, RONs are synthesised and packaged before micronemal proteins,
281 preventing them from interacting within the secretory system. Analogously in *P.*
282 *falciparum*, the interaction between Rh5 and its interacting partner, RIPR, is
283 observed on free merozoite apical tips, but segregated between micronemes and
284 rhoptries in non-segregated schizonts [42]. Further, analysis of the
285 transcriptome data of *P. falciparum* on PlasmoDB also indicates a small but
286 discrete delay between rhoptry neck and micronemal gene transcription, with
287 RON transcripts appearing and peaking in abundance earlier.
288
289 The key interaction between AMA1 and RON2 has supported the hypothesis that
290 invading Apicomplexans provide both the ligand and its cognate receptor for TJ
291 formation [7]. However, a conditional knockdown of AMA1 in *Plasmodium*
292 *berghei* using the flippase recombinase-flippase recognition target FLP-FRT
293 inducible knockdown system found that sporozoite invasion of hepatocytes was
294 not impaired *in vivo* or *in vitro*, although the functional loss of AMA1
295 subsequently prevented merozoite invasion of erythrocytes [58]. By contrast,
296 similar loss of RON4 significantly decreased sporozoite invasion of hepatocytes.
297 These unexpected findings suggest that AMA1 is not universally required for
298 zoite invasion and is not a prerequisite for TJ formation, although it appears to
299 be required for merozoite invasion of erythrocytes.
300
301 In addition to adhesins that initiate invasion after host recognition, Zuccala *et al.*
302 applied a bioinformatics approach to identify rhoptry proteins putatively
303 involved in TJ-mediated invasion, which they have termed 'invasins' [37].
304 Amongst several candidate invasins, this *in silico* approach identified
305 components of the TJ-forming RON complex as well as the Apical Sushi Protein
306 (ASP). ASP is a rhoptry neck protein that may be orthologous to *T. gondii* RON1
307 [59] or *Cryptosporidium parvum* PRP1 [60], although the overall sequence
308 similarity is poor, and only *Plasmodium* ASP is GPI-anchored. Intriguingly, the
309 host cell receptor for Rh4, complement receptor 1 (CR1), also contains tandem
310 sushi domains that may interact with Rh4 to facilitate invasion [61]. However,
311 the function of ASP and its sushi domains remain unknown.

312

313 Several other proteins that localize to the *Plasmodium* rhoptry neck remain to be
314 functionally characterized, including Pf34, AARP and RON6 (Figure 1, reviewed
315 in [46]). Whether these proteins are involved in adhesion or invasion requires
316 further investigation, but at the moment leaves open the possibility that rhoptry
317 neck proteins may engage in even more diverse functions.

318

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

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

332

333 The rhoptry bulb contains two well-characterized protein complexes identified
334 through immunoprecipitation experiments on rhoptry extracts: the RAP and
335 RhopH complexes. Members of these complexes are all highly abundant proteins
336 and are conserved amongst the malaria species, although in *P. falciparum*, the
337 number of ROP genes has expanded due to gene duplication events. For example,
338 RAP2 and RAP3 are paralogues in *P. falciparum*, whereas other *Plasmodium*
339 species harbor only a single RAP2/3 gene. Gene duplication has also occurred in
340 the RhopH complex; RhopH1 is a product of a multigene family of which there
341 are five known variants in *P. falciparum* (clag 2, 3.1, 3.2, 8 and 9) [68, 69].
342 Interestingly, other malaria species have only two RhopH1 genes: a clag9
343 orthologue and a clagx gene that shares similarities with other clag genes.

