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**Title:**

**An Alveolata secretory machinery adapted to parasite-host cell invasion**

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26 Apicomplexa are unicellular eukaryotes and obligate intracellular parasites, including

27 *Plasmodium*, the causative agent of malaria and *Toxoplasma*, one of the most widespread

28 zoonotic pathogens. Rhoptries, one of their specialized secretory organelles, undergo

29 regulated exocytosis during invasion<sup>1</sup>. Rhoptry proteins are injected directly into the host cell

30 to support invasion and subversion of host immune function<sup>2</sup>. The mechanism by which they

31 are discharged is unclear and appears distinct from those in bacteria, yeast, animals or plants.

32 Here we show that rhoptry secretion in Apicomplexa shares structural and genetic elements

33 with the exocytic machinery of ciliates, their free-living relatives. Rhoptry exocytosis

34 depends on intramembranous particles in the shape of a rosette embedded into the plasma

35 membrane of the parasite apex. Formation of this rosette requires multiple Non-discharge

36 (Nd) proteins conserved and restricted to Ciliata, Dinoflagellata, and Apicomplexa, that

37 together constitute the superphylum Alveolata. We identified Nd6 at the site of exocytosis in

38 association with an apical vesicle. Sandwiched between the rosette and the tip of the rhoptry,

39 this vesicle appears as a central element of the rhoptry secretion machine. Our results

40 describe a conserved secretion system that was adapted to provide defense for free-living

41 unicellular eukaryotes and host cell injection in intracellular parasites.

42

43 Apicomplexan parasites are invasive and defined by the presence of an apical complex used

44 to recognize and gain entry into host cells. It includes two secretory organelles: micronemes

45 and rhoptries<sup>3</sup>. Microneme proteins are secreted to the parasite surface and mediate motility,

46 host cell recognition and invasion<sup>4</sup>. Rhoptry proteins are injected directly into the host cell<sup>2</sup>,

47 where they anchor the machinery propelling the parasite into the host cell<sup>5</sup>, facilitate nutrient

48 import<sup>6-8</sup>, interfere with the immune response, and modulate gene expression to promote  
49 infection<sup>9</sup>. Rhoptry secretion requires a trigger in the form of microneme proteins binding to  
50 host cell receptors<sup>10,11</sup>. Importantly, rhoptry proteins not only cross the plasma membrane of  
51 the parasite (exocytosis) but also that of the host<sup>1,12</sup>. Rhoptry exocytosis factors identified to  
52 date<sup>11,13,14</sup> are mostly specific to Apicomplexa, suggesting that these cells depend on unique  
53 lineage-restricted secretory mechanisms. Consistent with this, no eukaryotic SNAREs, the  
54 main drivers for fusing vesicles to target membrane in eukaryotic system, have so far been  
55 associated with rhoptry exocytosis. Apicomplexa also lack genes encoding prokaryotic  
56 secretion systems<sup>15</sup>. Thus, how rhoptry effectors are delivered into the host cytoplasm  
57 remains unclear.

58         To explore the mechanisms of rhoptry secretion, we searched for examples of  
59 regulated secretion in organisms phylogenetically closely related to Apicomplexa<sup>16</sup>. All  
60 Alveolata bear alveolar sacs beneath the plasma membrane, which give the superphylum its  
61 name. They contain elaborate membrane-bounded secretory organelles with shared  
62 evolutionary origin<sup>6</sup> but different morphologies and functions. Known as trichocysts in the  
63 Ciliata *Paramecium*, they function in defense—docking at the plasma membrane and  
64 discharging in response to predation<sup>17</sup>. Exocytic membrane fusion occurs at plasma  
65 membrane sites consisting of rosettes of 8-9 intramembranous particles (IMPs)<sup>18,19</sup>. Previous  
66 electron microscopy (EM) studies have described similar rosettes at the apex of several  
67 apicomplexan parasites<sup>20-23</sup>. Though suggested to be involved in exocytic fusion<sup>20</sup>, the  
68 function of the IMPs rosette has never been experimentally addressed in Apicomplexa.  
69 Analysis of *Paramecium tetraurelia* mutants defective in both trichocyst exocytosis and  
70 rosette assembly<sup>24,25</sup> led to identification of *nd* (non-discharge) genes<sup>26-29</sup> (Supplementary  
71 Table 1). To determine if similar factors could be involved in exocytosis of secretory  
72 organelles in Apicomplexa, we first searched for Nd homologs in the tree of life. Genome

73 mining for *nd* genes and phylogenetic analyses revealed that *nd6* and *nd9* are conserved in  
74 Ciliata, but also in Dinoflagellata, Chromerida and Apicomplexa (Extended data Fig. 1 and  
75 Extended Data 1). We also found Nd9 homolog in the Perkinsozoa, a sister group of  
76 Dinoflagellata. Altogether, this analysis suggested a conserved evolution and common  
77 function of *nd* genes across Alveolata.

78 To define the parasite localization of Nd proteins, we tagged *nd6* (TGGT1\_248640)  
79 and *nd9* (TGGT1\_249730) at the endogenous loci in *Toxoplasma gondii*, an experimentally  
80 tractable apicomplexan (Extended data Fig. 2). Both proteins displayed a punctuate signal  
81 throughout the cytoplasm, but in addition *TgNd6* accumulated at the apical tip of the parasite  
82 (Fig. 1a, b). A closer look at the apical tip by immuno-EM localized *TgNd6* at the site of  
83 rhoptry exocytosis, in association with the parasite plasma membrane and an underlying  
84 membranous spheroid known as the “apical vesicle (AV)” (Fig. 1c). Despite previously  
85 observed by EM in *Toxoplasma*<sup>21,30</sup> and suggested to be linked to rhoptries<sup>22,30</sup>, the  
86 composition and function of the AV remain unknown.

87 Because *TgNd6* and *TgNd9* were predicted to be fitness-conferring genes<sup>31</sup>, in order  
88 to investigate their function we generated inducible knockdown mutants using an auxin-  
89 inducible degron for *TgNd6*<sup>32</sup> and tetracycline-induced repression for *TgNd9*<sup>33,34</sup> (Extended  
90 data Fig. 2). Parasites conditionally depleted of *TgNd6* or *TgNd9* showed reduced plaque  
91 formation on fibroblast monolayers, indicating the inability of both mutants to efficiently  
92 complete the lytic cycle (Extended Data Fig. 3a). *Tgnd6*-iKD and *Tgnd9*-iKD mutants  
93 showed no detectable defects in their intracellular replication, egress, conoid protrusion,  
94 motility, or host cell attachment (Extended Data Fig. 3b-g). In contrast, invasion was severely  
95 impaired for both mutants (Fig. 1d).

96 To understand the mechanistic basis of this invasion defect, we evaluated microneme  
97 and rhoptry secretion in the *Tgnd6*-iKD and *Tgnd9*-iKD mutants. Release of the micronemal

98 protein AMA1 into the supernatant was unimpeded by the absence of *TgNd6* or *TgNd9* (Fig.  
99 1e). In contrast, rhoptry secretion was significantly impaired by the loss of *TgNd6* or *TgNd9*  
100 as revealed by quantifying the release of rhoptry protein ROP1 into host cells using  
101 immunofluorescence assays (IFA)<sup>35</sup> (Extended Data Fig. 3h), or the delivery of Cre  
102 recombinase fused to the rhoptry protein toxofilin into the nucleus of a suitable reporter cell<sup>36</sup>  
103 (Fig. 1f). This was not due to defects in rhoptry biogenesis, as the mutants showed normal  
104 organelle formation and apical positioning, as judged by IFA and EM (Extended Data Fig. 4).  
105 Analysis by freeze fracture EM of the apex of *TgNd6*-iKD mutants showed no significant  
106 reduction in the presence of the apical rosette (Fig. 1g). In contrast, *T. gondii* depleted of  
107 *TgNd9* displayed a strong decrease in cells with a rosette at the parasite apex (Fig. 1g). This  
108 last result link rosette formation with rhoptry secretion and support an evolutionarily  
109 conserved mechanism of regulated exocytosis in Alveolata.

