A Structural and Functional Analysis of *Toxoplasma gondii* Perforin-like Protein1

by

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DEDICATION

This dissertation is dedicated to my family with much gratitude for their encouragement, support, and love and during its completion.

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Chapter 1
Introduction

Molecular mechanisms of parasite egress

Intracellular microorganisms are uniquely adapted to grow and develop within the confines of a host cell. This niche affords partial protection from host immune defenses and provides the microbe a ready source of nutrients. The Apicomplexa are a group of obligate, intracellular protozoan parasites that includes many species of medical and veterinary significance. During acute infection, these parasites use strategies that minimize the vulnerable time spent outside of the cell, in some cases abandoning an expired host cell for a fresh one in only a few seconds. Tools used to successfully make this transition include actin-myosin based gliding motility and specialized apical secretory organelles, which have been characterized largely from their role in host cell invasion. Gliding motility drives active cell invasion, which is assisted by secretory proteins that promote host cell adhesion and formation of a parasitophorous vacuole (PV) wherein the parasite replicates.

Egress by intracellular pathogens is much less well understood than cell invasion. Parasite escape from the host cell requires the breaching of multiple barriers including the parasitophorous vacuole membrane (PVM), host cytosolic organelles, the host cytoskeleton, and the host plasma membrane (HPM). Ideally, the parasite times the disruption of these barriers with completion of its replicative cycle to emerge from one infected cell fully prepared to infect a neighboring host cell. Several recent studies support egress as an active phenomenon and have identified parasite proteins required for egress including the contribution of a pore-

forming protein (PFP) for *Toxoplasma gondii* exit. A role for host G-protein coupled receptors and associated down-stream signaling pathways including calpain proteases, have also been established recently, highlighting a novel host contribution to parasite egress. Here I discuss these and other recent developments and build a model of the emerging view of *Toxoplasma* host cell egress.

Attack of the tachyzoites

Toxoplasma gondii is an obligate intracellular protozoan parasite. A hallmark of acute infection by *Toxoplasma* is the repeated cycle of cell invasion, intracellular replication, and parasite egress [1]. Cell invasion begins with attachment of the tachyzoite, the replicative form, to the host cell surface and orientation of the apical end of the parasite towards the host plasma membrane [2]. Secretion of proteins from the apical microneme and rhoptry organelles leads to formation of a moving junction, a structure composed of parasite proteins and host components that span the host cell membrane while maintaining contact with the parasite plasma membrane [3-5]. The parasite uses its actin-myosin motor system to slide through the moving junction, thereby invaginating the HPM and encapsulating itself within the PV in the host cytosol. While the PV originates from the HPM, it is extensively modified by the parasite during asexual replication [6, 7]. Finally, during egress the parasite secretes proteins into the PV, becomes motile, and actively exits the cell in search of a new target cell.

Sensing signs and signaling

Video microscopy observations of parasite egress indicate that it is a rapid, and therefore tightly regulated, event [8, 9]. Controlling the timing of departure is beneficial to the parasite, especially for *Plasmodium* where premature egress would be disastrous since the intermediate replicative stages are not invasive. *Toxoplasma* has somewhat greater flexibility in the timing of egress since daughter parasites are continually produced during intracellular replication. Studies in *Toxoplasma* have

established a role for K^+ and Ca^{2+} ion fluxes in parasite motility and egress (reviewed in Lavine *et al.*, 2007) [10]. The parasite senses K^+ levels in its surroundings, maintaining a non-motile state while bathed in the high K^+ intracellular environment, and activating its motility system upon experiencing a drop in external K^+ [8]. Precisely how the parasite senses K^+ is not known; however, phospholipase C, parasite intracellular calcium, and several Ca^{2+}-responsive proteins play a role (Table 2), suggesting regulation by Ca^{2+}-based signaling [8]. Indeed, several studies have shown that parasite intracellular Ca^{2+}, released from intracellular stores via several distinct signaling pathways [11], regulates motility by activating both the glideosome motility system and apical secretion of micronemal transmembrane adhesins, which engage the motor and act as a drive train to transduce power into motion [10]. Potassium sensing ensures the motility system is off during intracellular replication, but can be rapidly initiated for a hasty exit. Whether K^+ flux is a cue for natural egress at the end of the replicative cycle is not known. Metabolic or mechanical strain in parasite-laden infected host cells could cause K^+ to flood out of the cell, thereby activating exit. Interestingly, parasites have also been observed to exit host cells in the absence of parasite motility or K^+ flux [12]. Here, the authors observed that osmotic stress and host cell membrane tension had an effect on egress. Thus, perhaps the parasite uses K^+ sensing mainly for emergency purposes when, for example, under immune attack. Supporting this notion, Persson *et al* [13] have shown that *Toxoplasma* rapidly exits from cells undergoing apoptosis due to natural killer (NK) cell engagement of Fas ligand or delivery of perforin and granzymes. Rather than perishing in the dying cell the parasite remarkably reverses its fate by infecting the NK cell that instigated the attack [13]. This establishes that the parasite can respond to extrinsic cues to exit a damaged or dying cell and therefore escape immune attack.

Under normal circumstances, however, it is more likely that the parasite uses intrinsic cues to orchestrate its escape. One clue to how this might happen came with the identification of the egress-signaling molecule called abscisic acid (ABA)

[14]. In plants, ABA acts as a hormone that mediates growth and responses to environmental cues through cyclic ADP-ribose (cADPR) and calcium fluxes. In *Toxoplasma*, ABA induces the production of cADPR, which activates Ca^{2+} release from an internal membrane-bound pool (probably the ER), and this in turn triggers the secretion of microneme proteins and presumably also starts the parasite motor. Although the parasite genome has candidate genes for ABA synthesis, their precise role remains to be determined. Reflecting its plant heritage, ABA is most likely synthesized in the apicoplast, an organelle derived from an algal endosymbiont. Interestingly, ABA levels remain low during parasite replication, but rapidly spike just prior to parasite egress. Thus, steep production of ABA at the end of the replicative cycle might serve as an intrinsic cue for egress. The herbicide fluridone inhibited ABA synthesis and blocked parasite egress, but not growth or invasion. Furthermore, fluridone-treated mice survived inoculation with a lethal dose of *Toxoplasma*. Fluridone has also been shown to be effective against malaria, indicating common ABA pathways in apicomplexans. However, blocking ABA signaling in *Toxoplasma* tissue culture samples not only blocked egress, but also led to differentiation to bradyzoites, a parasite stage responsible for chronic infection. This suggests the absence of normal egress signals in the lytic cycle results in tachyzoite to bradyzoite differentiation and may regulate switching between these developmental stages.

