Title

***In situ* structure and priming mechanism of the rhoptry secretion system in *Plasmodium* revealed by cryo-electron tomography**

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Abstract

Apicomplexan parasites secrete the contents of rhoptries into host cells to permit their invasion and establishment of an infectious niche. The rhoptry secretory apparatus (RSA), which is critical for rhoptry secretion, was recently discovered in *Toxoplasma* and *Cryptosporidium*. It is positioned at the cell apex and associates with an enigmatic apical vesicle (AV), which docks one or two rhoptries at the site of exocytosis. The interplay among the rhoptries, the AV, and the parasite plasma membrane for secretion remains unclear. Moreover, it is unknown if a similar machinery exists in the deadly malaria parasite *Plasmodium falciparum*. In this study, we use *in situ* cryo-electron tomography to investigate the rhoptry secretion system in *P. falciparum* merozoites. We identify the presence of an RSA at the cell apex and a morphologically distinct AV docking the tips of the two rhoptries to the RSA. We also discover two new organizations: one in which the AV is absent with one of the two rhoptry tips docks directly to the RSA, and a second in which the two rhoptries fuse together and the common tip docks directly to the RSA. Interestingly, rhoptries among the three states show no significant difference in luminal volume and density, suggesting that the exocytosis of rhoptry contents has not yet occurred, and that these different organizations likely represent sequential states leading to secretion. Using subtomogram averaging, we reveal different conformations of the RSA structure corresponding to each state, including the opening of a gate-like density in the rhoptry-fused state. These conformational changes of the RSA uncover structural details of a priming process for major rhoptry secretion, which likely occur after initial interaction with a red blood cell. Our results highlight a previously unknown step in the process of rhoptry secretion and indicate a regulatory role for the conserved apical vesicle in host invasion by apicomplexan parasites.

Introduction

Plasmodium parasites, the causative agents of malaria, were responsible for an estimated 627,000 deaths in 2020 with most deaths attributable to *Plasmodium falciparum*¹. Despite significant progress in disease control over the last few decades, deaths increased by 12% from 2019². *Plasmodium* spp. belong to a larger phylum of important disease-causing parasites called Apicomplexa, including *Toxoplasma* and *Cryptosporidium* that impact a large portion of the global population^{3–5}. Invasive stages of these parasites, such as *Plasmodium* merozoites, contain a defining apical complex that harbors specialized structures and secretory organelles — micronemes and rhoptries — dedicated to parasite motility and host cell invasion^{6,7}. Micronemes first secrete their proteins onto the parasite surface, permitting parasite motility, host cell recognition, and downstream invasion events^{6,8}. Upon recognition of a host red blood cell, a pair of club-shaped rhoptries in the *Plasmodium* merozoite secrete their contents into the cytosol of the host, permitting the merozoite's invasion and development within^{7,9}. The mechanism by which rhoptries secrete their contents, and how proteins from inside the rhoptries are relocated across the rhoptry membrane, parasite plasma membrane, and host plasma membrane remain largely unknown¹⁰.

Earlier electron tomography studies of merozoites undergoing red blood cell invasion demonstrated that the two rhoptries fuse together following host contact coupled with a reduction in volume, indicating secretion of contents into the host cell¹¹. Similarly, traditional electron microscopy of merozoites invading red blood cells revealed that the merozoite's two rhoptry necks are fused together¹², or only one rhoptry (likely a fully fused rhoptry pair) is present^{9,13,14}, with electron-translucent regions. Moreover, a pore has been seen at the interface between the fused rhoptry tips and the parasite plasma membrane (PPM), likely to provide a portal through which rhoptry proteins can relocate^{13,15}. The red blood cell surface protein glycophorin A (GlyA) has been demonstrated to distinctly trigger rhoptry secretion in isolated *P. falciparum* merozoites, however the mechanism of how this occurs remains unknown¹⁶. Recently, multiple non-discharge (Nd) proteins were discovered in *T. gondii* and *P. falciparum* that are implicated in the mechanism by which rhoptries secrete¹⁷. Calcium is likely involved in

72 rhoptry fusion and secretion, as NdP1 and Fer2, two proteins containing calcium-binding C2
73 domains, are involved in rhoptry secretion and have been localized to the apical tip in *T. gondii*¹⁷.
74 Moreover, fluorescence microscopy has revealed a calcium spike at the extreme apex of the
75 merozoite just prior to penetration of the red blood cell^{18,19}. Despite these observations,
76 however, the molecular mechanism by which this process occurs remains unclear.

77
78 Recently, cryo-electron tomography (cryo-ET) of *T. gondii* and *C. parvum* has revealed an
79 elaborate molecular assembly, called the rhoptry secretory apparatus (RSA), that docks the
80 rhoptries and an apical vesicle (AV) atop the rhoptries' tips to the parasite apex^{17,20}. Knockdown
81 of the Nd9 protein disrupted the RSA structure in *T. gondii* and abolished rhoptry secretion in
82 both *T. gondii* and *P. falciparum*, linking the Nd-NdP proteins, the RSA, and rhoptry secretion
83 together. However, whether *P. falciparum* harbors an RSA and/or AV at the rhoptry tips has yet
84 to be determined. Furthermore, if the AV was genuinely part of this unique eukaryotic secretion
85 system, then this would imply that there are two passages (one between the rhoptry and AV;
86 another between the AV and PPM) that need to open for rhoptry secretion. The evidence for,
87 purpose and regulation of the passages are unclear since there have been scarce hints as to what
88 the role of the AV might be¹⁰.

89
90 Here we performed high-resolution cryo-ET imaging of isolated *P. falciparum* merozoites to
91 investigate the ultrastructure of their rhoptry secretion system. We consistently observed an RSA
92 in all the imaged merozoites and an AV in nearly two-thirds of them. We further resolved the RSA
93 in detail through subtomogram averaging, a process that improves resolution by aligning and
94 averaging multiple copies of the same structure in 3-dimensions (3-D). The elaborate structure
95 of the RSA is similar to that of *T. gondii* and *C. parvum*²⁰, all of which anchor into the PPM and
96 form connections to the AV held below. Furthermore, we resolved two additional distinct states
97 of the rhoptry secretion system that lack the AV: 1) with one of the two rhoptries directly docked
98 at the RSA; and 2) with the two rhoptries fused together and the common tip docked at the RSA.
99 Analyses of rhoptry volumes and rhoptry lumen densities revealed no significant changes across
100 the three morphological states, suggesting that major rhoptry secretion has not occurred. We

furthermore resolved different RSA conformations corresponding to each state through classified subtomogram averaging analyses, revealing multiple structural changes including the opening of a gate-like density in the RSA. Altogether, the different states likely reveal sequential events in rhoptry secretion, beginning with the opening of the passage between the AV and one of the two rhoptries, followed by fusion of the two rhoptries and gate opening in the RSA, suggesting a role for the AV in regulating rhoptry fusion to prime their secretion.