110 To determine whether other Apicomplexa require *nd* genes for rhoptry function, we  
111 analysed Nd9 in *Plasmodium falciparum*, the causative agent of the deadliest form of  
112 malaria. We confirmed that *P. falciparum* intracellular merozoites possess a fusion rosette of  
113 8 IMPs (Fig. 2a). As *nd9* is also predicted to have a fitness cost in *P. falciparum*<sup>36</sup>, we used  
114 the rapamycin-inducible dimerizable Cre recombinase (DiCre) system<sup>37</sup> to conditionally  
115 excise the low expressed *Pfnd9* gene (PF3D7\_1232700) (Extended Data Fig. 5a-d). DiCre  
116 mediated ablation of *Pfnd9* resulted in substantial reduction in parasite proliferation (Fig. 2b),  
117 which was due to the inability of *Pfnd9*-iKO mutants to reinvade host cells while their  
118 intracellular development and egress were unaffected (Fig. 2b, c; Extended Data Fig. 5e). As  
119 in *T. gondii*, microneme secretion was unaltered in *Pfnd9*-iKO parasites (Fig. 2d) but rhoptry  
120 secretion was affected (Fig. 2e).

121 To gain a more complete understanding of the molecular composition of the rhoptry  
122 secretion machinery, we searched for Nd interacting proteins in *T. gondii*. *TgNd9* displays

123 two Armadillo repeats while *TgNd6* shows homology with GDP/GTP exchange factors  
124 (GEFs) known to activate GTPases (Fig. 3a). We used *TgNd9* for immunoprecipitation (IP)  
125 experiments as we found *TgNd6* to be largely insoluble (Extended data Fig. 2g). Mass  
126 spectrometry analysis revealed robust interaction of *TgNd9* with *TgNd6* and with *TgFER2*  
127 (TGGT1\_260470), a member of the ferlin calcium sensor family, known to be essential for  
128 *Toxoplasma* rhoptry secretion<sup>13</sup> (Fig. 3a and Supplementary Table 2). The *TgNd9* IP also  
129 enriched TGGT1\_222660, a protein harboring Armadillo repeats and Leucine Rich Repeats,  
130 named hereafter *TgNdP1* (Nd Partner 1) and TGGT1\_316730 (*TgNdP2*: Nd Partner 2), a  
131 protein with a C2 calcium lipid binding domain (Fig. 3a). Both genes are broadly shared  
132 among Alveolata (Extended Data Fig. 1c, d and Supplementary Data 1) and predicted to be  
133 fitness-conferring in *Toxoplasma*<sup>31</sup> and *Plasmodium*<sup>38</sup>. In contrast, *TgNd9*-IP interactor  
134 TGGT1\_277840, a GTPase, is restricted to Apicomplexa and Dinoflagellata, and  
135 TGGT1\_253570 is only found in the apicomplexan subgroup Coccidia. Importantly, all these  
136 proteins are also recovered when using reverse IP with tagged-*TgNdP1* protein  
137 (Supplementary Table 3).

138 To learn more about the new conserved partners of *TgNd9*, we generated *T. gondii*  
139 lines in which *TgNdP1* or *TgNdP2* was tagged by an epitope and could be ablated  
140 conditionally (Extended Data Fig. 6). Both proteins appear as punctate cytoplasmic staining  
141 (Fig. 3b) but *TgNdP2*—like *TgNd6*—also appears as a dot at the apical tip of the parasite,  
142 although consistently with lower intensity. Depletion of *TgNdP1* or *TgNdP2* resulted in a  
143 profound growth defect (Extended Data Fig. 7a) that we linked to impairment of host cell  
144 invasion and rhoptry secretion (Fig. 3c, d and Extended Data Fig. 7). Again, loss of rhoptry  
145 secretion went hand-in-hand with loss of the rosette in *Tgndp1*-iKD and *Tgndp2*-iKD  
146 parasites (Fig. 3e).

147 To further validate our conservation data and broaden our discoveries back to Ciliata,  
148 we generated knockouts for orthologues of both NdP1 (TTHERM\_01287970, *TtnΔndp1*) and  
149 NdP2 (TTHERM\_00498010; *TtnΔndp2*) in the free-living Ciliata *Tetrahymena thermophila*  
150 (Extended Data Fig. 8a, b). The homologous organelles to *Paramecium* trichocysts in  
151 *Tetrahymena* are called mucocysts and are non-essential for laboratory growth. We found  
152 that *TtnΔndp1* and *TtnΔndp2* cells were defective in mucocyst secretion, which was triggered  
153 by exposure to dibucaine<sup>39</sup> (Fig. 3f). We further showed that the impairment of exocytosis  
154 was not due to defects in mucocyst biogenesis, given that mucocyst maturation (as measured  
155 by processing of mucocyst pro-proteins) and trafficking (monitored by IFA) remained  
156 unaltered (Extended Data Fig. 8c, d). Taken together, we identified a complex of proteins  
157 essential for organellar exocytosis and rosette assembly, conserved across Alveolata.

158 The exact position of the rosette relative to the apical tip of the rhoptry and the  
159 enigmatic AV remained elusive, since freeze-fracture technique—used to image the rosette  
160 on the membrane—does not capture the internal structures at the same time. To overcome  
161 this limitation, we imaged the *Toxoplasma* apex by cryo-electron tomography (cryo-ET)—a  
162 technique combining the advantages of 3D imaging with molecular resolution to reveal  
163 ultrastructure *in situ* in its native biological context. We were able to simultaneously visualize  
164 and define three linked elements — 1) the rosette (dark blue), 2) the AV (magenta), and 3)  
165 the apical tip (cyan) of the rhoptry (orange) (Fig. 4a and b, Extended Fig. 9). The rosette  
166 showed an 8-fold rotational symmetry around a central axis (Fig. 4c and d) and extended  
167 under the parasite plasma membrane (light blue) to interact with the AV (Fig. 4c). Thus, the  
168 rosette is tightly sandwiched between the AV and the plasma membrane and extensively  
169 interacts with both membranes. The AV in turn sits over the rhoptry tip (Fig. 4a and b). The  
170 contiguity of all elements from the rhoptry tip to the plasma membrane supports the idea that  
171 the rosette and the AV are integral part of the rhoptry secretion machinery. Interestingly, the



172 connection between AV and rosette is sometimes observed even in the absence of a docked  
173 rhoptry (Fig. 4e) suggesting that they assemble independently of rhoptry docking. Supporting  
174 this hypothesis, when we imaged the *Toxoplasma* ARO mutant in which rhoptries fail to dock  
175 and are dispersed in the cytoplasm<sup>40</sup>, both the rosette (seen by freeze-fracture; Fig. 4f) and the  
176 AV (seen by EM; Fig. 4g) were still present at the apex of the parasite. Altogether, these  
177 results positioned the rosette and AV at the heart of the exocytic machinery in Apicomplexa  
178 and implied that rhoptries do not make contact and fuse directly with the plasma membrane.

179 Our work breaks ground on the molecular and structural mechanisms for rhoptry  
180 exocytosis in Apicomplexa. We defined the apical rosette of IMPs as the site for rhoptry  
181 exocytosis in Apicomplexa, and we characterized an Alveolata-specific Nd complex  
182 necessary for the assembly of the rosette. In *Paramecium*, physiological studies predicted  
183 *PtNd6* to be active at the plasma membrane<sup>24</sup> while *PtNd9* appeared to be a diffusible  
184 cytoplasmic component interacting with both trichocyst and plasma membranes<sup>41,42</sup>.  
185 However, both the localization and identity of partners in Ciliata remained unknown. We  
186 localized *TgNd9* to the cytoplasm and *TgNd6* to the site of exocytosis in *Toxoplasma*. We  
187 found both proteins to form a complex that included *TgNdP1* and *TgNdP2*, which we  
188 demonstrated to be essential for organelle exocytosis in both Ciliata and Apicomplexa. This  
189 complex further includes proteins with C2 domains (*TgFER2* and *TgNdP2*), a homolog of the  
190 membrane fusion Ferlin family (*FER2*)<sup>13</sup>, a GTPase (*TGME49\_277840*), and a putative GEF  
191 protein (*TgNd6*). These proteins and their domains yield a regulatory model in which calcium  
192 signaling and nucleotide binding and hydrolysis constitute key steps that control rosette  
193 assembly and/or organelle discharge (more discussion in Supplementary text).