Microneme secretion is essential for parasite egress and invasion. Interestingly, rhoptry secretion is not required for egress [15, 16]. The requirement for microneme secretion during egress and invasion is well demonstrated by the defects of a parasite strain with a temperature-dependent mutation in a double C2 (DOC2) protein homologue, which presumably functions by recruiting membrane fusion machinery for microneme exocytosis [17]. However, it appears that different signaling pathways coordinate microneme secretion during egress and invasion. The calcium-dependent protein kinases, CDPK1 and CDPK3, mediate calcium-regulated signaling leading to microneme secretion and motility [18-21]. Both kinases

are required during parasite egress; however, CDPK3 is not required for invasion. This suggests that the parasite utilizes multiple pathways to respond to different cues and has a dedicated signaling pathway involving CDPK3 for parasite egress.

Breaking down barriers

Organisms throughout the tree of life use pore-forming proteins (PFP) to breach membranes, usually those of another cell. The discovery that MACPF (membrane attack complex/perforin) proteins have structural homology to bacterial cholesterol dependent cytolysins [22-24] provided a wealth of mechanistic insight into MACPF pore formation, and by extension, how apicomplexan parasites might use PFPs during infection. A brief overview of PFP function is given here and we refer the reader to the recent in-depth reviews for more information [24-27]. Most PFPs are secreted as monomers that bind to receptors on the target membrane, oligomerize, and insert to form a pore. PFPs are used for both host defense and microbial aggression. For example, pores created by the membrane attack complex (MAC, i.e., terminal components of the complement cascade) and perforin, aid in host immunity to microbial infections and malignancy. Bacteria also use PFPs on host membranes to escape from a vacuole after entry (e.g., *Listeria* [28]) or to deliver bacterial factors into the host cell (e.g., *Shigella* and *Yersinia*; [29, 30]). With the exception of *Cryptosporidium*, potential PFPs have been identified in all apicomplexan genomes by sequence homology to the MACPF domain [31]. The *Plasmodium* genome has five different MACPF-domain containing proteins, termed PPLP1-5 (*Plasmodium* perforin-like protein), and the *Toxoplasma* genome has two, TgPLP1 and TgPLP2 (*Toxoplasma gondii* perforin-like protein).

Permeabilization of the HPM and PVM a few minutes prior to *Toxoplasma* egress has been previously reported [8, 32], and this membrane permeabilization was found to be calcium- and parasite-dependent. Membrane permeabilization was observed by leakage of a small molecule out of host cells prior to parasite egress[8]. The isolation of mutants resistant to calcium-ionophore treatment indicated the

membrane permeabilization was parasite-dependent[32]. However, the mechanism of membrane permeabilization remained unknown. The basis of membrane permeabilization was revealed upon showing that TgPLP1 is crucial for rapid parasite egress [33]. Initial observations of TgPLP1-deficient parasite cultures revealed the presence of parasites trapped within large spherical structures floating in culture media. Such structures were not seen in cultures of WT parasites or mutant parasites with restored expression of TgPLP1. Further examination of the spheres showed parasites enveloped by one or two membranes i.e., the PVM and/or HPM. Upon induced egress with a calcium ionophore, TgPLP1-deficient parasites activated motility and secreted microneme proteins but were significantly delayed in their time to host cell exit. Video microscopy of knockout parasites upon induced egress showed parasites became motile in the PV and actively attempted to cross the PVM, but with limited success. Eventual escape appears to be principally due to motility-based mechanical rupture of the PVM since parasite vigorous movements and probing of the PVM with its extruded conoid immediately preceded eventual egress. Moreover, TgPLP1 expression was necessary for PVM permeabilization since a fluorescent protein expressed in the parasite PV failed to escape the PV upon egress induction in the knockout, in contrast to WT or genetically complemented parasites where fluorescence was observed throughout the host cytosol. Whether TgPLP1 itself forms a pore has not been established, but it has all of the key structural features used for pore formation by MACPF/CDC proteins including two α-helical elements that convert to amphipathic β-sheets to form a large oligomeric β-barrel pore. Co-infection, video microscopy, and immunofluorescence experiments revealed WT parasites can donate TgPLP1 to aid in escape of knockout parasites from the same cell. This indicates that TgPLP1 can function in *trans* and from either side of the PVM, suggesting that the receptor for TgPLP1 pore formation is present on both sides of the membrane. It was proposed that TgPLP1 promotes rapid parasite egress by sufficiently weakening the PVM to allow escape of motile parasites (Figure 1, step 2). Alternatively or additionally, TgPLP1 pores could

provide a conduit for other egress effector proteins, such as PV resident or secreted proteases and lipases, to access the host cytosol and disrupt the cytoskeleton and plasma membrane, thereby compromising the integrity of these other barriers to exit. TgPLP1-deficient parasites are highly attenuated (~5-log decrease in LD_{100}) in the mouse model of toxoplasmosis, highlighting the importance of rapid egress in the normal course of infection and disease[33].

The helpful host cell

As noted earlier, immune cell attack of *Toxoplasma* infected cells can induce rapid parasite egress. Both host perforin and Fas-ligand stimulated parasite egress, likely through calcium fluxes from perforin-mediated membrane damage or downstream apoptotic signaling. Escaping a perishing host cell promotes parasite survival, and parasites may even invade the attacking immune cell [13,34]. However, the course of the *Toxoplasma* lytic cycle *in vivo* varies depending on the cell infected. In the intraperitoneal infection model, parasites infecting mesothelial cells appeared to follow a similar course of replication as in tissue culture, with multiple rounds of replication prior to egress; but parasites infecting macrophages often underwent externally triggered egress (ETE) prior to replication [35]. ETE resulted in closely spaced rounds of parasite egress and invasion. While this led to macrophage turnover by macrophages dying upon parasite egress, it also limited parasite replication by "haven disruption" as parasites that egressed more frequently were more likely to encounter activated macrophages, thus controlling the infection. Parasites undergoing rapid cycles of egress and invasion may also be more susceptible to immune attack if they are unable to replenish their stocks of secretory proteins required for egress and invasion. Nevertheless, mice succumb to the lethal infection despite this proposed host defense mechanism. From another perspective it is possible that the rapid rounds of host cell cytolysis promotes increased inflammation, which contributes to a lethal outcome.

