



HAL
open science

Unraveling the Elusive Rhoptry Exocytic Mechanism of Apicomplexa

Daniela Sparvoli, Maryse Lebrun

► **To cite this version:**

Daniela Sparvoli, Maryse Lebrun. Unraveling the Elusive Rhoptry Exocytic Mechanism of Apicomplexa. *Trends in Parasitology*, 2021, 37 (7), pp.622-637. 10.1016/j.pt.2021.04.011 . inserm-03654113

HAL Id: inserm-03654113

<https://inserm.hal.science/inserm-03654113v1>

Submitted on 28 Apr 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

2

3 Daniela Sparvoli¹ and Maryse Lebrun^{1*}

4

5 **Affiliations**

6 ¹LPHI UMR5235, Univ Montpellier, CNRS, F-34095 Montpellier, France

7 *corresponding author: maryse.lebrun@umontpellier.fr

8 ORCIDs:

9 Daniela Sparvoli: 0000-0002-5992-3753

10 Maryse Lebrun: 0000-0002-0962-0156

11

12

13 **Keywords:** Apicomplexa, rhoptry, trichocyst, mucocyst, exocytosis, rosette

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 Ca²⁺ 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)P₂), 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)P₂
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 Ca²⁺ [72] excluding a Ca²⁺-
276 dependent role in rhoptry secretion for these proteins. Nevertheless, Ca²⁺ 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

391 **Acknowledgments**

392 The authors are grateful to Aaron P. Turkewitz for his critical reading of the manuscript and
393 valuable suggestions. We thank Jean-François Dubremetz and Eleonora Aquilini for EM images
394 B and C of **Figure 1**, respectively; Julien Marcetteau for the precious graphical design of the
395 figures. We apologize for all the relevant studies that could not be discussed and directly cited due
396 to length constraints and are therefore included in the bibliography of the listed reviews. M.L. is

397 supported by the French Government (Agence Nationale de la Recherche) Investissement
398 d’Avenir programme, Laboratoire d’Excellence (LabEx) 'French Parasitology Alliance for Health
399 Care' (ANR-11-LABX-0024-PARAFRAP), and the European Research Council (ERC advanced
400 grant number 833309 KissAndSpitRhoptry). D.S. was supported by the European Research
401 Council (ERC) under the European Union’s Horizon 2020 research and innovation program under
402 Grant agreement no. 833309 to M.L. M.L. is an INSERM researcher.

403

404 **Declaration of Interests**

405 The authors declare no competing interests

406

407 **References**

- 408 1 World Health Organization (2020) World Malaria Report 2020. WHO
- 409 2 McAuley, J.B. (2014) Congenital Toxoplasmosis. *J. Pediatr. Infect. Dis. Soc.* 3 Suppl 1,
410 S30-S35
- 411 3 Striepen, B. (2013) Parasitic infections: Time to tackle cryptosporidiosis. *Nature* 503, 189–
412 191
- 413 4 Carruthers, V.B. and Sibley, L.D. (1997) Sequential protein secretion from three distinct
414 organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell*
415 *Biol* 73, 114–123
- 416 5 Bisio, H. and Soldati-Favre, D. (2019) Signaling Cascades Governing Entry into and Exit
417 from Host Cells by *Toxoplasma gondii*. *Annu. Rev. Microbiol.* 73, 579–599
- 418 6 Lebrun, M. *et al.* (2020), Chapter 14 *Toxoplasma* secretory proteins and their roles in
419 parasite cell cycle and infection. In *Toxoplasma gondii: the Model Apicomplexan -*
420 *Perspectives and Methods.* (Weiss, L.M. and Kim, K., eds), pp. 607–704, 471 Academic
421 Press
- 422 7 Kessler, H. *et al.* (2008) Microneme protein 8--a new essential invasion factor in
423 *Toxoplasma gondii*. *J Cell Sci* 121, 947–956
- 424 8 Singh, S. *et al.* (2010) Distinct external signals trigger sequential release of apical
425 organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog* 6, e1000746
- 426 9 Aikawa, M. *et al.* (1978) Erythrocyte entry by malarial parasites. A moving junction
427 between erythrocyte and parasite. *J Cell Biol* 77, 72–82

