

1 Structural basis of *Toxoplasma gondii* Perforin-Like Protein

2 membrane interaction and activity during egress

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10 **Abstract**

11 Intracellular pathogens must egress from the host cell to continue their infectious cycle.
12 Apicomplexans are a phylum of intracellular protozoans that have evolved members of the
13 membrane attack complex and perforin (MACPF) family of pore forming proteins to disrupt
14 cellular membranes for traversing cells during tissue migration or egress from a replicative
15 vacuole following intracellular reproduction. Previous work showed that the apicomplexan
16 *Toxoplasma gondii* secretes a perforin-like protein (*TgPLP1*) that contains a C-terminal Domain
17 (CTD) which is necessary for efficient parasite egress. However, the structural basis for CTD
18 membrane binding and egress competency remained unknown. Here, we present evidence that
19 *TgPLP1* CTD prefers binding lipids that are abundant in the inner leaflet of the lipid bilayer.
20 Additionally, solving the high-resolution crystal structure of the *TgPLP1* APC β domain within
21 the CTD reveals an unusual double-layered β -prism fold that resembles only one other protein of
22 known structure. Three direct repeat sequences comprise subdomains, with each constituting a
23 wall of the β -prism fold. One subdomain features a protruding hydrophobic loop with an
24 exposed tryptophan at its tip. Spectrophotometric measurements of intrinsic tryptophan
25 fluorescence are consistent with insertion of the hydrophobic loop into a target membrane. Using
26 CRISPR/Cas9 gene editing we show that parasite strains bearing mutations in the hydrophobic
27 loop, including alanine substitution of the tip tryptophan, are equally deficient in egress as a
28 strain lacking *TgPLP1* altogether. Taken together our findings suggest a crucial role for the

29 hydrophobic loop in anchoring *TgPLP1* to the membrane to support its cytolytic activity and
30 egress function.

31 **Author Summary**

32 *Toxoplasma gondii* has a complex life cycle that involves active invasion of the host cell, the
33 formation of a replicative compartment, and egress from the replicative niche. *T. gondii* encodes
34 a pore-forming protein, *TgPLP1*, that contains a C-terminal domain that is crucial for efficient
35 exit from both the parasite containing vacuole and the host cell. However, the mechanism by
36 which *TgPLP1* recognizes and binds to the appropriate membrane is unclear. Here we use a
37 combination of biochemistry, structural biology, and parasitology to identify the a preference of
38 *TgPLP1* for specific lipids and show that a loop within the structure of the C-terminal domain
39 inserts into the membrane and is necessary for egress from the parasite containing vacuole. Our
40 study sheds light into the determinants of membrane binding in *TgPLP1* which may inform the
41 overall mechanism of pore formation in similar systems

42 **Introduction**

43 Cellular egress from the host is a crucial step in the infectious cycle of intracellular
44 pathogens. Accordingly, such pathogens have evolved multiple exit strategies, which can be
45 divided into those that leave the host cell intact and those that rupture the host cell. Several
46 bacterial pathogens, including *L. monocytogenes*, use an actin-based protrusion mechanism that
47 allows a bacterium to enter a neighboring host cell without damaging the original host cell. ^[1]
48 Other bacteria have developed extrusion or expulsion mechanisms that also leave the host cell
49 intact. ^[2-5] Also, pyroptotic and apoptotic mechanisms leverage cell-death signaling as a means
50 for intracellular pathogens to exit the host cell. ^[6] Many apicomplexan parasites, including
51 *Toxoplasma gondii*, use a cytolytic mechanism of egress that obliterates the infected cell.
52 Cytolytic egress results in direct tissue destruction and indirect collateral damage from the
53 ensuing inflammatory response, a hallmark of acute infection by apicomplexan parasites and a
54 key aspect of disease. ^[7]

55 The apicomplexan phylum encompasses a variety of parasitic genera important to both
56 human and veterinary health including *Plasmodium*, *Cryptosporidium*, *Eimeria*, and
57 *Toxoplasma*.^[8-11] These parasites contain a unique set of apical secretory organelles, micronemes
58 and rhoptries, which discharge proteins involved in parasite motility, host cell manipulation, and

59 egress.^[12-15] *T. gondii* is capable of infecting and replicating asexually within virtually any
60 nucleated cell during the acute stage of infection. Its “lytic cycle” can generally be divided into
61 three steps: invasion where the parasite containing vacuole (parasitophorous vacuole, PV) is
62 formed, intracellular replication, and finally egress from the vacuole. Whereas invasion and
63 intracellular replication have garnered considerable attention, egress remains the least understood
64 component of the lytic cycle.

65 Efficient egress by *T. gondii* critically relies on micronemal secretion of *TgPLP1*, a
66 member of the membrane attack complex/perforin (MACPF) protein family ^[15]. MACPF
67 proteins play central roles in immunity (e.g., perforins and complement proteins), embryonic
68 development (*Drosophila* torso-like and mammalian astrotactins), fungal predation (Oyster
69 mushroom pleurotolysins A/B), and cell traversal or egress by apicomplexan parasites. The
70 domain arrangement for *TgPLP1* includes a central MACPF domain that is flanked by an N-
71 terminal domain (NTD) and a C-terminal domain (CTD). Both the NTD and CTD have
72 membrane-binding activity, but only CTD is crucial for proper *TgPLP1* function.^[16] Although
73 structural insight into the *TgPLP1* apicomplexan perforin β -domain (APC β), which comprises a
74 portion of the CTD, was reported recently,^[17] how structural features of this domain contribute to
75 the function of *TgPLP1* in egress has not been addressed.

76 Overall, the mechanism of MACPF proteins begins with membrane recognition by the
77 CTD.^[18] Following membrane binding, MACPF proteins oligomerize into ring or arc shaped
78 complexes and undergo a marked structural rearrangement of the MACPF domain where the so-
79 called CH1 and CH2 helices unfurl and become extensions of the central β -sheets to create a
80 large pore.^[19] Previous work has shown that *TgPLP1* and other apicomplexans share this general
81 mechanism of pore formation. However, the molecular determinants of membrane recognition
82 and binding remain poorly understood. Here, we present evidence that *TgPLP1* has a strong
83 preference for inner leaflet lipids and that binding to such membranes occurs via the CTD.
84 Additionally, we solved the 1.13 \AA resolution X-ray crystal structure of the *TgPLP1* APC β
85 domain and identified a hydrophobic loop that likely inserts into the target membrane. Finally,
86 we use CRISPR/CAS9 to insert mutations into the hydrophobic loop and show that it is critical
87 for egress competence.

88 **Results**

89 ***PLP1* preferentially binds inner leaflet lipids**

90 Previous work has identified PLP1 as an important egress factor that can bind membranes
91 through its N-terminal domain (NTD) and C-terminal domain (CTD); however, only the CTD
92 has been shown to be required for lytic activity.^[16] In the canonical pore forming mechanism,
93 MACPF/CDC proteins bind to the target membrane via the CTD as a first step. Membrane
94 interactions occur via specific protein^[20-22] or lipid^[23-26] receptors in the target membrane. To
95 test if *TgPLP1* is capable of binding lipid receptors, we generated liposomes that mimic the outer
96 leaflet (OL) or inner leaflet (IL) of the plasma membrane and tested the binding of both native
97 *TgPLP1* and a series of recombinant His-tagged constructs of *TgPLP1* (**Figure 1A**) via
98 membrane flotation. Native and recombinant full length *TgPLP1* (r*TgPLP1*) both showed a

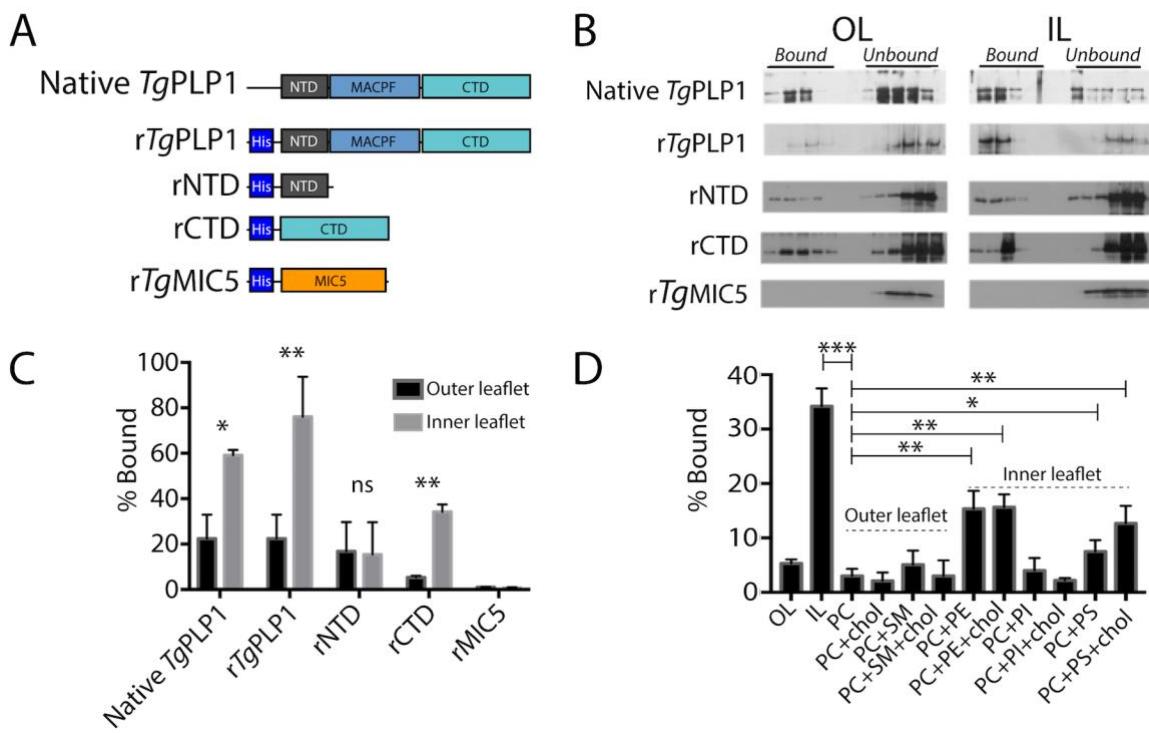


Figure 1. *T. gondii* PLP1 CTD prefers inner leaflet phospholipids. A. Schematic representation of the protein constructs used to test lipid binding. N-terminal, pore-forming, and C-terminal domains (NTD, MACPF, and CTD, respectively) are labeled for clarity. Micronemal protein r*TgMIC5* was included as a negative control. B. Representative western blots from membrane flotation assay. After ultracentrifugation aliquots were taken starting at the top of the ultracentrifuge tube. Material contained in the top half of the tube is considered bound to the liposomes while material contained in the bottom portion of the tube is considered unbound. Outer leaflet mimic lipid composition (OL) was as follows: 75% PC, 8.3% SM, 16.7% cholesterol; Inner leaflet mimic lipid composition (IL) was: 35.7% PE, 14.3% PS, 21.4% PI, 28.6% cholesterol. C. Quantification of western blots shown in panel B. Statistical significance determined by paired t-test of three biological replicates. D. Quantification of *TgPLP1*_{CTD} binding to liposomes of varying composition. Statistical significance determined by unpaired Student's t-test of three biological replicates.