Beyond triggering egress by immune attack, host cells facilitate egress by stimulating cytoskeletal remodeling. A requirement for a host calcium-dependent protease, calpain-1, for efficient parasite egress of *Plasmodium* and *Toxoplasma* was initially demonstrated (Figure 1, step 4) [36]. In host cells with calpain siRNA knockdown, *Toxoplasma gondii* egress was compromised, suggesting a requirement for host calpain. Infection of calpain-deficient cells led to the appearance of swollen vacuoles and a small plaque phenotype. While *P. falciparum* merozoites were never detected egressing erythrocytes lacking calpain-1, some *T. gondii* parasites were able to exit from calpain-deficient fibroblasts, indicating redundancy or non-essentiality in calpain-mediated egress by *Toxoplasma*. Since calpains are well known for their role in cytoskeletal remodeling [37], it is reasonable to think that they aid egress by helping to dismantle the host cytoskeleton.

Further siRNA screens identified numerous other host proteins involved in parasite egress [38]. The findings suggested a pathway potentially beginning with a parasite-derived ligand acting on a host G-protein coupled receptor (GPCR), leading to activation of phospholipase C and protein kinase C, which phosphorylated the cytoskeletal protein adducin. Adducin phosphorylation destabilizes the cytoskeleton, resulting in activation of the mechanosensitive membrane channel TRPC6 and an influx of calcium. The calcium influx activated calmodulin and calpain, further triggering cytoskeletal breakdown. In this way, the parasite taps into host signaling pathways to promote its escape.

Delineation of developments

Interfering with egress has been shown to be an effective method of disrupting parasite transmission and pathogenesis. The study of host cell exit by apicomplexans is expected to reveal conserved and unique roles for parasite and host proteins, thereby revealing potential new targets of therapeutic intervention. Although some distinctions due to the different infected cell types are likely to exist, the basic principles of parasite egress appear to be similar and can be grouped into

five steps (Figure 1). These steps may partially overlap and may not occur in the same sequence for all conditions. (1) The parasite produces an intrinsic egress cue, such as ABA, or senses an extrinsic signal to egress, such as immune attack or a drop in host K^+. (2) The parasite activates motility and secretes egress effector proteins. (3) The PVM is disrupted through the concerted action of pore-forming proteins, parasite motility, and perhaps other effector proteins. (4) Host and potentially parasite proteins act to disrupt the host cytoskeleton and HPM. (5) The parasite uses its motility system to escape from the dying host cell in search of a nearby host cell to infect.

Present puzzles pursued

While recent studies have shed new light on the molecular mechanisms of host cell egress by *Toxoplasma* and related organisms, many outstanding questions remain to be addressed. In the following investigations, I examine the function of TgPLP1 (termed PLP1 hereafter), the first identified secreted parasite protein involved in egress. Specific questions addressed are: 1) Is PLP1 sufficient for membrane damage and does it function through a conserved mechanism of membrane attack? 2) What is the role of each PLP1 domain in pore formation? 3) How is PLP1 activity regulated during egress and invasion? Answering these questions has provided new insight into the function of pore-forming proteins, the role of pore-formation in invasion and egress, and potential mechanisms regulating lytic activity.

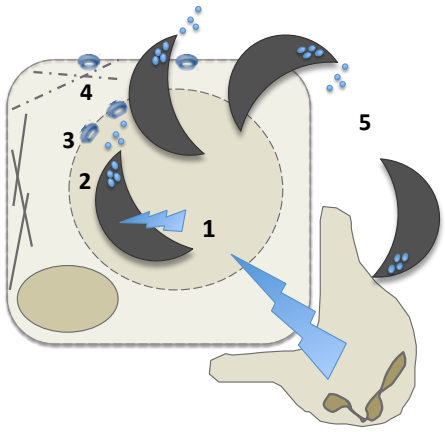

Figure 1. *Steps in Toxoplasma tachyzoite egress.* **(1)** Transmission of an egress signal. **(2)** Protein secretion from micronemes (*Toxoplasma*) and activation of the motility system. **(3)** Disruption of the PVM. **(4)** Disruption of the host cytoskeleton and HPM. **(5)** Active exit from the host cell and migration to a neighboring cell.

Egress Inducers	Abbreviation	Mode of action	References
A23187[a]	A23	Calcium ionophore	Black *et al.*, 2000b
Zaprinast[a]	Zap	cGMP-specific phosphodiesterase inhibitor	Lourido *et al.*, 2012
Dithiothreitol[a]	DTT	Nucleotide triphosphatase activation?	Stommel *et al.*, 1997
Nigericin[a]		Potassium ionophore	Fruth and Arrizabalaga, 2007
Ethanol[a]	EtOH	Calcium flux, possibly by activation of phospholipase C	Carruthers *et al.*, 1999
sodium nitroferricyanide (III) dihydrate[a]	SNP	Nitric oxide release	Ji *et al.*, 2013
Streptolysin O[a]	SLO	Permeabilization of HPM	Moudy *et al.*, 2001
S. aureus α-toxin[a]		Permeabilization of HPM	Moudy *et al.*, 2001
Abscisic acid[b]	ABA	Calcium flux via production of cADPR	Nagamune *et al.*, 2008b
Natural killer cell[b]	NK	Induction of apoptosis	Persson *et al.*, 2009