Results

Cryo-ET reveals an apical vesicle at the rhoptry tips in P. falciparum

Using cryo-ET, we imaged the apical region of isolated *P. falciparum* merozoites (Fig. 1a, b). We were able to resolve all the defining features of the apical complex, including two preconoidal rings, the apical polar ring, the inner membrane complex (IMC), rhoptries, micronemes, and subpellicular microtubules (Fig. 1b and Supplementary Fig. 1a-d). In addition, we observed a distinct AV atop the tips of the two rhoptries, whose presence has not been reported in *Plasmodium* (Fig. 1b, c) and is a distinct compartment from the two rhoptries that are docked just below, as evidenced by the significantly lower electron density within the AV versus the rhoptries (Fig. 1d, e and Supplementary Fig. 1e, f). As is the case with *T. gondii* and *C. parvum*, the AV in *P. falciparum* merozoites is docked at the PPM via the RSA (see details below). However, the AV of *P. falciparum* is smaller and its size and docking parameters are more variable compared to *T. gondii* and *C. parvum*²⁰ (Fig. 1f-k and Supplementary Fig. 2). Of the 39 AVs analyzed, 34 (87%) had two rhoptries docked, four (10%) had just one rhoptry docked, and one (3%) had no rhoptry docked (Fig. 1l and Supplementary Fig. 1g-i). Of the AVs with two docked rhoptries, one rhoptry is always more aligned to the AV's long (major) axis than the other (referred to as rhoptry 1 and rhoptry 2, respectively; Fig. 1g, i). In summary, our data reveal that the AV is a conserved feature across many apicomplexan parasites, docking the rhoptry tips to the RSA at the PPM.

P. falciparum merozoites display multiple rhoptry fusion morphologies

In addition to the discovery of the AV in *P. falciparum* (Fig. 1), we observed other rhoptry secretion system morphologies in a subset of merozoite tomograms analyzed (69/218; Fig. 2). Among them, 33 (15%) lacked the AV and instead had one of the two rhoptries docked directly to the RSA (Fig. 2b and Supplementary Fig. 3a-c). Rhoptries from merozoites displaying this morphology were generally more twisted and contorted as compared to rhoptry pairs from merozoites with an AV. Additionally, 36 (17%) merozoites display fusing rhoptries; again, these parasites lack an AV and instead the fused rhoptry tip is docked directly at the RSA. The fusion between the rhoptries ranged from fusion just at the rhoptry tips (Fig. 2c) to complete fusion to the base of the rhoptry bulbs (Fig. 2d and Supplementary Fig. 3d-g). Importantly, in all cases with fused rhoptries, no AV was observed. This suggests that the disappearance of the AV precedes the fusion of the rhoptry pair.

Rhoptry fusion has been previously observed during merozoite invasion of red blood cells when the rhoptries secrete their contents^{11,12}. We therefore sought to determine if the different rhoptry morphologies observed correspond to premature secretion even in the absence of host cells, although such an event is not expected during the merozoite's normal invasion process. We identified 9-10 tomograms of each morphological class (separated rhoptry pair with an AV; separated rhoptry pair without an AV; or fused rhoptry pair without an AV) with entire rhoptries captured in the field of view. We then used EMAN2 neural networks^{21,22} to segment the rhoptries in each tomogram and subsequently measured the volume of the rhoptry segmentations in UCSF Chimera²³. The results show no significant difference in the average rhoptry volume among the different morphological classes (Fig. 2e). Similarly, density profiles of the rhoptry lumen also show no significant difference across the different classes (Fig. 2f). These observations suggest that there is no major secretion occurring upon rhoptry fusion in the absence of a host red blood cell. However, these data do not rule out the possibility of initial, small-scale secretions of specific rhoptry contents in preparation for a subsequent major secretion event.

In attempts to visualize rhoptry secretion and understand the triggers for these morphological changes, some of the merozoites were treated with GlyA prior to freezing on EM grids for cryo-

ET imaging. We found that GlyA treatment did not affect the ratio of rhoptry morphologies observed (Fig. 2g), again supporting that these morphologies are not related to active secretion. Interestingly, we discovered that accidental rupture of the PPM (that likely happened during cryo-ET sample preparation) caused a significant decrease in the proportion of the morphology with an AV (from 81% to 58%; Fig. 2h). This suggests that the AV's disappearance may be regulated by signals whose accessibility to the rhoptry secretion system can be influenced by the integrity of the PPM.

Subtomogram averaging reveals an elaborate structure of the P. falciparum RSA

In all tomograms of merozoite apical complexes containing an AV, we found that the AV was always anchored to the PPM via the RSA (Figs. 1b, c, 2a, and Supplementary Fig. 1). A rosette of 8+1 intramembranous particles has been reported to be part of the *T. gondii* and *C. parvum* RSAs²⁰ and linked to rhoptry secretion¹⁷. A similar rosette, albeit at lower resolution, has been previously reported by freeze-fracture electron microscopy at the apex of *Plasmodium* merozoites^{17,24} and other apicomplexan parasites^{25,26} as well. Resolving the overall RSA structure in *P. falciparum* merozoites will therefore be crucial to our understanding of the apicomplexan rhoptry secretion system and, in particular, species-specific adaptation in *Plasmodium*. We performed subtomogram averaging using all the RSAs associated with an AV (RSA-A) to boost signal over noise to analyze structural details of this large molecular assembly. The resulting average of the *P. falciparum* RSA revealed a density of much greater complexity than what was observed in individual tomograms (Fig. 3, Supplementary Fig. 4b, c, and Movie 1). As part of the RSA, we resolved an apical rosette of 8 membrane anchors (Anchor-I [A-I]) surrounding a central density (CD) at the PPM (Fig. 3b, c[i], and d[top]). Densities extend from each A-I towards the AV (Anchor-II [A-II] and Anchor-III [A-III]; Fig. 3b), showing a clear 8-fold arrangement in the intracellular space (Fig. 3c, d[side], f). The A-II connects to A-I in the PPM (Fig. 3b), and A-III connects to the AV (Fig. 3c[v] and Supplementary Fig. 5). Furthermore, in the center of the 8-fold A-II/III densities lies a central channel (CC) that sits atop the tip of the AV (Fig. 3b[iii], c[iii], and e). The CC connects to another set of 8 anchors (Anchor-IV [A-IV]; Fig. 3b[ii], c[i]) in the PPM via the radiating spokes (rS; Fig. 3c[ii], e). We also resolved a density at the interface of CC and the

AV, termed the gate density (gD; Fig. 3b[iii], e[bottom], f[bottom]). All the structural details revealed by subtomogram averaging of the *P. falciparum* RSA docked with the AV are summarized in 3-D in Movie 2.