- 428 10 Besteiro, S. *et al.* (2011) The moving junction of apicomplexan parasites: a key structure
429 for invasion. *Cell Microbiol* 13, 797–805
- 430 11 Tyler, J.S. *et al.* (2011) Focus on the ringleader: the role of AMA1 in apicomplexan
431 invasion and replication. *Trends Parasitol* 27, 410–20
- 432 12 Ghosh, S. *et al.* (2017) The *Plasmodium* rhoptry associated protein complex is important
433 for parasitophorous vacuole membrane structure and intraerythrocytic parasite growth. *Cell*
434 *Microbiol* 19,
- 435 13 Hakimi, M.-A. *et al.* (2017) *Toxoplasma* Effectors Targeting Host Signaling and
436 Transcription. *Clin. Microbiol. Rev.* 30, 615–645
- 437 14 Melo, M.B. *et al.* (2011) *Toxoplasma gondii* effectors are master regulators of the
438 inflammatory response. *Trends Parasitol* 27, 487–95
- 439 15 Boothroyd, J.C. and Dubremetz, J.F. (2008) Kiss and spit: the dual roles of *Toxoplasma*
440 rhoptries. *Nat Rev Microbiol* 6, 79–88
- 441 16 Delorme-Walker, V. *et al.* (2012) Toxofilin upregulates the host cortical actin cytoskeleton
442 dynamics, facilitating *Toxoplasma* invasion. *J Cell Sci* 125, 4333–42
- 443 17 Sangaré, L.O. *et al.* (2019) In Vivo CRISPR Screen Identifies TgWIP as a *Toxoplasma*
444 Modulator of Dendritic Cell Migration. *Cell Host Microbe* 26, 478-492.e8
- 445 18 Saeij, J.P. *et al.* (2007) *Toxoplasma* co-opts host gene expression by injection of a
446 polymorphic kinase homologue. *Nature* 445, 324–7
- 447 19 Denkers, E.Y. *et al.* (2012) An inside job: hacking into Janus kinase/signal transducer and
448 activator of transcription signaling cascades by the intracellular protozoan *Toxoplasma*
449 *gondii*. *Infect Immun* 80, 476–82
- 450 20 Nguitragool, W. *et al.* (2011) Malaria parasite *clag3* genes determine channel-mediated
451 nutrient uptake by infected red blood cells. *Cell* 145, 665–77
- 452 21 Huang, B.Q. *et al.* (2004) *Cryptosporidium parvum* attachment to and internalization by
453 human biliary epithelia in vitro: a morphologic study. *J. Parasitol.* 90, 212–221
- 454 22 Hanssen, E. *et al.* (2013) Electron tomography of *Plasmodium falciparum* merozoites
455 reveals core cellular events that underpin erythrocyte invasion. *Cell Microbiol* 15, 1457–72
- 456 23 Paredes-Santos, T.C. *et al.* (2012) Dynamics and 3D organization of secretory organelles of
457 *Toxoplasma gondii*. *J Struct Biol* 177, 420–30

- 458 24 Nishi, M. *et al.* (2008) Organellar dynamics during the cell cycle of *Toxoplasma gondii*. *J*
459 *Cell Sci* 121, 1559–68
- 460 25 Shaw, M.K. *et al.* (1998) Acidic compartments and rhoptry formation in *Toxoplasma*
461 *gondii*. *Parasitology* 117 (Pt 5), 435–43
- 462 26 Dubremetz, J.F. (2007) Rhoptries are major players in *Toxoplasma gondii* invasion and host
463 cell interaction. *Cell Microbiol* 9, 841–8
- 464 27 Tomavo, S. (2014) Evolutionary repurposing of endosomal systems for apical organelle
465 biogenesis in *Toxoplasma gondii*. *Int J Parasitol* 44, 133–8
- 466 28 Ben Chaabene, R. *et al.* (2020) Biogenesis and discharge of the rhoptries: key organelles
467 for entry and hijack of host cells by the Apicomplexa. *Mol. Microbiol.* DOI:
468 10.1111/mmi.14674
- 469 29 Venugopal, K. and Marion, S. (2018) Secretory organelle trafficking in *Toxoplasma gondii*:
470 A long story for a short travel. *Int. J. Med. Microbiol. IJMM* 308, 751–760
- 471 30 Tomavo, S. *et al.* (2013) Protein trafficking through the endosomal system prepares
472 intracellular parasites for a home invasion. *PLoS Pathog* 9, e1003629
- 473 31 Carruthers, V.B. *et al.* (1999) Ethanol and acetaldehyde elevate intracellular [Ca²⁺] and
474 stimulate microneme discharge in *Toxoplasma gondii*. *Biochem J* 342 (Pt 2), 379–86
- 475 32 Carruthers, V.B. and Sibley, L.D. (1999) Mobilization of intracellular calcium stimulates
476 microneme discharge in *Toxoplasma gondii*. *Mol Microbiol* 31, 421–8
- 477 33 Brown, K.M. *et al.* (2016) Serum Albumin Stimulates Protein Kinase G-dependent
478 Microneme Secretion in *Toxoplasma gondii*. *J. Biol. Chem.* 291, 9554–9565
- 479 34 Nichols, B.A. *et al.* (1983) Secretion from the rhoptries of *Toxoplasma gondii* during host-
480 cell invasion. *J Ultrastruct Res* 83, 85–98
- 481 35 Hakansson, S. *et al.* (2001) *Toxoplasma* vacuoles: a two-step process of secretion and
482 fusion forms the parasitophorous vacuole. *Embo J* 20, 3132–3144
- 483 36 Miller, L.H. *et al.* (1979) Interaction between cytochalasin B-treated malarial parasites and
484 erythrocytes. Attachment and junction formation. *J Exp Med* 149, 172–84
- 485 37 Bannister, L.H. *et al.* (1986) Lamellar membranes associated with rhoptries in erythrocytic
486 merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion. *Parasitology* 92 (
487 Pt 2), 291–303