Egress Inhibitors[c]	Abbreviation	Target	References
U73122		Phospholipase C	Moudy *et al.*, 2001
Cytochalasin D[d]	CytD	Actin	Schwartzman and Pfefferkorn, 1983 Black *et al.*, 2000b Lavine *et al.*, 2007
Wortmannin	Wort	Phosphoinositide 3-kinase	Caldas *et al.*, 2007
Staurosporine		Unknown serine/threonine kinase(s)	Caldas *et al.*, 2007
Genistein		Unknown tyrosine kinase(s)	Caldas *et al.*, 2007
KT5926		Calcium-dependent protein kinase1	Kieschnick *et al.*, 2001 Moudy *et al.*, 2001
DCG04		Host calpain 1, 2	Chandramohanadas *et al.*, 2009
Dimethylamiloride	DMA	Sodium/Hydrogen exchanger 1	Arrizabalaga *et al.*, 2004
Compound 1		cyclic GMP-dependent protein kinase (PKG)	Lourido *et al.*, 2012
GF120918		P-glycoprotein	Bottova *et al.*, 2010

Table 1. *Egress inducers and inhibitors.* [a]Artificial egress inducer; [b]Natural egress inducer; [c]Artificial egress inhibitors; [d]Blocks artificially induced egress and delays natural egress

Egress Factors	Abbreviation	Function	References
Sodium/Hydrogen Exchanger1	NHE1	Calcium homeostasis	Arrizabalaga *et al.*, 2004
Apical complex lysine methyltransferase	AKMT	Regulation of motility	Heaslip *et al.*, 2011
Calcium-dependent protein kinase1	CDPK1	Induction of microneme secretion and motility	Moudy *et al.*, 2001; Lourido *et al.*, 2010
Calcium-dependent protein kinase3	CDPK3	Induction of microneme secretion and motility	Lourido *et al.*, 2012; Garrison *et al.*, 2012; McCoy *et al.*, 2012
Cyclic GMP protein kinase	PKG	Induction of microneme secretion and motility	Gurnett *et al.*, 2002; Wiersma *et al.*, 2004; Donald *et al.*, 2006
Double C2 protein 2.1	DOC2.1	Microneme exocytosis	Farrell *et al.*, 2012
MyosinA	MyoA	Gliding motility motor	Meissner *et al.*, 2001
Profilin	PRF	Actin polymerization	Plattner *et al.*, 2008
Perforin-like Protein1	PLP1	Permeabilization of PVM (and HPM?)	Kafsack *et al.*, 2009
Host calpain 1, 2	CAPN1,2	Cytoskeletal remodeling?	Chandramohanadas *et al.*, 2009
Host G-protein coupled receptors	GPCRs	Cytoskeletal remodeling?	Millholland *et al.*, 2013

Table 2. *Egress associated factors.*

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Chapter 2

PLP1 is sufficient for membrane permeabilization and displays a conserved mechanism of membrane attack

Introduction:

Pore-forming proteins involved in infection have been classically viewed as aiding pathogen invasion into host cells and tissues or promoting pathogen clearance by the host immune system. For example, the gram-positive bacterium, *Listeria monocytogenes,* utilizes listeriolysin O (LLO) to escape its initial endosomal vacuole to access its replication niche in the host cytosol [1]. Also, *Plasmodium* sporozoites utilize pore-forming proteins to promote tissue migration during multiple stages of their lifecycle [2, 3]. To combat infection, mammalian hosts circulate components of the complement cascade, which upon activation culminates in the assembly of the membrane attack complex (MAC) on target microbes, such as the gram-negative bacterium *Neisseria meningitidis* [4]. Additionally, perforin (PF), a pore-forming protein secreted by cytotoxic T lymphocytes (CTL) delivers proteases into virus-infected or transformed cells to initiate programmed cell death [5]. Pore-forming proteins are speculated to have functions not only in pathogenesis but also in development, and are found in diverse organisms throughout the tree of life [6].

Our group recently identified a perforin-like protein (PLP1) that is crucial for rapid host cell exit of the protozoan parasite, *Toxoplasma gondii* [7]. *T. gondii* is the etiological agent of toxoplasmosis, which causes severe disease when acquired congenitally or reactivated in immune-compromised individuals, manifesting as tissue destruction from unchecked lytic growth [8-10]. *T. gondii* belongs to the

phylum Apicomplexa, which includes other pathogens of human and veterinary significance such as *Plasmodium, Cryptosporidium*, *Eimeria* and *Theileria* [11-15]. These microorganisms have a unique set of apical secretory organelles termed micronemes and rhoptries. Proteins secreted from these organelles are involved in parasite gliding motility, cell invasion, and manipulation of the host cell to protect the intracellular replication compartment, the parasitophorous vacuole (PV) [16-19]. Thus, the majority of characterized secretory proteins function at the point of cell entry.

The *Toxoplasma* lytic cycle occurs through multiple rounds of cell entry, replication and egress. While molecular mechanisms of cell entry are well characterized, those governing cell exit are largely unknown [20, 21]. The discovery that a micronemal protein, PLP1, is required for rapid egress revealed that the parasite produces specific factors for cell exit. PLP1 is a lytic protein with a unique post-replicative function in niche escape rather than a pre-replicative function to access a replication site as seen for the aforementioned pathogens' pore-forming proteins. This and other studies [22-24] support an emerging hypothesis of egress as an event the parasite actively regulates instead of a strictly passive process occurring upon exceeding a critical capacity. PLP1-deficient parasites failed to rapidly egress from the PV after calcium ionophore treatment, which induces microneme secretion and motility [25, 26]. Additionally, whereas wild-type parasites effectively permeabilized the PV membrane (PVM), PLP1-deficient parasites retained an intact surrounding membrane [7]. These results suggested that PLP1 functions during egress by disrupting the PVM, which allows parasites to rapidly cross this physical barrier. PLP1-knockout parasites were also noted to have an invasion defect; however, the contribution of PLP1 to invasion remained unknown.