194 In this study, we also shed new light on the enigmatic AV. Described in  
195 *Toxoplasma*<sup>22,30</sup>, it is also visible in early micrographs from other apicomplexan  
196 parasites<sup>20,43,44</sup> (Extended Data Fig. 10). We show that the AV is on one side tightly

197 connected with the plasma membrane via the rosette, and from the other sits on the tip of the  
198 rhoptry, precluding a direct link and fusion between the rhoptry and the plasma membrane. In  
199 Ciliata, where no comparable AV is present, the docking and fusion of the trichocysts to the  
200 plasma membrane shapes the rosette. Diverging from this scenario, we also showed that the  
201 docking of the rhoptries in *Toxoplasma* is dispensable for rosette formation (Fig. 4e-g), which  
202 suggests that the assembly of the rosette in *Toxoplasma* might be instead induced by docking  
203 of the AV.

204         While *Paramecium* trichocysts are discharged into the environment to thwart  
205 predators, apicomplexan rhoptries translocate their contents directly into the cytoplasm of  
206 host organisms to infect and parasitize. The difference in terms of presence or absence of the  
207 AV (Fig. 4h) suggests that the AV is an adaptation in Apicomplexa for parasitism and cell  
208 invasion. Its presence may reflect additional complexity of the secretory machinery in  
209 apicomplexans, in which exocytosis must be coupled with injection of rhoptry content  
210 through the barrier of a host cell membrane. In support of this hypothesis, a similar vesicle is  
211 present at the apex of *Perkinsus marinus* (previously named *Dermocystidium marinum*)<sup>45</sup>.  
212 This organism possesses rhoptries and invades and parasitizes the cells of oysters.  
213 Phylogenetically, *Perkinsus* falls between Dinoflagellata and Apicomplexa and is viewed as a  
214 basal taxon and example of early adaptation to parasitism.

215         The rosette and the set of Nd proteins highlight a common ancestry for the fusion  
216 machinery linked to secretory organelles in two groups of protists that diverged hundreds of  
217 millions of years ago and have adopted radically different lifestyles. The architecture and  
218 molecular composition of the rhoptry system that enables the intracellular parasitism of  
219 Apicomplexa is now revealed. Future studies combining high resolution structural biology  
220 approaches with the genetics and cell biology of *Toxoplasma* will allow a full understanding

221 of how this fascinating mechanism unfolds and is regulated. Such an understanding may find  
222 application in designing strategies against malaria, cryptosporidiosis and toxoplasmosis.

223

## 224 **Methods**

225

### 226 **Parasite Immunofluorescence microscopy**

227 Immunofluorescence assays (IFAs) on intracellular *T. gondii* parasites were conducted as  
228 previously described<sup>46</sup>. Briefly, cell monolayers were washed and fixed in 4%  
229 paraformaldehyde (PFA) in Phosphate Saline Buffer (PBS) for 20 min. After three washes  
230 with PBS, cells were permeabilized with 0.1% (v/v) Triton X-100 or with saponin 0.1% (v/v)  
231 (for invading parasites) in PBS for 5 min, blocked with 10% fetal bovine serum in PBS  
232 (PFBS) for 45 min, incubated with primary Abs diluted in 2% PFBS, washed three times, and  
233 then incubated with secondary antibody. The coverslips were mounted onto microscope  
234 slides using Immunomount (Calbiochem).

235 For IFAs on *P. falciparum*, thin blood smears of highly synchronized DMSO and rapamycin-  
236 treated *PfNd9*-iKO schizonts were air-dried, fixed in 4% (w/v) PFA and permeabilized in  
237 0.1% (v/v) Triton X-100. After blocking in 1.5% BSA, samples were probed for 1 h with the  
238 suitable antibodies: rabbit anti-*PfAMA1*<sup>47</sup> antibody (1:1000); mouse anti-*PfMSP1*<sup>48</sup> antibody  
239 (1:1000); mouse anti-*PfRAP2*<sup>49</sup> antibodies (1:500). The secondary antibodies used were  
240 Alexa Fluor 488 and 594-conjugated antibodies against mouse or rabbit IgG (highly cross-  
241 adsorbed) both diluted 1:4000 (Molecular Probes). Samples were then counterstained with  
242 Hoechst and slides mounted with Vectashield® antifade mounting medium.

243 Except when specified, observations were performed with a Zeiss Axioimager Z1  
244 epifluorescence microscope equipped with a Zeiss AxioCam MRm CCD camera and  
245 100X/1.4 Oil Plan Apochromat objective. Images were processed using Zen Blue 2.3 pro

246 (Zeiss) software. Imaging of *TgNd6*, *TgNd9*, *TgNdP1* and *TgNdP2* co-localization with  
247 rhoptry protein *TgARO* (Fig. 1 and Fig. 3) was performed on a ZEISS confocal LSM880,  
248 equipped with an Airyscan detector and a 63X/1.4 Oil Plan Apochromat objective. Zen Black  
249 (Zeiss) was used for image airyscan processing. Z-stack images and z-projection images were  
250 denoised, adjusted in brightness and contrast, and colored with the program Fiji<sup>50</sup>.

251

252 Adjustments for brightness and contrast were applied uniformly on the entire images and  
253 when required, matching pairs of images were recorded with the same exposure time and  
254 processed identically. All optical images were collected at the Montpellier Ressources  
255 Imagerie facility of the University of Montpellier (MRI, [www.mri.cnrs.fr](http://www.mri.cnrs.fr)).

256

## 257 **Invasion assays**

258 *Toxoplasma* plaque assays, replication assays, and two-color invasion assays were performed  
259 as previously described<sup>35,51</sup>. Briefly, to synchronize invasion, freshly egressed tachyzoites ( $5$   
260  $\times 10^6$ ), pre-treated for 48 h  $\pm$  ATc (*Tgnd9*-iKD and *Tgndp1*-iKD) or 24 h  $\pm$  IAA (*Tgnd6*-iKD  
261 and *Tgndp2*-iKD), were harvested and settled on ice for 20 min on HFF monolayer grown on  
262 coverslips in 24-well plates. Invasion was allowed for 5 min at 38 °C and stopped by fixation  
263 with 4% PFA in Hank's Balanced Salt Solution (HBSS) for 20 min at room temperature. To  
264 detect extracellular parasites, immuno-detection was performed with the mouse mAb T4 1E5  
265 anti-SAG1 antibody (dilution 1:1000) in 2% FCS/HBSS, without previous permeabilization.  
266 After permeabilization with 0.01% saponin for 15 min, a second IFA was performed using  
267 rabbit anti-ROP1 antibody (dilution 1:1000) to label the parasitophorous vacuole of  
268 intracellular parasites. Extracellular and intracellular parasites were counted by microscopic  
269 examination of at least 20 fields per coverslip (n=3 coverslips). Graphs show the mean of  
270 three independent invasion assays.

271

## 272 **Microneme secretion assay**

273 Microneme secretion in *T. gondii* was assayed by monitoring the release into the culture  
274 medium. Freshly egressed *T. gondii* tachyzoites, pre-treated for 48 h ± ATc (*Tgnd9*-iKD and  
275 *Tgndp1*-iKD) or 24 h ± IAA (*Tgnd6*-iKD and *Tgndp2*-iKD), were harvested by centrifugation  
276 at 600 g, and washed twice in intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM MgCl<sub>2</sub>,  
277 2 mM EGTA, 5.6 mM glucose and 25 mM HEPES, pH 7.2), prewarmed to 37°C. Parasites  
278 were resuspended in DMEM (supplemented with 2 mM glutamine) ± propranolol 500 µM,  
279 and incubated at 37°C for 20 min to induce microneme secretion. Parasites were pelleted at  
280 1000 g for 5 min at 4°C, washed once in PBS and stored at -20°C. Supernatants were  
281 centrifuged at 2000 g for 5 min, at 4°C and used as ESA (excreted/secreted antigen). Pellets  
282 and ESA samples were analysed for micronemal protein (AMA1) by Western blot.