99 striking preference for IL liposomes (**Figure 1B & C**). Recombinant *TgPLP1* CTD (rCTD) also
100 showed a preference for IL liposomes whereas the recombinant NTD (rNTD) bound equally to
101 IL and OL liposomes (**Figure 1B & C**). As a control, we included an unrelated His-tagged
102 recombinant micronemal protein (*rTgMIC5*), which failed to bind liposomes.

103 Since rCTD appears to bind preferentially to liposomes that mimic the IL of the plasma
104 membrane we tested if rCTD prefers binding liposomes composed of individual lipids or
105 combinations thereof. Consistent with our previous observation, rCTD does not bind to
106 liposomes comprised of OL phospholipids, namely phosphatidylcholine (PC) and sphingomyelin
107 (SM), in the presence and absence of cholesterol (**Figure 1D & Figure S1**). Consistent with its
108 preference for certain IL lipids, rCTD bound to liposomes composed of
109 phosphatidylethanolamine (PE) or phosphatidylserine (PS), but not phosphatidylinositol (PI)
110 (**Figure 1D**). However, none of the liposomes prepared with individual IL liposomes fully
111 recapitulate the binding to IL mimic liposomes. Together these findings suggest that the
112 preference of *TgPLP1* for IL lipids is conferred by its CTD and involves amalgamated binding to
113 PS and PE.

114 ***The TgPLP1 APC β domain contains a hydrophobic loop that likely inserts into membranes***

115 *TgPLP1* contains a well-conserved central MACPF domain that is flanked by a poorly
116 conserved NTD and CTD (**Figure 2A**). The CTD includes the apicomplexan specific APC β
117 domain, which consists of 3 direct repeats with 4 highly conserved cysteines in each repeat and a
118 C-terminal tail (CTT) that includes a basic patch. To better understand the molecular
119 determinants that govern membrane binding and lipid specificity in this system we expressed,
120 purified, and crystallized the *T. gondii* PLP1 CTD. Despite using a construct that encompassed
121 the entire CTD, the resulting crystals contained only the APC β domain. Whether this
122 discrepancy is due to a lack of electron density or enzymatic cleavage of the CTT remains
123 unclear.

124 The *TgPLP1* APC β domain crystallized in the C₁₂₁ space group with two monomers in
125 the asymmetric unit. The structure was solved using iodine soaks and single-wavelength
126 anomalous dispersion and subsequently refined to 1.13 Å resolution. The crystallographic data
127 and refinement statistics are summarized in **Table 1**. The *TgPLP1* APC β structure shows that the
128 β -rich repeat region is comprised of a single globular domain with internal pseudo threefold

129 symmetry wherein each β -rich repeat forms a subdomain (**Figure 2B & C**). Each of the three
130 subdomains contains an internal antiparallel β -sheet and an outer β -hairpin (**Figure S2A & B**).
131 The inner and outer layers of each subdomain are held together, in part, by two disulfide bonds
132 between the highly conserved cysteines. An overlay of the three subdomains highlights the
133 similarity in the core of each subdomain. Indeed subdomains 1 and 2 (**Figure S2C** red and
134 green) overlay with an RMSD of 0.728 \AA . Subdomain 3 (**Figure S2C** blue), however, aligns less
135 well with subdomain 1 (RMSD 3.207 \AA) and subdomain 2 (RMSD 3.175 \AA), with the majority
136 of the alignment differences attributed to two loops that protrude from the “bottom” of
137 subdomain 3. The longer of the two loops has hydrophobic character, and thus is termed the
138 hydrophobic loop (**Figure 2B**, colored cyan), whereas the shorter of the two loops has basic
139 character, termed the basic loop (**Figure 2B**, colored orange).

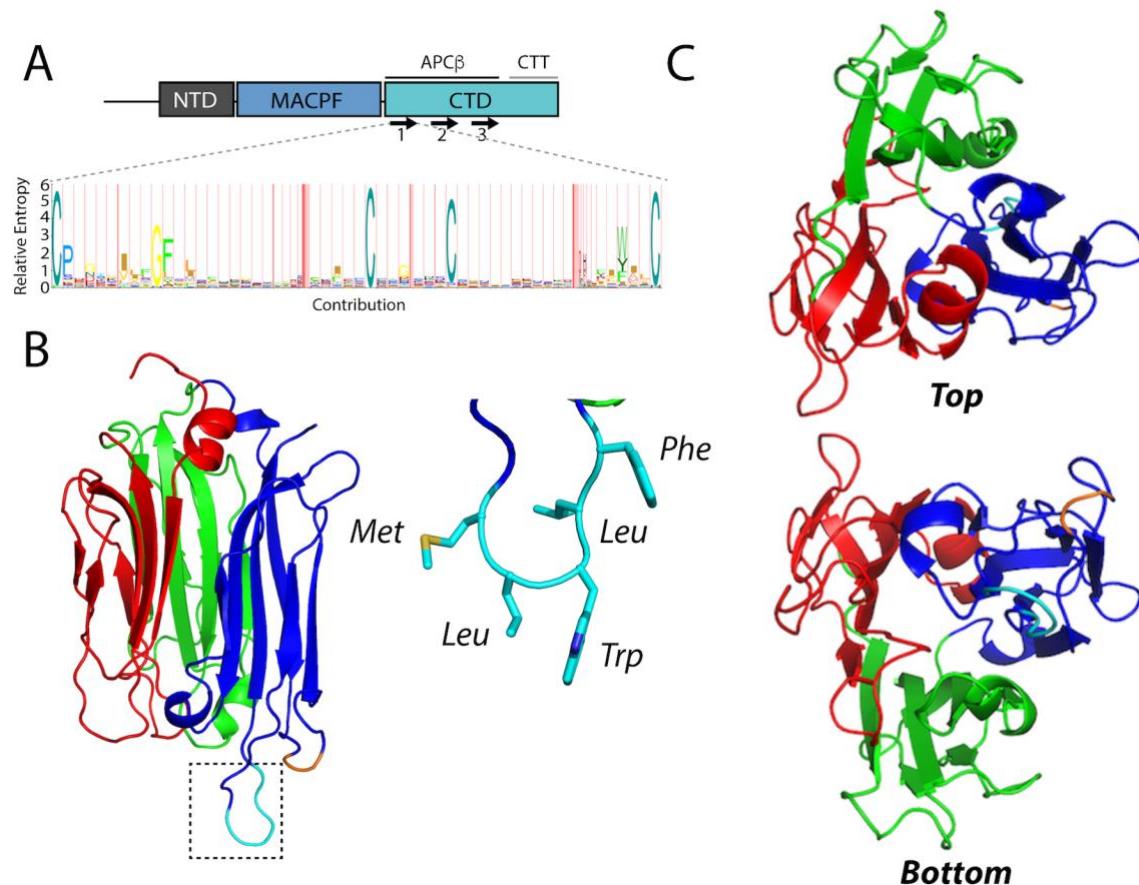


Figure 2. The 1.13 \AA resolution crystal structure of the TgPLP1 APC β domain. A. Schematic representation of the three domains in TgPLP1. The CTD contains a three β -rich sequences (denoted by arrows) and a C-terminal tail (CTT). A Hidden Markov Model consensus display of the β -rich repeat sequences from 51 apicomplexan CTD sequences. B. 1.13 \AA resolution crystal structure of the APC β domain. The crystal structure is made up of three subdomains (red, green, and blue) and contains a basic loop (orange) and a hydrophobic loop (cyan, inset) that protrude from the bottom of one subdomain. C. Top and bottom view of the TgPLP1 APC β domain highlights the internal pseudo threefold symmetry.

140 To determine commonality of double-layered β -prism fold we searched for proteins with
141 structural similarity to the APC β using the Dali server. ^[27] The Dali server measures similarity
142 by a sum-of-pairs method that outputs a Dali-Z score. Structures with a Dali-Z score above 2 are
143 considered to have structural similarity. With the exception of a recently published structure of a
144 similar *TgPLP1* APC β construct (Dali-Z score 48.5), ^[17] the top scoring result from the Dali
145 server for the *TgPLP1*_{APC β} structure is the C-terminal domain of human proprotein convertase
146 subtilisin/kexin type 9 (PCSK9 V-domain, Dali-Z score 6.0; **Table S1**). Indeed, this domain
147 contains similar internal pseudo threefold symmetry and is a double-layered β -prism fold with