Recent work on pore-forming proteins has demonstrated conservation of structure between the cholesterol-dependent cytolysins (CDCs) and membrane attack complex/perforin family (MACPF) proteins [27-29]. The conservation of structure leads to a proposed conservation in mechanism of membrane

permeabilization [6, 30, 31]. The pore-forming protein is secreted as a soluble monomer and binds a receptor on the target cell membrane. Following membrane binding, monomers oligomerize into ring-like structures and undergo a structural rearrangement to create a lesion in the target membrane [32, 33]. While many pore-forming proteins, such as perforin and LLO, are sufficient for membrane damage, others, such as the membrane attack complex, require assembly of multiple proteins in a specific order [34]. Thus, it is important to identify whether PLP1 is sufficient for membrane damage, or requires other parasite or host factors.

A potential co-factor for parasite egress is the other perforin-like protein in the *Toxoplasma* genome, perforin-like protein 2 (PLP2). Previous results determined that PLP2 transcription is not upregulated in the PLP1 knockout, suggesting a lack of functional compensation [7]. However, it is possible that PLP2 has an accessory role during parasite egress, such as seeding pore-formation, as observed for assembly of the membrane attack complex.

Here we investigated the contribution of PLP1 to host cell invasion, PLP1 sufficiency in membrane damage, the general mechanism of membrane attack, and the possible involvement of PLP2 in tachyzoite egress. We find that genetic ablation of PLP1 leads to a secondary invasion defect, indicating that PLP1 function is dedicated to parasite egress. PLP1 is sufficient for membrane damage and displays a conserved mechanism of membrane attack. Furthermore, loss of PLP2 does not reduce egress of wild-type parasites, or further exacerbate the egress defect of the PLP1 knockout, which suggests PLP2 is not required for rapid egress of tachyzoites.

Materials and methods:

Ethics statement

This study was carried out in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and AAALAC accreditation guidelines. The protocol was approved by the University of Michigan's Committee

on the Use and Care of Animals (Animal Welfare Assurance # A3114-01, protocol #09482). All efforts were made to minimize suffering.

Parasite culture

Parasites were maintained in human foreskin fibroblasts (HFF) in DMEM supplemented with 10% calf serum, 10 mM HEPES, 2 mM L-glutamine, and 50 µg/ml Pen/Strep.

Invasion assays

Parasites were inoculated at a high (~300 µl) or low (~50 µl) passage in T25 flasks of HFF. Following 2 days' growth, parasites in high passage flasks had egressed, while parasites in low passage flasks remained intracellular. Egressed parasites were filter-purified in Endo buffer (44.7 mM K_2SO_4, 106 mM sucrose, 10 mM $MgSO_4$, 20 mM Tris-H_2SO_4 (pH 8.2), 5 mM glucose, 3.5 mg/ml BSA) [35] and a red-green invasion assay was performed as previously described [36]. Low passage flasks were washed twice with room temperature Endo buffer, scraped, and syringe-passed prior to filter-purification in Endo buffer and subsequent invasion assay. Immunofluorescence was performed as previously described [36] and 15 fields of view (400x total magnification) per strain were examined on a Zeiss Axio inverted microscope for attached and invaded parasites.

Egress assays

Parasites were inoculated into HFF in an 8 well chamber slide, and incubated at 37°C at 5% CO_2 for 30 h. Wells were washed twice with warm PBS and egress was tested by adding 120 µl/well of vehicle (DMSO) or 2 µM A23187 in egress assay buffer (EAB; Hanks buffered salt solution containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES) and incubated for 2 min in a 37°C water bath. Egress was stopped by addition of 2x fixative (8% formaldehyde in 2x PBS). Immunofluorescence was performed to stain parasites (SAG1) and the parasitophorous vacuole membrane

(PVM) (GRA7). At least 10 fields of view (400x magnification) per condition were imaged on a Zeiss Axio inverted microscope, and a minimum of 100 vacuoles, with 4 or more parasites per vacuole, was scored as occupied or unoccupied. Graphs depict the average and standard deviation of 3 independent experiments.

Parasite strains

The RH, *plp1ko*, *m2apko*, *Δhxg*, and RH*Δku80Δhxg* strains have been previously described [7,36,46,47]. PLP1 was genetically ablated in the RH*Δku80Δhxg* strain using the same approach described previously [7]. The RH*Δku80Δhxg*, RH*Δku80Δplp1* strains were transfected with 50μg of the dsRed expression plasmid, which carries a chloramphenicol resistance marker [37], linearized within the *tubulin* promoter. DsRed-expressing strains were maintained with chloramphenicol selection.

The *plp2ko* plasmid was generated by amplifying the 5' and 3' genomic flanks of *plp2* from RH genomic DNA and cloning them into sites flanking the *dihydrofolate reductase* (*dhfr*) gene to generate the vector, pMDC11-Cr_(PLP2 KO_DHFR). RH*Δku80Δhxg::dsRed*, RH*Δku80Δplp1::dsRed* strains were transfected with the *plp2ko* plasmid and transgenic parasites were selected for with pyrimethamine, cloned by limiting dilution and confirmed by PCR.

Recombinant protein cloning, expression and purification

PLP1 sequences were PCR amplified from RH cDNA and cloned into the pET15b expression vector by restriction enzyme digestion and ligation. The expression vector was transformed into BL21 Codon+ *E. coli*. Expression was induced with 1 mM IPTG at OD 0.4-0.6 at room temperature for 4-10 h and confirmed by Coomassie stain and immunoblot. Mature recombinant PLP1 was largely insoluble and was isolated by extracting inclusion bodies as described by the manufacturer (Novagen). Briefly, inclusion bodies were denatured with 6M guanidine-hydrochloride, allowed to bind to the Ni-NTA resin for 1 h at room

temperature or 4°C overnight, washed and eluted with non-denaturing buffers. All recombinant protein samples were buffer-exchanged into 50 mM NaCl, 10 mM Tris-HCl, pH 8.0. Primer sequences are shown in Table 3.

Recombinant M2AP was generated as described previously [38]. The recombinant LLO expression vector was a kind gift from Mary O'Riordan [39]. LLO was expressed and purified in the same manner as mature recombinant PLP1.

Recombinant PLP2 N- and C-terminal domain boundaries were determined by aligning the MACPF domains of PLP2 and PLP1. The N-terminal domain contains amino acids 52-221 and the C-terminal domain covers amino acids 570-854. The constructs were amplified from RH tachyzoite cDNA, cloned into the pET22b expression vector, and expressed in *E. coli*. Recombinant PLP2 constructs were purified by Ni-NTA affinity chromatography.

