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1 **Unraveling the elusive rhoptry exocytic mechanism of Apicomplexa**

2

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4

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14

15 **Abstract**

16 Apicomplexan parasites are unicellular eukaryotes that invade the cells in which they proliferate.

17 The development of genetic tools in *Toxoplasma*, and then in *Plasmodium*, in the 1990s allowed

18 the first description of the molecular machinery used for motility and invasion, revealing a crucial

19 role for two different secretory organelles, micronemes and rhoptries. Rhoptry proteins are injected

20 directly into the host cytoplasm not only to prompt invasion, but also to manipulate host functions.

21 Nonetheless, the injection machinery has remained mysterious, a major conundrum in the field.

22 Here we review recent progress in uncovering structural components and proteins implicated in

23 rhoptry exocytosis, and explain how revisiting early findings and considering the evolutionary

24 origins of Apicomplexa contributed to some of these discoveries.

25

## 26 **The rhoptry, a compelling weapon at the heart of Apicomplexa pathogenesis**

27 The phylum **Apicomplexa** (see **Glossary**) comprises more than 5000 unicellular eukaryotes and  
28 includes some of the most important pathogenic parasites of man and animals. The deadliest is the  
29 malaria parasite *Plasmodium falciparum*, responsible for almost half a million human deaths per  
30 year in many tropical and subtropical countries [1]. Human pathogens also include *Toxoplasma*  
31 *gondii*, a prominent cause of human congenital infections [2], and *Cryptosporidium*, one of several  
32 important pathogens responsible for severe diarrhea in infants [3]. These human parasites are  
33 obligatory intracellular pathogens that have developed unique active invasion mechanisms.  
34 Invasion induces the formation of a parasitophorous vacuole (PV), which often offers a safe niche  
35 in which the parasite replicates until the progeny eventually exit to invade a new host. During this  
36 sequence of steps the parasite sequentially discharges the contents of three distinct secretory  
37 organelles unique to Apicomplexa, called micronemes, rhoptries and dense granules [4].  
38 Micronemes and rhoptries are part of an apical complex found in all Apicomplexa; this reflects  
39 evolutionary innovations in the common ancestor of these parasites. Microneme **secretion** starts  
40 while the parasite is still a resident of the PV, just prior to egress (see review [5]), while **rhoptry**  
41 secretion occurs after the parasite has emerged and likely when it comes into contact with what  
42 will become its next host cell. Factors secreted by micronemes are, among other functions  
43 (reviewed in [6]), required to trigger downstream rhoptry secretion [7,8], which results in the  
44 injection of rhoptry contents directly into the host cytoplasm (**Figure 1**). Rhoptry proteins are  
45 targeted to several locations inside the host [6]. A subset are restricted to the invasion site where  
46 they promote host-cell anchoring and establish the machinery that propels the parasite into the host  
47 cytoplasm [9–11]. Other rhoptry proteins clearly play post-invasion roles. Proteins associated with  
48 the PV membrane in *Plasmodium* contribute to building the vacuole [12]; for *Toxoplasma*, injected  
49 factors promote virulence by facilitating evasion of immune factors (for compelling reviews see  
50 [13–15]). Other known functions include remodeling the host's actin cytoskeleton to favor parasite  
51 invasion [16] and transmigration [17], and transcriptional regulation by nucleus-targeted factors  
52 to subvert host immune responses [18,19]. Finally, in *Plasmodium* some injected proteins are  
53 redistributed to host cell membranes where they drive nutrient uptake [20]. Thus, rhoptries are  
54 exceptionally important for both establishing and controlling the infection.

55 Rhoptries are present in most stages of the apicomplexan life cycle, though their abundance  
56 varies between species and life stages. *Cryptosporidium* spp. have a single rhoptry [21], *P.*

57 *falciparum* merozoites display two [9,22], whereas the coccidian subgroup of Apicomplexa  
58 (*Besnoitia*, *Eimeria*, *Neospora*, *Sarcocystis*, and *Toxoplasma*) have multiple rhoptries – a dozen in  
59 *T. gondii* [23]. Located in the anterior half of the parasite, rhoptries are fusiform organelles that  
60 display distinct structural polarity with a bulbous body connected to an elongated neck that is  
61 oriented towards the parasite's apex. Rhoptries are assembled *de novo* during cell replication,  
62 specifically when the daughter parasites develop within the mother [24]. They arise as immature  
63 compartments called pre-rhoptries, which are recognizable by electron-microscopy as spherical  
64 organelles located in nascent parasites [25]. Pre-rhoptries elongate just prior to cytokinesis to form  
65 mature rhoptries, which subsequently migrate to the apexes of daughter cells [26]. Recent findings  
66 have highlighted how apicomplexans parasites repurposed their endosomal system for the  
67 biogenesis of apical organelles, including rhoptries [27]. For more exhaustive reviews on the  
68 biogenesis of secretory organelles in *Toxoplasma*, and on factors important for their organization  
69 and apical positioning, see [26,28–30].

70 Rhoptry secretion does not result in extracellular release of rhoptry contents as in  
71 conventional **exocytosis**; instead, the contents are transferred across the host cell membrane  
72 (export). The coupling of 'regulated exocytosis' of secretory granules with 'injection of their  
73 contents into a second cell is unique in cell biology, and it has been named 'Kiss and Spit' [15].  
74 The underlying mechanisms remain largely unresolved, in stark contrast to our detailed  
75 understanding of rhoptry formation and the functions of rhoptry effectors.

76 In this review we focus mainly on rhoptry exocytosis, a poorly-studied stage of rhoptry  
77 biology, with a particular emphasis on the apicomplexan model *T. gondii*. While rhoptry research  
78 remains challenging, we discuss recent approaches combining decades-old pioneering  
79 ultrastructural observations in Apicomplexa with data on regulated secretion from the sister  
80 lineage **Ciliata**. Together these have advanced our understanding of rhoptry exocytosis.

81

## 82 **The elusive mechanism of rhoptry secretion**

83 The mechanisms underlying rhoptry secretion have puzzled scientists for decades. The process has  
84 been challenging to study, in part due to the absence of known inducers of rhoptry exocytosis. By  
85 contrast, the availability of such compounds for micronemes greatly facilitated biochemical  
86 approaches and accelerated the characterization of microneme exocytosis [31–33]. In addition, the  
87 reliance of rhoptry secretion on host cell attachment and prior microneme release [4] has presented

88 severe challenges to designing genetic screens to detect rhoptry-specific defects, since the  
89 sequential secretory events are tightly linked.

90

### 91 *Ultrastructure of rhoptry secretion*

92 Our understanding of rhoptry secretion has relied mainly on ultrastructural observations from  
93 Electron Microscopy (EM) studies in the 1980s. Rhoptry exocytosis cannot be understood as a  
94 'full-fusion' phenomenon since following discharge, the organelle's membrane remains at the apex  
95 as an empty saccule (**Figure 1**). The saccules become more ovoid than elongated and sometimes  
96 appear bifurcated in both *Toxoplasma* and *Plasmodium*, suggesting that rhoptries have undergone  
97 fusion with one another as well as with the parasite plasma membrane (PPM), so-called compound  
98 exocytosis [9,22,34]. Another insight from ultrastructure was the occurrence of clusters of smooth  
99 irregular vesicles in the cytoplasm of the host cell around the advancing tip of the parasite [34,35].  
100 Although a more comprehensive analysis of such vesicles is definitely required, they are imagined  
101 to partially derive from rhoptry contents – by a process yet to be determined – rapidly fusing with  
102 the host cell's plasma membrane (PM) during the invasion process and eventually becoming  
103 incorporated into the parasitophorous vacuole membrane (PVM) [34,35].