344

345 Previously, the RAP complex has been implicated in invasion, as antibodies and
346 peptides specific for RAP1 can partially block *in vitro* invasion of RBCs. RAP1 is
347 also immunogenic, as anti-RAP1 can be detected in the sera of naturally infected
348 hosts, although vaccines directed against this protein do not completely protect
349 against challenge infection (reviewed in [9]. This lack of protection is consistent
350 with what we now know about the sequence of invasion, which has revealed that
351 ROPs are actually secreted into the PV/PVM after formation of the TJ [53].
352 Certainly, the immunogenicity of RAP1 can be accounted for by abortive invasive
353 events, whereby attached merozoites release rhoptry contents without invasion
354 of a host cell, or phagocytosis of erythrocytes infected by early stage parasites.
355 The timing of ROP release now also fails to explain the role of these proteins
356 during invasion as suggested by studies that have demonstrated the binding of
357 various bulb proteins such as RhopH3 to the RBC surface. These may reflect
358 potential interactions that would occur between ROPs and RBC proteins
359 transferred to the PVM during invasion, or are the result of non-specific binding
360 of sequences to lipid membranes, which constitute both the RBC surface and the
361 PVM.
362
363 To better understand the function of the ROPs, loss-of-function studies have
364 been performed via gene disruption in *P. falciparum* (Table 1) and *P. berghei*.
365 Disruption of RAP1 was only possible in one particular *P. falciparum* strain that
366 utilizes alternative parasite ligands for invasion [25]. Interestingly, although no
367 *in vitro* phenotype was observed, RAP2 targeting to the rhoptry was disturbed in
368 the RAP1 knockout. In *P. falciparum*, RAP3 is not essential for merozoite invasion,
369 most likely because RAP2 can complement the loss of RAP3 [70]. In *P. berghei*
370 which only has a single RAP2/3 gene, a successful knockout of RAP2/3 has not
371 been achieved [71]. This suggests that the RAP complex may indeed be crucial
372 for parasite survival in an *in vivo* setting.
373
374 For the RhopH complex, which is transferred to the host cell cytoplasm and
375 plasma membrane post-invasion, several functions have been assigned (Figure
376 1) [72-76]. The *P. falciparum* RhopH1/clag9 has been implicated in the
377 cytoadherance of infected RBCs to the microvasculature, preventing clearance

378 through the spleen, factors that contribute to severe malaria [72]. Recently
379 RhopH1 has been associated with establishing new permeation pathways in the
380 RBC; the clag3 variants, which are inserted into the RBC membrane, alter host
381 cell permeability to a diverse range of solutes and thus potentially aid in nutrient
382 acquisition [76]. Additionally, clag3 null mutants display a significant growth
383 defect *in vitro*, with no invasion phenotype observed [77]. The function of clag2
384 and clag8 in *P. falciparum* is unknown, although it is likely they have some
385 degree of functional redundancy with clag3. In keeping with this, the single
386 orthologues of *P. falciparum* clag9 and clag 2/3/8 in *P. berghei* are refractory to
387 gene deletion [78], which implies they have a crucial functional role *in vivo*.
388
389 While these studies have highlighted novel roles for *P. falciparum* ROPs, several
390 questions remain unanswered, especially concerning the timing of secretion and
391 trafficking of RhopH proteins. Although RhopH1 proteins (clag3 and clag 9
392 variants) are implicated in functions downstream of invasion, their expression
393 peaks in schizonts. Another RBC-membrane interacting protein (ring-infected
394 erythrocyte surface antigen; RESA) that is also maximally expressed in schizonts,
395 is instead found in the dense granules. By contrast, the knob-associated histidine
396 rich protein (KAHRP), which is also implicated in cytoadherence, demonstrates a
397 peak level of expression after invasion during ring stages. Whilst RESA and
398 KAHRP are most likely translocated across the PVM by a multimeric protein
399 machinery termed the *Plasmodium* translocon for exported proteins (PTEX) [79,
400 80], PTEX is unlikely to transport RhopH as a complex. As the RhopH complex
401 initially localizes to the PVM after invasion, and antibodies to RhopH2 later label
402 the Maurer's Clefts [73], it remains to be examined whether the RhopH complex
403 is exported into the RBC via initial cleft formation or via another mechanism.
404
405 **Concluding remarks**
406 The biological functions of many *Plasmodium* rhoptry proteins remain largely
407 unknown due to the lack of appropriate and/or robust methodologies to study
408 products of genes essential to parasite survival within RBCs [81]. The ordered
409 and sequential release of proteins from the three apical organelles was thought
410 to reflect the function of each organelle's contents; micronemes secreted

411 proteins required for invasion and the establishment of the TJ, rhoptry proteins
412 were implicated in PV and PVM formation, and dense granules proteins were
413 required to modify the host cell after invasion had been completed. However,
414 this model is now under challenge, as Rh5 is suggested to be part of the
415 attachment process, RON2/RON4 are implicated in TJ formation with the
416 micronemal protein AMA1, and ROPs are implicated downstream of PVM
417 formation, in processes that include altering host cell permeability to enhance
418 nutrient acquisition.
419

420 Table 1. Gene deletion studies in *P. falciparum*.

Gene	Findings	Ref
RAMA	Refractory to deletion	[29]
RAP1	Gene disruption, no *in vitro* phenotype in D10 parasite line	[25]
RAP3	Gene disruption, no *in vitro* phenotype	[70]
RhopH1/Clag3	Reduced *in vitro* growth	[77]
RhopH3	Refractory to deletion	[82]
RALP	Refractory to deletion	[10]

421

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656 **Box 1. Sorting of protein cargo in eukaryotes**