The RSA displays a conserved ultrastructure among apicomplexans

We next compared the subtomogram average of the RSA and AV of *P. falciparum* with that of *T. gondii* and *C. parvum* previously resolved by our group²⁰. Side views of the three RSA structures show a similar organization: the RSAs are anchored to the PPM intracellularly and holds the AV just below (Fig. 4a). Consistent with the greater variability in size and positioning of the *P. falciparum* AV compared to that of the two other organisms (Supplementary Fig. 2), only a small portion of the AV closer to the RSA was resolved in *P. falciparum* (Fig. 4a[i]), while more of the AV and its additional connections to the RSA were resolved in *T. gondii* and *C. parvum* (Fig. 4a[ii], 4a[iii]). Additionally, the membrane bound by the RSA displays significant curvature in both *T. gondii* and *C. parvum* while remaining flat in *P. falciparum*. Top views at the PPM display a conserved rosette of particles in all three parasites, however *P. falciparum* appears to contain another inner rosette of A-IV particles (Fig. 4b). The CD in both *P. falciparum* and *C. parvum* is positioned at the PPM (Fig. 4a[i], b[i], a[iii], and b[iii]), while that of *T. gondii* extends into the extracellular space (Fig. 4a[ii], b[ii]). Additionally, the outer rosette of A-I particles appears differently shaped between the three parasites: in *P. falciparum* they are circular (Fig. 4b[i]), whereas in *T. gondii* they appear elongated (Fig. 4b[ii]) and in *C. parvum* they appear teardrop shaped (Fig. 4b[iii]). The top views of the A-I, the CC and the A-II/III densities reveal a conserved 8-fold organization with some differences in the shape of the anchors (Fig. 4b, c). The shapes of each component can be better understood with a 3-D view of each RSA (Fig. 4d). From this view, we observe a similar arrangement of A-I densities at the PPM in all three parasites (Fig. 4d[top row]) with differences in the extent to which they protrude from the membrane, as well as differences in membrane curvature. From the side views (Fig. 4d[bottom row]), we can see that both *P. falciparum* and *T. gondii* have relatively globular A-II/III densities with limited or flexible (therefore largely averaged out in the subtomogram averages) contacts with the AV (Fig. 4d[i, bottom], d[ii, bottom]). On the other hand, the *C. parvum* RSA makes extensive, rigid contacts

with the AV that are resolved in the average and contribute to the full inclusion of the AV in the final average (Fig. 4d[iii, bottom]). Additionally, the *C. parvum* RSA contains a posterior central channel that sits within the AV (Fig. 4d[iii, bottom]) that is not seen in both *P. falciparum* and *T. gondii* AVs. Together, we show that the three apicomplexan rhoptry secretion systems contain an ultrastructurally conserved secretory apparatus with notable species-specific alterations in the docking of the AV at the PPM.

RSA structure changes with rhoptry fusion

To investigate if rhoptry fusion with the AV or fusion between the two rhoptries (Fig. 2) induces conformational changes in the RSA structure, we performed separate subtomogram averaging of the RSA particles classified under these different AV-rhoptry morphologies: those with an AV docked and two separate rhoptries (RSA-A; Fig. 2a and Movies 1, 2), those with no AV and two separate rhoptries (RSA-2R; Fig. 2b, Supplementary Fig. 4d-g, and Movie 3), and those with no AV and fusing rhoptries (RSA-1R; Fig. 2c, d, Supplementary Fig. 4h-k, and Movie 4). The central slice from the side view of each structure shows similar docking of the AV to the RSA (RSA-A) or of the rhoptry tip to the RSA (RSA-2R and RSA-1R), but with different membrane curvatures at the interface (Fig. 5a). Multiple RSA components appear different among the three structures. First, from the top view at the level of the PPM, there is a significant reduction of the CD and disappearance of the A-IV densities in the RSA-1R structure compared to the other two (Fig. 5b[iii]). Additionally, the diameter of the rosette of A-I particles decreases by ~3.5 nm between the RSA-A and RSA-2R structures, and by another ~1 nm between the RSA-2R and RSA-1R structures (Fig. 5b). At a slice through the CC, we observe significantly less electron densities within the channel of the RSA-1R structure compared with the other two (Fig. 5d). To ensure this finding is not due to contrast differences among the averages, we plotted the normalized pixel intensity across the CC from each structure and show a clear decrease in relative pixel intensities within the RSA-1R CC (Fig. 5d, e [blue line]). Remarkably, from the side views of the structures, we identified the main cause for such a density decrease: loss of the gD at the interface between the CC and the fused rhoptry tip (Fig. 5f). Similarly, consistent with the top views, the CD was largely reduced in the PPM of the RSA-1R structure from the side view (Fig. 5f). Finally, the side

views of the A-II/III densities revealed a ~3.5 nm shift of them towards the PPM in the RSA-1R structure compared with the RSA-A structure (Fig. 5g). This upward shift of the A-II/III densities is also reflected in the top view slice through the rS density (Fig. 5c): we observe only A-II densities around the rS in the RSA-A and RSA-2R structures, whereas both A-II and A-III densities are seen in the RSA-1R structure. Moreover, we observe an additional connection formed between the A-III and the PPM (termed Anchor-V [A-V]; Fig. 5g) while the A-II density connecting to A-I appears largely reduced (Fig. 5a, g). Altogether, the results revealed intricate conformational changes of the RSA related to the sequential AV and rhoptry fusion activities. The detailed structural differences are illustrated in 3-D in Movie 5.

Discussion

The AV is a conserved feature of the apicomplexan rhoptry secretion system

Previous cryo-ET imaging of *Plasmodium* revealed important insights into parasite motility and ultrastructure^{11,27}, however the information was limited due to thick samples and the inherent low contrast of biological samples. Cryo-ET is rapidly evolving and recent advancements in contrast enhancing transmission electron microscopy imaging technologies have allowed for the visualization of parasitic structures at molecular resolution in a native, frozen-hydrated state with cellular contexts^{20,28,29}. For example, the use of contrast-enhancing phase plates and improved detectors have permitted novel insights into the conoid and rhoptry secretion system of invasive-stage *T. gondii* and *C. parvum* parasites^{17,20,29}, while focused ion beam milling has allowed cryo-ET imaging of *P. falciparum*-infected red blood cells³⁰. Here, we apply these technologies to image the apical complex of isolated *P. falciparum* merozoites and successfully resolve multiple defining features, including the rhoptry secretion system and multiple cytoskeletal components (Fig. 1, Supplementary Fig. 1a-d). Similar to previous reports^{8,11,13,31,32}, we observe two rhoptries with necks that extend through the preconoidal rings (Fig. 1b). Unlike previous studies that have reported on the AV in *T. gondii*, *C. parvum*, and *Sarcocystis tenella*^{20,26}, no study has reported on this vesicle in *P. falciparum*. We report for the first time that the *P. falciparum* rhoptry tips indeed dock at a distinct AV in most merozoites (Fig. 1), similar to that of *T. gondii* and *C. parvum*²⁰. The AV exhibits a small, ellipsoid shape with an electron-translucent interior. The rhoptry tips dock at