- 488 38 Suss-Toby, E. *et al.* (1996) *Toxoplasma* invasion: the parasitophorous vacuole is formed
489 from host cell plasma membrane and pinches off via a fission pore. *Proc Natl Acad Sci U A*
490 93, 8413–8
- 491 39 Mital, J. *et al.* (2005) Conditional expression of *Toxoplasma gondii* apical membrane
492 antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion.
493 *Mol Biol Cell* 16, 4341–9
- 494 40 Sidik, S.M. *et al.* (2016) A Genome-wide CRISPR Screen in *Toxoplasma* Identifies
495 Essential Apicomplexan Genes. *Cell* 166, 1423-1435 e12
- 496 41 Broncel, M. *et al.* (2020) Profiling of myristoylation in *Toxoplasma gondii* reveals an N-
497 myristoylated protein important for host cell penetration. *eLife* 9,
- 498 42 Gilberger, T.W. *et al.* (2003) The cytoplasmic domain of the *Plasmodium falciparum* ligand
499 EBA-175 is essential for invasion but not protein trafficking. *J Cell Biol* 162, 317–27
- 500 43 Ayong, L. *et al.* (2007) Identification of *Plasmodium falciparum* family of SNAREs. *Mol*
501 *Biochem Parasitol* 152, 113–22
- 502 44 Bisio, H. *et al.* (2020) The ZIP Code of Vesicle Trafficking in Apicomplexa:
503 SEC1/Munc18 and SNARE Proteins. *mBio* 11,
- 504 45 Aquilini, E. *et al.* (2021) An Alveolata secretory machinery adapted to parasite host cell
505 invasion. *Nat. Microbiol.* 6, 425–434
- 506 46 Dubremetz, J.F. and Torpier, G. (1978) Freeze fracture study of the pellicle of an *eimerian*
507 sporozoite (Protozoa, Coccidia). *J. Ultrastruct. Res.* 62, 94–109
- 508 47 Knoll, G. *et al.* (1991) Quenched flow analysis of exocytosis in *Paramecium* cells: time
509 course, changes in membrane structure, and calcium requirements revealed after rapid
510 mixing and rapid freezing of intact cells. *J Cell Biol* 113, 1295–304
- 511 48 Plattner, H. *et al.* (1973) Membrane specializations in the form of regular membrane-to-
512 membrane attachment sites in *Paramecium*. A correlated freeze-etching and ultrathin-
513 sectioning analysis. *J Cell Sci* 13, 687–719
- 514 49 Porchet-Hennere, E. and Nicolas, G. (1983) Are rhoptries of Coccidia really extrusomes? *J*
515 *Ultrastruct Res* 84, 194–203
- 516 50 Goodenough, U. *et al.* (2018) Epiplasts: Membrane Skeletons and Epiplastin Proteins in
517 Euglenids, Glaucophytes, Cryptophytes, Ciliates, Dinoflagellates, and Apicomplexans.
518 *mBio* 9,

519 51 Rosati, G. and Modeo, L. (2003) Extrusomes in ciliates: diversification, distribution, and
520 phylogenetic implications. *J Eukaryot Microbiol* 50, 383–402

521 52 Ruehle, M.D. *et al.* (2016) *Tetrahymena* as a Unicellular Model Eukaryote: Genetic and
522 Genomic Tools. *Genetics* 203, 649–65

523 53 Plattner, H. (2017) Trichocysts-*Paramecium*'s Projectile-like Secretory Organelles:
524 Reappraisal of their Biogenesis, Composition, Intracellular Transport, and Possible
525 Functions. *J Eukaryot Microbiol* 64, 106–133

526 54 Turkewitz, A.P. (2004) Out with a bang! *Tetrahymena* as a model system to study secretory
527 granule biogenesis. *Traffic Cph. Den.* 5, 63–68

528 55 Satir, B. *et al.* (1972) Membrane reorganization during secretion in *Tetrahymena*. *Nature*
529 235, 53–54

530 56 Tiedtke, A. (1976) Capsule shedding in tetrahymena. *Naturwissenschaften* 63, 93

531 57 Vayssié, L. *et al.* (2000) Molecular genetics of regulated secretion in *paramecium*.
532 *Biochimie* 82, 269–288