104 In some images of *Toxoplasma* tachyzoites or *Plasmodium* merozoites invasion, the  
105 rhoptry membrane appears to be fusing with the parasite membrane at a site lined up with an  
106 opening to the host cell, although the limited resolution precludes ascertaining whether a channel  
107 links the rhoptry lumen and host cytosol [22,34,36]. Intriguingly, until the end of invasion the apex  
108 of the parasite remains in close contact with the host cell's membrane (or newly formed PV  
109 membrane), with a connection formed by undefined material that appears continuous with the rim  
110 of the rhoptry [22,36,37]. This connection might represent the path by which the rhoptry content  
111 is transferred across the host cell membrane. The same structure might be responsible for a feature  
112 of **freeze-fracture** images of the host membrane at the *Toxoplasma*-contact site, namely a small  
113 depression resembling a 40 nm membrane pore [26] (**Figure 1B**). This depression is discernible  
114 after secretion occurs, and it is not clear whether it reflects an open pore that channels the rhoptry  
115 discharge during the entire invasion, or instead corresponds to the scar left after the formation of  
116 a transient pore. Independent support for such a pore came from electrophysiology, which  
117 consistently detected a transient spike in host cell membrane conductance when the apical end of  
118 the *T. gondii* parasite attaches to the host cell [38]. This spike was interpreted as the opening of a

119 pore towards the host cell cytoplasm, allowing discharge of rhoptry contents into the host cell.  
120 However, the existence of a pore for rhoptry export has never been verified.

121

### 122 ***Signaling for rhoptry secretion***

123 The molecular mechanisms responsible for the tight coupling of microneme and rhoptry release  
124 are unknown. One idea is that a signaling cascade follows the initial contact of the parasite with  
125 the host, generated by the interaction of microneme proteins (MICs) with host cell receptors. In *P.*  
126 *falciparum* increased intracellular  $Ca^{2+}$  stimulates translocation to the PPM of the transmembrane  
127 microneme protein EBA175, which can then bind to glycophorin A (GPA) on red blood cells  
128 (RBCs) [8]. This restores the cytosolic  $Ca^{2+}$  to basal levels, and also promotes the release of rhoptry  
129 proteins [8]. MIC secretion is also important for triggering rhoptry discharge in *T. gondii*, where  
130 the relevant microneme factors include transmembrane protein AMA1 [39], MIC8 [7], possibly  
131 CLAMP [40], and MIC7 [41]. The roles of MICs in this process is not limited to extracellular  
132 binding, since in *P. falciparum*, the cytoplasmic tail of PfEBA175 was found to be required for a  
133 GPA-dependent host-cell invasion pathway [42]. Similarly, the cytosolic domain of *Toxoplasma*  
134 MIC8 is essential for its function in rhoptry secretion [7]. These studies support a model in which  
135 interactions between MICs and host cell receptors result in a signal transduction cascade generated  
136 by the cytosolic tails of MICs, leading to rhoptry release. The mechanistic details of such signaling  
137 pathways remain unexplored.

138

### 139 **Rhoptry Exocytosis: lessons from old studies in Ciliata**

140 Members of the Apicomplexa are distantly related to Opisthokonts (including mammals, yeast,  
141 flies) and Archaeplastida (plants, algae), where most cell biological studies have been pursued.  
142 Thus, many eukaryotic models (*Saccharomyces cerevisiae*, *Drosophila melanogaster*,  
143 *Caenorhabditis elegans*, and *Arabidopsis thaliana*, to name a few) offer limited guidance to  
144 deciphering rhoptry secretion machinery. An intriguing example of such limitations is the role of  
145 SNAREs, which are universal mediators of membrane fusion across eukaryotes. SNAREs are  
146 found in Apicomplexa, but not associated with rhoptry exocytosis [43,44]. One possibility is that  
147 rhoptry-related SNAREs exist but have diverged in sequence so profoundly that they are no longer  
148 recognizable as homologs of the animal or plant proteins. However, more closely related  
149 eukaryotes may offer better models for Apicomplexa. In order to dissect the mechanisms

150 underlying rhoptry secretion, Aquilini et al. [45] took advantage of studies on regulated secretion  
151 in organisms phylogenetically closer to Apicomplexa, the sister lineage Ciliata. In particular, they  
152 used decades-old pioneering ultrastructural observations in Apicomplexa and Ciliata, showing  
153 striking structural analogies at the exocytic sites of their respective secretory organelles (see  
154 description in the following text) [46–49]. Based on this ultrastructural similarity, they  
155 hypothesized that genes important for exocytosis in the Ciliata might provide important clues for  
156 rhoptry exocytosis in *Toxoplasma* and *P. falciparum*.

157 Apicomplexa belong to the **Alveolata** branch of the Stramenopiles–Alveolata–Rhizaria  
158 (SAR) supergroup, together with protists in the phyla Ciliata and **Dinoflagellata** (See glossary).  
159 Alveolates are all unicellular eukaryotes whose name derives from a shared element, a membrane-  
160 bound organelle underlying the PM that is named the 'alveolus' in Ciliata and 'inner membrane  
161 complex (IMC)' in Apicomplexa [50]. Alveolates are also characterized by the presence of  
162 regulated secretory organelles often showing elaborate morphologies, collectively called  
163 extrusomes [51]. **Box 1** highlights the common evolution of ciliate and apicomplexan secretory  
164 organelles.

165 Ciliate extrusomes undergo stimulus-dependent exocytosis [51], a pathway primarily  
166 investigated in *Tetrahymena thermophila* and *Paramecium tetraurelia*, two ciliate models that  
167 have contributed to groundbreaking discoveries in molecular and cell biology [52]. *Tetrahymena*  
168 and *Paramecium* evolved homologous secretory organelles called **mucocysts** and **trichocysts**,  
169 respectively [53,54]. Their function in *Tetrahymena* is poorly understood, but in *Paramecium*  
170 trichocyst secretion appears to be a mechanism of predator evasion. The vast majority of mature  
171 trichocysts or mucocysts are docked at specific sites of the cortex, and thus, heavily concentrated  
172 in the cell periphery (**Figure 2**). Upon sensing an external stimulus, they fuse with the PM and  
173 release their content at incredible speed (<1ms) (for a review see [53]). As observed by freeze-  
174 fracture EM, the docking site in *Paramecium* appears as a 300 nm double-ring of around 80  
175 **intramembranous particles (IMPs)**, surrounding a rosette of 8 IMPs located in the center of the  
176 ring [47,48,55] (**Figure 2**). The ring represents the site of docking of newly synthesized  
177 trichocysts. Their docking induces the formation of the rosette and the accumulation of an electron-  
178 dense fibrous material known as 'connecting material', which links the trichocyst membrane to the  
179 rosette (for a review on the subject see [53]). These structures, and the steps associated with  
180 trichocyst–rosette assembly and fusion, are described in **Figure 2**.

181 Trichocysts and mucocysts are dispensable for cell growth under laboratory conditions,  
182 facilitating studies of mutants in their formation or exocytosis. It was also useful that global  
183 synchronous trichocyst or mucocyst discharge could be induced by commercially available  
184 secretagogues [53,56]. These features made such organelles an attractive platform for studying  
185 regulated exocytosis in eukaryotes, and indeed, in the 1970s and 1980s, facilitated screening in  
186 ciliates for exocytosis-defective mutants following chemical mutagenesis (for details on the  
187 different phenotypes of *P. tetraurelia* mutants see [57]). Some mutants were specifically deficient  
188 in the last step(s) of membrane fusion, and were called *nd* mutants (for non-discharged) in  
189 *Paramecium* [58–60]. They lack rosettes and connecting material, supporting the importance of  
190 these two structures in ciliates exocytosis. The *nd* gene products were predicted to be either integral  
191 components of the two structures or regulators of their assembly. However, these ideas were left  
192 untested even as more tools for molecular analysis became available in ciliates, and in total only a  
193 small number of genes were characterized [61–63]. For details of the *nd* genes characterized in  
194 *Paramecium* see **Box 2**.