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

684

685 **Box 2. Poorly characterised bulb proteins**

686 Several proteins are thought to localize to the rhoptry bulb but require further
687 characterization to confirm this positioning. Repetitive organellar protein
688 (ROPE) has characteristics similar to mammalian spectrin and is thought to

689 function in invasion by interacting with the RBC cytoskeleton [84]. Several
690 *Plasmodium* spp. encode an integral stomatin-like protein (band 7-related
691 protein) [85]. This stomatin orthologue, which partially co-localises with
692 RhopH2, is delivered to the cytoplasmic face of the PVM and is proposed to play a
693 role in PVM formation. Rhoptry-associated leucine-zipper like protein (RALP1)
694 contains four heptad repeats of leucines, resembling a leucine zipper [10]. RALP1
695 colocalises with RAP1 in merozoites, but as it is not observed in newly invaded
696 cells, its function is unknown.
697
698

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.

732
733 Parasitophorous vacuole (PV): vacuole found in infected host cells where
734 most Apicomplexan parasites reside and develop.
735 Tight junction (TJ): a dynamic junction formed between the apical end of the
736 invading merozoite and the host erythrocyte and hence is often also referred
737 to as the moving junction. The *Plasmodium* merozoite TJ is predicted to be
738 composed of the RON complex (RON2, RON4, RON5) and the micronemal
739 protein AMA1.
740
741 RAP complex: a low molecular weight protein complex localized to the
742 rhoptry bulb, comprised of heterodimers of rhoptry associated protein 1
743 (RAP1) and RAP2, or RAP1 and RAP3.
744
745 RhopH complex: a high molecular weight protein complex comprising
746 RhopH1, RhopH2 and RhopH3.
747
748 Rhoptry-associated membrane antigen (RAMA): an essential and abundant
749 glycophosphatidyl inositol (GPI)-anchored protein that accumulates in the ER
750 and Golgi compartments in lipid rafts prior to rhoptry development,
751 implicated in rhoptry biogenesis.
752
753 R1 inhibitory peptide: a 20-residue peptide that is able to inhibit invasion of
754 *P. falciparum* merozoites by binding to AMA1 and preventing TJ formation.
755
756 Sushi domain: cysteine-rich domains that typically regulate complement
757 ligands, also known as short complement repeats (SCRs). These domains are
758 found in the rhoptry neck protein ASP, as well as CR1, the erythrocyte
759 receptor for Rh4.
760
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763

764 **Figure 1. Rhoptry neck and bulb proteins during and after invasion**
765 RONs predominantly localise to the merozoite surface adhesion site or tight
766 junction during attachment and invasion, although the function and final
767 localisation of several RONs remain unknown. ROP proteins are secreted after
768 the parasite has fully entered the erythrocyte, and may be involved in forming
769 the PVM. Some ROPs have been detected at the Maurer's clefts (MC) and even the
770 erythrocyte plasma membrane (ePM) after invasion. Again, the final localisation
771 of some ROPs remain unknown. A, adhesion site; D, dense granule; M,
772 microneme; R, rhoptry; N, nucleus; TJ, tight junction; nPVM, nascent
773 parasitophorous vacuole membrane; PVM, parasitophorous vacuole membrane;
774 MC, Maurer's clefts; ePM, erythrocyte plasma membrane.
775
776
777 **Figure 2. Model of protein sorting to the rhoptries in *Plasmodium*.**
778 Proteins destined for the rhoptries are co-translationally inserted through Sec61
779 at the endoplasmic reticulum (ER) via their signal sequence and trafficked to the
780 Golgi in COPII-coated vesicles. At the Golgi exit face, the GPI-anchored protein
781 RAMA aggregates with other rhoptry bulb cargo such as the RAP complex within
782 detergent resistant membranes, and these regions ultimately bud off as rhoptry-
783 destined vesicles (CCV; clathrin coat vesicles). The Sortilin-like receptor
784 (SORTLR) potentially acts as a rhoptry escorter, facilitating an interaction with
785 adapter proteins (AP) and the cytosolic trafficking machinery. The CCVs fuse at
786 the target membrane to release cargo into nascent rhoptries. Here, proteolytic
787 processing of the rhoptry cargo facilitates their release from the trafficking
788 complex, with a bulb-retention motif in the RAP complex preventing dispersal
789 throughout the entire rhoptry as it matures. The Armadillo Repeats-Only (ARO)
790 protein is targeted to the rhoptry membrane; whether this is predetermined at
791 the Golgi or once it has trafficked at the rhoptry remains to be elucidated. The
792 GPI-anchored rhoptry neck protein Pf34 may perform a similar role to RAMA.
793 Whilst DrpB is also involved in rhoptry biogenesis, its localization and function
794 in rhoptry biogenesis and trafficking is yet to be revealed.
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797