Unraveling the Elusive Rhoptry Exocytic Mechanism of Apicomplexa

Daniela Sparvoli, Maryse Lebrun

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Daniela Sparvoli[1] and Maryse Lebrun[1]*

Affiliations

[1]LPHI UMR5235, Univ Montpellier, CNRS, F-34095 Montpellier, France

*corresponding author: maryse.lebrun@umontpellier.fr

ORCIDs:

Daniela Sparvoli: 0000-0002-5992-3753

Maryse Lebrun: 0000-0002-0962-0156

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Abstract

Apicomplexan parasites are unicellular eukaryotes that invade the cells in which they proliferate. The development of genetic tools in *Toxoplasma*, and then in *Plasmodium*, in the 1990s allowed the first description of the molecular machinery used for motility and invasion, revealing a crucial role for two different secretory organelles, micronemes and rhoptries. Rhoptry proteins are injected directly into the host cytoplasm not only to prompt invasion, but also to manipulate host functions. Nonetheless, the injection machinery has remained mysterious, a major conundrum in the field. Here we review recent progress in uncovering structural components and proteins implicated in rhoptry exocytosis, and explain how revisiting early findings and considering the evolutionary origins of Apicomplexa contributed to some of these discoveries.

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

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

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

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

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

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

The elusive mechanism of rhoptry secretion

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

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

Ultrastructure of rhoptry secretion

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

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

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

Signaling for rhoptry secretion

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

Rhoptry Exocytosis: lessons from old studies in Ciliata

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

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

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

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

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

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

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

Reconsidering the frame of rhoptry exocytosis

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

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

parasites or *Plasmodium* spp.; not even in the most comprehensive ultrastructural study of rhoptry biology of *P. falciparum* [22]. This study described direct fusion between rhoptries and the PPM, a scenario initially postulated also for *Toxoplasma* [34]. Fusion events are quite challenging to capture; thus, the step in which the AV membrane fuses with one of the underlying rhoptry during invasion might be difficult to catch, the AV – becoming contiguous with the rhoptry neck – would be no longer recognizable. Moreover, the question of whether the two fusion events are simultaneous or sequentially ordered is open, and deserves further investigation. Although the exact function of the Nd factors is unknown, the association of TgNd6 with AV, their protein domains, as well as the predicted cellular structures associated with the ciliate counterparts (described in **Box 2**), support a model in which these proteins form part of the connecting material, interact to stabilize the IMPs of the rosette, and contribute to the fusion of the AV (or trichocyst/mucocyst in *Paramecium*/*Tetrahymena*) with the PM (**Box 3** and **Figure 3, Key Figure**). In this model, the fusion of rhoptries with the AV would involve apicomplexan-specific proteins such as the recently discovered rhoptry protein RASP2 (Rhoptry Apical Surface Protein 2) which accumulates on the cytoplasmic face of rhoptry apexes. RASP2 was identified in a bioinformatic search for new rhoptry proteins and proteins related to rhoptry biogenesis and secretion [72]. It is specifically required for rhoptry secretion in both *Toxoplasma* and *Plasmodium*, while its interacting partners RASP1 and RASP3, are dispensable. In a parallel study, *P. falciparum* RASP2, named *P. falciparum* Cytosolically Exposed Rhoptry Leaflet Interacting protein 1 (PfCERLI1), was also shown to be essential for rhoptry secretion and invasion, although characterized as a rhoptry-bulb protein [73]. Its homolog, PfCERLI2, appears to be required for rhoptry protein processing and the formation of morphologically wildtype rhoptries, and is consequently important for parasite invasion [74]. In *Toxoplasma,* degenerate Ca^{2+} lipid-binding-like (C2) and Pleckstrin Homology-like (PH) domains in TgRASP2 bind specifically to phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), and this is crucial for rhoptry secretion [72]. Interestingly, exocytosis of dense-core vesicles in mammals also depends on interactions between effectors containing C2 and PH domains with PA and $PI(4,5)P_2$ [75,76]. This analogy led to the proposal that RASP2 is involved in the docking and/or fusion of the rhoptries to the PPM upon host-parasite contact. With the new knowledge that rhoptries directly contact the AV [45], RASP2 might rather function at the rhoptry-AV interface. Further work is required to support this model, particularly because of conflicting results regarding the