195 During the same period, a similar rosette in the plasma membrane was discovered at the  
196 very apical tip of several apicomplexans (**Figure 1A**). It was observed specifically on extracellular  
197 *Toxoplasma* [49], *Eimeria* [46], *Sarcocystis* [64], and on *Plasmodium* sporozoites within the  
198 salivary gland [65,66], and intracellular merozoites [67]. Thus, rosettes are present in two  
199 Alveolate branches, suggesting an ancestral and persistent association with exocytosis in this  
200 lineage, but not (as far as we are aware) in any other group of organisms. Consistent with this, the  
201 *Paramecium nd6* and *nd9* genes [62,63] – shown to be essential for rosette assembly and  
202 exocytosis – are conserved across Apicomplexa, and clearly restricted to Alveolates [45]. *T. gondii*  
203 and *P. falciparum nd6* and *nd9* homologs are essential for rhoptry secretion and invasion;  
204 moreover, *nd9* is required for rosette assembly in *Toxoplasma*, substantiating that the rosette has  
205 a key role also in rhoptry secretion [45]. In *Toxoplasma*, TgNd9 is part of a complex including  
206 TgNd6, and two other Alveolata-specific proteins named TgNdP1 and TgNdP2 (for Nd partners),  
207 which are equally important for rosette assembly and rhoptry exocytosis in *Toxoplasma* [45]. This  
208 same analysis demonstrated that *Tetrahymena* homologs of these *ndp* genes are essential for  
209 mucocyst secretion [45]. Other factors identified by proteomic analysis include Ferlin 2 (TgFer2),  
210 a rhoptry secretion factor identified in an independent study [68], which can therefore be  
211 considered part of this complex and implicated in exocytosis. TgFer2 was shown to be associated



212 with the cytoplasmic side of both the IMC and rhoptries in intra- and extracellular tachyzoites,  
213 respectively [68], suggesting that it may play additional roles during rhoptry secretion. Altogether,  
214 these findings argue that the apicomplexan elements underlying rosette formation are shared with  
215 the sister-lineage Ciliata, and in both they play critical roles in exocytosis.

216

### 217 **Reconsidering the frame of rhoptry exocytosis**

218 Rhoptries are membrane-bound organelles. In parasites like *Toxoplasma* with multiple copies, up  
219 to four rhoptry necks may reach the inside of the conoid [23], a hollow cone-shaped cytoskeletal  
220 structure in coccidians [69]. Among those inside the conoid, only one or two rhoptries appear to  
221 be secreted. Pioneering EM studies suggested a connection between those rhoptries with a ~50nm  
222 spherical element positioned at the apex of the parasite – initially known as the apical vesicle (AV)  
223 [64], (**Figure 1C,D**) – and more recently named as porosome-like structure [23]. The possibility  
224 that the AV might be connected to both rosette and rhoptry exocytosis was previously speculated  
225 [23,49]. However, how the rhoptries were linked to the AV and related to the rosette was unclear  
226 in these earlier images because the freeze-fracture technique – used to image the rosette on the  
227 plasma membrane – precludes the simultaneous visualization of the rosette and the internal  
228 elements of the secretion apparatus (including AV and rhoptries). These questions were recently  
229 revisited and resolved by Cryo-Electron tomography (cryo-ET) and 3D reconstitution of *T. gondii*  
230 tachyzoite apices [45]. Remarkably, this analysis showed that the AV is connected on one side to  
231 the tip of the rhoptries and on the other side to the rosette. By cryo-ET the rosette displays the  
232 distinct eight fold rotational symmetry around a central density which extends under the PPM and  
233 connects to the AV, suggesting that the IMPs of the rosette are part of a more complex secretory  
234 ensemble than previously understood [45], whose molecular players and detailed organization  
235 remain to be explored.

236 These new data prompt a rethink about the mechanism of secretion of rhoptries in  
237 *Toxoplasma*. Rhoptry discharge has been broadly seen as a two-interconnected-steps process: (i)  
238 fusion of the rhoptry tip with the PPM and (ii) crossing of the host PM. Based on our new  
239 understanding of the structure of the *Toxoplasma* secretion apparatus, two fusion events likely  
240 exist: one between rhoptry/ies and the AV, and the other between the AV and the PPM. It is not  
241 yet clear if the AV is present at the apex of all tachyzoites, and in all Apicomplexa. It has been  
242 observed in other coccidian parasites [46,70,71] but not yet documented in *Cryptosporidium*

243 parasites or *Plasmodium* spp.; not even in the most comprehensive ultrastructural study of rhoptry  
244 biology of *P. falciparum* [22]. This study described direct fusion between rhoptries and the PPM,  
245 a scenario initially postulated also for *Toxoplasma* [34]. Fusion events are quite challenging to  
246 capture; thus, the step in which the AV membrane fuses with one of the underlying rhoptry during  
247 invasion might be difficult to catch, the AV – becoming contiguous with the rhoptry neck – would  
248 be no longer recognizable. Moreover, the question of whether the two fusion events are  
249 simultaneous or sequentially ordered is open, and deserves further investigation. Although the  
250 exact function of the Nd factors is unknown, the association of TgNd6 with AV, their protein  
251 domains, as well as the predicted cellular structures associated with the ciliate counterparts  
252 (described in **Box 2**), support a model in which these proteins form part of the connecting material,  
253 interact to stabilize the IMPs of the rosette, and contribute to the fusion of the AV (or  
254 trichocyst/mucocyst in *Paramecium/Tetrahymena*) with the PM (**Box 3** and **Figure 3, Key**  
255 **Figure**). In this model, the fusion of rhoptries with the AV would involve apicomplexan-specific  
256 proteins such as the recently discovered rhoptry protein RASP2 (Rhoptry Apical Surface Protein  
257 2) which accumulates on the cytoplasmic face of rhoptry apices. RASP2 was identified in a  
258 bioinformatic search for new rhoptry proteins and proteins related to rhoptry biogenesis and  
259 secretion [72]. It is specifically required for rhoptry secretion in both *Toxoplasma* and  
260 *Plasmodium*, while its interacting partners RASP1 and RASP3, are dispensable. In a parallel study,  
261 *P. falciparum* RASP2, named *P. falciparum* Cytosolically Exposed Rhoptry Leaflet Interacting  
262 protein 1 (PfCERLI1), was also shown to be essential for rhoptry secretion and invasion, although  
263 characterized as a rhoptry-bulb protein [73]. Its homolog, PfCERLI2, appears to be required for  
264 rhoptry protein processing and the formation of morphologically wildtype rhoptries, and is  
265 consequently important for parasite invasion [74]. In *Toxoplasma*, degenerate Ca<sup>2+</sup> lipid-binding-  
266 like (C2) and Pleckstrin Homology-like (PH) domains in TgRASP2 bind specifically to  
267 phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>), and this is crucial  
268 for rhoptry secretion [72]. Interestingly, exocytosis of dense-core vesicles in mammals also  
269 depends on interactions between effectors containing C2 and PH domains with PA and PI(4,5)P<sub>2</sub>  
270 [75,76]. This analogy led to the proposal that RASP2 is involved in the docking and/or fusion of  
271 the rhoptries to the PPM upon host-parasite contact. With the new knowledge that rhoptries  
272 directly contact the AV [45], RASP2 might rather function at the rhoptry-AV interface. Further  
273 work is required to support this model, particularly because of conflicting results regarding the

274 locations and functions of PfCERLI proteins [73,74]. The C2 domains in *Toxoplasma* and  
275 *Plasmodium* RASP2 are degenerated, and TgRASP2 do not bind Ca<sup>2+</sup> [72] excluding a Ca<sup>2+</sup>-  
276 dependent role in rhoptry secretion for these proteins. Nevertheless, Ca<sup>2+</sup> may be important for the  
277 fusion of AV with the PPM given the presence of C2 domains in both Fer2 and NdP2; however, it  
278 has not yet been established if these C2 domains are functional [45,68].

279 Another protein recently associated with rhoptry secretion in *Toxoplasma* is TgPL3, a  
280 patatin-like phospholipase [77]. TgPL3 contains a phospholipase A2 (PLA2) domain whose  
281 activity is essential for rhoptry secretion, and also a microtubule-binding domain that might  
282 determine its localization at the apical cap of the parasite, just beneath the conoid, which becomes  
283 even more pronounced in extracellular parasites. Interestingly, 4-bromophenacyl bromide (4-  
284 BPB), an inhibitor of phospholipase A2 blocks rhoptry secretion in *Toxoplasma* [78], but further  
285 investigation is required to understand if TgPL3 is the target of 4-BPB.

286

### 287 **An Alveolata-conserved fusion machinery with lineage specificities**

288 The identification of functionally conserved homologous genes involved in regulated exocytosis  
289 across Alveolata strongly supports the existence of a common secretory machinery in these protists  
290 in which individual lineages diverged hundreds of millions of years ago to sustain radically  
291 different lifestyles. Currently, homologous machinery provides a defensive mechanism for free-  
292 living Ciliata and supports host-cell invasion and intracellular replication for parasites. Although  
293 many of the molecular 'gears' regulating secretion are similar, there are also lineage-restricted  
294 features specific to exocytosis in ciliates versus apicomplexan parasites.