Antibody production

Recombinant PLP1 was used to generate antibodies in mice as described previously with 50µg recombinant protein per injection [40] and in rabbits by a commercial service (Cocalico). Furio Spano (Istituto Superiore di Sanità, Rome, Italy) and members of his lab produced antibodies for PLP2. Recombinant PLP2 Nterm (amino acids 52-221) and Cterm (amino acids 570-854) were used to generate antibodies in mice and rabbits.

Lytic activity

Lytic activity on host cells was tested by incubating HFF in an 8 well chamber slide with 100 nM recombinant protein (M2AP, PLP1 or LLO) in EAB/12.5 µM propidium iodide/10 mM dithiothreitol. Cells were incubated for 10 min at 37°C, washed twice with warm PBS and fixed with formaldehyde. Immunofluorescence was performed for PLP1 and nuclei were stained with DAPI. Hemolysis was tested by incubating washed sheep red blood cells at 2% final hematocrit in PBS with varying concentrations of recombinant protein. RBCs were incubated for 1 h at

37°C and pelleted by centrifugation at 500g for 5 min. Supernatant was collected and absorbance at 540 nM read in a 96-well plate reader. The assay was performed with triplicate wells, normalized to 1% Triton-X-100 lysis.

Membrane flotation

Membrane flotation was performed on host cell debris isolated from fully egressed cultures. Parasites were separated from host cell debris by centrifugation at 1000g for 10 min. Supernatant was collected and centrifuged again. The second supernatant was subjected to ultracentrifugation at 84,000g rpm for 1 h in a Thermo Scientific Sorvall WX Ultra Series centrifuge. The high-speed pellet (HSP) was washed once by resuspension in PBS and centrifugation as in the previous step. The pellet was overlaid with a sucrose density gradient. The pellet was resuspended in 750 µl of 85% sucrose and overlaid with 3 ml 65% sucrose and 1 ml 10% sucrose. Sucrose solutions were prepared in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4. Samples were centrifuged at 100,000g for 16 h at 4°C in a Thermo Scientific Sorvall WX Ultra Series centrifuge. Fractions (0.5 ml each) were collected from the top of the gradient and applied to SDS-PAGE. Blots were probed for PLP1, GRA4, or MIC5.

SDS-Agarose gel electrophoresis

SDS-agarose gels were prepared by boiling 1% (w/v) agarose in SDS-running buffer (25 mM Tris base, pH 8, 250 mM glycine, 0.1% (w/v) SDS), poured into 15x17 cm vertical glass plates with a 1.5 mm gap and cooled to room temperature. Filtered parasite lysate or HSP were electrophoresed at 5-8 mA and transferred to PVDF membrane in a semi-dry transfer machine at 20 V for 70 min. Titin from mouse muscle lysate was used as a size standard and membranes were briefly stained with Coomassie prior to immunoblotting for PLP1.

Hexafluoroisopropanol treatment

HSP was TCA-precipitated and resuspended in 500 µl of buffer (50 mM NaCl, 10 mM Tris, pH 7.4) or HFIP. Samples were allowed to dry in a speed-vac and pellets were resuspended in sample buffer prior to SDS-PAGE and immunoblot for PLP1.

Results:

Loss of PLP1 leads to a secondary invasion defect

PLP1 has been previously identified as an important egress factor [7]. In addition to the striking defect in rapid egress observed on the level of individual infected cells, we show here that this defect is observed on a population level (Figure 2E). The majority of PLP1-deficient parasites remain trapped in the PV two minutes after ionophore addition while other strains such as RH (the original parent for all of the strains used in this study), *Δhxg*, and m2apko efficiently exit from cells. While PLP1 had been noted to have an invasion defect [7], the contribution of PLP1 to parasite invasion was unclear. Video microscopy revealed that *plp1ko* parasites that were able to egress after a delay appeared to utilize persistent gliding motility to eventually break through the PVM and escape from the cell [7]. Accordingly, we reasoned that *plp1ko* parasites might exhaust limited resources such as the microneme contents in their prolonged attempts to breach the PVM, thus leaving them less competent for cell invasion. To this end, we compared cell invasion of naturally egressed (Figure 2F) and mechanically liberated parasites (Figure 2G). The ~40% decrease in invasion of naturally egressed *plp1ko* is similar to that observed for *m2apko*, a parasite strain with a previously reported invasion defect [36]. However, mechanically liberating the parasites restored *plp1ko* invasion to that of the parental strain, but did not rescue *m2apko* invasion, consistent with the *plp1ko* invasion defect being secondary to the egress defect. We also noted a 5- to 7-fold increase in cell invasion for mechanically liberated parasites over naturally egressed parasites. The increased invasiveness of mechanically liberated parasites could be due to preserving limiting components that would normally be depleted

during egress and extracellular existence. The findings support a model in which economical egress promotes efficient cell invasion.