the AV like in *T. gondii*, rather than being already fused to the AV membrane like that of *C. parvum* (Supplementary Fig. 2a). Whereas *C. parvum* sporozoites contains one rhoptry³³, *P. falciparum* merozoites have two and *T. gondii* tachyzoites have 8-12^{34,35} and thus docking at the AV, rather than being pre-fused, may represent a mode of regulation that permits secretion of multiple rhoptries at once. Furthermore, the presence of the posterior central channel within the *C. parvum* AV (Fig. 4a[iii], d[iii]) and the pre-fused rhoptry-AV membranes reflects the possibility that this organism exhibits an alternate mode by which it regulates fusion between the rhoptry tip and the AV. Alternatively, this may represent an intermediate stage with the same mode of secretion regulation that has already been partly activated in *C. parvum*. With this discovery of the AV in *P. falciparum*, we can now observe its presence in earlier cryo-ET images of merozoites^{11,30}. The AV in *P. falciparum* merozoites has likely been overlooked due to its small size and flexibility (Fig. 1 f-h, j, k and Supplementary Fig. 5) compared to that of other apicomplexan parasites^{17,20}. Recent comparative analyses between the apicomplexan rhoptry secretion system and the extrusome system in the related, free-living Ciliata phylum³⁶ suggests that the AV is an adaptation in Apicomplexa for parasitism, likely involved in the invasion of a host cell^{10,17}. By resolving the apical vesicle in *P. falciparum* merozoites, we provide further evidence that this enigmatic vesicle is a conserved feature among apicomplexan parasites that may play a role in host cell invasion.

Conserved RSA features suggest a similar activity and regulation of rhoptry secretion across Apicomplexa

Our recent report discovered the elaborate structures of the RSA in *T. gondii* and *C. parvum*, which exhibits complex interactions with the AV and the PPM to ultimately guide the rhoptries to the parasite apex via the AV (Fig. 4)²⁰. Here, the extensive connections observed between the *P. falciparum* RSA and PPM and between the RSA and the AV (Fig. 3) again support the function of the RSA as one that docks the AV to the PPM; the AV in turn guides the rhoptries to the proper site for exocytosis. Detailed differences among the RSA structures from these organisms (e.g., the *P. falciparum* RSA contains two concentric membrane rosettes [A-I and A-IV densities], while *T. gondii* and *C. parvum* contain only one [Fig. 4b]) may reflect adaptations in the molecular

mechanisms for their specific parasitic activities. Further cryo-ET imaging of these parasites in the context of host cell invasion will permit the species-specific dissection of RSA-mediated rhoptry secretion, thus, to understand the roles of each common or unique structural component of the RSAs. Identification of the molecular components that comprise the RSA is also necessary to further elucidate the mechanisms of secretion. The recent discovery of Nd proteins, which are necessary for rhoptry secretion in *T. gondii* and *P. falciparum* and may contribute to RSA function¹⁷, provide a hopeful avenue to begin probing RSA-mediated secretion by cryo-ET. For example, knockdown of the Nd9 protein abolishes rhoptry secretion and cryo-ET revealed this was due to altered RSA structure in *T. gondii*²⁰. Further cryo-ET analysis of related mutants and/or conditions to map molecules onto the RSA structures will permit a more comprehensive understanding of this unique molecular machine.

The AV may regulate rhoptry fusion to prime for secretion

Earlier electron micrographs of *Plasmodium* merozoites in the act of red blood cell invasion revealed the formation of a pore between the rhoptry membrane and the PPM^{13,37,38}. Additionally, fusion between the two rhoptries during rhoptry secretion has been observed^{11–13}. It has therefore been hypothesized that the rhoptries fuse together and with the PPM during secretion³⁹, allowing the discharge of two rhoptries simultaneously. Our observation that the AV is only present at the tips of non-fused rhoptry pairs but always absent when the rhoptries were seen fused (Fig. 2a-d and Supplementary Fig. 3) suggests that the AV and its disappearing may participate in the regulation or initiation of rhoptry fusion in *P. falciparum*. A recent cryo-ET study of developing merozoites within schizonts showed images of the apical complex in which the AV was present at the rhoptry tips (Supplementary Fig. 1j)³⁰, but was overlooked and instead marked as part of the rhoptry (Supplementary Fig. 1k, l). This indicates that AV biogenesis occurs during parasite development within the red blood cell, and that the rhoptry morphologies observed in merozoites lacking an AV are likely subsequent states to the AV-containing state. Moreover, in the morphology that lacks the AV while the two rhoptries are separated, the two rhoptry necks often twist around each other or display bulges that extend towards one another (Fig. 2b, Supplementary Fig. 3a-c). This may be indicative of the initiation of fusion between the two

332 rhoptries, hinting at a role for the AV's disappearance in triggering this activity. Indeed, similar
 333 observations of possible rhoptry fusion can be observed in earlier electron micrographs of *T.*
 334 *gondii* during host invasion, where the secreting rhoptries display branched morphologies that
 335 may be representative of two rhoptries fused together along the necks^{34,35,40}. Although it has not
 336 been proven if the rhoptries are indeed fused in these data, the fact that no AV could be observed
 337 in any of the invading *T. gondii* parasites again supports a role of the AV's disappearance in
 338 rhoptry function during host invasion.

339
 340 Interestingly, volumetric and density analyses revealed that no major secretion occurs across all
 341 the rhoptry morphologies captured in our cryo-ET data (Fig. 2a-f), thus uncoupling rhoptry fusion
 342 from the major secretion event. This suggests that the fusion between the two rhoptries could
 343 be an independent event upstream of major content secretion and likely has its own functional
 344 purpose. The different RSA conformations revealed by subtomogram averaging for each rhoptry
 345 morphology further showed molecular rearrangements at the rhoptry exocytosis site correlating
 346 with the AV and rhoptry fusion activities. Importantly, the gD is absent, the CD is largely reduced,
 347 and the CC interior is less electron dense in the rhoptry-fused state (RSA-1R; Fig. 5e, f), seemingly
 348 to clear up the most direct path for rhoptry contents to leave the merozoite. Together, these
 349 data reveal a previously unknown step prior to secretion of rhoptry contents that may function
 350 as a priming mechanism to prepare the merozoite for major rhoptry secretion upon host contact.
 351 Despite no significant reduction in rhoptry volume or luminal density during this priming step
 352 (Fig. 2e, f), the possibility of small-scale secretions of specific components required for red blood
 353 cell invasion cannot be excluded. Indeed, previous studies have demonstrated that proteins
 354 important for invasion, such as Rh5, translocate from the rhoptries to the merozoite surface prior
 355 to full rhoptry secretion^{31,41}. Furthermore, the trigger for this priming step remains unknown as
 356 GlyA-treated parasites (GlyA was previously shown to be a trigger for rhoptry secretion¹⁶)
 357 displayed similar proportions of merozoites with and without the AV (Fig. 2g). Given the intimate
 358 link between calcium signaling and rhoptry secretion^{16,18,19,42}, the priming activity of rhoptries
 359 captured in our samples may be due to changes in intracellular calcium concentrations as the
 360 proportion of merozoites with primed rhoptries (the morphologies that lack the AV) increases

with PPM rupture that likely causes entry of extracellular calcium into the cell (Fig. 2h). In accordance with this, various proteins required for the secretion of rhoptries are linked to calcium, including Rasp2⁴³, Fer2, and NdP2¹⁷. Further analysis is necessary to confirm whether these fusion events are linked to calcium.