283 Microneme secretion in *P. falciparum* was assayed by monitoring the surface translocation of  
284 AMA1. Highly synchronized DMSO and rapamycin-treated *Pfnd9*-iKO schizonts were  
285 exposed to 10 µM E64 to block egress. IFAs were then performed with anti-*Pf*AMA1  
286 antibodies to analyse AMA1 translocation to the merozoite surface.

287

## 288 **Rhoptry secretion assays**

289 To evaluate the efficiency of rhoptry secretion in *T. gondii*, we expressed in the *Tgnd*-iKD  
290 and *Tgndp*-iKD lines a rhoptry secretion reporter protein consisting of the rhoptry protein  
291 toxofilin fused with Cre-recombinase, a nuclear localization signal (NLS), and a myc tag,  
292 collectively called Secreted Cre, Epitope-tagged (SeCrEt)<sup>36</sup>. The reporter strains generated  
293 were called *Tgnd9*-iKD\_SeCrEtUPRT, *Tgndp1*-iKD\_SeCrEtUPRT, and *Tgnd6*-  
294 iKD\_SeCrEtHXGPRT. Ds Red cells, constitutively expressing DsRed and able to switch to  
295 eGFP expression upon Cre-mediated recombination, were used as reporter cells<sup>36</sup>. DsRed

296 Cells were maintained at < 50% confluence (regardless of the flask size) and plated the day  
297 before parasite infection, at a density of  $2 \times 10^5$  cells /ml (in T25 flasks). Tachyzoites pre-  
298 treated for 48 h  $\pm$  ATc (*Tgnd9*-iKD and *Tgndp1*-iKD) or 24 h  $\pm$  IAA (*Tgnd6*-iKD) were  
299 collected and used to infect the DsRed cells at a multiplicity of infection (MOI) of 3. 24 hours  
300 post-invasion (hpi) infected DsRed cells were trypsinized and examined by fluorescence-  
301 activated cell sorting (FACS) to assess the percentage of Ds-Red (rhoptry secretion  
302 impairment) and GFP (successful rhoptry secretion) expressing cells. Number of GFP  
303 positive cells were normalized as the percentage compared to 100% in the control (-IAA or -  
304 ATc).

305 Additionally, rhoptry secretion has been also quantified by classical e-vacuole assay<sup>35</sup>.

306 Freshly egressed parasites, pre-treated for 48 h  $\pm$  ATc (*Tgnd9*-iKD and *Tgndp1*-iKD) or 24 h  
307  $\pm$  IAA (*Tgnd6*-iKD), were preincubated with 1  $\mu$ M of cytochalasin D (cytD) for 10 min and  
308 then incubated with HFF cells in the presence of cytD for 15 min. IFAs were then performed  
309 with anti-ROP1 (rhoptry secretion) and anti-SAG1 (parasite surface), and the number of  
310 ROP1 stainings per field was determined by microscopic examination of at least 20 fields per  
311 coverslip (n = 3 coverslips).

312 To quantify rhoptry secretion in *P. falciparum*, the same amount of DMSO and rapamycin-  
313 treated *Pfnd9*-iKO purified schizonts were arrested with 1.5  $\mu$ M C2 for 4 h before being  
314 washed twice in order to allow them to egress. Parasites were then incubated for 30 min in  
315 complete medium with 1  $\mu$ M cytD in the presence of red blood cells (RBCs). IFAs were then  
316 performed with anti-*Pf*RAP2 antibodies to visualise rhoptry secretion events ('spits' of RAP2  
317 export into the RBC). The number of secretion events were counted over the total of RBCs  
318 by microscopic examination of 11 fields (~3000 events analysed).

319

## 320 **Freeze-fracture**

321 Cells used for the freeze-fracture were harvested, pelleted and fixed in 2.5% glutaraldehyde  
322 in 0.1 M phosphate buffer at room temperature for 2 h. Following primary fixation, the  
323 samples were rinsed in the same buffer, and kept overnight in 30% glycerol 0.1 M phosphate  
324 solution. The cells were then quickly frozen by immersion in liquid nitrogen under vacuum.  
325 The frozen samples were fractured in a BAL-TEC BAF 060 apparatus; subsequently the  
326 fracture face was shadowed by evaporating platinum 45° (3.2nm) and Carbon 90° (25nm).  
327 The replicas were then washed in 6.5% sodium hypochlorite, rinsed in a chloroform (2/3) -  
328 (1/3) solution, rinsed in distilled water and mounted on copper grids. In order to interpret the  
329 replicas obtained in freeze-fracture technique one must keep in mind that the portion of the  
330 lipid bilayer associated with the exterior of the cell is termed the E-face, or extracellular face.  
331 The portion associated with the interior of the cell is termed the P-face, or protoplasmic  
332 face<sup>52</sup>.

333

#### 334 ***In vitro* growth assay of *P. falciparum* parasite asexual development**

335 The growth capability of *Pfnd9*-iKO mutant parasites was assessed by comparing it with the  
336 *p230p* DiCre parental line<sup>53</sup> ± rapamycin in biological triplicates. DMSO (vehicle control)  
337 and rapamycin-treated cultures were synchronized at ring stages by sorbitol synchronization  
338 and parasitaemia adjusted to 0.2% to follow growth over 3 cycles. Parasites around 30 hpi  
339 were collected at each cycle to determine parasitaemia by counting Giemsa-stained parasites  
340 on at least 1000 RBCs per culture.

341

#### 342 ***P. falciparum* induced egress and time-lapse microscopy**

343 *P. falciparum* egress was imaged using C2 compound to tightly synchronize egress. DMSO  
344 and rapamycin-treated *Pfnd9*-iKO parasites were highly synchronized and blocked 40 hpi  
345 (mature schizonts) with 1.5 μM C2 compound to prevent egress. Four hours later parasites

346 were washed twice with warm RPMI 1640 medium (Gibco), to remove C2, and placed into a  
347 35 mm Dish (MatTek). The parasites suspension was sealed by adhering a 22 x 22 mm  
348 square coverslip to the Dish and the preparation was introduced on a temperature-controlled  
349 microscope stage at 37°C. Bright field images were collected 5 min after washing off the C2  
350 at 1 s intervals over a total of 15 min using a ZEISS AxioObserver Microscope fitted with a  
351 coolsnap HQ2 digital camera and 63X/1.4 Oil Plan Apochromat objective. Images were  
352 exported to MOV movies using ZEN 2 (blue edition) software. Number of egress events in  
353 15 min were normalized as the percentage compared to 100% in the control.

354

### 355 ***Tetrahymena* dibucaine assay to quantify mucocyst secretion**

356 *Tetrahymena* cells were grown to stationary phase ( $10^6$  cells/ml) in 25 ml SPP (2% proteose  
357 peptone, 0.1% yeast extract, 0.2% dextrose and 0.003% ferric-EDTA supplemented with 250  
358  $\mu$ g/ml penicillin G, 250  $\mu$ g/ml streptomycin sulfate, and 0.25  $\mu$ g/ml amphotericin B  
359 fungizone) for 48 h, and then concentrated by centrifugation into a loose 1.5ml pellet. Cells  
360 were stimulated with 2.5 mM dibucaine, vigorously mixed for 30 s and diluted to 15 ml with  
361 10 mM Hepes, pH 7.5, and 5 mM CaCl<sub>2</sub>. After gently mixing, the culture was centrifuged at  
362 1,200 g for 2 min, resulting in the formation of a cell pellet/flocculent bilayer. Quantification  
363 of mucocysts secretion has been done by weighing the flocculent layer overlying the pellet of  
364 cells.