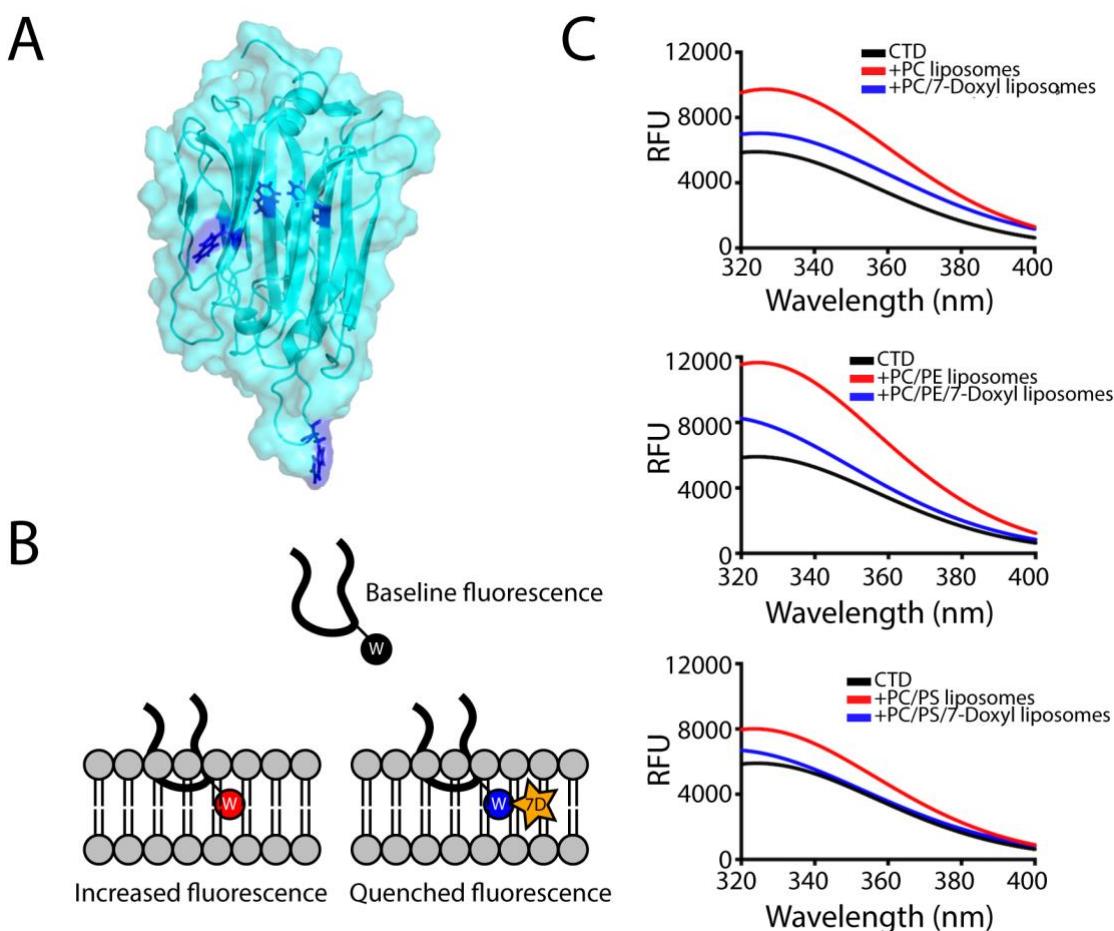


Figure 3. Intrinsic tryptophan fluorescence spectra are indicative of insertion of tryptophan into liposomes. A. Location of the tryptophan side chains in the *TgPLP1*_{APC β} structure. Only two tryptophan residues are exposed at the surface of *TgPLP1*_{APC β} . B. Cartoon schematic of the hydrophobic loop inserting into a lipid bilayer. In the tryptophan fluorescence experiment the intrinsic fluorescence of the tryptophan sidechain increases when moving from an aqueous environment to a more hydrophobic environment in the lipid bilayer. Liposomes were also prepared replacing 10% of the PC lipids with a PC lipid conjugated to a collisional quencher (7-Doxyl, 7D). This results in a quenching of the fluorescence. C. Intrinsic tryptophan fluorescence emission spectra of *TgPLP1*_{CTD} in the absence (black curves) or presence of PC, PC/PE, or PC/PS liposomes (red curves). In each case the fluorescence emission increases in the presence of liposomes. The increase is less pronounced in the presence of 7-Doxyl (blue curves). Curves are from the averages of three biological replicates each with 3 technical replicates. Liposome composition as follows: PC: 100% PC; PC/7-Doxyl: 90% PC, 10% 7-Doxyl; PC/PE: 50% PC, 50% PE; PC/PE/7-Doxyl: 40% PC, 50% PE, 10% 7-Doxyl; PC/PS: 50% PC, 50% PS; PC/PS/7-Doxyl: 40% PC, 50% PE, 10% 7-Doxyl.

148 disulfide bonds that link the inner and outer layers. However, the number of outer layer β -sheets
149 is variable within the domain (**Figure S3**). Despite the similarities in the overall fold the APC β
150 structure has very poor structural alignment with the PCSK9 V-domain (RMSD 15.706 Å).
151 These findings suggest that APC β adopts an uncommon variation of the β -prism fold, which,
152 together with the PCSK9 V-domain, constitutes a new subgroup of the β -prism family.

153 Given the hydrophobic character of the loop at the bottom of the APC β structure we
154 reasoned that this loop has the potential to insert into the membrane upon binding. To test this,
155 we took advantage of the intrinsic fluorescence of tryptophan, which is augmented upon
156 exposure to a hydrophobic environment. The *TgPLP1* CTD houses four tryptophan residues of
157 which only two are surface exposed (**Figure 3A**). We recorded the tryptophan fluorescence
158 spectrum of purified rCTD in the presence or absence of liposomes of varying compositions.
159 Incubation with liposomes resulted in an increase in the emission spectrum. Replacement of 10%
160 of PC lipids with PC lipids modified with 1-palmitoyl-2-stearoyl-(7-doxyl)-sn-glycero-3-
161 phosphocholine (7-Doxyl), a collisional quencher, attenuated the increased fluorescence (**Figure**
162 **3B & C**). These observations are consistent with insertion of the hydrophobic loop into the lipid
163 bilayer.

164 ***Integrity of the hydrophobic loop is necessary for parasite egress***

165 We next investigated the importance of the hydrophobic loop in *TgPLP1* function. We
166 used CRISPR/CAS9 gene editing to generate mutant parasites expressing *TgPLP1* with a loop
167 that is symmetrically shortened by two or four amino acids (PLP1_{MWF} and PLP1_w, respectively)
168 as well as a mutant lacking the loop entirely (PLP1_{Δloop}) (**Figure 4A**). Immunofluorescence
169 microscopy confirmed the micronemal sub-cellular localization of the hydrophobic loop deletion
170 mutants (**Figure S4**). Additionally, all three loop mutants are secreted in a calcium-dependent
171 manner as evidenced by excreted/secreted antigen (ESA) assays conducted in the presence or
172 absence of the calcium chelator BAPTA-AM (**Figure 4B**).

173 Next, we tested the egress competence of these loop deletion mutants using four general
174 criteria that have been seen in the *TgPLP1* knockout strain ($\Delta plp1$): (1) the presence of spherical
175 structures in egressed cultures representing failed egress events; (2) formation of smaller plaques
176 relative to wild type parasites; (3) inability to permeabilize the PV membrane (PVM) after
177 calcium ionophore induction; and (4) delayed egress after calcium ionophore induction.

178 Spherical structures were observed in egressed cultures of PLP1_w, PLP1_{MWF}, and PLP1_{Δloop}
 179 similar with those seen in $\Delta plp1$ (**Figure 5A**). PLP1_w, PLP1_{MWF}, and PLP1_{Δloop} parasites form
 180 smaller plaques compared to the parental (WT) strain, consistent to those observed for $\Delta plp1$
 181 (**Figure 5B & C**). Previous studies have shown that $\Delta plp1$ parasites immobilized with
 182 cytochalasin D treatment fail to permeabilize the PVM after induced egress with a calcium
 183 ionophore as compared to WT parasites. We therefore tested the ability of the hydrophobic loop
 184 deletion strains to permeabilize the PVM under the same conditions. PLP1_w, PLP1_{MWF}, and