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

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

An Alveolata-conserved fusion machinery with lineage specificities

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

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

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

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

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

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

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

Concluding remarks

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

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

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

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

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

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Declaration of Interests

The authors declare no competing interests

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Glossary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In *Toxoplasma*, TgFer2, TgNd9 and TgNdP1 are all found in undefined punctate structures throughout the cytosol of the parasites, with TgFer2 being also associated with the IMC and rhoptries in intra- and extracellular tachyzoites, respectively, while TgNd6 and TgNdP2 show, in addition to this dispersed pattern, a distinct accumulation at the apical end of the parasite [45,68]. Despite this intriguing apical localization, TgNd6 and TgNdP2 do not have any transmembrane domains and are thus unlikely to be members of the IMP rosette. Instead they might be part of the density observed underneath the rosette by cryo-ET [45], a structure likely equivalent to the fibrous 'connecting material' described in Ciliata. Unlike TgNd9, TgNdP1, and TgNdP2, TgNd6 appears not to be necessary for rosette assembly based on knockdown analysis by Aquilini et al. [45]. Both its role in rhoptry secretion and its localization in proximity to the AV and PPM raise the possibility that TgNd6 contributes to AV–PPM fusion. *Paramecium* Nd6 also functions at the exocytic site, where it was predicted to control the organization of the IMPs and to promote their interaction with the underlying trichocyst [60], probably regulating the fusion of the organelle with the PM. In contrast, TgFer2 is the only protein among the partners of the Nd complex to have a transmembrane domain. To test whether this factor is important for rosette assembly further 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^{2+}sensing C2 domain. TgFer2 has homology to the ferlin family
798 of double C2 domain-containing Ca^{2+} 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