295 Some of the *Paramecium nd* genes (**Box 2**), predicted to be associated with the trichocyst  
296 compartment, are not found in Apicomplexa parasites. Indeed, some like *Ptnd7* do not have clear  
297 homologs even in other ciliates, highlighting the existence of species-specific adaptations in the  
298 assembly of the secretion apparatus. Conversely, there are factors associated with TgNd9 and  
299 TgNdP1 that are found only in apicomplexans, or restricted to *Toxoplasma*, which may represent  
300 adaptations specific to parasitism [45]. Lineage-specific proteins must contribute to the distinctive  
301 differences in shape and composition of alveolate extrusomes, as well as to differences in the  
302 distinctive docking arrangements, which – in apicomplexans but not ciliates – involves an  
303 intermediary vesicle between the secretory organelle and the PPM. Diversification in exocytic  
304 mechanisms among Alveolata is likely to extend to the triggers and signaling pathways for

305 inducing exocytosis. In Ciliata, exocytosis can be stimulated by encounters with predators, or  
306 chemically, while rhoptry discharge depends on parasite attachment to its host and microneme  
307 release (**Figure 3**). The requirement for upstream MICs/host receptor interactions is obviously  
308 specific to Apicomplexa parasites but nonetheless differs between species and even between stages  
309 that invade different cell types. For example, MIC8 is specific to coccidian parasites [7], and  
310 EBA175 is present in *P. falciparum* merozoites but absent at the sporozoite stage, where the  
311 signaling cascade may instead require other MICs like 6-cys proteins, and host factors such as  
312 CD81 and SRB-1 [79].

313 In *Paramecium*, the rosette only appears upon docking of the secretory organelles, while  
314 in Apicomplexa the rosette and the AV are present even in the absence of docked rhoptries [45].  
315 An attractive idea is that the apicomplexan rosette forms upon docking of the AV, but this has  
316 been difficult to test in the absence of known AV-defective mutants. Interestingly, the AV is  
317 already present in daughter tachyzoites that have not developed their PPM yet (**Figure 1C**). Since  
318 they acquired their PPM from the mother just prior to cytokinesis (**Figure 1D**), one can argue that  
319 rosette formation occurs very late during parasite division. Intriguingly, the rosette is not always  
320 observed in extracellular *Toxoplasma* by free-fracture and when it is spotted it is rarely made of  
321 all eight particles [49]. Aquilini et al. confirmed that the rosette is visible only in a fraction (~60%)  
322 of the tachyzoites analyzed [45]. This observation might reflect technical limitations of the freeze-  
323 fracture, for example, the challenge of identifying the rosette among the background of IMPs  
324 covering the parasite apex, or the fact that the rosette can be associated with either the protoplasmic  
325 P-phase or exoplasmic E-phase of the membrane. Thus, when the partition is random between the  
326 eight particles, the circular symmetry may disappear and the structure may no longer be  
327 recognized. Alternatively, the rosette-null parasites may correspond to parasites that have already  
328 discharged their rhoptries.

329 Curiously, *Toxoplasma* has not only multiple rhoptries at the apical end but also several  
330 round vesicles aligned along a pair of microtubules within the conoid (**Figure 1D, Figure 3**)  
331 [23,49,80]. Thus, it is quite tempting to think that these vesicles represent a reservoir of AVs.  
332 Following rhoptry discharge, the docking of a new vesicle would stimulate re-formation of rosette  
333 and anchoring of a new rhoptry. The rebuilding of the secretion apparatus would ensure successive  
334 secretion of rhoptries by the same parasite before committing to invasion. This process would

335 permit the hijacking of cells that the parasite does not invade in order to limit host responses  
336 [81,82].

337 The AV is found in coccidians [23,46,49,70,71] but not yet confirmed in other  
338 Apicomplexa, as previously mentioned. However, a similar structure was also described in  
339 organisms considered very basal to Apicomplexa, such as *Perkinus marinus* [83] and *Colpodella*  
340 *vorax* [84]. *Perkinsus* is part of a group of intracellular parasites belonging to the taxon  
341 Perkinsozoa, a sister lineage to free-living dinoflagellates [85]. Like apicomplexans, *P. marinus*  
342 deploys rhoptries when invading mollusk cells. In contrast, *Colpodella* spp. are free-living  
343 organisms that, together with Chromerida, represent the nearest sister lineages to the obligate  
344 apicomplexan parasites [86,87]. Their phylogenetic position suggests that they reflect early  
345 adaptations to parasitism. The free-living predator *C. vorax* attaches via its apical end to its prey.  
346 Subsequently, in a process called myzocytosis or cellular vampirism, the cytoplasm of the prey is  
347 withdrawn and digested [84]. It is tempting to speculate that such a combination of free-living and  
348 parasite-like attributes was characteristic of ancestral Apicomplexa. The apparent lack of an AV  
349 in Ciliata – although the existence of such element cannot be conclusively excluded in under-  
350 represented ciliate models – suggests that this structure might be important for systems that  
351 evolved to coordinate secretion with the transfer of material between cells, namely the host cell  
352 cytoplasm for parasites or prey cytoplasm for free-living predators.

353

### 354 **Concluding remarks**

355 The past 3 years have seen spectacular progress in our understanding of rhoptry secretion in the  
356 apicomplexan model *Toxoplasma* and, to some extent, in *P. falciparum*, at both molecular and  
357 structural levels [45,68,72–74,77]. These recent studies strongly suggest that *Toxoplasma* rhoptries  
358 do not directly contact the PPM but instead do so via an AV tightly connected to a rosette of eight  
359 IMPs embedded in the PPM. The implication is that expulsion of rhoptry content requires two  
360 fusion events in *Toxoplasma*: rhoptries to the AV, and the AV to the PPM. Factors involved in  
361 these two stages have been identified and partially characterized, offering a unique opportunity to  
362 further investigate the mechanistic details underpinning these steps.

363 Recent work also revealed molecular correlates for the long-observed morphological and  
364 functional similarities between sites of exocytosis in Ciliata and Apicomplexa. These structural  
365 and molecular studies suggest that features of regulated exocytosis are evolutionarily conserved

366 within Alveolata, and distinct from those in mammals. The mechanisms that underlie rhoptry  
367 exocytosis in Apicomplexa must reflect adaptations following the evolutionary transition from a  
368 free lifestyle to parasitism, and include responsiveness to different stimuli, and the requirement to  
369 deliver rhoptry effectors into the cytoplasm of a targeted cell. Many questions remain about these  
370 critical steps in infection, and we have listed some to promote further investigation (see  
371 **Outstanding Questions**).

372  
373 In recent years, new powerful tools have been developed to assay rhoptry exocytosis and  
374 function [88] but all of these rely on quantifying the export of rhoptry proteins into the host cell,  
375 the last stage in the process. Developing specific assays to follow in a stepwise fashion the complex  
376 process of rhoptry secretion, as well as identifying artificial triggers to induce rhoptry discharge  
377 independent of a host cell, would certainly advance the field. As we describe in the previous text,  
378 comparative studies have proven to be transformative for revealing fusion machinery shared  
379 between Apicomplexa and Ciliata. One implication is that ciliates should now be more  
380 aggressively exploited to explore Apicomplexan biology. The secretory organelles in *Tetrahymena*  
381 are large and abundant and are genetically accessible since they are nonessential for cell viability  
382 under laboratory conditions. With its well developed genetic and bioinformatics tools,  
383 *Tetrahymena* offers a versatile platform to study exocytosis and should attract the interest of a  
384 broader audience of scientists studying rhoptry secretion as well as the biogenesis of Apicomplexa  
385 secretory organelles.

386 Looking ahead, we believe that uncovering the molecular mechanisms underlying rhoptry  
387 secretion will provide a better picture of how evolutionary changes within the eukaryotic secretory  
388 pathway promoted the transition from a free-living to a parasitic lifestyle, and also favor the long-  
389 term development of new strategies to fight diseases caused by the Apicomplexa diseases.