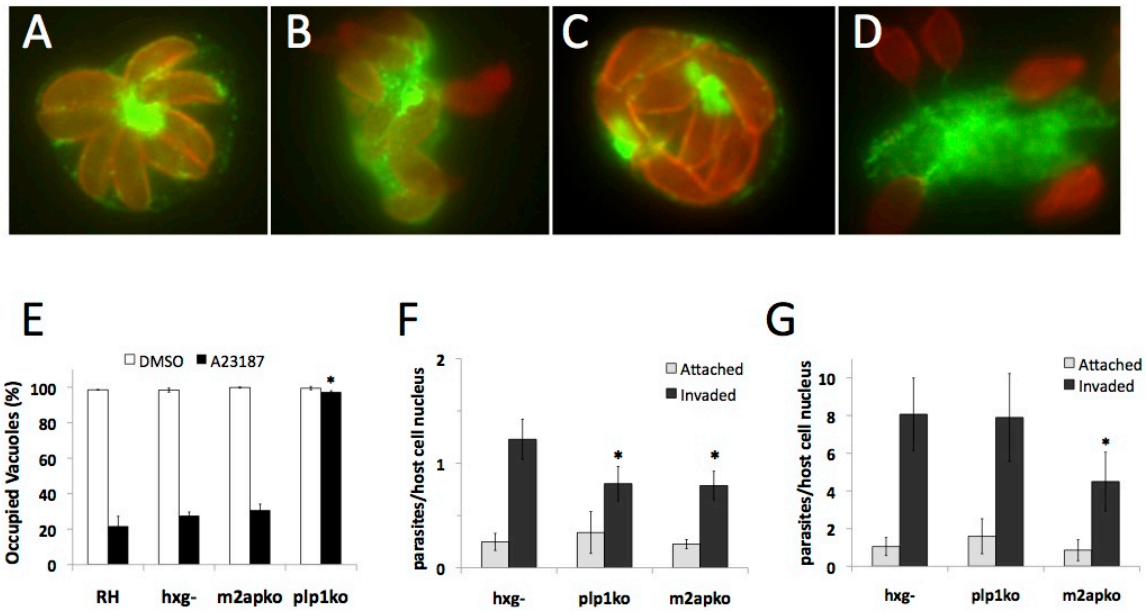

Figure 2. *Loss of PLP1 leads to a primary egress defect and a secondary invasion defect.* **A-E**) 30 hour vacuoles are treated with vehicle (DMSO, white bars) or ionophore (A23187, black bars) for 2 minutes and >100 vacuoles in >10 fields of view scored by IFA as occupied or unoccupied vacuoles. RH is the parental strain for all mutants used in these studies. *Δhxg* is the parental strain for the *plp1ko. m2apko* is a previously characterized strain with an invasion defect. **A-D)** Representative immunofluorescence images of vacuoles. Parasites are stained for surface antigen, SAG1 (red) and parasitophorous vacuole is stained for GRA7 (green). Images A-C are scored as occupied vacuoles; D is scored as an unoccupied vacuole. **A)** Parasite rosette characteristic of intracellular growth. **B)** Parasites initiating egress. **C)** Disrupted rosette typical of *plp1ko* parasites after A23187 treatment. **D)** Parasites egressed from vacuole. **E)** Quantification of egress by indicated strains. Naturally egressed **(F)** or mechanically lysed **(G)** parasites were tested for invasion defects by red/green invasion assay; light grey bars= attached parasites, dark grey bars= invaded parasites.

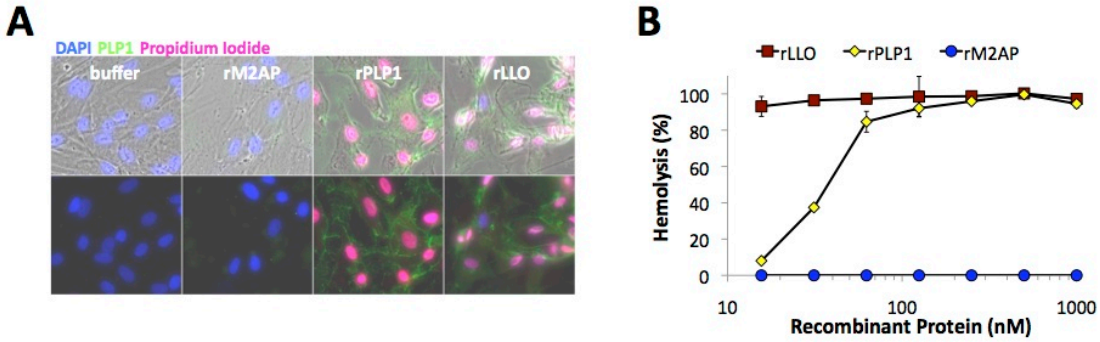

Figure 3. *PLP1 is sufficient for lytic activity on host cells and erythrocytes.* **A)** Host cells were incubated for 10 minutes at 37C with buffer or 100 nM recombinant M2AP, PLP1, or Listeriolysin O (LLO) and propidium iodide; pink nuclei indicate membrane permeabilization (The PLP1-antibody cross-reacts with LLO.) **B)** Cytolytic activity was also observed by hemolysis assay with varying concentrations of recombinant protein and a dose-dependent hemolysis for PLP1 is observed.

PLP1 is sufficient for membrane permeabilization, likely via a canonical mechanism of membrane attack

Kafsack et al. [7] showed that PLP1 is necessary for ionophore-induced PVM permeabilization, so we next asked if PLP1 activity was sufficient for membrane damage. We produced recombinant and refolded PLP1 in *E. coli* and tested it for lytic activity on host cells and erythrocytes. Recombinant protein was incubated with host cells in the presence of propidium iodide (PI), which stains nuclei of cells with membrane damage. Host cells incubated with PLP1 or listeriolysin O (LLO) exhibited PI-stained nuclei, while those incubated with buffer or M2AP excluded PI (Figure 3A). PLP1 and LLO were both hemolytic, while M2AP failed to lyse red blood cells (Figure 3B). These assays confirm that PLP1 is sufficient for membrane damage.

Previous work on pore-forming proteins such as the CDCs, complement and perforin suggest a common mechanism for pore formation. Pore-forming proteins are generally secreted as monomers that bind to a receptor(s) on the target cell, oligomerize, and undergo a structural rearrangement to cross the target cell membrane, creating a large transmembrane lesion [33].