365

### 366 **Transmission electron microscopy**

367 *Tgnd9*-iKD and *TgARO*-iKD, together with the  *$\Delta$ ku80*-TATi parental strain, were treated  
368 with ATc for 72 h. *Tgnd6*-iKD parasites, and  *$\Delta$ ku80*-Tir1 parental strain were treated with  
369 IAA for 24 h. Extracellular parasites were collected in the culture medium after natural egress  
370 and fixed by adding an equal volume of phosphate buffer 0.1 M containing 5% of



371 glutaraldehyde for one hour at RT. Parasites were then centrifuged and resuspended in 1 ml  
372 of fresh buffer with 2.5% glutaraldehyde for 2 h before being kept at 4°C until further  
373 processing. Each of the following steps were performed in suspension followed by  
374 centrifugation steps in a tabletop microcentrifuge. Sample were post-fixed with 1% OsO<sub>4</sub> and  
375 1.5% potassium ferricyanide in 0.1 M phosphate buffer for 1 hour at RT, washed in water and  
376 then incubated in 2% uranyl acetate in water overnight at 4°C. Tachyzoites were then  
377 dehydrated in growing concentration of acetonitrile, followed by impregnation in Epon118:  
378 acetonitrile 50:50 for 2 hours, 90:10 for 2 additional hours and then overnight in pure epon.  
379 Pellets were polymerized in fresh Epon for 48 h at 60°C. 70 nm ultrathin sections were cut  
380 with a Leica ultracut (Leica microsystems), counterstained with uranyl acetate and lead  
381 citrate.

382

### 383 **Immunoelectron microscopy**

384 *Tgnd6*-HA<sub>3</sub> parasites, infected fibroblast monolayers were trypsinized and fixed with equal  
385 volume of 8% formaldehyde (FA) in phosphate buffer overnight at 4°C and resuspended in  
386 4% fresh FA until further processing. Cells were then incubated with 0.1 % glycine in  
387 phosphate buffer, pelleted and embedded in 12% gelatin, cut in small blocks (< 1 mm) and  
388 infused in 2.3 M sucrose on a rotating wheel for 24 h at 4°C. Gelatin blocks were mounted on  
389 specimen pins and frozen in liquid nitrogen. Cryo-sectioning was performed on a Leica UC7  
390 cryo-ultramicrotome, 70 nm cryosections were picked-up in a 1:1 mixture of 2.3 M sucrose  
391 and 2% methylcellulose in water and stored at 4°C. For on-grid immunodetection, grids were  
392 floated on 2% gelatin in PBS for 30 min at 37°C to remove methylcellulose/sucrose mixture,  
393 then blocked with 1% skin-fish gelatin (SFG, Sigma) in PBS for 5 min. Successive  
394 immunolabeling steps were performed on drops as follows: 1) rat monoclonal anti-HA  
395 antibodies (clone 3F10, Roche) in 1% BSA, 2) rabbit polyclonal anti-rat IgG antibody

396 (Sigma) in 1% BSA, 3) Protein A-gold (UMC Utrecht) in 1% BSA. Four washes (2 min  
397 each) with 0.1% BSA were performed between steps. After Protein A treatment, grids were  
398 washed four times for 2 min each with PBS, fixed 5 min in 1% glutaraldehyde in water, and  
399 washed six times for 2 min each with distilled water. Grids were then incubated with 2%  
400 methylcellulose: 4% uranyl acetate 9:1 on ice in the dark for 15 min, picked-up on a wire  
401 loop and air-dried.

402 All chemicals were from Electron Microscopy Sciences (USA), solvents were from Sigma.  
403 Observations and image acquisition were performed on a Jeol 1200 EXII transmission  
404 electron microscope at the Electron Microscopy Platform of the University of Montpellier  
405 (MEA; <http://mea.edu.umontpellier.fr>). Transmission electron microscopy images were  
406 processed with Fiji for contrast optimization and the Image J plugin was used to make the  
407 EM panels.

408

#### 409 **Cryo-electron-tomography (Cryo-ET)**

410 Freshly isolated *Toxoplasma gondii* cells were suspended in HBSS along with fiducials (10  
411 nm colloidal gold from Ted Pella for alignment of tilt series). A 4  $\mu$ l drop of the suspension  
412 was applied onto EM grids, excess liquid blotted away, and plunge frozen in a liquid  
413 ethane/propane mixture (pre-cooled using liquid nitrogen) using a EM GP2 automatic  
414 plunger (Leica Microsystems, Wetzlar, Germany)<sup>54</sup>. The blotting chamber was set to 95-  
415 100% relative humidity at 37°C and blotting was done either from the sample side of the grid  
416 or from the back using Whatman filter paper #1. Plunge-frozen grids were subsequently  
417 loaded into autogrid cartridges (ThermoFisher). EM cartridges containing frozen grids were  
418 stored in liquid nitrogen and maintained at  $\leq -170^\circ\text{C}$  throughout storage, transfer and cryo-ET  
419 imaging.

420 Cryo-ET data collection was performed on a ThermoFisher Krios G3i 300 keV field emission  
421 gun cryo-TEM equipped with a 6k x 4k K3 direct electron detector (Gatan, Inc.) at the  
422 Beckman Center for Cryo-Electron Microscopy in the Singh Center for Nanotechnology,  
423 University of Pennsylvania. The camera was operated in electron counting mode to enable  
424 motion correction. An energy filter (Gatan Imaging Filter, Gatan, Inc.) with a slit width of 20  
425 eV was used to increase the contrast of the projection images. Additionally, a Volta phase  
426 plate was used to boost the image contrast at defoci of negative 2-3  $\mu\text{m}$ . A magnification of  
427 33,000X with corresponding pixel sizes of 2.65  $\text{\AA}$  was used for imaging. SerialEM software<sup>55</sup>  
428 was used for all imaging. Cells were first assessed at lower magnifications for suitability of  
429 ice thickness and outer membrane integrity (the parasites sometimes tend to vesiculate their  
430 outer membrane possibly in response to blotting). Once the target cells were identified and  
431 marked, anchor maps were used to revisit these locations and collect tilt-series in an  
432 automated fashion. Each tilt-series was collected from negative 60° to positive 60° with an  
433 increment of 2° in an automated fashion using the low dose functions of tracking and  
434 focusing. The cumulative dose of each tilt-series ranged between 100 and 150  $\text{e}^-/\text{\AA}^2$ . Once  
435 acquired, tilt-series were binned twice or four times before aligned using the 10 nm colloidal  
436 gold as fiducials and reconstructed into tomograms by our in-house automated computation  
437 pipeline utilizing the IMOD software package<sup>56</sup>. For presentation purposes, tomograms were  
438 favorably oriented in three dimensions before averaging a few slices around the slice of  
439 interest (to enhance contrast) using the slicer window in IMOD. In a few cases (Figs. 4b-e,  
440 contrast was further enhanced using the non-linear anisotropic diffusion filter in IMOD.

441

#### 442 **Immunopurification and mass spectrometry analysis**

443 Immunopurification was performed using anti HA magnetic beads (Pierce R88836) as  
444 previously described<sup>57</sup>. Purified proteins were loaded on a SDS-PAGE and digested in gel (2

445 bands per sample) as previously described<sup>58</sup>. Samples were loaded onto a 25 cm reversed  
446 phase column (75 mm inner diameter, Acclaim Pepmap 100® C18, Thermo Fisher Scientific)  
447 and separated with an Ultimate 3000 RSLC system (Thermo Fisher Scientific) coupled to a Q  
448 Exactive HFX (Thermo Fisher Scientific). MS/MS analyses were performed in a data-  
449 dependent mode. Full scans (375 – 1,500 m/z) were acquired in the Orbitrap mass analyzer  
450 with a resolution of 60,000 at 200 m/z. For the full scans, 3e6 ions were accumulated within a  
451 maximum injection time of 60 ms. The twelve most intense ions with charge states  $\geq 2$  were  
452 sequentially isolated (1e5) with a maximum injection time of 45 ms and fragmented by HCD  
453 (Higher-energy collisional dissociation) in the collision cell (normalized collision energy of  
454 28%) and detected in the Orbitrap analyzer at a resolution of 30,000.  
455 Raw spectra were processed using the MaxQuant<sup>59</sup> using standard parameters with label-free  
456 quantification (LFQ) and match between runs<sup>60</sup>. MS/MS spectra were matched against the  
457 UniProt Reference proteomes of *T. gondii* and Human (respectively Proteome ID  
458 UP000001529 and UP000005640) and 250 frequently observed contaminants as well as  
459 reversed sequences of all entries (MaxQuant contaminant database). Statistical analysis were  
460 done using Perseus on LFQ data<sup>61</sup>.