In summary, cryo-ET imaging of isolated *P. falciparum* merozoites has revealed a priming mechanism for the secretion of rhoptries (Fig. 6), in which the enigmatic AV may serve as a trigger to initiate rhoptry fusion. We have further resolved multiple RSA structures that correspond to the disappearance of the AV and fusion of the rhoptries, providing a structural framework to understand the mechanism at the molecular level. Through comparative analysis of the *P. falciparum* RSA to those of *T. gondii* and *C. parvum*, we strengthen the support for the AV's role in host invasion by apicomplexan parasites in addition to evidence for conservation of this novel secretion system across Apicomplexa. Discovery of proteins required for rhoptry secretion that localize to the AV and RSA will permit the mapping of components onto this molecular machine *in situ* for fine dissection of the secretion mechanism that will provide novel targets for the treatment of this deadly parasite.

Methods

Culture of *P. falciparum* merozoites

P. falciparum 3D7 parasites were maintained at 37°C in a gas mixture of 5% O₂, 5% CO₂, and 90% N₂ in a 2% suspension of human erythrocytes (donated from the Children's Hospital of Philadelphia) in complete medium (RPMI-1640 completed with 27 mM NaHCO₃, 11 mM glucose, 5 mM HEPES, 0.01 mM thymidine, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 10 µg/mL gentamicin (all aforementioned culture media components were purchased from Sigma Aldrich), and 5 g/L Albumax (Thermo Fisher Scientific)). Parasite cultures were synchronized over 3-4 cycles prior to merozoite isolation. Ring-stage parasite cultures were incubated with 5% (w/v) sorbitol (Sigma Aldrich) for 5 min at 37°C. Schizont-stage cultures were centrifuged in a 70% (v/v) isotonic Percoll (Sigma Aldrich) solution and the late-stage parasites were collected after treatment.

390

391 **Preparation of *P. falciparum* merozoites and cryo-ET grid preparation**

392 For merozoite preparation, cultures were scaled up to 60 mL at 5% hematocrit. Late-stage
 393 schizonts were purified from highly synchronous cultures by magnetic activated cell sorting
 394 (MACS). Isolated schizonts were treated with 10 μ M E64 for 6-8 hours, followed by passage
 395 through 1.2 μ m syringe filters (preincubated in 1% BSA + PBS for 20 minutes) to mechanically
 396 isolate merozoites⁴⁴. Isolated merozoites were spun down at 2200 *g* for 15 minutes and
 397 resuspended in 20 μ L complete media. 10 nm colloidal gold fiducials (Ted Pella, Redding, USA)
 398 were added to the suspension (for alignment during tomogram reconstruction from tilt series).
 399 4 μ L of suspended merozoites were applied onto Quantifoil 200 mesh copper R2/2 holey carbon
 400 EM grids, excess liquid blotted away, and plunge frozen into a liquid ethane/propane mixture
 401 (pre-cooled with liquid nitrogen) using an EM GP2 automatic plunger (Leica Microsystems,
 402 Wetzlar, Germany)⁴⁵. The blotting chamber was set to 95% relative humidity at 24°C and blotting
 403 was done from the sample side of the grid using Whatman filter paper #1. Plunge-frozen grids
 404 were subsequently loaded into autogrid c-clip rings (Thermo Fisher). The autogrid box containing
 405 frozen grids were stored in liquid nitrogen and maintained at $\leq -170^{\circ}\text{C}$ throughout storage,
 406 transfer, and cryo-ET imaging.

407

408 **Drug treatment of *P. falciparum* merozoites**

409 For treatment with glycyphorin A, mechanically isolated merozoites (prior to plunge freezing)
 410 were resuspended in buffer mimicking extracellular ionic conditions (140 mM NaCl, 5 mM KCl, 1
 411 mM CaCl_2) containing 1 mg/mL glycyphorin A (Sigma Aldrich) and incubated at 37°C for 5 – 15
 412 minutes¹⁶.

413

414 **Cryo-electron tomography (cryo-ET)**

415 Cryo-ET was performed on a Thermo Fisher Krios G3i 300 keV field emission cryo-transmission
 416 electron microscope. Dose-fractionated imaging was performed using the SerialEM software⁴⁶
 417 on a K3 direct electron detector⁴⁷ (Gatan Inc., Pleasanton, CA, USA) operated in electron-counted
 418 mode. Motion correction of images was done using the Alignframe function in IMOD⁴⁸. Imaging

was done using a Volta phase plate⁴⁹ to increase contrast without high defocus, and the Gatan Imaging Filter (Gatan Inc., Pleasanton, CA, USA) with a slit width of 20 eV to increase contrast by removing inelastically scattered electrons⁵⁰. After initially assessing cells at lower magnifications for suitability of ice thickness and plasma membrane integrity, tilt series were collected with a span of 100° (-50° to +50°; dose-symmetric scheme⁵¹) or 120° (-60° to +60°; dose-symmetric or bi-directional scheme) with 2° increments at a magnification of 33,000x (with a corresponding pixel size of 2.65 Å) and a defocus range of -1 to -4 μm. Each tilt series was collected with a cumulative dose of around 140 e⁻/ Å². Once acquired, tilt series were aligned using the 10 nm colloidal gold as fiducials and reconstructed into tomograms by our in-house automated computation pipeline utilizing the IMOD software package⁴⁸.

Quantification, statistics, and reproducibility

We obtained a total of 218 *P. falciparum* tomograms (151 untreated and 67 GlyA-treated) from nine frozen grids prepared from six biological replicates (four untreated and two GlyA-treated) that were imaged over six multiple-day sessions. Each of the following quantifications is from a subset of 39 tomograms that resolved the feature of interest. We note that ~50% of parasites imaged had a broken plasma membrane, resulting in a greater resolution of intracellular features compared with completely intact merozoites. We also note that parasites flattened on the grid, likely due to blotting. However, this flattening did not reflect on the shape of individual rhoptries, the AV, or the RSA. Flattening could have added subtle variations to the relative positions of these features, but their organizational patterns were evident despite the presence of such a potential effect.

Rhoptry distance from the AV

Shortest distance between the AV membrane and the rhoptry tip membrane for each rhoptry.

θ (orientation parameter for rhoptries at the AV)

Orientation of rhoptry 1 (θ_{Rh1}) and rhoptry 2 (θ_{Rh2}) necks with respect to the major axis of the AV (see below under 'vesicle dimensions' for more details). Angle was measured between the major

axis of the AV and the line connecting the rhoptry tip membrane to the center point of the AV major axis.