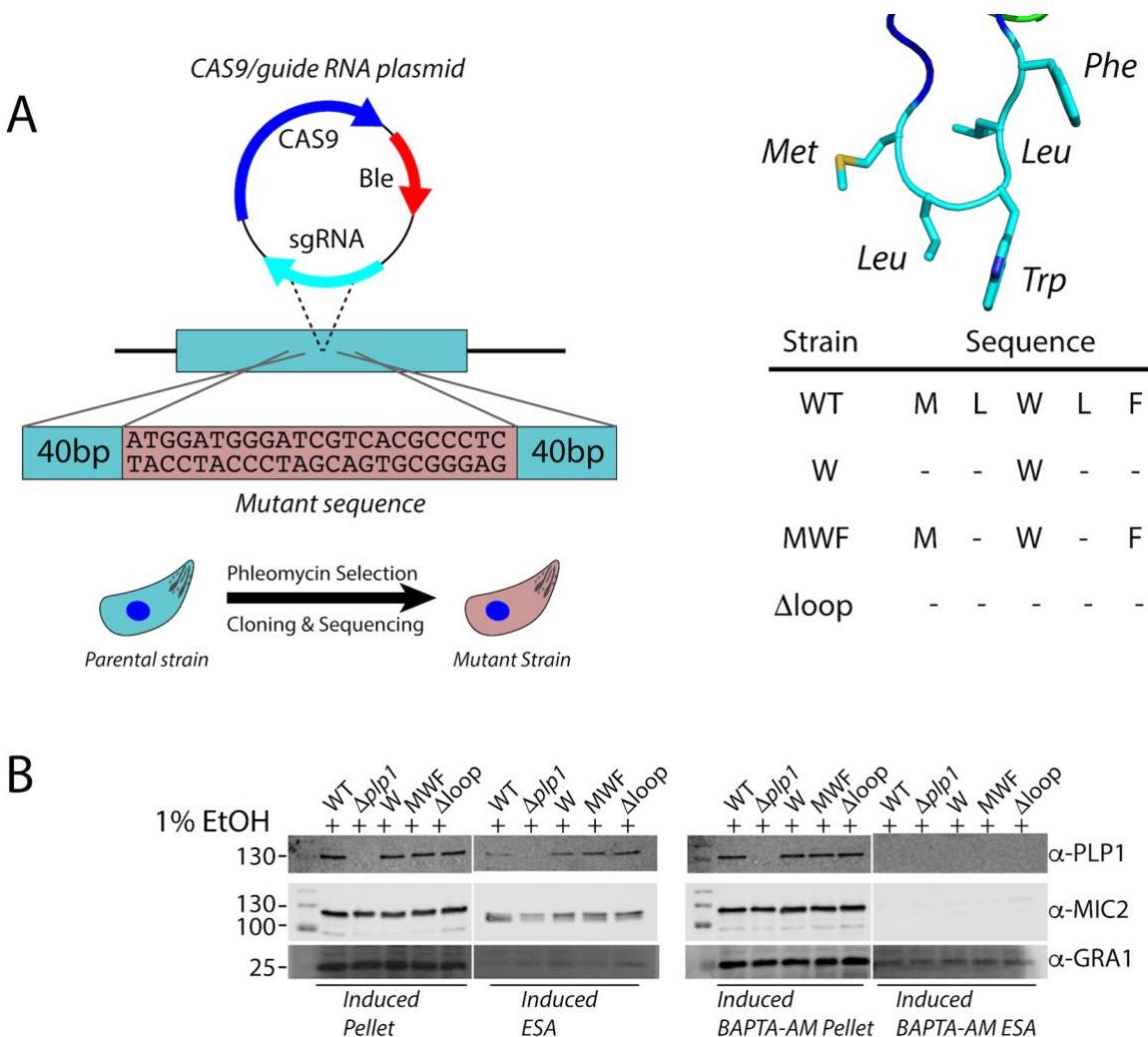


Figure 4. Generation of amino acid deletion mutants in the PLP1 hydrophobic loop A. Schematic representation of the implementation of CRISPR/CAS9 to generate the mutant strains in this study. A sgRNA was used to generate a double stranded break at the hydrophobic loop and a repair template that encoded the appropriate mutation was supplied. The wild type sequence (MLWLF) was truncated to generate shorter loops with the sequences shown on the right. B. Representative western blot of an ESA assay of amino acid deletions in the hydrophobic loop of *Tg*PLP1, strains are labeled with remaining amino acid sequence as above. Pellet fractions are comprised of parasites recovered after induction. ESA fractions include soluble proteins that have been secreted by the parasites. All strains secrete PLP1 in a calcium dependent manner after induction with 1% ethanol. MIC2 and GRA1 serve as loading controls.

185 PLP1_{Δloop} parasites all fail to permeabilize the PVM after the addition of 200 μ M zaprinast, a
 186 phosphodiesterase inhibitor that activates the parasite protein kinase G to induce egress,
 187 consistent with what is observed for $\Delta plp1$ parasites (**Figure 5D**). Finally, we monitored the
 188 extent to which deletions to the hydrophobic loop affect egress from the host cell. To address
 189 this, we infected HFF monolayers in a 96-well plate with WT, $\Delta plp1$, and each of the loop
 190 mutants. Thirty hours post-infection the infected monolayers were treated with zaprinast to
 191 induce egress. Culture supernatants were assayed for lactate dehydrogenase (LDH), which is
 192 released from infected host cells upon parasite egress. $\Delta plp1$, PLP1_W, PLP1_{MWF}, and PLP1_{Δloop}
 193 showed a marked decrease in LDH release compared to cells infected with WT parasites (**Figure**
 194 **5E**). These data suggest that integrity of the hydrophobic loop is critical for *TgPLP1* function.

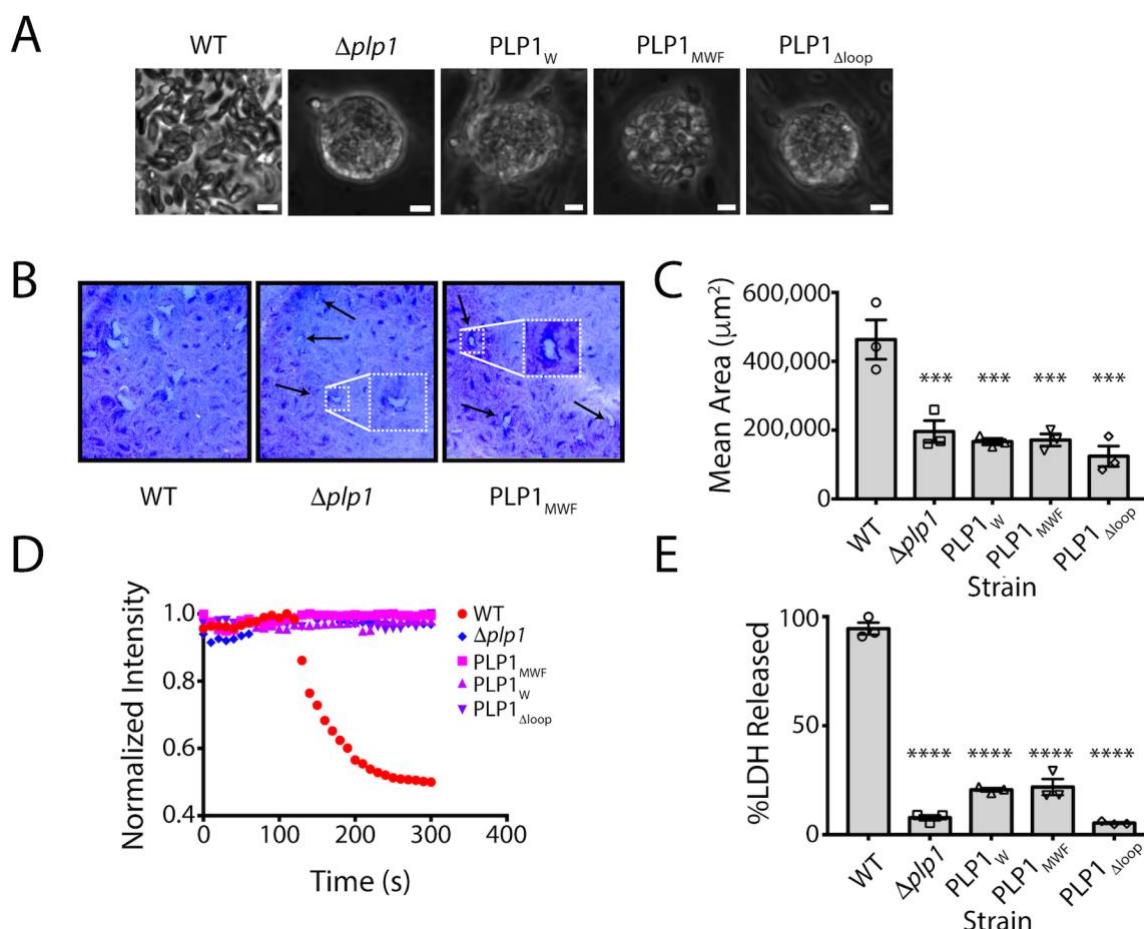


Figure 5. Shortening of the hydrophobic loop mimics the egress phenotype of the *plp1* knockout strain. A. Phase contrast images of parasite cultures. Scale bar, 5 μm . B. Representative images from one of three biological replicate plaque assays for WT, $\Delta plp1$, and hydrophobic loop amino acid deletion mutants C. Quantification of plaque area. Statistical significance determined by one-way ANOVA with Dunnett correction of three biological replicates. D. Fluorescence intensity tracings of DsRed escape from the PV. Infected monolayers were treated with 1 μM cytochalasin D and 200 μM zaprinast and observed by fluorescence microscopy. E. Egress of wild type and mutant parasites as measured by LDH release upon addition of 200 μM zaprinast and incubation for 20 min. Statistical significance determined by one-way ANOVA with Dunnett correction of three biological replicates.

195 ***Amino acid identity in the hydrophobic loop is important for TgPLP1 function in egress***

196 Since shortening the hydrophobic loop resulted in an egress defect that mimics $\Delta plp1$ parasites, we tested how the amino acid composition of the loop influences function. We again 197 used CRISPR/CAS9 to generate four mutant strains including a leucine to valine substitution 198 (PLP1_{MLWVF}) as well as three alanine substitution mutants (PLP1_{MAAAF}, PLP1_{MAWAF}, and 199 PLP1_{MLALF}) to probe the importance of the leucine and tryptophan residues in the hydrophobic 200 loop. We then tested the subcellular localization of these constructs by immunofluorescence 201 microscopy and confirmed that all mutant strains have micronemal localization of *TgPLP1* 202 (**Figure S5**). Additionally, all four mutants showed calcium-dependent secretion (**Figure 6A**). 203 Next, we tested the egress competence of these mutant strains using the same criteria described 204 above. Whereas the most conservative mutant (PLP1_{MLWVF}) was not retained within spherical 205 structures, all of the alanine substitution mutants (PLP1_{MLALF}, PLP1_{MAWAF}, and PLP1_{MAAAF}) 206 were entrapped in such structures, consistent with defective egress (**Figure 6B**). Next, we 207 analyzed the plaque size of the mutants. PLP1_{MLWVF} formed large plaques similar to those of WT 208 parasites (**Figure 6C**). PLP1_{MLALF}, PLP1_{MAWAF}, and PLP1_{MAAAF} parasites, however, formed 209 small plaques akin to $\Delta plp1$ (**Figure 6C & D**). In PVM permeabilization assays, PLP1_{MLWVF} was 210 the only mutant that retained activity after induction with zaprinast. Neither PLP1_{MLALF}, 211 PLP1_{MAWAF}, nor PLP1_{MAAAF} parasites permeabilize the PVM after induction, thus resembling the 212 defect observed in $\Delta plp1$, PLP1_w, PLP1_{MWF}, PLP1_{Δloop} strains (**Figure 6E**). Finally, we tested 213 the extent to which amino acid substitutions to the hydrophobic loop affect egress from the host 214 cell using the LDH assay described above. Consistent with the above findings, PLP1_{MLWVF} is the 215 only point mutant that retained the ability to egress from host cells. PLP1_{MLALF}, PLP1_{MAWAF}, 216 PLP1_{MAAAF} failed to egress in the duration of the experiment (**Figure 6F**). Taken together these 217 results indicate that amino acid identity and hydrophobic character of the loop are important for 218 proper *TgPLP1* function. 219

220 **Discussion**

221 This paper provides new insight into the structure and function of APC β , an 222 apicomplexan specific membrane-binding domain associated with parasite egress and cell 223 traversal. Previous studies established that the *TgPLP1* CTD, which includes APC β , has 224 membrane-binding activity and is crucial for *TgPLP1* function in egress.^[16]; however, the lipid