533 58 Beisson, J. *et al.* (1976) Genetic analysis of membrane differentiation in *Paramecium*.
534 Freeze-fracture study of the trichocyst cycle in wild-type and mutant strains. *J Cell Biol* 69,
535 126–43

536 59 Bonnemain, H. *et al.* (1992) Interactions between genes involved in exocytotic membrane
537 fusion in paramecium. *Genetics* 130, 461–70

538 60 Lefort-Tran, M. *et al.* (1981) Control of exocytotic processes: cytological and physiological
539 studies of trichocyst mutants in *Paramecium tetraurelia*. *J Cell Biol* 88, 301–11

540 61 Froissard, M. *et al.* (2004) Novel secretory vesicle proteins essential for membrane fusion
541 display extracellular-matrix domains. *Traffic* 5, 493–502

542 62 Froissard, M. *et al.* (2001) ND9P, a novel protein with armadillo-like repeats involved in
543 exocytosis: physiological studies using allelic mutants in *paramecium*. *Genetics* 157, 611–
544 20

545 63 Gogendeau, D. *et al.* (2005) Nd6p, a novel protein with RCC1-like domains involved in
546 exocytosis in *Paramecium tetraurelia*. *Eukaryot Cell* 4, 2129–39

547 64 Porchet, E. and Torpier, G. (1977) [Freeze fracture study of *Toxoplasma* and *Sarcocystis*
548 infective stages (author's transl)]. *Z Parasitenkd* 54, 101–24

- 549 65 Meszoely, C.A. *et al.* (1995) *Plasmodium vivax*: freeze-fracture studies on the ultrastructure
550 of the sporozoites within the salivary gland of the mosquito *Anopheles stephensi*. *Am. J.*
551 *Trop. Med. Hyg.* 52, 443–449
- 552 66 Dubremetz, J.F. *et al.* (1979) Structure of pellicule of the *Plasmodium yoelii* sporozoite:
553 Freeze-fracture study. *CR Acad Sci Paris* 228, 623–626
- 554 67 Dubremetz, J.F. and Entzeroth, R. (1993) Exocytic events during cell invasion by
555 Apicomplexa. *Adv. Cell Mol. Biol. Membr. Membr. Traffic Protozoa Ed AM Tartakoff H*
556 *Plattner* 2A, 83–98
- 557 68 Coleman, B.I. *et al.* (2018) A Member of the Ferlin Calcium Sensor Family Is Essential for
558 *Toxoplasma gondii* Rhoptry Secretion. *MBio* 9,
- 559 69 Dos Santos Pacheco, N. *et al.* (2020) Evolution, Composition, Assembly, and Function of
560 the Conoid in Apicomplexa. *Trends Parasitol.* 36, 688–704
- 561 70 Sheffield, H.G. (1966) Electron microscope study of the proliferative form of *Besnoitia*
562 *jellisoni*. *J Parasitol* 52, 583–94
- 563 71 Varghese, T. (1975) Fine structure of the endogenous stages of *Eimeria labbeana*. I. The
564 first generation merozoites. *J Protozool* 22, 66–71
- 565 72 Suarez, C. *et al.* (2019) A lipid-binding protein mediates rhoptry discharge and invasion in
566 *Plasmodium falciparum* and *Toxoplasma gondii* parasites. *Nat Commun* 10, 4041
- 567 73 Liffner, B. *et al.* (2020) PfCERLI1 is a conserved rhoptry associated protein essential for
568 *Plasmodium falciparum* merozoite invasion of erythrocytes. *Nat. Commun.* 11, 1411
- 569 74 Liffner, B. *et al.* (2020) Pfcerli2, a duplicated gene in the malaria parasite *Plasmodium*
570 *falciparum* essential for invasion of erythrocytes as revealed by phylogenetic and cell
571 biological analysis, *Microbiology*.
- 572 75 Martin, T.F. (2015) PI(4,5)P(2)-binding effector proteins for vesicle exocytosis. *Biochim*
573 *Biophys Acta* 1851, 785–93
- 574 76 Tanguy, E. *et al.* (2020) Role of Phospholipase D-Derived Phosphatidic Acid in Regulated
575 Exocytosis and Neurological Disease. *Handb. Exp. Pharmacol.* 259, 115–130
- 576 77 Wilson, S.K. *et al.* (2020) A *Toxoplasma gondii* patatin-like phospholipase contributes to
577 host cell invasion. *PLoS Pathog.* 16, e1008650
- 578 78 Ravindran, S. *et al.* (2009) 4-Bromophenacyl bromide specifically inhibits rhoptry secretion
579 during *Toxoplasma* invasion. *PLoS One* 4, e8143

580 79 Loubens, M. *et al.* (2020) *Plasmodium* sporozoites on the move: Switching from cell
581 traversal to productive invasion of hepatocytes. *Mol. Microbiol.* DOI: 10.1111/mmi.14645