Vesicle dimensions

The AV was approximated to an ellipsoid using only two axes (major and minor) instead of three for simplicity. The longest axis of each AV in 3-D was marked as the major axis (labeled as AV_{maj}) while the shortest axis orthogonal to the major axis and intersecting it at the centroid was marked as the minor axis (labeled as AV_{min}).

Eccentricity

$(1-b^2/a^2)^{1/2}$, where ' a ' is the semi-major axis, and ' b ' is the semi-minor axis.

AV_{dist} (anchoring distance of AV)

Shortest distance between the AV membrane and the apex of the parasite plasma membrane, represented by the CD of the RSA.

Ψ (orientation parameter for AV)

Angle formed between the major axis of the AV and the line formed between the apex of the parasite plasma membrane and the centroid of the AV.

Quantifications were performed on IMOD-generated models. All model files were exported for analysis and plotting using Numpy, Matplotlib, and Seaborn libraries in Python 3.7. The corresponding graphs report the mean $\pm$ standard deviation values. For skewed distributions (θ and Ψ) we instead report the median value alone or boxplots displaying data quartiles.

Pixel value analysis

To analyze the pixel values across line profiles through AVs, rhoptries, and RSAs, 3-D volumes were opened in IMOD and, for the AVs and rhoptries, 5 layers of voxels spanning a thickness of 5.3 nm were averaged along the Z axis. TIFF images of desired slices were subsequently imported

into FIJI⁵² where a Gaussian filter was applied to smoothen the images. A line with a thickness of 10 pixels was drawn across the AVs and rhoptries, extending a few pixels beyond each membrane. Pixel values along these line profiles were exported for further analysis. For analysis of the RSA central channel, no filter was applied to the images in FIJI. Pixel values were inverted by subtracting each value from 255 so that electron dense regions appeared as peaks when plotted. Analysis and plotting of pixel values across each line were performed using Numpy, Matplotlib, and Seaborn libraries in Python 3.7.

Rhoptry volume analysis

29 tomograms (10 parasites with an AV, 9 lacking the AV and with two distinct rhoptries, and 10 lacking the AV and with fused rhoptries) were analyzed in EMAN2^{21,22} and UCSF Chimera²³. Tomograms were imported into the EMAN2 tomogram annotation workflow and a neural network was trained to recognize and segment density corresponding to rhoptries within the tomogram. Segmented rhoptries were subsequently imported into UCSF Chimera, where the segmentations were cleaned up using volume eraser to erase false positive noise, a dome cap was maintained at the top and bottom of rhoptry segmentations following the rhoptry curvature to account for the missing wedge effect, and the volumes of the rhoptry segmentations were measured. Plotting of the rhoptry volumes was performed using Matplotlib and Seaborn libraries in Python 3.7.

Generation of RSA subtomogram averages

Subtomogram alignment and averaging were performed using Dynamo^{53,54}. In IMOD, we performed manual inspections of merozoite tomograms at the apical end and identified RSA ultrastructures. Subtomogram boxes were centered in the middle of the RSA central channel and manually rotated to the same orientation among RSA ultrastructures. Model points and manual orientations were imported in Dynamo using custom scripts. Subtomogram averaging was initially performed using 149 RSAs without rotational symmetry. Densities with 8-fold rotational symmetry were seen throughout the RSA in the average, from the extracellular region to the AV (Supplementary Fig. 3). We therefore utilized this symmetry during particle picking, thus yielding

8 times more subtomograms, increasing the total number to 1,192 to further enhance the signal-to-noise ratio. The result was a better-resolved average that revealed a more elaborate ensemble of components than what was directly observed in the tomograms or from the unsymmetrized average.

RSAs from cells with different rhoptry morphologies (RSA-A: two rhoptries with an AV; RSA-2R: two rhoptries without an AV; RSA-1R: fused rhoptries without an AV) were manually sorted and then aligned and averaged independently from each other using Dynamo software (1,192 subtomograms from 149 RSA-As, 264 subtomograms from 33 RSA-2Rs, and 288 subtomograms from 36 RSA-1Rs). To generate an initial reference, each group of subtomograms was averaged using the manual orientations and without any computational alignment. An ellipsoid mask that covered the RSA and part of the plasma membrane was used in conjunction with each reference that was used to align all subtomograms. The alignment procedure started with a cone range (Euler angles Z and X) of 6° and an azimuth range (Euler angle Z') of 22.5°, followed by 6 refinement iterations, each of which used half the angular search range of the previous iteration. A new reference was generated with the best two-thirds of aligned subtomograms, and the process was repeated two more times. This process was then repeated three more times with an initial azimuth range of 6° (in total, 6 iterations of alignment). We then incorporated 8-fold rotational symmetry of the RSAs by picking 8 subtomograms from each RSA particle (by iteratively rotating each particle by 45°) and performing another subtomogram alignment with cone and azimuth ranges of 6° for 3 iterations, yielding the final average for each kind of RSA (RSA-A, RSA-2R, and RSA-1R). Fourier shell correlation plots were calculated using the “Adaptive bandpass filtering” function in Dynamo.

Figures presentation, modeling, and segmentations

Tomograms were oriented in 3-D using IMOD's Slicer window such that the desired tomogram section was in view for presentation. To enhance contrast, 5-10 layers of voxels spanning thicknesses of 5.3-10.6 nm were averaged around the section of interest. Figures were prepared using Inkscape 1.1. Manual rhoptry segmentations were generated in IMOD and imported into

535 ChimeraX⁵⁵ for presentation. Segmentations of the RSA-A subtomogram average was done
536 utilizing the Segger function in ChimeraX. Subsequent visualization and animations of the RSA
537 were also generated using ChimeraX.

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Author contributions

M.M., M.L., and Y.-W.C. conceptualized and designed the experiments. M.M. cultured and isolated parasites provided by A.O.J. M.M.C. provided a protocol and consultations for the efficient isolation of merozoites. P.M. provided further training and useful insights towards *P. falciparum* culture and merozoite isolation. M.M. prepared frozen grids and performed cryo-ET, with training from S.K.M., using an automated data-processing pipeline for on-the-fly tomogram reconstruction that was established by W.D.C., who also provided additional computational support during data collection, processing, and management. M.M. analyzed the tomograms, performed subtomogram averaging, and analyzed the RSA structure. W.D.C. performed data analysis of rhoptry volumes. S.K.M., A.G., M.M.C., and M.L. provided important insights for the interpretation of data. M.M. prepared the manuscript with critical inputs and revisions from all authors.