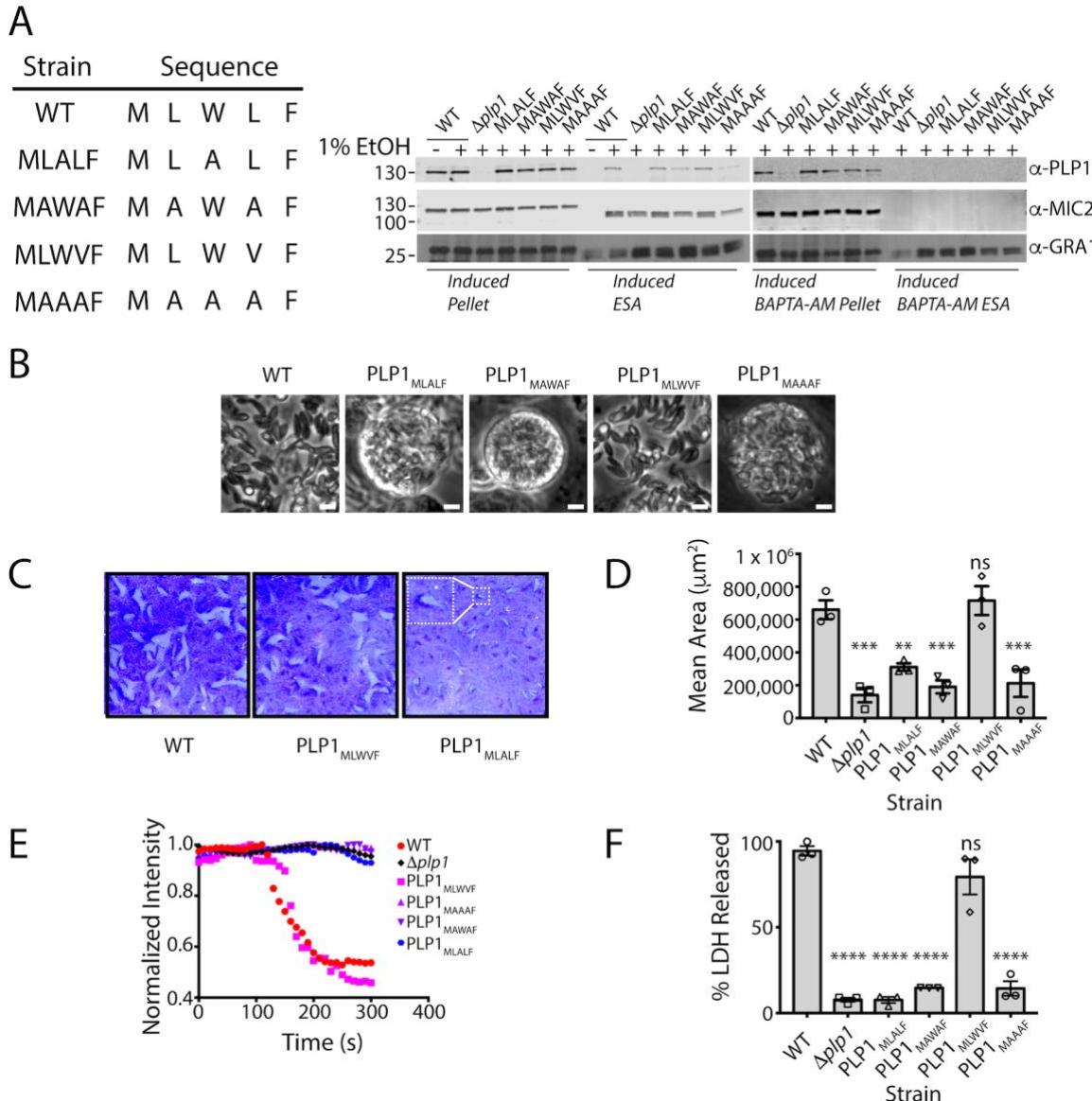


Figure 6. Alanine substitution mutants mimic the deletion strain egress phenotype. A. Representative western blot of an ESA assay of amino acid substitutions in the hydrophobic loop of *TgPLP1*. The wild type sequence (MLWLF) was mutated to generate various amino acid substitution mutant strains. Strains are labeled with the generated amino acid sequence. Pellet fractions are comprised of parasites recovered after induction. ESA fractions include soluble proteins that have been secreted by the parasites. MIC2 and GRA1 serve as control for calcium-dependent and calcium-independent secretion, respectively. B. Phase contrast images of WT and mutant strains. Scale bar, 5 μ m. C. Representative images from triplicate plaque assays of WT, *Δapl1*, and hydrophobic loop amino acid substitution mutants. Inset shows an enlargement of a plaque for a small plaque mutant that exemplifies the other small plaque mutants. D. Quantification of plaque area from three biological replicate experiments. Statistical significance determined by one-way ANOVA with Dunnett correction. E. Fluorescence intensity tracings of DsRed escape from the PV. Infected monolayers were treated with 1 μ M cytochalasin D and 200 μ M zaprinast and observed by fluorescence microscopy. Data shown is representative of that from three biological replicates F. Egress as measured by LDH release upon addition of 200 μ M zaprinast and incubation for 20 min. These data were collected simultaneously with the data shown in Figure 5E. Statistical significance determined by one-way ANOVA with Dunnett correction of three biological replicates.

225 binding specificity and structure of this domain remained unknown. The structure solved herein
 226 is essentially identical to the *TgPLP1* APC β domain reported recently by Ni et al. [17], but our
 227 work additionally defines the lipid binding specificity, provides evidence for insertion of the

228 hydrophobic loop into membranes, and establishes a critical role for this loop in *TgPLP1*
229 function during parasite egress.

230 We found that the *TgPLP1* CTD has a preference for binding IL phospholipids,
231 particularly PS and PE, and that it fails to efficiently recognize OL phospholipids such as PC and
232 SM. These results suggest a working model of directional activity wherein phospholipid
233 accessibility enhances *TgPLP1* activity during egress and limits activity during subsequent
234 invasion. In this model, micronemal secretion of *TgPLP1* during egress delivers it to the PVM
235 for putative binding to PS and PE. Upon escape from the PV, additional secretion of *TgPLP1*
236 targets PS and PE in the IL of the host plasma membrane to facilitate parasite exit from the
237 infected cell. The model further posits that a lack of preferred phospholipid receptors on the OL
238 of the target cell limits *TgPLP1* activity during invasion, thereby allowing formation of the PV.
239 That *T. gondii* is capable of wounding host cells in a *TgPLP1*-dependent manner when
240 microneme secretion and *TgPLP1* activity are enhanced in low pH medium supports this model.
241 However, the leaflet-specific phospholipid composition of the PVM remains unknown. Although
242 the initial topology of this membrane upon invasion is such that PE and PS would be exclusively
243 in the cytosolic leaflet, reports have suggested extensive lipid remodeling of the PVM after
244 invasion and during intracellular replication. ^[28, 29] Also, *T. gondii* is known to secrete into the
245 PV a PS decarboxylase, which converts PS to PE, implying the presence of PS in the PVM ^[30].
246 We attempted to create transgenic *T. gondii* that secrete a PS binding probe (lactadherin C2-
247 GFP) into the PV, but the protein is largely retained in the parasite endoplasmic reticulum. An
248 additional limitation of the working model is that the extent to which *TgPLP1* acts upon the host
249 plasma membrane during egress remains unknown. Nevertheless, other examples of directional
250 activity exist including the predatory fungus *Pluerotus ostreatus* (Oyster mushroom), which
251 targets nematodes by secreting pleurotolysin A/B, a two-component MACPF pore-forming toxin.
252 The pleurotolysin A subunit requires SM and cholesterol for binding, ^[31-33] which is consistent
253 with its action, together with the MACPF B subunit, on exposed membranes of the target
254 nematode. Perforin-1 also exhibits directional activity, but its specificity for a target membrane is
255 influenced by the spacing of phospholipids from unsaturation of acyl chains rather than by
256 recognition of head groups.^[34] Defining the phospholipid binding specificity and the role of acyl
257 chain spacing for other MACPF proteins might reveal additional examples of leaflet preference
258 consistent with a directional activity model.

259 Identifying molecular determinants of membrane recognition by apicomplexan PLPs is
260 necessary to understand cytolytic parasite egress and cell traversal. To address this, we generated
261 an expression construct of the *TgPLP1* CTD and solved the 1.13 Å resolution crystal structure of
262 the APC β domain. Despite using a full-length CTD expression construct the diffraction data only
263 contains the APC β domain and lacks the CTT. Whether the lack of density is due to flexibility in
264 the CTT or enzymatic cleavage remains unclear. The *TgPLP1* APC β structure presented here is an
265 unusual double-layered β -prism with pseudo threefold symmetry. Analysis of the crystal
266 structure shows a hydrophobic loop that protrudes from one of the subunits, which we
267 interrogated for membrane insertion via fluorescence of a tryptophan located within the loop.
268 The observed increase in intrinsic tryptophan fluorescence in the presence of liposomes is
269 consistent with that seen for perfringolysin O and pneumolysin in the presence of liposomes. [35-
270 37] The *TgPLP1* APC β domain contains three other tryptophan side chains, but two are buried
271 within the hydrophobic core and their spectra is likely unchanged by the addition of liposomes.
272 The third tryptophan is surface exposed, but is located in between the inner and outer layer of the
273 beta prism, a position that is unlikely to insert into a lipid bilayer. Thus, we conclude that the
274 tryptophan located at the tip of the protruding hydrophobic loop is probably responsible for
275 changes in the fluorescence spectra reported here. These results are consistent with recently
276 published molecular dynamic simulations that theorize insertion of the protruding hydrophobic
277 loop into the membrane. [17] Insertion of the hydrophobic loop into the target membrane might be
278 required for *TgPLP1* to gain a strong foothold for the subsequent conformational rearrangements
279 that accompany pore formation.

280 To further probe the extent to which the hydrophobic loop is important to *TgPLP1*
281 function we generated parasite lines that were genetically engineered to harbor mutations that
282 probed the length of the hydrophobic loop as well as the importance of particular residues
283 therein. Previous work has shown that *TgPLP1* is required for efficient lysis of the PVM and the
284 subsequent egress from the host cell. [15] Additionally our previous work has also identified the
285 *TgPLP1* CTD as being critical for function. [16] The results presented here show that mutations
286 that affect the length and character of the hydrophobic loop recapitulate the egress defect seen
287 with $\Delta plp1$ parasites. Namely, these loop mutant strains form spheres in egressed cultures, form
288 small plaques relative to wild type parasites, fail to permeabilize the PVM when motility is
289 arrested, and have a delay in egress after induction. The most striking of these observations is the

290 single alanine substitution of the tryptophan located at the tip of the hydrophobic loop.
291 Tryptophan often contributes to the binding interface of membrane binding proteins because of
292 its properties as both a hydrophobic and polar molecule. Indeed, many MACPF/CDC proteins
293 utilize tryptophan residues for binding target membranes^[35, 38, 39] That a single tryptophan to
294 alanine substitution recapitulates the egress defect observed in parasites lacking *TgPLP1*
295 altogether underscores the importance of the hydrophobic loop for efficient egress. The extent to
296 which a similarly important loop is conserved in other apicomplexan PLPs remains unknown in
297 the absence of other structural studies.