461

462 **Amino acid sequence alignments and phylogenetic analyses, cell and parasite culture,**  
463 **parasite cloning strategies, parasite transfections, parasite immunoblots, *T. gondii***  
464 **plaque assays, *T. gondii* intracellular growth assay, *T. gondii* immunofluorescence-based**  
465 **induced egress assay, *T. gondii* conoid extrusion assay, *T. gondii* gliding assays, *T. gondii***  
466 **attachment assay, Ciliata culture conditions, Ciliata biolistic transformation, generation**  
467 **of *Tetrahymena* knockout strain, Ciliata immunoblots, Ciliata immunofluorescence**  
468 **microscopy, statistical analysis, reagents and antibodies are found in Supplementary**  
469 **Methods.**

470

471 **Data availability**

472 The datasets generated during and/or analysed during the current study are available from the  
473 corresponding author upon request.

474

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627

628

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652

### 653 **Author contributions**

654 E.A. performed most of the work on *Toxoplasma*. M.M.C generated the *Plasmodium* data and  
655 completed the work on *Toxoplasma* during revision. A.G. performed the phylogenetic  
656 analyses. E.A. D.S and R. N. generated the *T. thermophila* data. C.S. generated the donor  
657 plasmid for *Pfnd9*-iKO. A.N.G., N.D.S.P., R.N. and M.M. contributed to *Toxoplasma*  
658 phenotypic analyses. D.M.P.V. performed the IP analyses. S. U. performed the mass  
659 spectrometry analyses. E.A., L.B-S and J.F.D. performed electron-microscopy. E. A., P.R.F  
660 and J.F.D. performed the freeze fracture analyses. S.K.M. and Y.-W.C. performed the cryo-  
661 ET. A.N.G. and S.K.M. prepared the samples for cryo-ET. M.L. designed the study with the  
662 support of E.A. M.L. supervised the research. E.A. and M.L. wrote the paper with editorial  
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664 in the main manuscript and in the supplementary materials.

665

## 666 **Competing interests**

667 The authors declare no competing financial interests.

668

## 669 **Materials and Correspondence**

670 Correspondence and requests for materials should be addressed to M.L.

671

672 **Supplementary Information** is available for this paper.

673

## 674 **Fig. 1 | Rhoptry secretion is dependent on rosette formation.**

675 **a**, Immunofluorescence assay (IFA) of control untagged line and endogenously HA<sub>3</sub>-tagged

676 *TgNd6* and HA<sub>3</sub>-tagged *TgNd9* tachyzoites. The green arrow points to *TgNd6* apical puncta.

677 ARO is a marker for rhoptries. Shown are maximum intensity projections of confocal z-

678 stacks of fixed parasites. **b**, Super-resolution microscopy of the apical dot of *TgNd6*.

679 Schematic of apical end of tachyzoite. Maximum intensity projections of z-stacks of *TgNd6*-

680 HA<sub>3</sub> parasites transiently expressing RNG1\_GFP (top) and of *TgNd6*-HA<sub>3</sub> parasites

681 expressing centrin 2 (CEN2)-Ty<sub>2</sub> (bottom). The protein RNG1\_GFP marks the apical polar

682 ring (APR) and CEN2\_Ty marks pre-conoidal rings (PCR). Higher magnifications show that

683 *TgNd6*-HA<sub>3</sub> (red arrow) localizes above the apical polar ring (green arrow) and co-localizes

684 partially with CEN2. DIC: differential interference contrast. **c**, Immunogold labelling of

685 *TgNd6*-HA<sub>3</sub>. Right panel shows *TgNd6*-HA<sub>3</sub> on the AV. Insert panel: higher magnification of

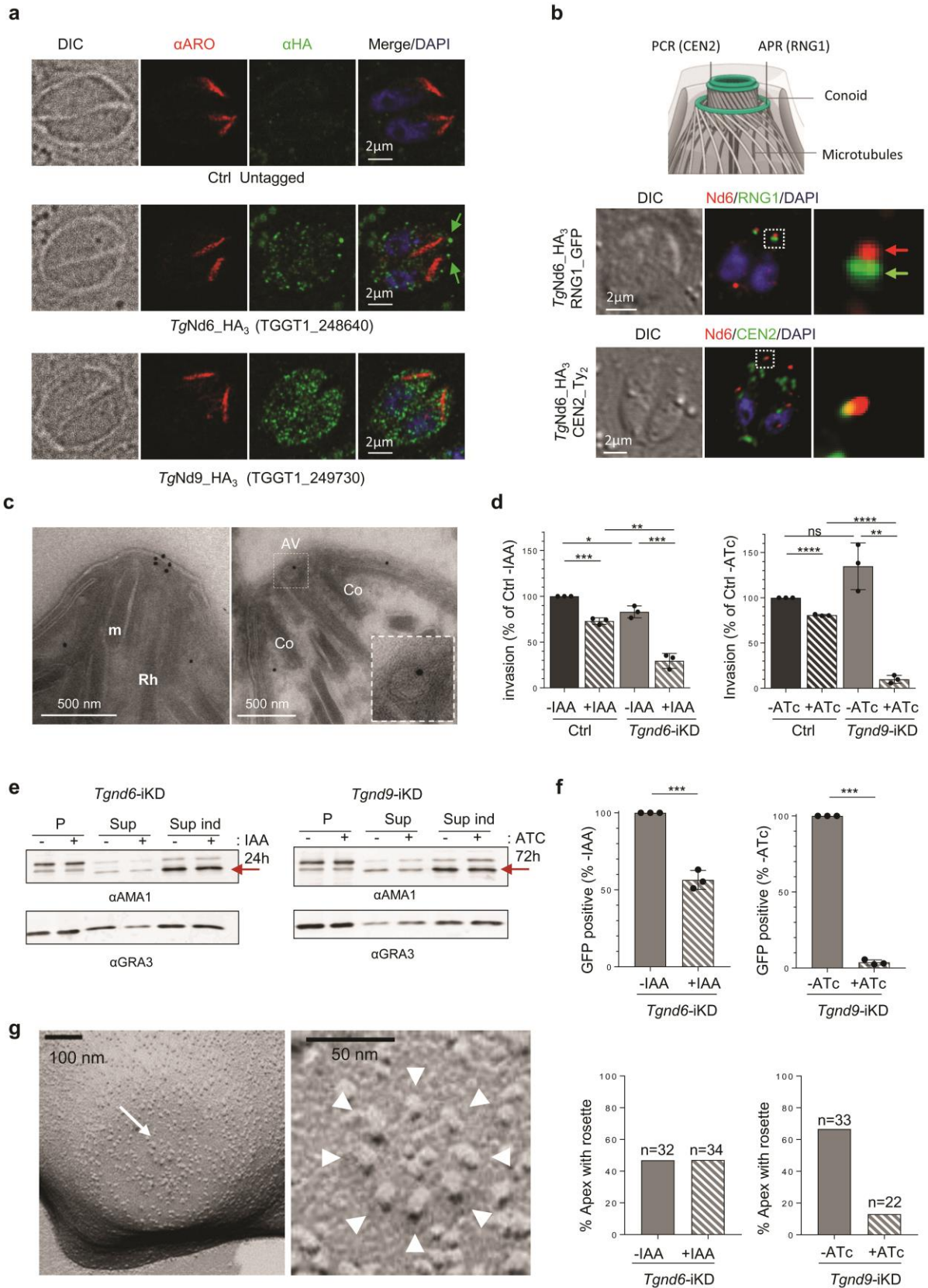
686 the AV. Micronemes (m) and rhoptries (Rh) are visible in transverse section of the conoid

687 (Co). **d**, Quantification of invasion after depletion of *TgNd6* and *TgNd9*. Mean  $\pm$  SD of n=3