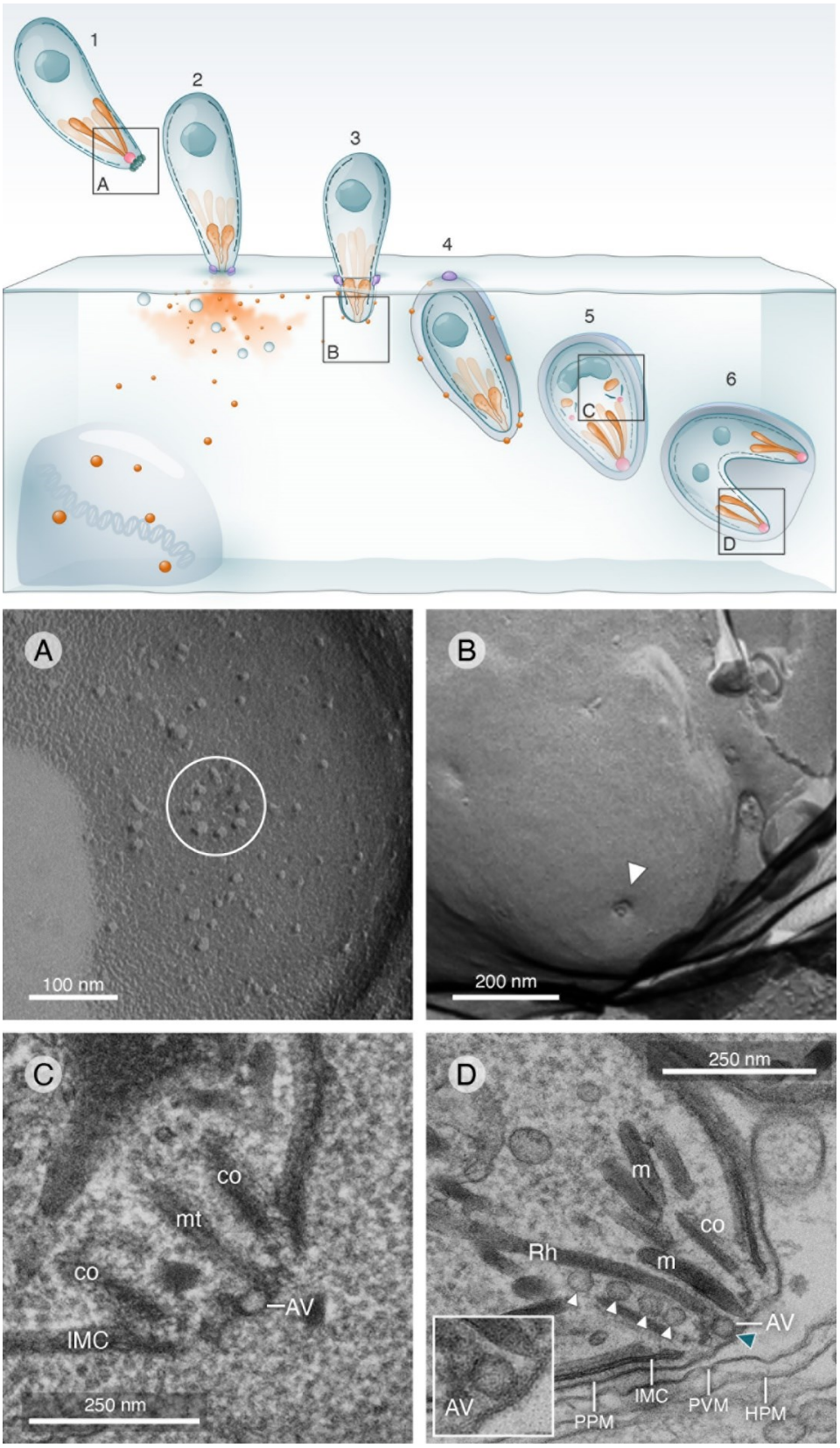
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Figure 1. The fate of the rhoptry secretion apparatus during invasion and intracellular development of *Toxoplasma gondii*. (**1**) An extracellular tachyzoite with two elongated rhoptries (orange organelles) docked to the apical vesicle (AV, pink)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