390

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403

#### 404 **Declaration of Interests**

405 The authors declare no competing interests

406

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663

## 664 **Glossary**

665 **Alveolata:** alveolates constitute a major clade within eukaryotes that arose ~850 million years ago.  
666 The group includes several thousand unicellular organisms (protists) with very different forms and  
667 environmental niches. They may have free-living or parasitic life-styles. The three major phyla are  
668 Ciliata, Dinoflagellata and Apicomplexa, but it also contains Perkinsozoa and Chromerida. The  
669 entire group is unified by the presence of flattened sacs underlying the plasma membrane that have

670 evolved specific functions in each taxon. In the Apicomplexa they anchor key components  
671 required for the motility and invasion of the parasites.

672 **Apicomplexa:** this phylum includes diverse intracellular parasites of vertebrates but also includes  
673 organisms that may infect non-vertebrate hosts. They typically have sexual life cycles in a defined  
674 host, though they may have multiple host species. The closest relatives to Apicomplexan parasites  
675 are the Chromerids, which include Colpodellids – predatory protozoa.

676 **Ciliata:** the ciliates are a heterotrophic and ecologically important group of free-swimming  
677 organisms, with an estimated 30000 morphologically diverse species. They owe their name to the  
678 presence of numerous motile cilia that partially or completely cover the cell surface. They are  
679 characterized by a dimorphic genetic organization, defined by the presence of a transcriptionally-  
680 silent micronucleus carrying the germline genome, and a macronucleus, that contains the somatic  
681 genome and sustains all gene expression during vegetative growth.

682 **Cryo-Electron tomography (cryo-ET):** is an emerging powerful electron microscopy imaging  
683 technique, that can reveal the structure of intact cells in a frozen-hydrated state in 3D to  
684 macromolecular (~4 nm) resolution. In cryo-ET, cells are plunge-frozen onto electron microscope  
685 grids and imaged at the temperature of liquid nitrogen at different angles, as they are tilted. This  
686 generates a series of projection images that allows the reconstruction of the entire cell or part of it  
687 in 3D, in an essentially native state.

688 **Dinoflagellata:** the dinoflagellates are mostly marine plankton, but they include freshwater and  
689 parasitic organisms. They possess two flagella. The phylum closest to Dinoflagellata is  
690 Perkinsozoa – organisms that also possess flagella, and which parasitize mollusks.

691 **Freeze-fracture:** a powerful technique for visualizing the internal organization of membranes. It  
692 consists of freezing and breaking a biological sample, the membranes of which are then visualized  
693 by vacuum-deposition of platinum-carbon and observed by transmission electron microscopy.

694 **Intramembranous particles (IMPs):** particles seen in freeze-fractured cellular membranes,  
695 presumed to be formed by integral membrane proteins.

696 **Mucocyst:** a membrane-bounded secretory organelle in *Tetrahymena*, homologous to the  
697 trichocysts. Like trichocysts, they are stably docked at the plasma membrane, underlying almost  
698 the entire cell surface. They store newly-synthesized cargo proteins, which are secreted when  
699 extracellular signals stimulate their exocytic fusion.



700 **Rhoptry:** a club-shaped organelle, connected to the parasite's apical end through an elongated  
701 neck. The organelle content is made of proteins and lipidic membranous structure and is secreted  
702 when the parasite interacts with the host cell.

703 **Secretion/exocytosis:** the term 'secretion' is associated with the membrane trafficking pathway  
704 common to all eukaryotes. It is characterized by the translocation of newly-synthesized proteins  
705 from the endoplasmic reticulum to the Golgi apparatus, and the vesicle-mediated transport towards  
706 the plasma membrane. In the secretory pathway 'exocytosis' represents the last step, when a vesicle  
707 fuses with the plasma membrane and releases its content in the extracellular space. The term  
708 "secretion" is often used to refer to the exocytic step itself. Here we use 'rhoptry secretion' to refer  
709 to the full process of injection of rhoptry proteins into the host cell, and "exocytosis" to refer to  
710 the fusion step occurring at the parasite plasma membrane.

711 **Trichocyst:** a membrane-bounded secretory organelle in *Paramecia*. It is named according to the  
712 Greek words, τρίχων (hair) and κύστη (vesicle, cyst). Trichocysts were the first cytoplasmic  
713 organelles described, decades before mitochondria and chloroplasts.

714

### 715 **Box 1. The similarity between *Tetrahymena* and *Paramecium* secretory organelles and** 716 **rhoptries in Apicomplexa**

717 Unlike apicomplexan parasites or raptorian ciliates, in which individual cells have multiple classes  
718 of secretory organelles, *Paramecium* and *Tetrahymena* each have a single type of granule –  
719 trichocysts and mucocysts, respectively. Mucocysts are roughly cylindrical, while trichocysts have  
720 a bulbous 'body' and a narrow 'tip' [53]. Trichocysts are thus reminiscent of rhoptries although  
721 much larger, with a size roughly corresponding to that of a *T. gondii* tachyzoite. Similarly to  
722 rhoptries, they show a differential segregation of proteins between the bulb and the tip regions  
723 [53]. *Tetrahymena* mucocysts also show differential segregation of tip proteins, but no structural  
724 differentiation is apparent at the level of ultrastructure [89]. Mucocysts and trichocysts, like  
725 rhoptries, go through a maturation process after budding from the Golgi [89]. Maturation involves  
726 proteolytic processing of precursor proteins [90–92]. Mucocysts and probably trichocysts, as well  
727 as rhoptries, are related to lysosome-related organelles (LROs) since their biogenesis involves a  
728 combination of secretory and endocytic trafficking [89]. It has also recently been proposed that  
729 mammalian dense core granules be considered a class of LROs [93]. For the Alveolata, molecular  
730 support for this classification comes from recent studies in *T. gondii*, *P. falciparum* and *T.*

731 *thermophila*, showing the involvement of lysosomal enzymes, sortilin receptors, early endocytic  
732 RabGTPases, adaptor proteins, SNAREs and the tethering complexes HOPS and CORVET, in  
733 rhoptry and/or mucocyst biogenesis [90,94–102]. Notably, these endocytic factors are also  
734 involved in microneme biogenesis. Finally, microtubules have been implicated in microneme  
735 [103,104], rhoptry [105], and trichocyst positioning [106]. Taken together, the mechanisms of  
736 biogenesis of mucocysts/trichocysts, and possibly of transport shared common evolutionary  
737 features with those of micronemes and rhoptries. However, unlike micronemes but like rhoptries,  
738 they are membrane-bound at the plasma membrane and rely on homologous exocytic machinery,  
739 as detailed in the main text. These features indicate that broadly similar mechanisms are used to  
740 generate specialized secretory organelles in Ciliata and Apicomplexa. Such secretory  
741 compartments have differentiated during evolution to fulfill the very different requirements for  
742 parasitic vs free-living protists but maintain clear molecular traces of their common origin.

743

#### 744 **Box 2. Non-discharged (nd) genes in *Paramecium***

745 The exocytosis-defective Nd mutants generated in *P. tetraurelia* are devoid of rosettes and  
746 connecting material. However, none of the *Nd* genes encodes a rosette component per se; instead,  
747 they are likely involved in its assembly by acting in the cytosol, or within the trichocyst or plasma  
748 membranes. In *Paramecium*, 31 alleles defining 17 *nd* genes were isolated, and a subset  
749 experimentally explored: *nd2/nd22p* [61]), *nd6* [63], *nd7* [61], *nd9* [62], and *nd169* [61]. A variety  
750 of functional complementation tests were used to directly determine which compartment was  
751 affected by a specific mutation. These tests included transfer-via-microinjection of cytoplasm or  
752 trichocysts between cells [60,107], and experiments based on phenotype rescue during conjugation  
753 when paired cells exchange cytoplasm. The results predicted that PtNd2, PtNd169 and PtNd7 are  
754 associated with the trichocyst [59,107,108] and this was confirmed for PtNd2, a transmembrane  
755 protein [61]. PtNd6 was predicted to be active at the PM. It contains a RCC1-like domain [63], a  
756 domain that in other proteins has been shown to have GEF activity towards small GTPases and to  
757 be involved in intracellular membrane fusion events [109]. The cytoplasmic component PtNd9  
758 interacts with both the PM and trichocyst membranes to assemble the rosette and connecting  
759 material [108]. PtNd9 has two domains. The N-terminal domain is predicted to be important for  
760 direct or indirect membrane binding, while the armadillo repeat-containing C-terminal domain  
761 may moderate protein–protein interactions [62]. The analysis of dominant negative forms of PtNd9

762 suggested that PtNd9 might act as a homopolymeric bridge between the trichocyst and PM. Studies  
763 on double *nd* mutants revealed interactions between PtNd9 with two other cytosolic factors,  
764 PtNd16 and PtNd18. These have not been characterized but are both implicated in rosette assembly  
765 [59,62]. These proteins may form a multimeric complex that contributes to the connecting material  
766 beneath the plasma membrane.