688 independent experiments. **e**, Immunoblot showing microneme secretion assessed by the

689 release of proteolytically cleaved AMA1 (arrow=processed/secreted *TgAMA1*) in *Tgnd6*-

690 iKD  $\pm$  IAA 24 h (left) and *Tgnd9*-iKD  $\pm$  ATC 72 h (right). P = pellet, Sup = supernatant, Sup  
691 ind = propanolol-induced supernatant. GRA3, loading control. **f**, Rhoptry secretion assay by  
692 Secreted Cre, epitope-tagged (SeCrEt). Rhoptry secretion quantification of *Tgnd6*-iKD (left)  
693  $\pm$  IAA and *Tgnd9*-iKD (right)  $\pm$  ATC. Mean  $\pm$  SD of n=3 independent experiments. **g**, Left:  
694 Freeze-fracture electron microscopy of a *T. gondii* tachyzoite (P face) showing a rosette of  
695 intramembranous particles (white arrow) at the middle of the apex. Middle: Higher  
696 magnification of the left panel. The white arrowheads point to the eight IMPs of the rosette.  
697 Right: Quantification of rosettes of IMPs in *Tgnd9*-iKD  $\pm$  ATc 72 h and *Tgnd6*-iKD  $\pm$  IAA  
698 24 h using freeze fracture. (**d**, **f**) Unpaired two tail student's *t* test: \*\*\*\* *p*-value < 0.0001,  
699 \*\*\* *p*-value < 0.001, \*\* *p*-value < 0.01, \* *p*-value < 0.05.



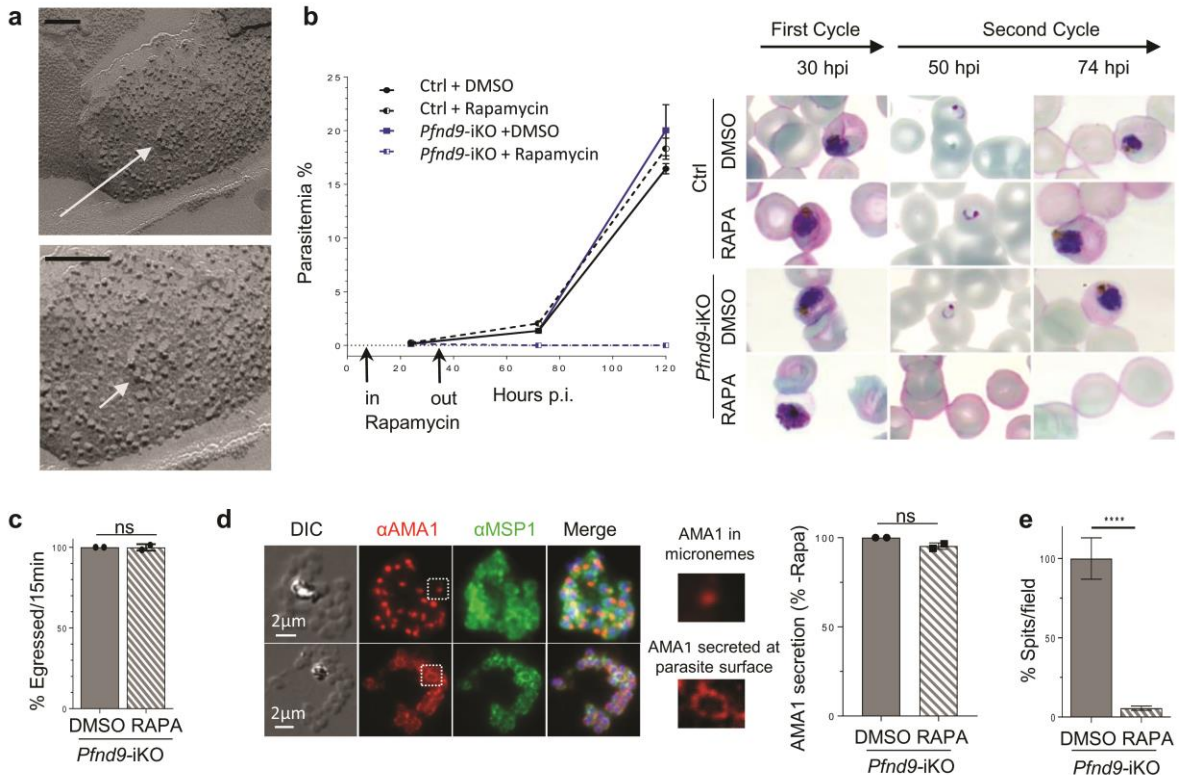
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703 **Fig. 2 | *Pf*ND9 is essential for rhoptry secretion in *P. falciparum*.**

704 **a**, Freeze-fracture electron microscopy of a *P. falciparum* merozoite (P face) showing a  
 705 rosette of intramembranous particles (white arrow). Higher magnification at the bottom. Bar  
 706 is 100 nm. **b**, Growth curves (parasitaemias) of *p230p* DiCre (Ctrl) and *Pfnd9*-iKO  
 707 mutant ± rapamycin shows that *Pf*ND9-depleted parasites have a growth defect. On the right:  
 708 Giemsa staining of the growth experiment illustrating development and reinvasion of *p230p*  
 709 DiCre (Ctrl) and *Pfnd9*-iKO asexual parasites (along 2 cycles) ± rapamycin treatment. **c**,  
 710 Quantification of egress of *Pfnd9*-iKO ± rapamycin schizonts. Data collected from 8 movies  
 711 of *Pfnd9*-iKO ± rapamycin. **d**, Left: IFA illustrating AMA1 protein stored in micronemes  
 712 (top) or secreted and translocated at the surface of the parasite prior to egress (bottom). . **e**,  
 713 Quantification of rhoptry secretion events in *Pfnd9*-iKO ± rapamycin-treated schizonts using  
 714 anti-*Pf*RAP2 antibodies to visualise rhoptry secretion events ('spits' of RAP2 exported into  
 715 the RBC). (e) Unpaired two tail student's *t* test: \*\*\*\* *p*-value < 0.0001.