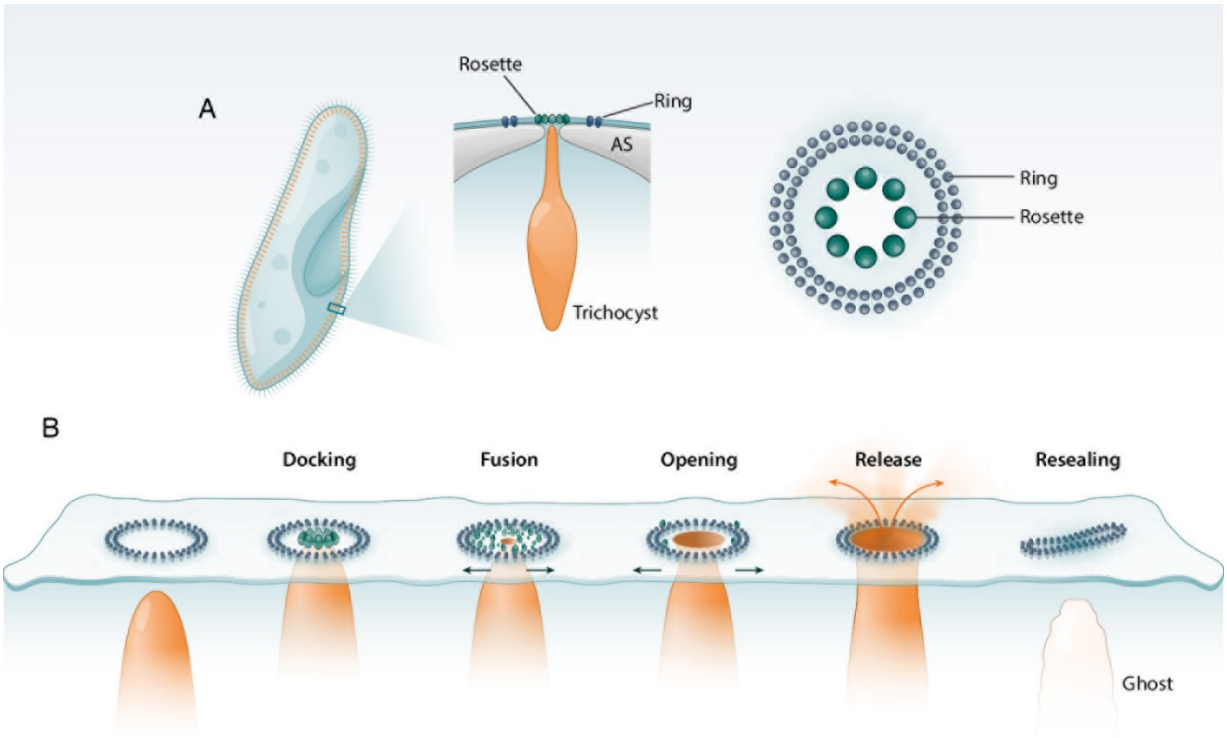

Figure 2. Remodeling of the exocytosis site during docking and stimulated discharge of *Paramecium* trichocysts. (**A**) On the left, schematic drawing of a *Paramecium* cell shown in cross-section, with cilia in blue covering the entire surface of the ciliate, and trichocysts (orange organelles) docked in an ordered fashion at the membrane. In the middle, schematic of the magnified secretory organelle positioned beneath the plasma membrane (PM), and docked at the exocytosis site between the alveolar sacs (AS). The rosette and ring of intramembranous particles (IMPs), embedded in the PM, appear where the trichocyst tip touches the PM. On the right, top view of the exocytosis site, characterized by a 300 nm double-ring of around 80 IMPs surrounding a 60 nm rosette of eight IMPs. (**B**) Sequence of docking and post-docking events following chemically-induced exocytosis of trichocyst. Upon docking of the trichocyst to the PM, in correspondence of the IMP-containing double-ring, a rosette of eight IMPs is formed in the center of the ring. In response to a chemical stimulus, membrane fusion takes place, leading to the formation, of a 20–30 nm pore in the center of the rosette [47,114] where the IMPs – initially very close to the opening site – disperse as the opening enlarges, and a new population of smaller particles appears, likely as a result of the dissociation of a protein complex [47]. The enlarging pore eventually covers the entire area within the large ring, enabling the release of trichocyst