767 Another gene involved in *Paramecium* exocytosis is calmodulin (*cam*), the most ubiquitous  
768 Ca<sup>2+</sup> mediator in eukaryotic cells. A screen of mutants affected in swimming behavior identified  
769 several mutations in the calmodulin gene (named *cam* mutants) [110]. One of them, *cam1*, was  
770 shown to be also defective in rosette assembly and formation of underlying connecting material,  
771 and thus in regulated exocytosis [111]. These observations argue for a role of calmodulin in the  
772 exocytic step, which may account in part for the well established role of Ca<sup>2+</sup> in trichocyst  
773 exocytosis [53].

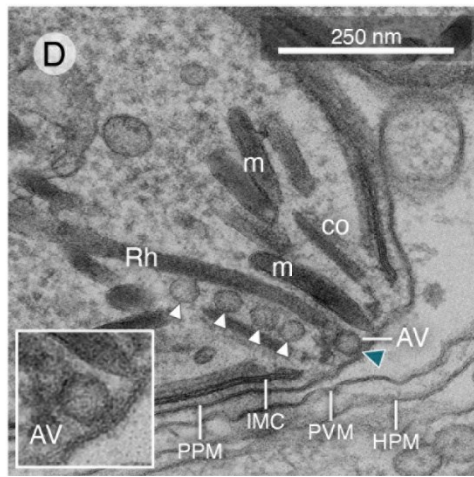
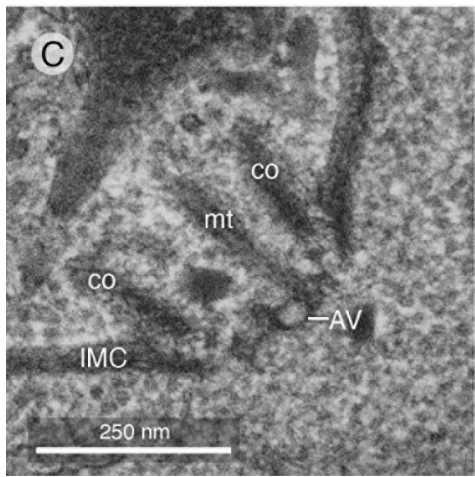
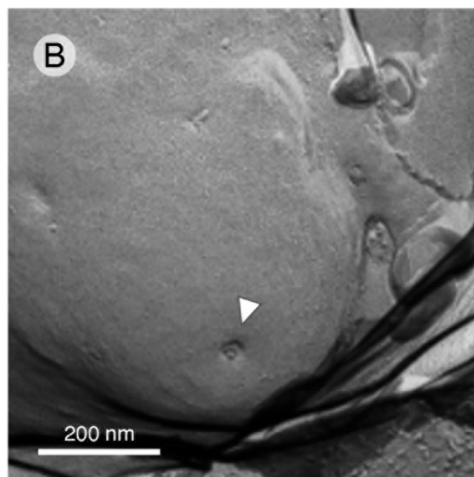
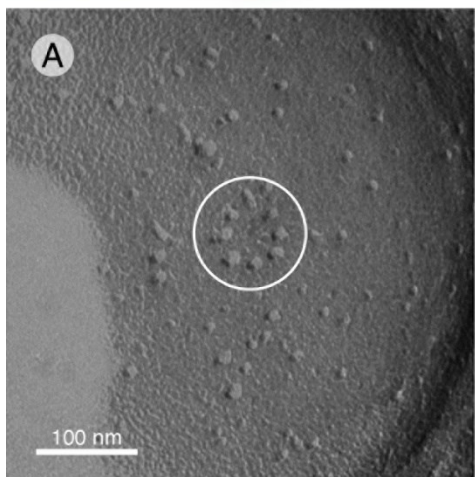
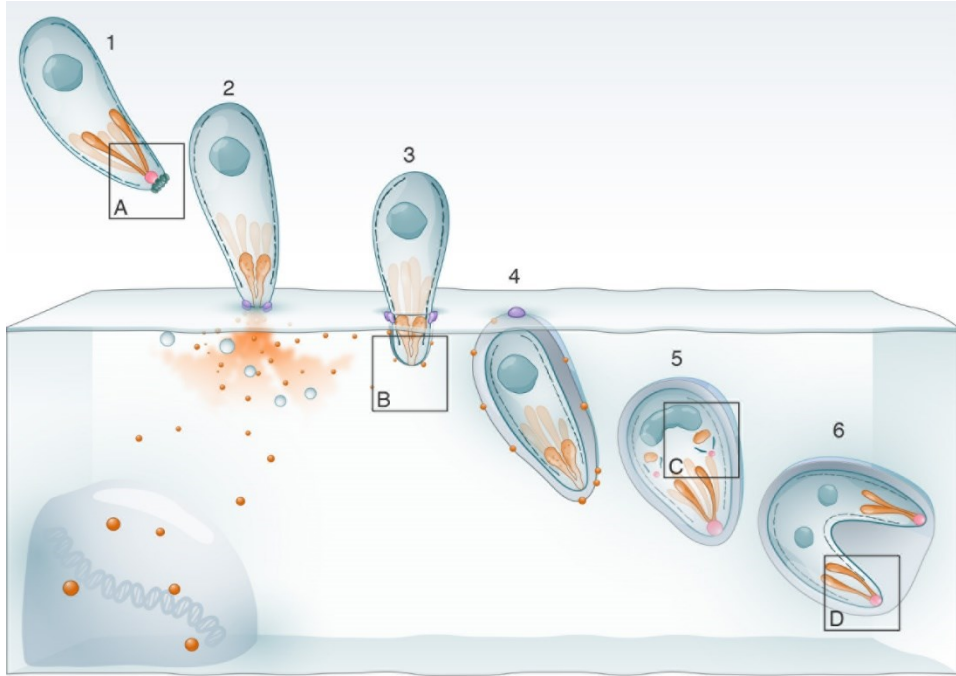
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### 775 **Box 3. A model for the role of apicomplexan Nd proteins**

776 In *Toxoplasma*, TgFer2, TgNd9 and TgNdP1 are all found in undefined punctate structures  
777 throughout the cytosol of the parasites, with TgFer2 being also associated with the IMC and  
778 rhoptries in intra- and extracellular tachyzoites, respectively, while TgNd6 and TgNdP2 show, in  
779 addition to this dispersed pattern, a distinct accumulation at the apical end of the parasite [45,68].  
780 Despite this intriguing apical localization, TgNd6 and TgNdP2 do not have any transmembrane  
781 domains and are thus unlikely to be members of the IMP rosette. Instead they might be part of the  
782 density observed underneath the rosette by cryo-ET [45], a structure likely equivalent to the fibrous  
783 'connecting material' described in Ciliata. Unlike TgNd9, TgNdP1, and TgNdP2, TgNd6 appears  
784 not to be necessary for rosette assembly based on knockdown analysis by Aquilini et al. [45]. Both  
785 its role in rhoptry secretion and its localization in proximity to the AV and PPM raise the possibility  
786 that TgNd6 contributes to AV–PPM fusion. *Paramecium* Nd6 also functions at the exocytic site,  
787 where it was predicted to control the organization of the IMPs and to promote their interaction  
788 with the underlying trichocyst [60], probably regulating the fusion of the organelle with the PM.  
789 In contrast, TgFer2 is the only protein among the partners of the Nd complex to have a  
790 transmembrane domain. To test whether this factor is important for rosette assembly further  
791 analysis will be required.