582 80 Lemgruber, L. *et al.* (2011) New details on the fine structure of the rhoptry of *Toxoplasma*
583 *gondii*. *Microsc Res Tech* 74, 812–8

584 81 Koshy, A.A. *et al.* (2012) *Toxoplasma* co-opts host cells it does not invade. *PLoS Pathog* 8,
585 e1002825

586 82 Chen, L. *et al.* (2020) The *Toxoplasma gondii* virulence factor ROP16 acts in cis and trans,
587 and suppresses T cell responses. *J. Exp. Med.* 217,

588 83 Perkins, F.O. (1976) Zoospores of the Oyster Pathogen, *Dermocystidium marinum*. I. Fine
589 Structure of the Conoid and Other Sporozoan-Like Organelles. *J. Parasitol.* 62, 959–974

590 84 Brugerolle, G. (2002) *Colpodella vorax*: ultrastructure, predation, life-cycle, mitosis, and
591 phylogenetic relationships. *Europ. J. Protistol.* 38, 113–125

592 85 Saldarriaga, J.F. *et al.* (2003) Multiple protein phylogenies show that *Oxyrrhis marina* and
593 *Perkinsus marinus* are early branches of the dinoflagellate lineage. *Int. J. Syst. Evol.*
594 *Microbiol.* 53, 355–365

595 86 Kuvardina, O.N. *et al.* (2002) The phylogeny of colpodellids (Alveolata) using small
596 subunit rRNA gene sequences suggests they are the free-living sister group to
597 apicomplexans. *J. Eukaryot. Microbiol.* 49, 498–504

598 87 Moore, R.B. *et al.* (2008) A photosynthetic alveolate closely related to apicomplexan
599 parasites. *Nature* 451, 959–63

600 88 Suarez, C. *et al.* (2020) Assessing Rhoptry Secretion in *T. gondii*. *Methods Mol. Biol.*
601 *Clifton NJ* 2071, 143–155

602 89 Briguglio, J.S. and Turkewitz, A.P. (2014) *Tetrahymena thermophila*: a divergent
603 perspective on membrane traffic. *J Exp Zool B Mol Dev Evol* 322, 500–16

604 90 Dogga, S.K. *et al.* (2017) A druggable secretory protein maturase of *Toxoplasma* essential
605 for invasion and egress. *Elife* 6,

606 91 Verbsky, J.W. and Turkewitz, A.P. (1998) Proteolytic processing and Ca²⁺-binding activity
607 of dense-core vesicle polypeptides in *Tetrahymena*. *Mol. Biol. Cell* 9, 497–511

608 92 Adoutte, A. *et al.* (1984) Proteolytic cleavage and maturation of the crystalline secretion
609 products of *Paramecium*. *J. Mol. Biol.* 180, 1065–1081

610 93 Delevoeye, C. *et al.* (2019) Lysosome-related organelles as functional adaptations of the
611 endolysosomal system. *Curr. Opin. Cell Biol.* 59, 147–158

612 94 Briguglio, J.S. *et al.* (2013) Lysosomal sorting receptors are essential for secretory granule
613 biogenesis in *Tetrahymena*. *J Cell Biol* 203, 537–50

614 95 Hallée, S. *et al.* (2018) The malaria parasite *Plasmodium falciparum* Sortilin is essential for
615 merozoite formation and apical complex biogenesis. *Cell. Microbiol.* 20, e12844

616 96 Kaur, H. *et al.* (2017) An endosomal syntaxin and the AP-3 complex are required for
617 formation and maturation of candidate lysosome-related secretory organelles (mucocysts)
618 in *Tetrahymena thermophila*. *Mol Biol Cell* 28, 1551–1564

619 97 Kremer, K. *et al.* (2013) An overexpression screen of *Toxoplasma gondii* Rab-GTPases
620 reveals distinct transport routes to the micronemes. *PLoS Pathog* 9, e1003213

621 98 Kumar, S. *et al.* (2014) An aspartyl cathepsin, CTH3, is essential for proprotein processing
622 during secretory granule maturation in *Tetrahymena thermophila*. *Mol Biol Cell* 25, 2444–
623 60

624 99 Morlon-Guyot, J. *et al.* (2015) *Toxoplasma gondii* Vps11, a subunit of HOPS and
625 CORVET tethering complexes, is essential for the biogenesis of secretory organelles. *Cell*
626 *Microbiol* 17, 1157–78

627 100 Sakura, T. *et al.* (2016) A Critical Role for *Toxoplasma gondii* Vacuolar Protein Sorting
628 VPS9 in Secretory Organelle Biogenesis and Host Infection. *Sci Rep* 6, 38842

629 101 Sloves, P.J. *et al.* (2012) *Toxoplasma* sortilin-like receptor regulates protein transport and is
630 essential for apical secretory organelle biogenesis and host infection. *Cell Host Microbe* 11,
631 515–27