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

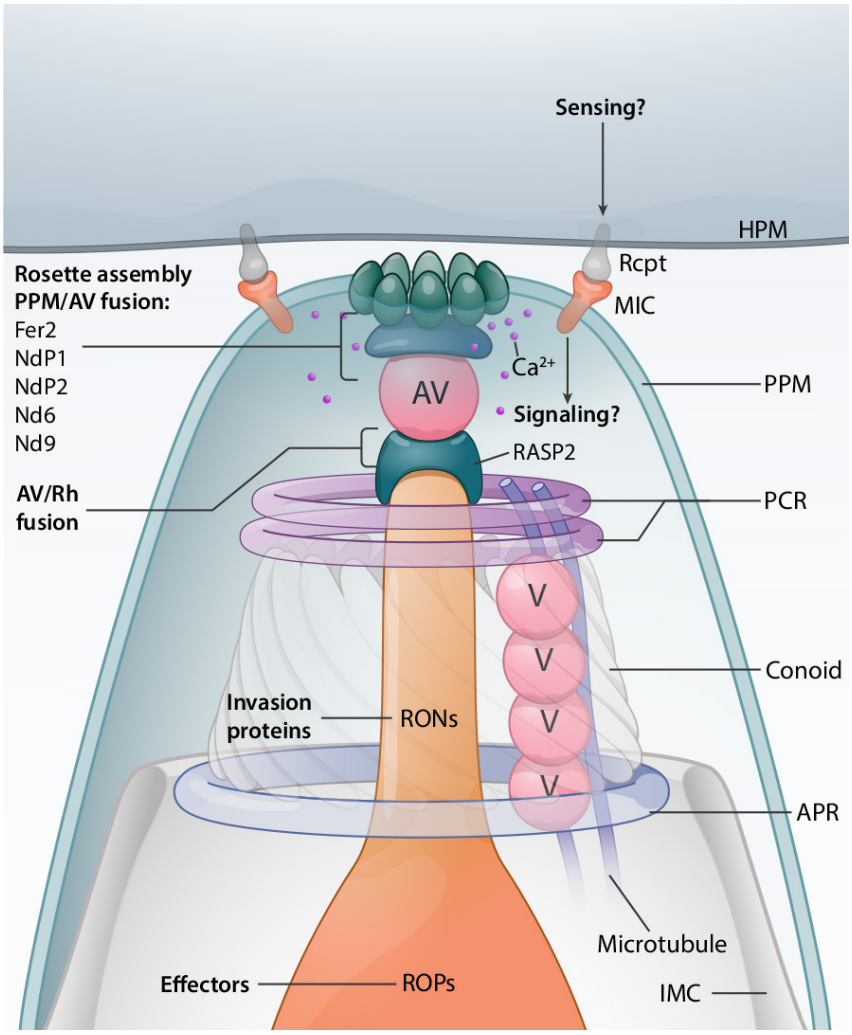

Figure 3. Schematic representation of the apical complex and the fusion events required for rhoptry discharge. Rhoptry exocytosis in *T. gondii* follows the contact of the parasite’s apex with the host cell plasma membrane (HPM), which is mediated by the interaction of microneme proteins (MIC), translocated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

cascade of signals inducing at least two fusion events, one between rhoptry/ies (Rh) and the apical vesicle (AV), and the other between the AV and the PPM, leading to the release of rhoptry neck proteins (RONs) and effectors (ROPs). The Rh/AV fusion might require the rhoptry protein RASP2, which accumulates on the cytoplasmic face of the rhoptry tip, and specifically binds to phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) via its Ca^{2+}-lipid-binding-like (C2) and Pleckstrin Homology-like (PH) domains, perhaps favoring lipid mixing during the fusion event. The rosette assembly and AV/PPM fusion might instead require a repertoire of proteins, Fer2, NdP1, NdP2, Nd6, and Nd9, which could be part of the connecting density observed by cryo-ET between the apical vesicle and the rosette [45]. This interpretation is based on the accumulation of Nd6 and NdP2 at the apical end of the parasite, and on the fact that they interact with Fer2, NdP1, and Nd9. However, additional functions for these proteins cannot be excluded since they all show a cytoplasmic localization [45,68]. In addition, Fer2 was shown to accumulate inside the conoid and the cytosolic face of the rhoptry membrane [68]. The RCC1-like domain (RLD) of Nd6 might act as guanine exchange factor towards small GTPases, as previously seen for proteins involved in vesicular membrane fusion [109]. Via its armadillo-like repeats the Nd9 protein might facilitate protein–protein interactions with other Nd members at the exocytic site in order to assemble the rhoptry secretion apparatus and to stabilize the intramembranous particles (IMPs) of the rosette. The C2 domain-containing proteins NdP2 and Fer2 might contribute to the Ca^{2+}-dependent fusion of the AV at the parasite's membrane. Upon fusion and rhoptry discharge, a new exocytosis machinery might be rebuilt at the parasite apex, by recruiting to the PPM a new apical vesicle from the reservoir of vesicles (V) inside the conoid. This event would stimulate the formation of another rosette and anchoring of a new rhoptry selected from the pool of undocked organelles present at the apex of the parasite. Rearming the secretion apparatus would ensure successive secretion of rhoptries, and thus successive injections of rhoptry effectors into the host, in order to hijack cells that the parasite does not invade. PCR, pre-conoidal ring in violet; APR, apical polar ring in light blue; IMC, inner membrane complex in white.

Highlights

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

Outstanding questions:

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