298 The work presented here along with that of Ni et al^[17] is a key first step in our
299 understanding of how apicomplexan PLPs recognize and bind to membranes. *T. gondii* encodes
300 two PLPs, *TgPLP1* and *TgPLP2*, but only *TgPLP1* has been shown to be important for cytolytic
301 egress. *TgPLP2* is not expressed in the tachyzoite stage. A related apicomplexan parasite and
302 causative agent of malaria, *Plasmodium spp.*, has a more complex life cycle and encodes an
303 expanded family of five PLPs, *PPLP1-5*.^[40-45] *Plasmodium* PLPs have been implicated in
304 cytolytic egress as well as in cell traversal, a process required for migrating through tissue.^[46-48]
305 Despite some sequence similarity between the *T. gondii* and *Plasmodium spp.* PLPs (55%
306 similarity between *TgPLP1* and *P. falciparum* PLP1) the molecular determinants that govern
307 membrane recognition and binding by *Plasmodium* PLPs remain unclear. More thorough
308 structural and functional studies on *Plasmodium* PLPs are needed to further our understanding of
309 how apicomplexan PLPs recognize their target membranes and shed light on how these differing
310 activities have evolved within a common protein scaffold.

311 Together with previous findings, the current work brings us closer to a molecular
312 understanding of how *TgPLP1* facilitates parasite egress. The work also provides a foundation
313 upon which future work on *TgPLP1* and other apicomplexan PLPs are expected to illuminate the
314 molecular basis of differential function in egress and cell traversal.

315

Table 1. Data collection and refinement statistics

<i>Data collection</i>	<i>TgPLP1_{APCβ}</i> PDB code: 6D7A
Resolution range	24.88 - 1.13 (1.17 - 1.13)
Space group	C 1 2 1
Unit cell	
a, b, c (Å)	101.98, 50.85, 105.34
α , β , γ (°)	90, 90.13, 90
Total reflections	851134 (10855)
Unique reflections	183401 (14245)
Multiplicity	4.6 (1.3)
Completeness (%)	94.92 (71.21)
Mean I/ σ (I)	7.99 (0.49)
Wilson B-factor	10.13
R-merge	0.07709 (1.266)
R-meas	0.08447 (1.735)
R-pim	0.03364 (1.179)
CC1/2	0.997 (0.276)
CC*	0.999 (0.658)
<i>Refinement</i>	
Reflections used in refinement	183401 (14245)
Reflections used for R-free	9505 (737)
R-work	0.1407 (0.3020)
R-free	0.1636 (0.3008)
CC(work)	0.968 (0.430)
CC(free)	0.963 (0.441)
Number of non-hydrogen atoms	4778
macromolecules	4120
ligands	4
solvent	654
Protein residues	531
RMS(bonds) (Å)	0.009
RMS(angles) (°)	1.38
Ramachandran favored (%)	97.15
Ramachandran allowed (%)	2.85
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.43
Clash score	2.08
Average B-factor (Å ²)	16.70
Macromolecules (Å ²)	14.44
Ligands (Å ²)	12.06
Solvent (Å ²)	30.99

Statistics for the highest resolution shell are shown in parentheses.

318 **Materials and Methods**

319 ***Liposome preparation***

320 Liposomes were prepared from 10 mg/mL stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-
321 phosphocholine (POPC, Avanti), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS,
322 Avanti), L- α -phosphatidylethanolamine (POPE, Avanti), Sphingomyelin (SM, Avanti), L- α -
323 phosphatidylinositol (PI, Avanti), and cholesterol (Avanti) in the ratios described in the figure
324 captions. Two μ mol total lipids were dried under a nitrogen stream. Dried lipids were incubated
325 in 1 mL of rehydration buffer (100 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 50 mM Tris pH 7.4)
326 for 30 min and subsequently vortexed until lipid film was completely dissolved. Hydrated lipid
327 suspension was subjected to 3 freeze/thaw cycles, alternating between dry ice/ethanol bath and
328 warm water bath. The rehydrated lipid solution was extruded through 400 nm pore-size filters
329 using a mini-extruder (Avanti) to produce liposomes.

330 ***Membrane Flotation***

331 Two hundred nmol of liposomes were incubated with 0.5-1 nmol of recombinant proteins at
332 37°C for 15 min in a final volume of 200 μ L. After incubation the reaction mixture was diluted
333 with 1 mL 85% sucrose and layered with 2.8 mL of 65% sucrose and 1 mL of 10% sucrose. The
334 reaction was centrifuged at 115,000g at 4°C for 16 h (Sorvall rotor AH-650). Fractions were
335 collected and analyzed by western blot. Band intensity was quantified using Image J. The
336 liposome binding efficiency was calculated as:

337

$$338 \% \text{ Bound} = \frac{I_{\text{Bound}}}{I_{\text{Bound}} + I_{\text{Unbound}}} \times 100 \quad (1)$$

339 ***Protein Production & Purification***

340 High-five or *Sf9* cells were expanded to six 1 L volumes. These were used to seed 20 L of media
341 (Expression Systems) at 2.0×10^6 cells/mL in a 36 L stir tank bioreactor. The culture was
342 infected with recombinant baculovirus at a multiplicity of infection of 5. The reactor was
343 incubated at 27°C with stirring and sparged with air for 72 h. The culture was pumped out and
344 centrifuged at 1000g and 4°C for 40 min to pellet the cells. The media was collected, and batch
345 bound with 0.5 mL per liter of Roche cOmplete His-Tag purification resin for 4 h at 4°C with

346 stirring. Resin was loaded onto a column and washed with 50 mM Tris (pH 8.0), 300 mM NaCl
347 and 20 mM imidazole. Protein was eluted with 50 mM Tris (pH 8.0), 300 mM NaCl and 250
348 mM imidazole. Wash and elution fractions were collected and run on SDS page to determine
349 purity and protein location. The poly-histidine tag was subsequently cleaved with TEV-protease
350 at 4°C overnight and separated from the CTD by immobilized metal affinity chromatography.
351 CTD fractions were further purified by anion exchange chromatography and concentrated to 22
352 mg/mL.

353 ***Crystallization & Data Acquisition***

354 *TgPLP1_{APC β}* crystals were obtained from the Molecular Dimensions Clear Strategy II screen set
355 up with 1 μ L of protein and 1 μ L of reservoir containing 100 mM sodium cacodylate at pH 6.5,
356 0.15 M KSCN, and 18% PEG 3350. Hanging drop plates were equilibrated at 20°C. The needle-
357 like crystals were mounted on a cryoloop and transferred to a cryoprotectant solution containing
358 the reservoir solution supplemented with 20% ethylene glycol. Native crystals were flash frozen
359 in liquid nitrogen for data collection. For phase problem solution data sets, a two-hour soak was
360 performed in a synthetic mother liquor supplemented with 20 mM KI and flash frozen in mother
361 liquor supplemented with 20% ethylene glycol. Data sets were collected at LS-CAT beamline
362 21ID-D and 21ID-G.

363 ***Structure determination***

364 The crystals belonged to the C₁₂₁ space group with cell dimensions of $a = 101.98 \text{ \AA}$, $b = 50.85 \text{ \AA}$,
365 $c = 105.34 \text{ \AA}$, $\alpha = 90^\circ$, $\beta = 90.13^\circ$, and $\gamma = 90^\circ$. Data reduction and scaling were performed with
366 autoPROC.^[49] Phasing was performed using the AutoSol in Phenix.^[50] Initial solution was
367 obtained by SAD on KI soaked crystals. The partial solution was then used as a molecular
368 replacement model for the high-resolution data sets. Model building was performed in COOT^[51]
369 and refinement in PHENIX. The *TgPLP1_{APC β}* crystal structure was refined to a crystallographic
370 R_{work} of 14.07% and a R_{free} of 16.36% The final structure was analyzed with validation tools in
371 MOLPROBITY. Structural visualization was performed via PyMOL.

372 ***Fluorescence Measurements***

373 Intrinsic tryptophan emission intensity was measured on a Biotek Snergy H1 equipped with
374 monochromators for both excitation and emission. The emission spectra were recorded between

375 320-400 nm with a step size of 5 nm. The excitation wavelength was set to 280 nm. The
376 emission intensity was recorded in the absence and presence of PC, PC/PE, PC/PS liposomes and
377 liposomes that had 10 mol% of the PC lipid replaced with 1-palmitoyl-2-stearoyl-7-doxyl)-sn-
378 glycero-3-phosphocholine (7-Doxyl) (Avanti).

379 ***Host cell and parasite culture***

380 All cells and parasites were maintained in a humidified incubator at 37°C and 5% CO₂. Human
381 foreskin fibroblast cells (HFF, ATCC CRL-1634) were maintained in Dulbecco's modified
382 Eagle's medium (DMEM) supplemented with 10% Cosmic Calf serum, 20 mM HEPES pH 7.4,
383 2 mM L-glutamine and 50 µg/mL penicillin/streptomycin. All *T. gondii* parasites were
384 maintained by serial passaging in HFF cells and were routinely checked for mycoplasma
385 contamination.

386 ***Parasite line generation***

387 PLP1 hydrophobic mutant strains were generated using CRISPR-Cas9 using RHΔ*ku80* as a
388 parental strain. 20 bp of *plp1*-specific guide RNA sequence targeting the hydrophobic loop was
389 inserted into pCRISPR-Cas-9-Ble using site-directed mutagenesis to generate the pAG1 plasmid.
390 An annealed synthetic oligonucleotide repair template pair that encoded the appropriate
391 mutations and a silent mutation to replace the NGG cut site (IDTDNA) was mixed with pAG1
392 and precipitated by addition of ethanol. Fifty million tachyzoites were transfected by
393 electroporation in a 4 mm gap cuvette using a Bio-Rad Gene Pulser II with an exponential decay
394 program set to 1500 V, 25 µF capacitance and no resistance and immediately added to a
395 confluent HFF monolayer in a T25 flask. Parasites were selected with 50 µg/mL phleomycin 24
396 h post transfection for 6 h and subsequently added to a confluent HFF monolayer in T25 flask.
397 Parasites were incubated for 36-48 h. Clonal populations were isolated and a 1 kb fragment
398 generated from extracted DNA was sequenced for confirmation of the correct mutation.