632 102 Sparvoli, D. *et al.* (2018) Remodeling the Specificity of an Endosomal CORVET Tether
633 Underlies Formation of Regulated Secretory Vesicles in the Ciliate *Tetrahymena*
634 *thermophila*. *Curr Biol* 28, 697-710 e13

635 103 Bannister, L.H. *et al.* (2003) *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-
636 1) is translocated within micronemes along subpellicular microtubules during merozoite
637 development. *J Cell Sci* 116, 3825–34

638 104 Leung, J.M. *et al.* (2017) Stability and function of a putative microtubule-organizing center
639 in the human parasite *Toxoplasma gondii*. *Mol Biol Cell* 28, 1361–1378

- 640 105 Lentini, G. *et al.* (2019) The roles of Centrin 2 and Dynein Light Chain 8a in apical
641 secretory organelles discharge of *Toxoplasma gondii*. *Traffic Cph. Den.* 20, 583–600
- 642 106 Plattner, H. *et al.* (1982) Cytoskeleton-secretory vesicle interactions during the docking of
643 secretory vesicles at the cell membrane in *Paramecium tetraurelia* cells. *J. Cell Biol.* 92,
644 368–377
- 645 107 Aufderheide, K.J. (1978) The effective sites of some mutations affecting exocytosis in
646 *Paramecium tetraurelia*. *Mol. Gen. Genet. MGG* 165, 199–205
- 647 108 Beisson, J. *et al.* (1980) Control of membrane fusion in exocytosis. Physiological studies on
648 a *Paramecium* mutant blocked in the final step of the trichocyst extrusion process. *J Cell*
649 *Biol* 85, 213–27
- 650 109 Hadjebi, O. *et al.* (2008) The RCC1 superfamily: from genes, to function, to disease.
651 *Biochim Biophys Acta* 1783, 1467–79
- 652 110 Kink, J.A. *et al.* (1990) Mutations in paramecium calmodulin indicate functional
653 differences between the C-terminal and N-terminal lobes in vivo. *Cell* 62, 165–174
- 654 111 Kerboeuf, D. *et al.* (1993) Calmodulin is essential for assembling links necessary for
655 exocytotic membrane fusion in *Paramecium*. *EMBO J.* 12, 3385–3390
- 656 112 Nouvian, R. *et al.* (2011) Exocytosis at the hair cell ribbon synapse apparently operates
657 without neuronal SNARE proteins. *Nat Neurosci* 14, 411–3
- 658 113 Bright, L.J. *et al.* (2010) Comprehensive analysis reveals dynamic and evolutionary
659 plasticity of Rab GTPases and membrane traffic in *Tetrahymena thermophila*. *PLoS Genet.*
660 6, e1001155
- 661 114 Olbricht, K. *et al.* (1984) Synchronous exocytosis in *Paramecium* cells. II.
662 Intramembranous changes analysed by freeze-fracturing. *Exp. Cell Res.* 151, 14–20

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^{2+} 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^{2+} in trichocyst
773 exocytosis [53].