792           The function of the other *Toxoplasma* Nd homologs cannot be inferred from localization  
793 data because, though they all appear to be required for rosette building, they are not detectible at  
794 the apical exocytic site. Nonetheless, the following observations argue for a cooperative role of  
795 these proteins at this location. In *Paramecium* Nd9 is part of a complex [59] and interacts with  
796 both the PM and trichocysts, to trigger rosette formation [108]. TgNdP2 is present at the apex and  
797 like TgFer2 contains a likely Ca<sup>2+</sup>-sensing C2 domain. TgFer2 has homology to the ferlin family  
798 of double C2 domain-containing Ca<sup>2+</sup> sensors, and in mammals, otoferlin regulates the release of  
799 neurotransmitters via fusion between synaptic vesicles and the PM [112]. It is thus tempting to  
800 hypothesize that, like Nd6, these factors contribute to the fusion of the AV, or  
801 mucocysts/trichocysts, at the PM (**Figure 3**). This step may be calcium-dependent, due to the  
802 involvement of Fer2 and NdP2, but also depend on the activation of small GTPases. Consistent  
803 with the latter, an Alveolata-conserved GTPase was found in the immunopurification of TgNd9  
804 [45], and Nd6 has a GEF domain [45,63]. RabGTPase–GEF interactions are well established  
805 regulatory mechanisms for several protein complexes that control membrane fusion by recruiting  
806 specific SNARE-dependent machinery to the target membrane. A GTPase present near mucocyst  
807 docking sites has been described in *Tetrahymena* cells but its contribution to exocytosis has not  
808 been explored [113].

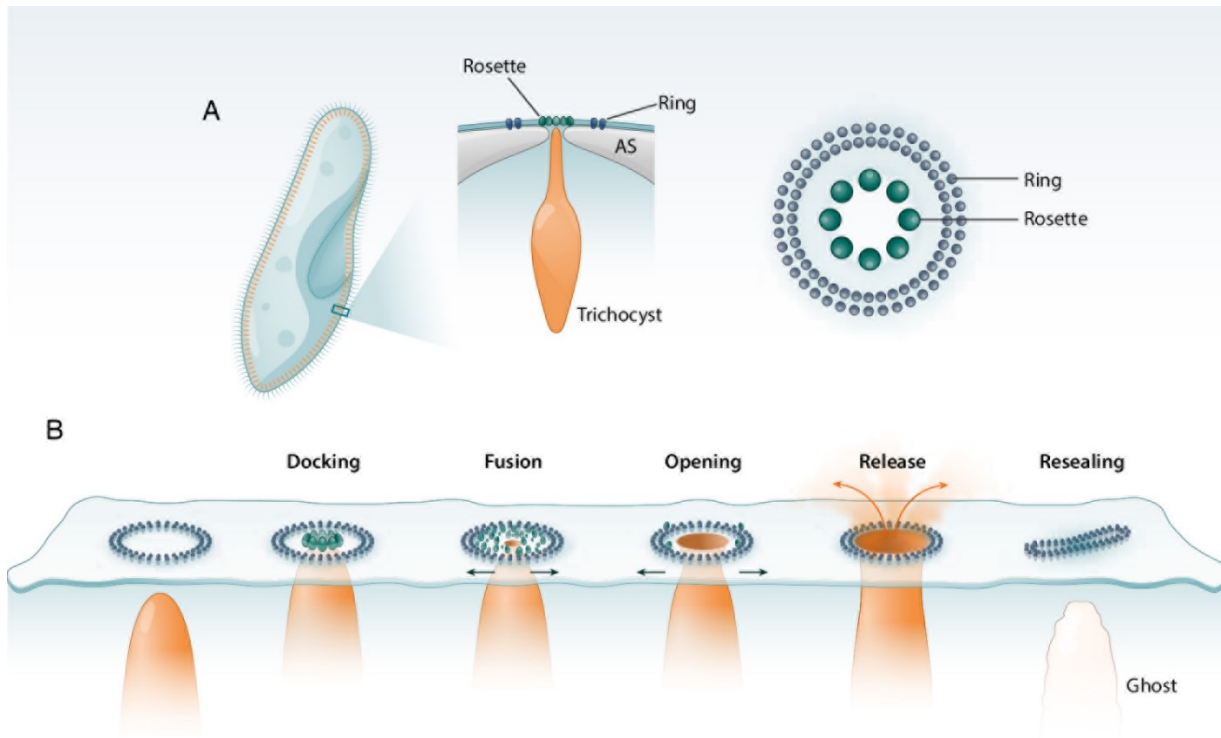
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810

811 **Figure 1. The fate of the rhoptry secretion apparatus during invasion and intracellular**  
812 **development of *Toxoplasma gondii*.** (1) An extracellular tachyzoite with two elongated rhoptries  
813 (orange organelles) docked to the apical vesicle (AV, pink) which is positioned beneath the PPM-  
814 embedded rosette (dark-green particles). (2) An extracellular parasite attached to the host cell, in  
815 the process of rhoptry secretion. The necks of two rhoptries fuse and the rhoptry material (proteins  
816 in orange and round-shaped vesicles in blue) is released inside the host cell. (3) A parasite in the  
817 process of active invasion. The rhoptry neck proteins (RONs) support the formation of the moving  
818 junction (MJ, violet) and parasite entry into the host. ROPs (orange) are effectors injected within  
819 the host cytoplasm and targeted to the nucleus, to subvert host cell function. (4) The parasite is  
820 intracellular, enclosed by the parasitophorous vacuole membrane (PVM), which provides a safe  
821 niche for the parasite to replicate. Discharged rhoptries disassembled following host cell entry.  
822 Some ROPs (orange dots) are also associated with the PVM. (5) During parasite replication,  
823 rhoptries assemble *de novo* as spherical pre-rhoptries in nascent parasites, which also display the  
824 apical vesicle at the apex, as shown in panel c. (6). Pre-rhoptries elongate to form mature rhoptries,  
825 which subsequently migrate to the apexes of daughter parasites and dock at the AV. The two new  
826 parasites acquire their PPM from the mother just prior cytokinesis, when rosette assembly likely  
827 occurs. Inset: (A) Freeze fracture-derived electron micrograph of the PPM-embedded rosette  
828 (white circle) at the apex of an extracellular parasite. (B) Freeze fracture-derived electron  
829 micrograph of a parasite apex of an invading parasite, where the parasitophorous vacuole  
830 membrane (PVM) at the apical tip of the parasite is exposed, and exhibits a small depression  
831 (arrow), that might correspond to the opening by which the rhoptry content is transferred inside  
832 the host. (C) Electron micrograph of the apex of a tachyzoite developing inside the mother. The  
833 nascent inner membrane complex (IMC), and the pair of central microtubules (mt) in the conoid  
834 (co) are visible. The AV is found on top of the microtubules in the absence of PPM. (D) Electron  
835 micrograph of the apex of an intracellular mature parasite showing the apical vesicle (AV) docked  
836 at the PPM, a mature rhoptry (Rh) docked at the apical vesicle (AV), and several vesicles aligned  
837 within the conoid (co). The expected position of the rosette at the PPM is indicated by a blue arrow.  
838 m, micronemes. PPM, parasite plasma membrane; HPM, host plasma membrane; IMC, inner  
839 membrane complex. Image (B) kindly provided by JF Dubremetz. Image (D) adapted from  
840 Aquilini and et al. [45].