399 ***Excreted secreted antigen***

400 Induced excreted secreted antigens (ESA) were performed as previously described. ^[52] Briefly, 2
401 x 10⁷ parasites were incubated at 37°C for 2 min in 1.5 mL Eppendorf tubes with DMEM
402 containing 10 mM HEPES, pH 7.4 and supplemented with 1% ethanol. Parasites were separated
403 by centrifugation (1000g, 10 min, 4°C). Samples of the pellet and supernatant were run on a 10%

404 SDS-PAGE gel and analyzed by western blot. ESAs were performed in the presence and absence
405 of 100 μ M BAPTA-AM.

406 ***Immunofluorescence staining***

407 Infected monolayers were fixed with 4% formaldehyde for 20 min and washed with PBS. Slides
408 were with 0.1% Triton X-100 for 10 min and blocked with 10% fetal bovine serum (FBS),
409 0.01% Triton X-100 in PBS). Slides were subsequently incubated with rabbit anti-PLP1 (1:500)
410 and mouse anit-MIC2 (6D10, 1:250) diluted in wash buffer (1% FBS, 1% Normal Goat Serum
411 (NGS), 0.01%Triton X-100 in PBS) for 1 h at RT. After three washes, slides were incubated for
412 1 h at RT with Alexa Fluor goat anti-rabbit and goat anti-mouse secondary antibodies (Invitrogen
413 Molecular Probes) diluted in wash buffer (1:1000). Slides were washed and mounted in Mowiol
414 prior to imaging.

415 ***Plaque assay***

416 Infected monolayers of HFF cells in T25 flasks were washed with phosphate-buffered saline
417 (PBS). Parasites were liberated by scraping the monolayer and passaging through a 27-gauge
418 needle. Liberated parasites were filtered through a 3 μ m filter (Millipore) and counted on a
419 hemocytometer. Monolayers of HFFs in individual 6-well plates were inoculated with 50
420 parasites and incubated, undisturbed, for one week and fixed with 2% (w/v) crystal violet. Plaque
421 size was quantified using ImageJ.

422 ***PV permeabilization***

423 Monolayers were infected with tachyzoites expressing DsRed and allowed to replicate for 30 h.
424 Infected monolayers were washed twice with warmed Ringer's buffer (155 mM NaCl, 3 mM
425 KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM,NaH₂PO₄, 10 mM HEPES, 10 mM Glucose, 1% FBS,
426 pH 7.40). Ionophore treatment was initiated by addition of an equal volume of 2 μ M
427 cytochalasin D and 400 μ M zaprinast. Fluorescence image time series were collected every 10
428 sec for 5 min.

429 ***LDH egress assay***

430 Infected monolayers of HFF cells in T25 flasks were washed with phosphate-buffered saline
431 (PBS) and liberated by scraping the monolayer and passaging through a 27-gauge needle.

432 Liberated parasites were filtered through a 3 μ m filter (Millipore), counted on a hemocytometer,
433 and centrifuged at 1000g for 10 min. Parasites were resuspended to a density of 5×10^5
434 parasites/mL and each well of a 96-well flat bottom culture plate was inoculated with 5×10^4
435 tachyzoites of the appropriate strain. Infected cells were washed with warm Ringer's buffer and
436 treated with zaprinast diluted to 200 μ M in Ringer's buffer, Ringer's with an equal volume of
437 dimethyl sulfoxide (DMSO), or cell lysis reagent (BioVision) diluted in Ringer's. Treated plates
438 were incubated at 5% CO₂ and 37°C for 20 min. Plates were placed on ice and 50 μ l of each well
439 was transferred to a 96-well round-bottom plate. Round-bottom plates were centrifuged at 500g
440 for 5 min. Ten μ l of solution was tested for lactate dehydrogenase (LDH) following the
441 manufacturer's protocol (Biovision).

442 **Data availability**

443 The atomic coordinates are available at the Protein Data Bank (PDB) under accession number
444 6D7A.

445 **End Matter**

446 ***Author Contributions and Notes***

447 Lipid binding experiments were designed by OZ and VBC and performed by OZ. JDP and WCB
448 expressed recombinant proteins and performed initial purification. CMEB purified recombinant
449 proteins and crystallized *TgPLP1_{APC β}* . Diffraction data was collected by CMEB, NMK, and ZW.
450 The *TgPLP1_{APC β}* structure was solved by NMK and refined by AJG. Tryptophan fluorescence
451 and mutational analysis was designed by AJG and VBC and performed by AJG. Mutant
452 characterization was performed by AJG and M-HH. AJG and VBC wrote the manuscript.

453

454 The authors declare no conflict of interest.

455 This article contains supporting information online.

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652 **Supplementary Figures & Tables**

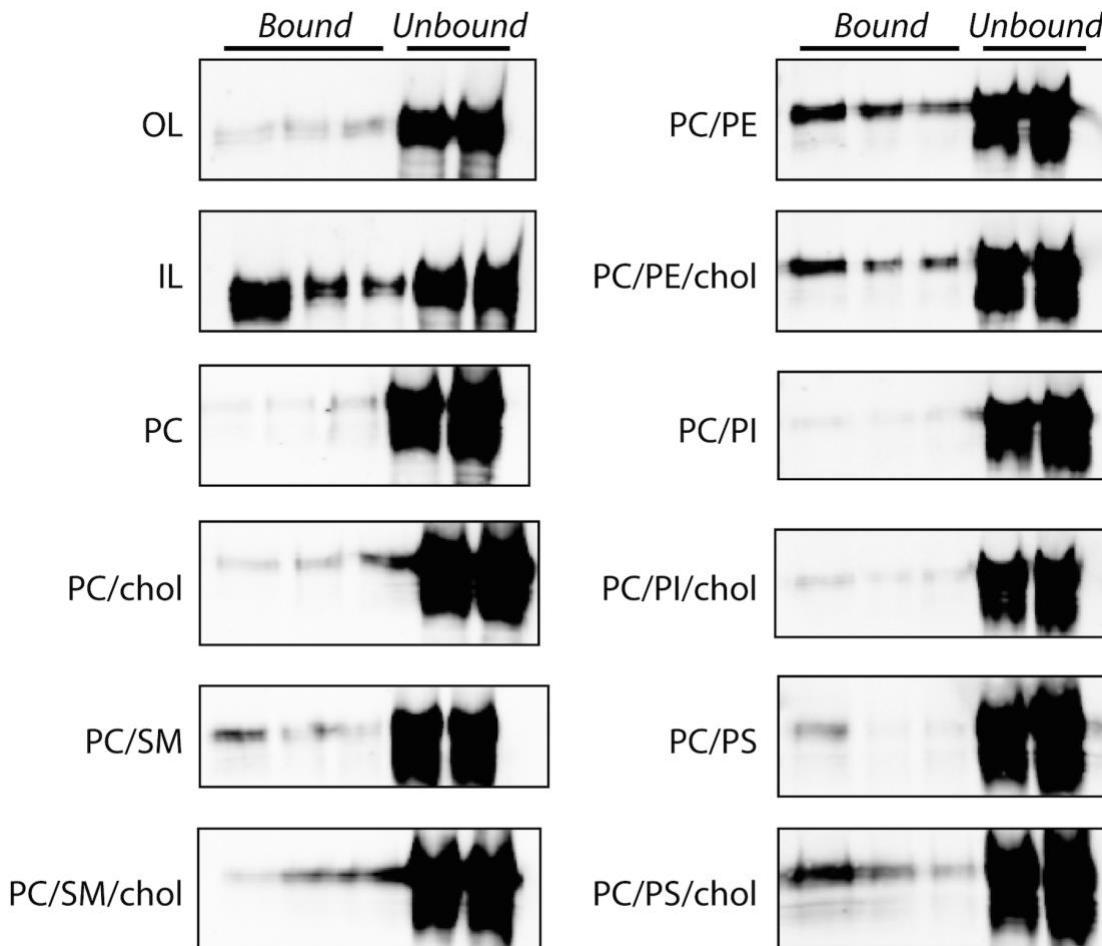


Figure S1. *TgPLP1*_{CTD} binding to liposomes of varying composition. Representative western blots of liposome membrane flotation assays. Liposome composition as follows: OL: 75% PC, 8.3% SM, 16.7% cholesterol; IL: 35.7% PE, 14.3% PS, 21.4% PI, 28.6% cholesterol; PC: 100% PC; PC/chol: 50% PC, 50% cholesterol; PC/SM: 50% PC, 50% SM; PC/SM/chol: 30% PC, 50% SM, 20% cholesterol; PC/PE: 50% PC, 50% PE; PC/PE/chol: 30% PC, 50% PE, 20% cholesterol; PC/PS: 50% PC, 50% PS; PC/PS/chol: 30% PC, 50% PS, 20% cholesterol; PC/PI: 50% PC, 50% PI; PC/PI/chol: 30% PC, 50% PI, 20% cholesterol. Quantification of these bands is shown in Figure 1D.