695 **Competing interests**

696 The authors have no competing financial interests.

697

698 **Materials and correspondence**

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700

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

702

Figure 1. *P. falciparum* merozoites harbor an apical vesicle that docks the rhoptries to the parasite plasma membrane. (a) Simplified schematic of a *P. falciparum* merozoite. **(b)** 2-D slice from a tomogram of the merozoite apical complex displaying two rhoptries (orange), rhoptry tip density (cyan) two preconoidal rings (brown), an apical polar ring (purple), the parasite plasma membrane (PPM; dark blue), the inner membrane complex (IMC; green), the apical vesicle (AV; pink), and the rhoptry secretory apparatus (RSA; light blue). **(c)** Zoom-in view of the merozoite apex from panel b without color overlays. The orange and blue lines crossing the rhoptry and the

711 AV, respectively, denote the locations of the pixel values shown in panel d. **(d)** Representative
 712 line profile plot of the inverted pixel values across the rhoptry (orange) and AV (blue). Arrows
 713 point to peaks corresponding to membranes. Shaded regions are the central 1/3 of pixels used
 714 to calculate the luminal density for the ratios shown in panel e. **(e)** Box plot showing the ratios of
 715 the luminal density to the corresponding membrane density (averaged) for each AV (blue) and
 716 docked rhoptry (orange). Mean value represented by the dotted black line. N = 12 AVs and 12
 717 rhoptries. **(f)** Summary schematic of AV dimensions and docking distance to the PPM. **(g)**
 718 Summary schematic of the docking parameters among the rhoptries, AV, and RSA/PPM. **(h)** Plot
 719 of the angle (Ψ) at which the major axis of the AV is oriented with respect to the line connecting
 720 the PPM apex to the AV centroid, measured from several cells. **(i)** Plot of the angles at which
 721 rhoptry one (θ_{Rh1}) and rhoptry two (θ_{Rh2}) are docked at the AV, with respect to the major axis of
 722 the AV. **(j)** Measurement of the shortest distance between the PPM apex and the AV membrane,
 723 and **(k)** Plot of the major versus minor axes of the AV. **(l)** Histogram of the number of rhoptries
 724 docked at the AV in each cell. N = 39 AVs for panels h, j-l and N = 34 AVs and 68 rhoptries for
 725 panel i. Scale bars = 100 nm.

Figure 2. Various observed states of the rhoptry secretion system. (a–d) Representative examples of a merozoite apical end displaying two separate rhoptries docked at the AV (a), two separate rhoptries docked directly at the RSA (b), and rhoptries fused at the tips (c) and along the necks (d). Left: Tomogram slice of the apical end with color overlay on the RSA (light blue), AV (pink), and rhoptries (orange). Middle: Zoom-in of the apical tip of the rhoptries, with and without color overlay. Right: 3-D segmentations of the rhoptries, AV, and RSA with the proportion of analyzed merozoites exhibiting each morphology denoted. **(e)** Rhoptry volumetric analysis of 3-D rhoptry segmentations among the three rhoptry morphological states. N = 10 rhoptry pairs (left and right columns) and N = 9 rhoptry pairs (middle column). **(f)** Rhoptry luminal density analysis of 2-D slices of one rhoptry among the three rhoptry states from each tomogram analyzed in panel e. N = 10 rhoptries (left column) and 9 rhoptries (middle and right columns). **(g)** Plot of the proportion of *P. falciparum* merozoites, stratified by Glycophorin A (GlyA) treatment, and **(h)** Plot of the proportion of merozoites, stratified by PPM integrity, displaying each rhoptry morphology. Numbers above each bar represent the number of merozoites displaying each morphology over the total number of merozoites in each set (i.e., with or without GlyA treatment [g] or an intact or ruptured PPM [h]). N = 211 for total analyzed merozoites. Scale bars = 100 nm.

Figure 3. *In situ* structure of the multicomponent rhoptry secretory apparatus of *P. falciparum*.

(a) Schematic of the RSA, AV, and PPM demonstrating the side view and top view orientations used to generate 2-D slices of the subtomogram average in panels b and c. **(b)** Side view slices of the RSA-A subtomogram average, from peripheral (left) to central (right) cross sections. The corresponding cross sections of the top view slices in panel c are denoted by the pink lines in b(iii). Colored outlines and overlays denote components of the RSA observed in these views: The

751 Anchors I-IV (A-I, A-II, A-III, A-IV), the central channel (CC), the central density (CD), and the gate
752 density (gD). **(c)** Top view slices of the RSA subtomogram average, from extracellular (left) to
753 intracellular (right) cross sections. Colored outlines and overlays denote components of the RSA
754 observed in these views: the radiating spokes density (rS) and other components as labeled in
755 panel b. **(d)** 3-D segmentation of the RSA displaying outer most components from top and side
756 views. **(e)** 3-D segmentation of the RSA displaying interior components from side views. **(f)** 3-D
757 segmentation of the RSA from a bottom view with the AV shown (top) and not shown (bottom)
758 to reveal the gD (bottom). Scale bars in panels b and c = 10 nm.

760 **Figure 4. Comparison of the RSA structures of *P. falciparum*, *T. gondii*, and *C. parvum*. (a)**
761 Central slices of the side view of each RSA subtomogram average (left – *P. falciparum*; middle –
762 *T. gondii*; and right – *C. parvum*) with color overlays of each corresponding component. **(b)** Top
763 view of each RSA, comparing the densities observed on the PPM surface. **(c)** Top view of each
764 RSA through a cross-sectional plane in the intracellular region, comparing CC, A-II, and A-III
765 densities. **(d)** 3-D isosurface of each RSA, with views from outside the PPM (top row) and from
766 the side (bottom row). The AV of *C. parvum* is made semi-transparent to reveal the posterior
767 central channel density inside (orange). Scale bars = 20 nm.

Figure 5. Different *P. falciparum* RSA conformations were resolved corresponding to different morphological states of the rhoptry secretion system. (a) Central slices of the side view of each RSA conformation (left – RSA-A; middle – RSA-2R; right – RSA-1R). Cross sections in panels b-d are denoted by the pink dashed lines. **(b)** Top view of each RSA conformation from outside the PPM. The CD and A-IV densities are observed in the RSA-A (i) and RSA-2R (ii) conformations but reduced in the RSA-1R conformation (iii). The diameter of the rosette formed by the A-I densities on the PPM surface is noted by the blue lines at the bottom. **(c)** Top view of each RSA conformation just below the PPM. A-II and A-III densities have color overlays to highlight their change in the RSA-1R conformation. **(d)** Top view of each RSA through an intracellular cross section. The green, orange, and blue lines across the CC of the RSA-A, RSA-2R, and RSA-1R, respectively, denote the location of the pixel values plotted in panel e. **(e)** Plot of normalized, inverted pixel values across the central channel of each RSA (RSA-A, green; RSA-2R, orange; RSA-1R, blue). A large dip is seen among the pixels of the RSA-1R CC. **(f)** Zoom in on panel a (i and iii) highlighting changes of the gD and CD between RSA-A and RSA-1R. **(g)** Zoom in on panel a (i and iii) highlighting changes in A-II/III between RSA-A and RSA-1R, including a diminished A-II connection to the A-I density and 3.5 nm upward shift of the A-II/III densities (curved red arrow), as well as the presence of a new Anchor-V (A-V) in the RSA-1R conformation. Scale bars = 10 nm in panels a–d and 5 nm in panels f, g.