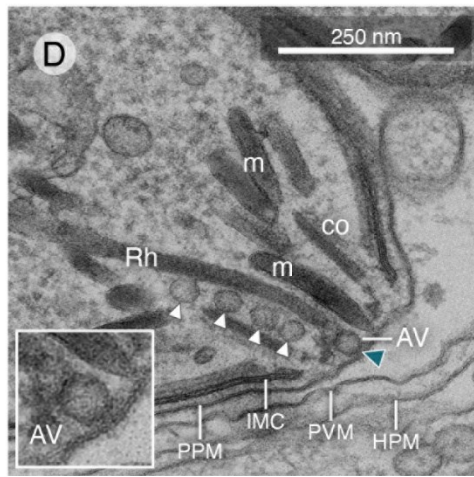
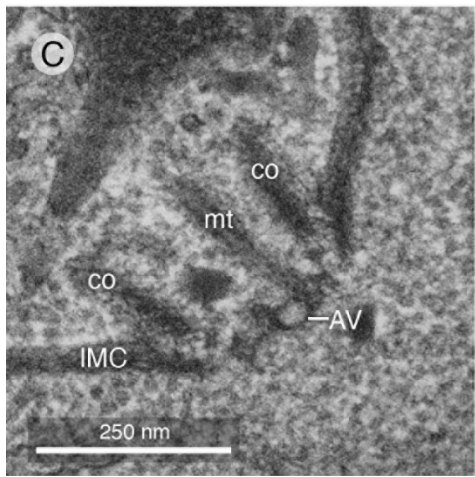
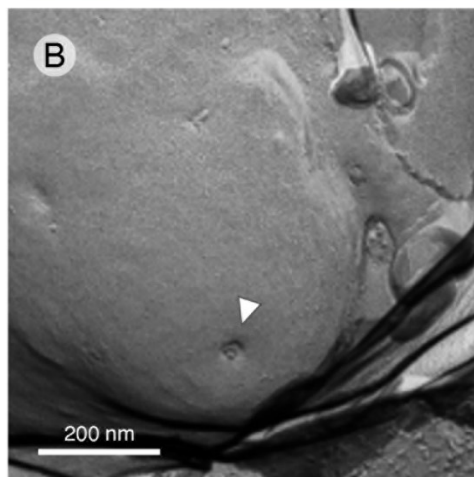
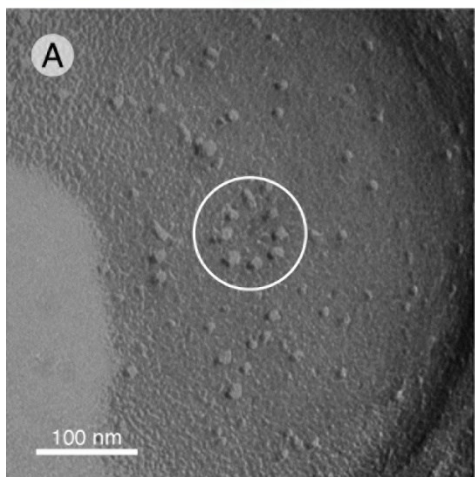
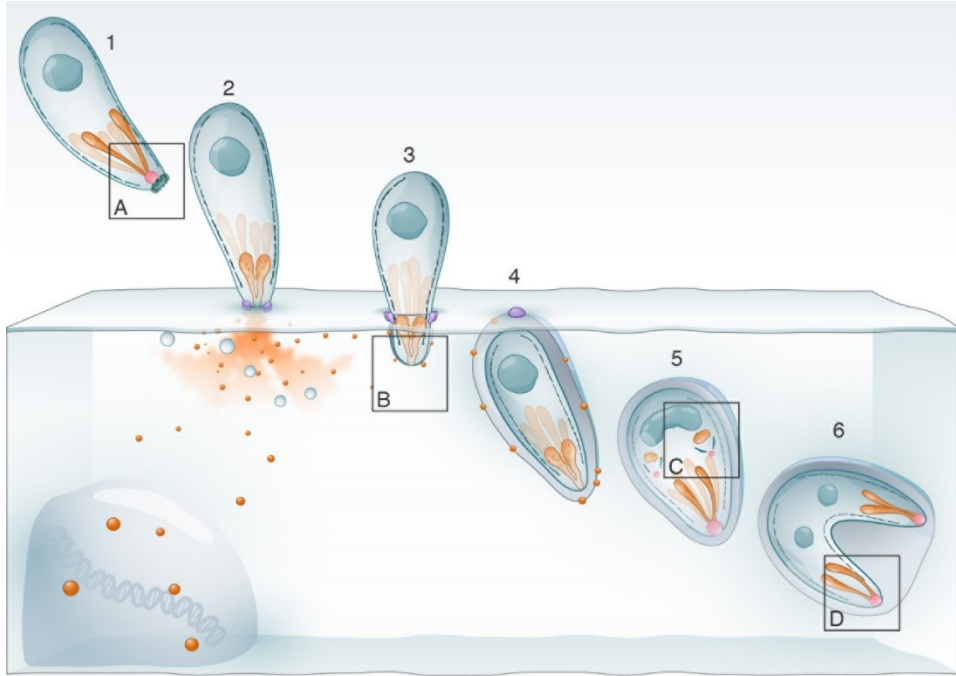
774

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 Ca²⁺-sensing C2 domain. TgFer2 has homology to the ferlin family
798 of double C2 domain-containing Ca²⁺ 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].