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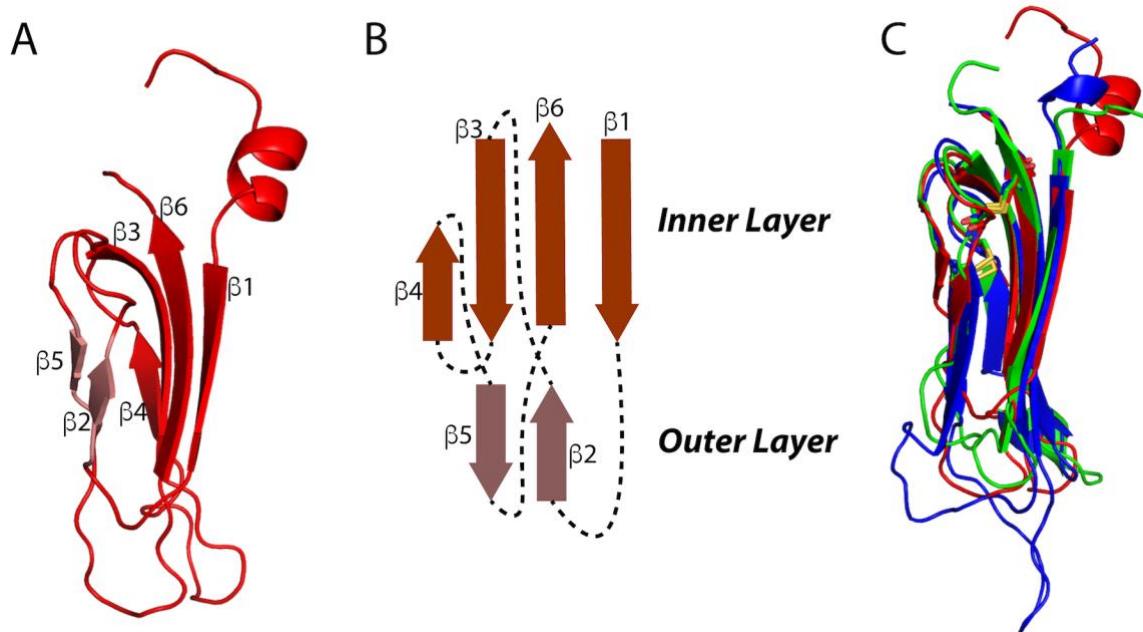


Figure S2. Structural details of the *TgPLP1* $\text{APC}\beta$ structure. A. Subdomain 1 of the $Tg\text{PLP1}_{\text{APC}\beta}$ crystal structure. Each subdomain is made up of an inner β -sheet of four anti-parallel strands and an outer β -sheet of two anti-parallel strands. B. Schematic representation of the inner and outer β -sheets. C. Overlay of the three subdomains of the $\text{APC}\beta$ crystal structure.

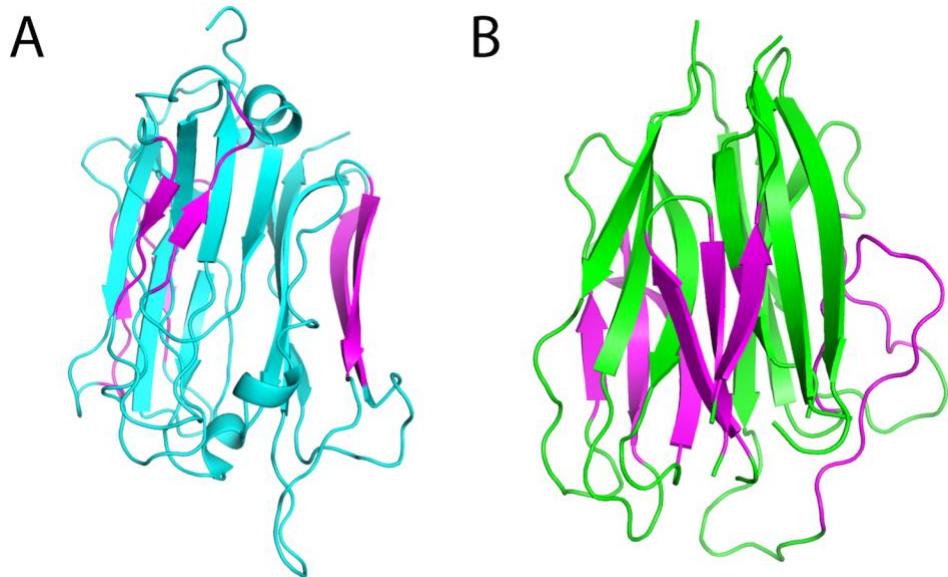


Figure S3. Comparison of the *TgPLP1* APC β crystal structure and the PCSK9 V-domain crystal structure A. Cartoon representation of *TgPLP1* APC β structure. B. Cartoon representation of the PCSK9 V-domain.

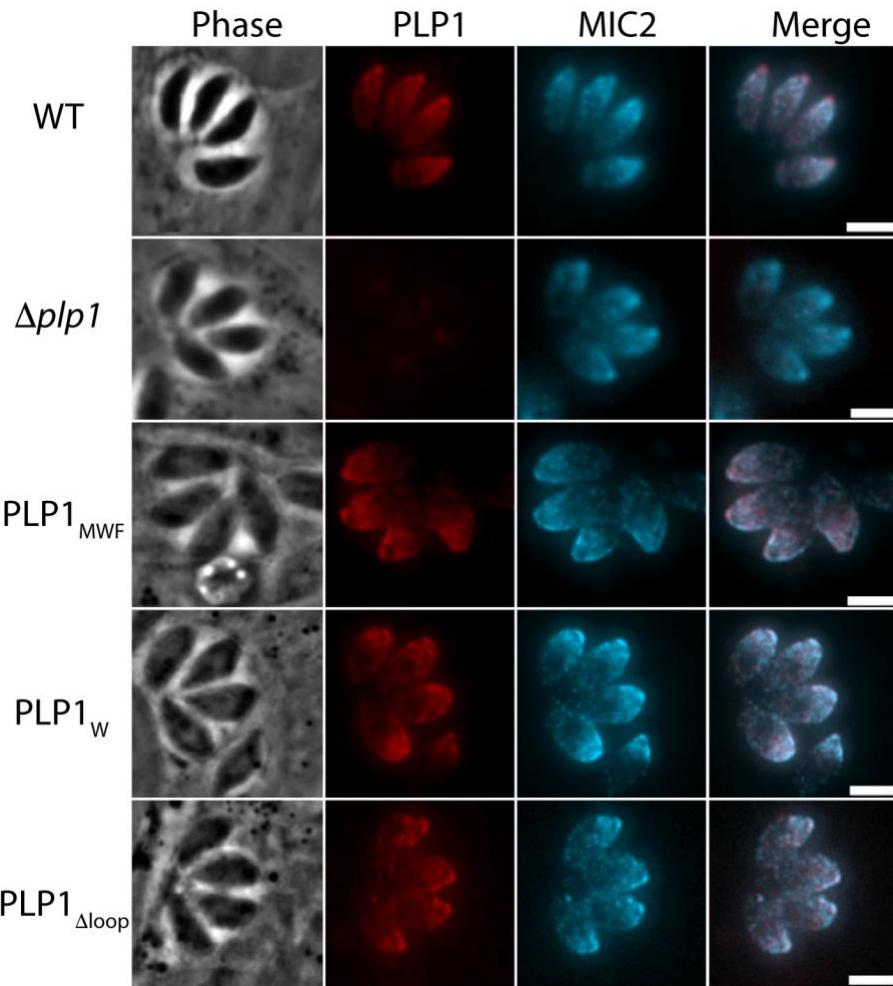


Figure S4. Indirect immunofluorescence of intracellular parasites with mouse anti-*TgMIC2* (cyan) and rabbit anti-*TgPLP1* (red) antibodies.

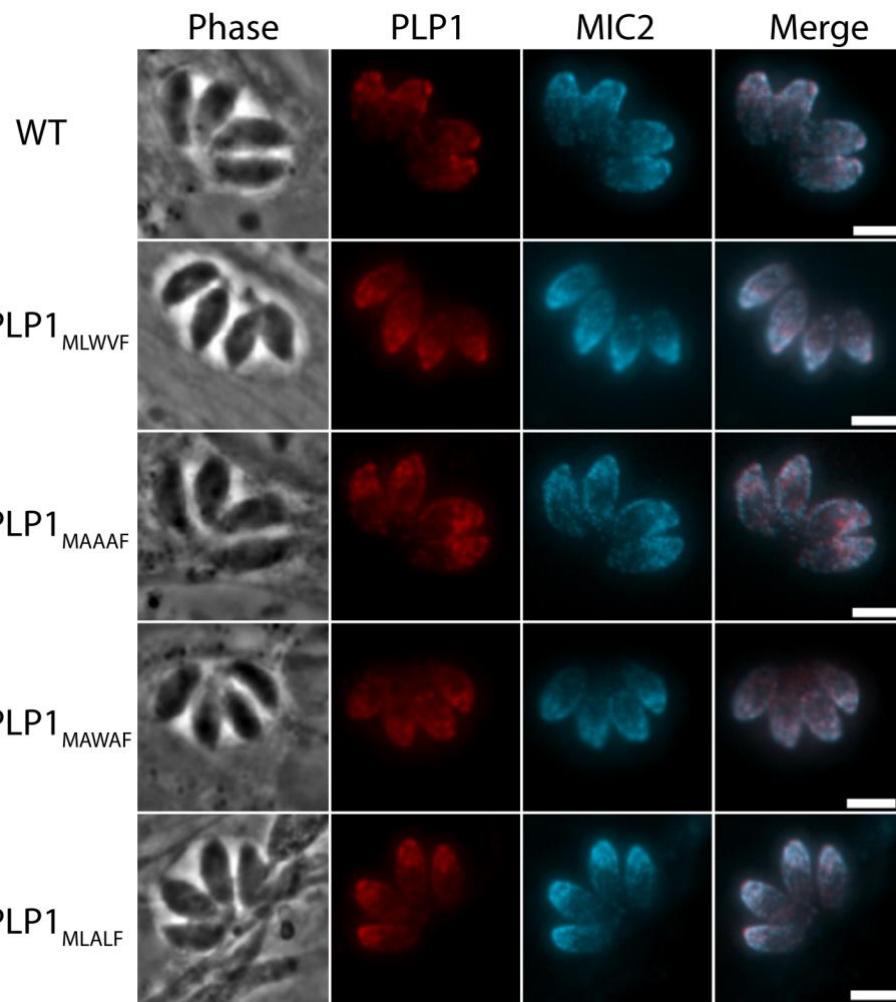


Figure S5. Indirect immunofluorescence of intracellular parasites with mouse anti-*Tg*MIC2 (cyan) and rabbit anti-*Tg*PLP1 (red) antibodies.

Table S1. Dali server search results for PDB entry 6D7A

Number	PDB-chain	Dali-Z	% ID	Molecule Description
1	5ouo-A	48.5	100	Perforin-like protein 1
2	5vlp-A	6.0	17	Proprotein convertase subtilisin/kexin type 9
3	5a3k-A	3.9	10	Putative pteridine-dependent dioxygenase

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