841



842

843 **Figure 2. Remodeling of the exocytosis site during docking and stimulated discharge of**

844 ***Paramecium* trichocysts. (A)** On the left, schematic drawing of a *Paramecium* cell shown in

845 cross-section, with cilia in blue covering the entire surface of the ciliate, and trichocysts (orange

846 organelles) docked in an ordered fashion at the membrane. In the middle, schematic of the

847 magnified secretory organelle positioned beneath the plasma membrane (PM), and docked at the

848 exocytosis site between the alveolar sacs (AS). The rosette and ring of intramembranous particles

849 (IMPs), embedded in the PM, appear where the trichocyst tip touches the PM. On the right, top

850 view of the exocytosis site, characterized by a 300 nm double-ring of around 80 IMPs surrounding

851 a 60 nm rosette of eight IMPs. (B) Sequence of docking and post-docking events following

852 chemically-induced exocytosis of trichocyst. Upon docking of the trichocyst to the PM, in

853 correspondence of the IMP-containing double-ring, a rosette of eight IMPs is formed in the center

854 of the ring. In response to a chemical stimulus, membrane fusion takes place, leading to the

855 formation, of a 20–30 nm pore in the center of the rosette [47,114] where the IMPs – initially very

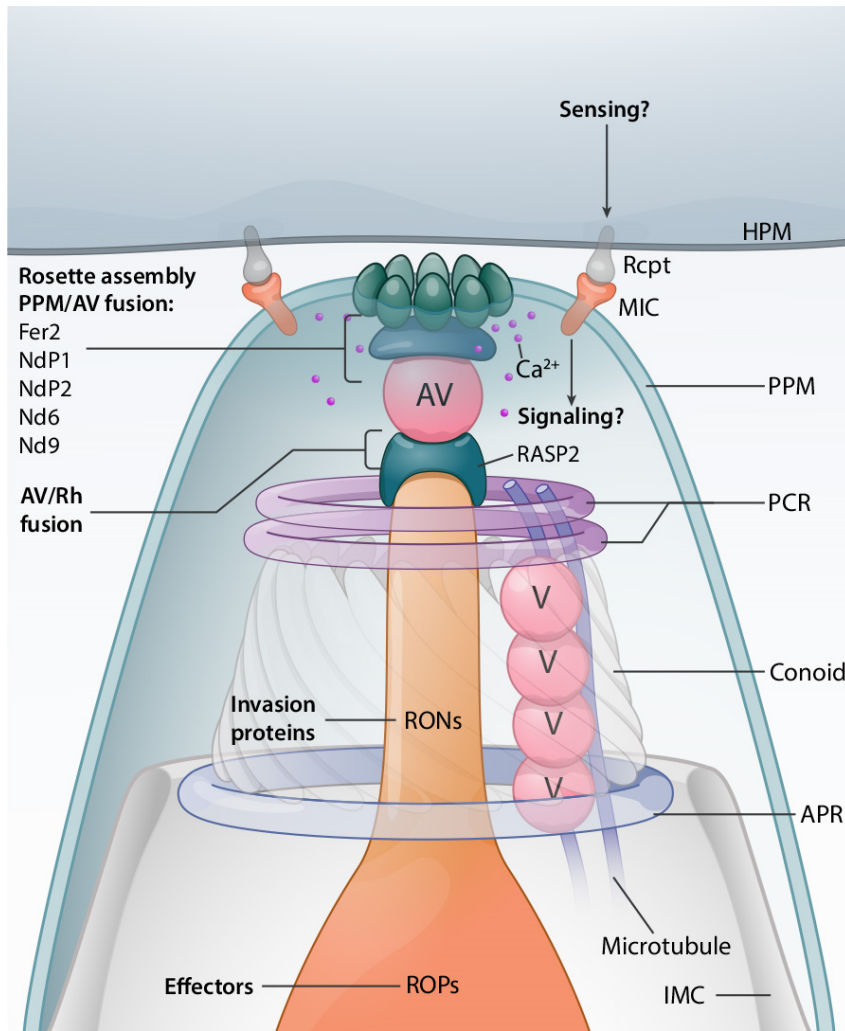
856 close to the opening site – disperse as the opening enlarges, and a new population of smaller

857 particles appears, likely as a result of the dissociation of a protein complex [47]. The enlarging

858 pore eventually covers the entire area within the large ring, enabling the release of trichocyst

859 content and, consequently, the formation of the so-called 'ghost' (residual empty trichocyst  
 860 membrane). Exocytosis is followed by the rapid resealing of the disrupted membrane which is not  
 861 accompanied by the formation of a new rosette, despite the presence of smaller IMPs. The outer  
 862 rings adopt a 'parenthesis' shape, and the surface is now free for the next docking event. When a  
 863 newly formed trichocyst reaches the cortex, the parenthesis develops into the large ring and a new  
 864 rosette is built at the center. Modified from [114].

865



866

867 **Figure 3.** Schematic representation of the apical complex and the fusion events required for  
 868 rhoptry discharge. Rhoptry exocytosis in *T. gondii* follows the contact of the parasite's apex with  
 869 the host cell plasma membrane (HPM), which is mediated by the interaction of microneme proteins  
 870 (MIC), translocated to the parasite plasma membrane (PPM in light green) upon secretion, with  
 871 host receptors (Rcpt). Upon such a 'sensing' event, a 'signaling' pathway is likely activated, with a



872 cascade of signals inducing at least two fusion events, one between rhoptry/ies (Rh) and the apical  
873 vesicle (AV), and the other between the AV and the PPM, leading to the release of rhoptry neck  
874 proteins (RONs) and effectors (ROPs). The Rh/AV fusion might require the rhoptry protein  
875 RASP2, which accumulates on the cytoplasmic face of the rhoptry tip, and specifically binds to  
876 phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) via its Ca<sup>2+</sup>-lipid-  
877 binding-like (C2) and Pleckstrin Homology-like (PH) domains, perhaps favoring lipid mixing  
878 during the fusion event. The rosette assembly and AV/PPM fusion might instead require a  
879 repertoire of proteins, Fer2, NdP1, NdP2, Nd6, and Nd9, which could be part of the connecting  
880 density observed by cryo-ET between the apical vesicle and the rosette [45]. This interpretation is  
881 based on the accumulation of Nd6 and NdP2 at the apical end of the parasite, and on the fact that  
882 they interact with Fer2, NdP1, and Nd9. However, additional functions for these proteins cannot  
883 be excluded since they all show a cytoplasmic localization [45,68]. In addition, Fer2 was shown  
884 to accumulate inside the conoid and the cytosolic face of the rhoptry membrane [68]. The RCC1-  
885 like domain (RLD) of Nd6 might act as guanine exchange factor towards small GTPases, as  
886 previously seen for proteins involved in vesicular membrane fusion [109]. Via its armadillo-like  
887 repeats the Nd9 protein might facilitate protein–protein interactions with other Nd members at the  
888 exocytic site in order to assemble the rhoptry secretion apparatus and to stabilize the  
889 intramembranous particles (IMPs) of the rosette. The C2 domain-containing proteins NdP2 and  
890 Fer2 might contribute to the Ca<sup>2+</sup>-dependent fusion of the AV at the parasite’s membrane. Upon  
891 fusion and rhoptry discharge, a new exocytosis machinery might be rebuilt at the parasite apex, by  
892 recruiting to the PPM a new apical vesicle from the reservoir of vesicles (V) inside the conoid.  
893 This event would stimulate the formation of another rosette and anchoring of a new rhoptry  
894 selected from the pool of undocked organelles present at the apex of the parasite. Rearming the  
895 secretion apparatus would ensure successive secretion of rhoptries, and thus successive injections  
896 of rhoptry effectors into the host, in order to hijack cells that the parasite does not invade. PCR,  
897 pre-conoidal ring in violet; APR, apical polar ring in light blue; IMC, inner membrane complex in  
898 white.

899

## 900 **Highlights**

- 901 • Rhoptries are connected to an apical vesicle which is docked to the plasma membrane (PM)  
902 and extends to a rosette of PM-embedded intramembranous particles via electron-dense  
903 connecting material. Rhoptry secretion thus involves multiple membrane-fusion events.
- 904 • The rosette is essential for rhoptry secretion but is dispensable for microneme exocytosis.
- 905 • Rosette formation and rhoptry exocytosis require conserved proteins that are restricted to the  
906 Alveolata.

907

908

909 **Outstanding questions:**

- 910 • How do microneme proteins contribute to rhoptry secretion?
- 911 • What are the signaling pathways regulating rhoptry discharge?
- 912 • When and how is the rhoptry secretion machine assembled?
- 913 • Is rhoptry fusion regulated by evolutionary divergent SNAREs/Synaptotagmins?
- 914 • [Is the apical vesicle conserved in all Apicomplexa?](#)
- 915 • Which are the constituents of the rosette IMPs and the connecting material? [How such](#)  
916 [constituents are organized in the parasite plasma membrane-vesicle interface?](#)
- 917 • How are exocytosis and rhoptry protein injection into the host cell cytoplasm coordinated?
- 918 • How does rhoptry content cross the plasma membrane of the host cell?
- 919 • Does opening of a pore eventually contribute to the export of rhoptry material?
- 920 • How do host factors contribute to rhoptry secretion, signaling and pore formation?
- 921 • What is the role of the apical vesicle in rhoptry secretion?

922

923

924