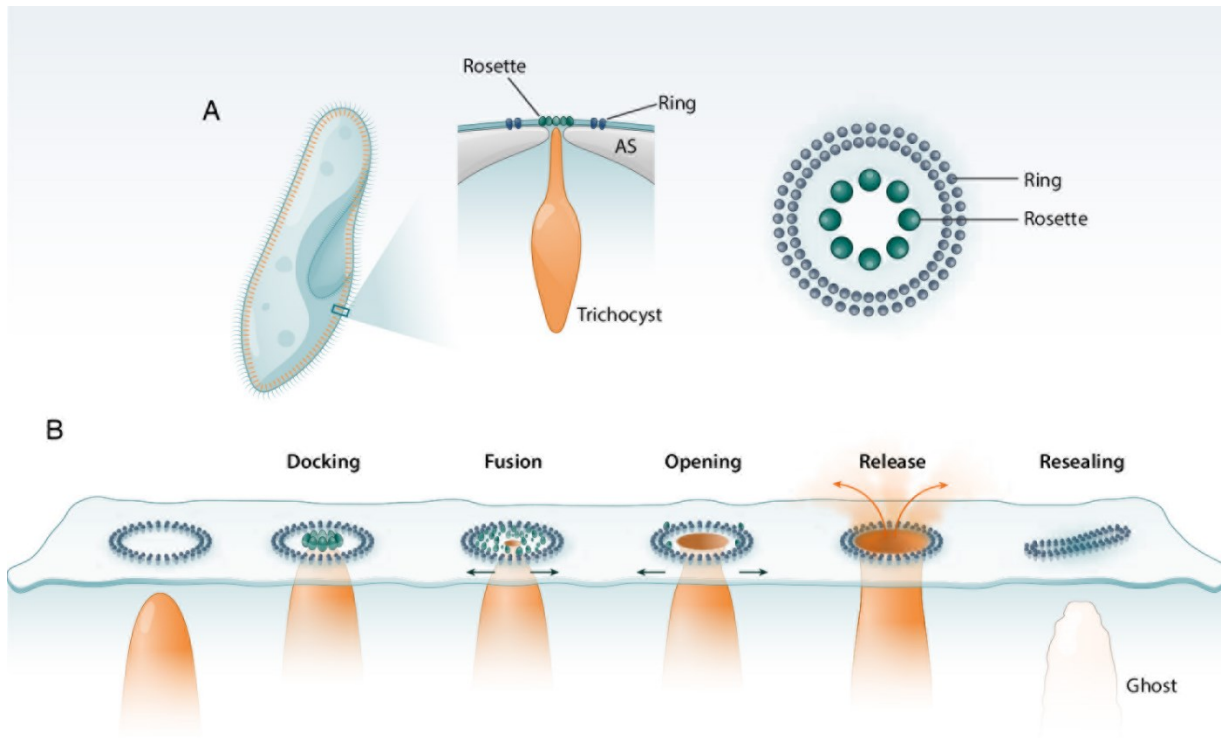
809



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].

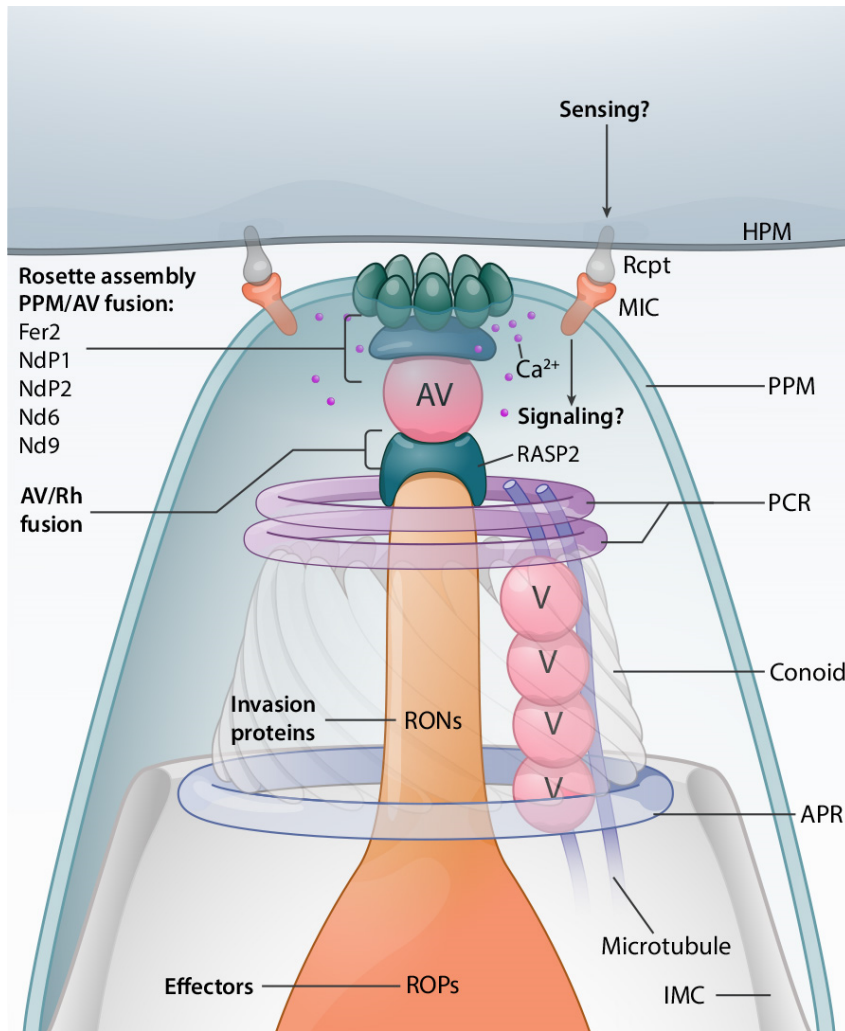
841



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)P₂) via its Ca²⁺-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 Ca²⁺-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