Figure 6. Working model of the priming of rhoptry secretion in *P. falciparum* merozoites. Prior to invasion signals, the AV docks to the RSA and guides the two rhoptries to their proper exocytosis site in preparation for secretion. Upon exposure to proper (unknown) signals, the AV fuses with one rhoptry (labeled Rh 1 for rhoptry 1), a process that may be mediated by calcium, creating the first passage opening for rhoptry secretion (i.e., between the rhoptry and AV). One rhoptry then docks directly at the RSA and prepares to fuse with the second rhoptry (labeled Rh 2 for rhoptry 2). During this process, the diameter of the A-I rosette in the PPM decreases and the membrane adopts a curvature around the CD. The rhoptries then fuse together partially (along the necks) or fully, resulting in a loss of the gD and a second passage opening, between the rhoptry and the RSA. This is accompanied by a further decrease in A-I rosette diameter, loss of the A-IV densities, formation of the A-V densities connecting the A-III densities to the PPM, and a large reduction of the CD density.

Supplementary Figure 1. The apical complex and apical vesicle of *P. falciparum* merozoites. (a) Simplified schematic of a *P. falciparum* merozoite apical region. **(b–d)** 2-D slices through a tomogram of a top-down view of the merozoite apical complex with color overlays of all the

806 observed apical end components: apical vesicle (AV; pink), parasite plasma membrane (PPM;
807 blue), rhoptry (orange), rhoptry secretory apparatus (RSA; light blue), microneme (yellow), inner
808 membrane complex (IMC; green), preconoidal rings (brown), apical polar ring (purple), and
809 subpellicular microtubules (cyan). The cross-sectional planes used in b-d are arranged such that
810 they start at the parasite apex and move inwards into the cell. Scale bar = 100 nm. **(e, f)** Additional
811 examples of 2-D slices of the AV and rhoptry (left). Blue and orange lines across the AV and
812 rhoptry, respectively, denote the locations of pixel values plotted on the right. Arrows in the pixel
813 plots point to peaks in values corresponding to membranes. Scale bars = 50 nm. **(g-i)** 2-D slices
814 from tomograms showing either one rhoptry (g, h) or no rhoptries (i) docked at the AV. Scale bars
815 = 50 nm. **(j)** 2-D slice from a *P. falciparum* merozoite apical end tomogram within a mature
816 schizont, adapted from [30]. **(k)** The same 2-D slice from panel j with the original color overlay
817 of the rhoptry of interest, according to [30]. **(l)** The same 2-D slice from panel j with an updated
818 color overlay of the rhoptry of interest, the AV, and the RSA, based on our analysis. Scale bars =
819 200 nm.

Supplementary Figure 2. Comparison of the apical vesicles from *P. falciparum*, *T. gondii*, and *C. parvum*. **(a)** Representative 2-D slices of tomograms displaying the AV from each organism (left – *P. falciparum*; middle – *T. gondii*; and right – *C. parvum*), highlighting the PPM (dark blue), RSA (light blue), AV (pink), and rhoptries (orange). Scale bars = 50 nm. **(b)** Plots of the shortest distance (AV_{dist}) between the PPM apex and the AV membrane from several cells each organism, showing the mean $\pm$ sd. **(c)** Plots of the major (AV_{maj}) versus minor axis (AV_{min}) of the AV from each organism, showing mean $\pm$ sd. **(d)** Plots of the angle (Ψ) at which the major axis of the AV is oriented with respect to the line connecting the PPM apex and the AV centroid each organism. The median value is shown. N = 39 AVs for *P. falciparum*, 25 AVs for *T. gondii*, and 28 AVs for *C. parvum*.

832 **Supplementary Figure 3. Various fusion states of the *P. falciparum* rhoptries. (a–c)** 2-D slices
 833 through tomograms of apical ends showing the absence of the apical vesicle at the rhoptry tips
 834 and either bulging of one rhoptry towards the other (a, b) or twisting of the rhoptries (c). Dashed
 835 orange lines in panel c denote the portion of the rhoptry neck that is out of view. A mesh of
 836 electron-dense material (green) can be observed between rhoptries (orange) that display bulging
 837 towards the other. One rhoptry is docked directly at the RSA (purple). **(d, e)** Examples in which
 838 the two rhoptries are fused together and docked directly at the RSA. **(f, g)** Examples in which only
 839 one, very large rhoptry with no discernable neck region was observed and docked directly at the
 840 RSA. Scale bars = 100 nm.

h RSA-1R (non-symmetrized) – Side views

i RSA-1R (non-symmetrized) – Top views

j RSA-1R (8-fold symmetrized) – Side views

k RSA-1R (8-fold symmetrized) – Top views

843 **Supplementary Figure 4. Subtomogram averages of the *P. falciparum* RSA. (a)** Schematic of the
844 RSA, apical vesicle, and PPM demonstrating the top view and side view orientations used to
845 generate 2-D slices of the subtomogram averages. **(b, c)** Side views (b) and top view (c) of the
846 subtomogram average of the RSA-A structure before applying 8-fold symmetry. Left to right
847 panels: from peripheral to central cross sections (b) or from extracellular to intracellular cross
848 sections (c). **(d–g)** Side views (d, f) and top view (e, g) of the subtomogram average of the RSA-
849 2R structure, before and after applying 8-fold symmetry. **(h–k)** Side views (h, j) and top views (i,
850 k) of the subtomogram average of the RSA-1R structure, before and after applying 8-fold
851 symmetry. **(i)** Gold standard Fourier shell correlation plot of the final, 8-fold symmetrized
852 subtomogram averages of the three RSA structures. Scale bars = 10 nm.

854 **Supplementary Figure 5. Representative cryo-ET images of the *P. falciparum* AV and RSA. (a-j)**
855 2-D slices with of merozoite apices (left panels) with color overlays (right panels) highlighting the
856 RSA (light blue), the AV (pink), and the connections from the RSA A-II/III densities to the AV
857 (encircled in red dashed lines). Scale bars = 50 nm.

Movie 1. Subtomogram average of the *P. falciparum* RSA-A structure slicing first through the top view from extracellular to intracellular and back, followed by slicing through the side views.

Movie 2. 3-Dimensional view of the subtomogram average of the *P. falciparum* RSA-A structure.

Movie 3. Subtomogram average of the *P. falciparum* RSA-2R structure slicing first through the top view from extracellular to intracellular and back, followed by slicing through the side views.

Movie 4. Subtomogram average of the *P. falciparum* RSA-1R structure slicing first through the top view from extracellular to intracellular and back, followed by slicing through the side views.

Movie 5. 3-Dimensional view of the *P. falciparum* RSA structure, highlighting conformational changes between the RSA-A and RSA-1R states.