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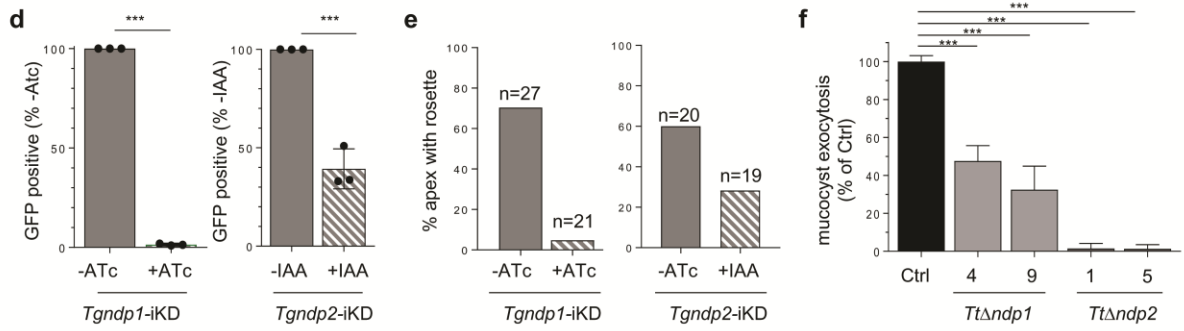
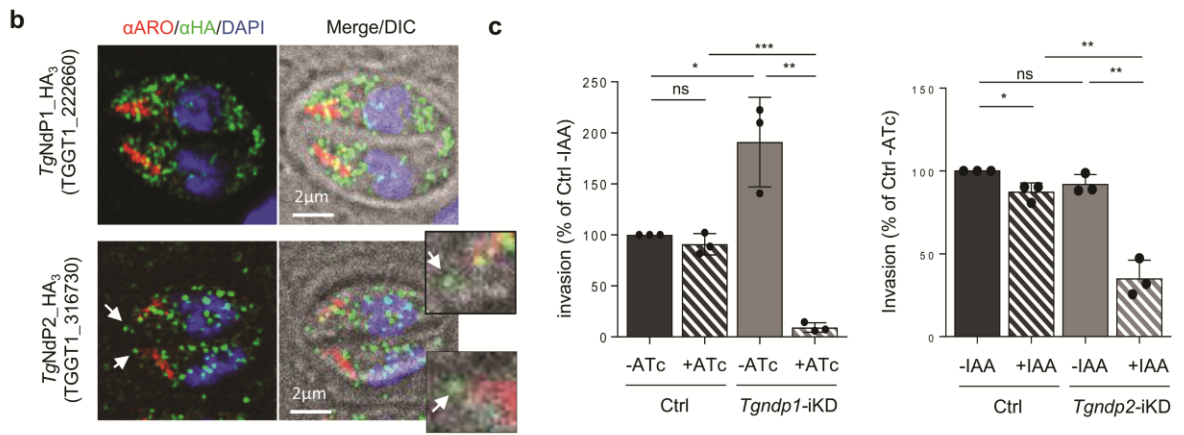
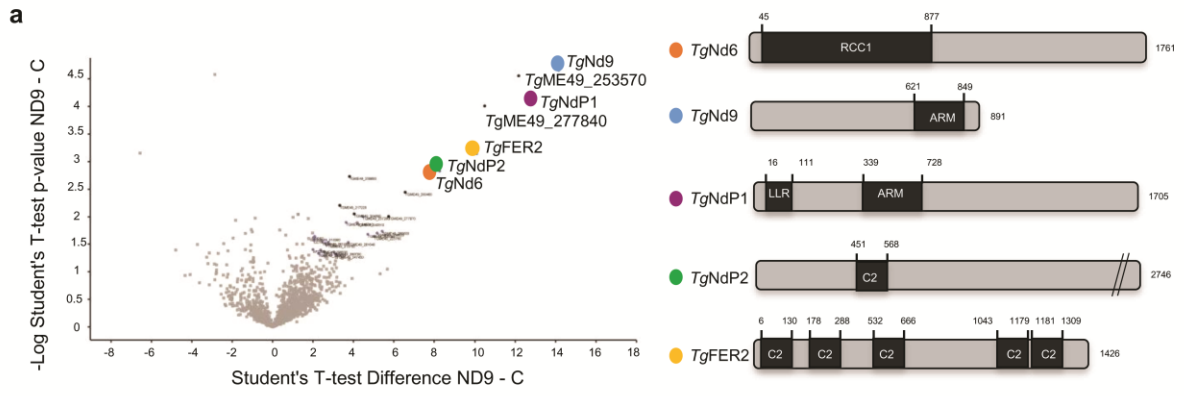


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718 **Fig. 3 | Nd6 and Nd9 are part of an Alveolata complex essential for organelle secretion**

719 **in *T. gondii* (Apicomplexa) and *T. thermophila* (Ciliata)**

720 **a**, Mass spectrometry analysis of immuno-isolated *TgNd9*-HA<sub>3</sub>. Left: Volcano Plot of  
721 proteins differentially enriched in *TgNd9* vs control IP. This plot presents the fold change  
722 (Difference) and significance (-Log p) obtained from a t-test of three independent IPs using  
723 LFQ intensity values. Right: Schematic representation of *TgNd* proteins using  
724 SUPERFAMILY<sup>62</sup>. RCC1: regulator of chromosome condensation 1-like domains (RLDs), a  
725 versatile domain that performs many different functions, including guanine nucleotide  
726 exchange on small GTP-binding proteins<sup>63</sup>. LRR: Leucine Rich Repeat domain. ARM:  
727 Armadillo Repeat domain. C2: lipid-calcium binding domain. **b**, Immunofluorescence (IF) of  
728 endogenously HA<sub>3</sub>-tagged *TgNdP1* and *TgNdP2* tachyzoites. The white arrow points to  
729 *TgNdP1* apical dots of two adjacent parasites, which are magnified on the right. ARO:  
730 rhoptry marker. DAPI: DNA marker. DIC: differential interference contrast. **c**, Quantification  
731 of invasion after depletion of *TgNdP1* (left; using ATc) and *TgNdP2* (right; using IAA) along  
732 with negative control strains. Mean ± SD of n=3 independent experiments. **d**, Rhoptry  
733 secretion quantification of *TgndP1*-iKD (left) ± ATc and *TgndP2*-iKD (right) ± IAA. Mean ±  
734 SD of n=3 independent experiments. **e**, Quantification of rosettes of IMPs in *TgndP1*-iKD ±  
735 ATc 72 h and in *TgndP2*-iKD ± IAA 24 h using freeze-fracture. **f**, Quantification of  
736 mucocyst exocytosis by dibucaine assay. Data collected from three experiments. (**c-f**)  
737 Unpaired two tail student's *t* test: \*\*\*\* *p*-value < 0.0001, \*\*\* *p*-value < 0.001, \*\* *p*-value <  
738 0.01, \* *p*-value < 0.05.



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743 **Fig. 4 | The rhoptry secretion machinery includes the apical vesicle and the rosette**

744 **a**, A slice through a tomogram showing a side view of the apical complex – conoid (brown),  
745 pre-conoidal apical rings (PCR; gray; two in number), micronemes (yellow), plasma  
746 membrane (PM; light blue) and the rhoptry secretion system consisting of the rosette (dark  
747 blue), apical vesicle (AV; magenta), rhoptry (orange) and rhoptry tip density (cyan). Original  
748 image (right) is annotated with color overlays (left). **b**, Left: magnified image of the boxed  
749 region in (a) showing the connections between the rhoptry, rhoptry tip density, AV, rosette  
750 and the PM. The rhoptry tip is 9 nm distant from the AV. Right: a pair of 3-dimensional  
751 segmentations from the data on the left. The PM is rendered transparent in one of these  
752 segmentations to see the rosette. **c**, Magnified image of the boxed region in (b) showing the  
753 side view of the rosette. The AV is 14 nm distant from the PM. **d**) Top view of the rosette  
754 from a horizontal tomogram section perpendicular to the plane in (c), showing an 8-fold  
755 rotational symmetry and a diameter of ~67 nm. **e**) AV connected with the PM via a rosette in  
756 the absence of docked rhoptry. All measurements are made in 3D. Images in (b-e) are  
757 computationally filtered to boost contrast (see Methods). The images in (c) and (d) are from  
758 two different cells oriented differently on the EM grid resulting in better resolved side view  
759 and top view, respectively. **f**, Quantification of apical rosettes in *TgARO*-iKD mutants  $\pm$  ATc  
760 72 h. **g**, Left: Ultrastructure of wild type (RH strain type I) tachyzoites with the AV  
761 positioned beneath the plasma membrane, and above the tip of the rhoptry neck. Right: In  
762 *TgARO*-iKD ATc-treated tachyzoites (72 h), the AV is still properly positioned at the apex  
763 (presumably under the rosette), while rhoptries are not docked on the AV. Inset shows a  
764 magnification of the vesicles. co: conoid; m: micronemes; Rh: rhoptry; APR: apical polar  
765 ring; PCR: pre-conoidal rings; AV: apical vesicle; ICM: pair of intraconoidal microtubules.  
766 **h**, Schematic of similarities and differences of the exocytic machinery between Ciliata and

767 Apicomplexa. Exocytosis in Alveolata (Ciliata, Dinoflagellata and Apicomplexa) is outlined  
768 by the presence of a rosette of particles embedded in the outer membrane, defining the site of  
769 exocytosis. In Ciliata, organelles discharge can have a defensive or predatory function.  
770 Rhoptry exocytosis in Apicomplexa is one of the critical steps of host cell invasion and  
771 therefore fundamental for parasitism. In Apicomplexa, but not in Ciliata, an apical vesicle  
772 (AV) of unknown function is present between the tip of the rhoptry and the plasmalemma,  
773 plausibly involved in the injection of rhoptry proteins into the host cell. PM, plasma  
774 membrane; PVM, parasitophorous vacuole membrane; AS, alveolar sac [in Ciliata], which is  
775 homologous to the IMC (inner membrane complex) of Apicomplexa.