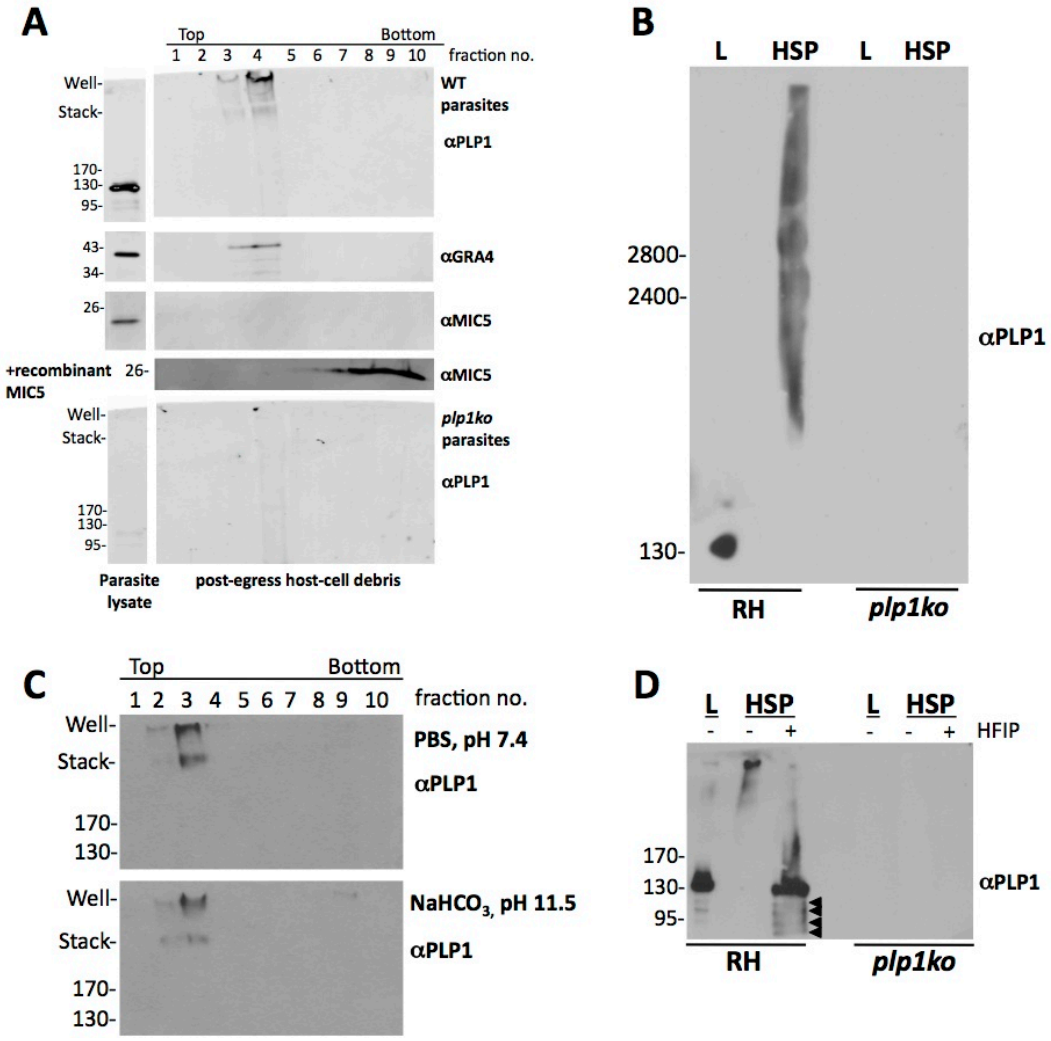

Figure 4. *PLP1 forms high molecular weight complexes on cell debris after parasite egress.* **A)** Membrane flotation of cell debris after parasite egress reveals PLP1 and GRA4 signal associated with lighter density fractions, while endogenous MIC5 is not detected due to lack of membrane binding activity and addition of recombinant MIC5 leads to MIC5 signal associated with the higher density fractions, also indicative of no membrane binding. Low-density, high molecular weight PLP1 signal is absent in the PLP1 knockout sample, supporting signal specificity. **B)** SDS-agarose gel electrophoresis demonstrates cell-debris associated PLP1 is in high molecular weight complexes exceeding 2800 kDa, which are absent in the *plp1ko*. L = parasite lysate. HSP = high-speed pellet. **C)** Membrane-associated PLP1 is resistant to alkaline extraction, indicative of membrane integration, and TCA precipitation leads to removal of sample membranes and loss of low-density PLP1 signal. **D)** Treatment of HSP with HFIP (hexafluoroisopropanol) disrupts high MW PLP1 and reveals the major component to be monomeric PLP1, although some processed forms are also present (arrowheads).

To examine PLP1 membrane binding and oligomerization *in vivo*, we isolated host cell debris in a high-speed pellet (HSP) from naturally egressed cultures of RH and *plp1ko*. We tested for PLP1 membrane association via flotation in a sucrose-density gradient. In an immunoblot of gradient fractions, PLP1 signal was observed in the lighter density fractions along with another PVM protein, GRA4 (Figure 4A). MIC5 is a secreted protein with no known membrane binding capacity; accordingly, no MIC5 signal was observed in the HSP (not shown) or in the membrane-associated fractions. Recombinant MIC5 added to the bottom of the gradient with the HSP remained associated with the higher density fractions, confirming a lack of membrane association. Treatment of the HSP with sodium bicarbonate, pH 11.5, did not extract PLP1 from the membrane fractions, suggesting it is embedded in membranes (Figure 4C).

Interestingly, whereas monomeric PLP1 in parasite lysate migrates at 130 kDa, we observe membrane-associated PLP1 signal in the well and at the interface between the stacking gel and separating gel (Figure 4A,C). This severely retarded migration suggests that PLP1 forms large, highly stable oligomeric complexes that are resistant to boiling in SDS-PAGE sample buffer. To resolve these putative oligomers further, we performed SDS-agarose gel electrophoresis (SDS-AGE). PLP1 in parasite lysate migrated as a single band of ~130 kDa while PLP1 associated with host cell debris in the HSP migrated as multiple, high molecular weight complexes (Figure 4B). These complexes were not observed in samples from *plp1ko* parasites, confirming the specificity of the antibody. Since PLP1 is partially processed upon secretion in extracellular parasites [41], we next asked if PLP1 is proteolytically processed in these large molecular weight complexes. We treated the HSP with hexafluoroisopropanol (HFIP) to disrupt protein-protein interactions and applied the samples to SDS-PAGE. HFIP treatment resulted in the loss of PLP1 signal from the well and stacking gel along with the concurrent appearance of a major band at 130 kDa, similar to that observed in parasite lysate. We also observed multiple minor, processed forms of PLP1 upon HFIP treatment, which suggests some

processed PLP1 is incorporated into the high MW complexes, though the majority of the complex is composed of mature PLP1 (Figure 4D). We conclude that PLP1 forms large, membrane-embedded oligomers, consistent with the established mechanism of oligomerization and membrane insertion by other members of the MACPF and CDC protein families.

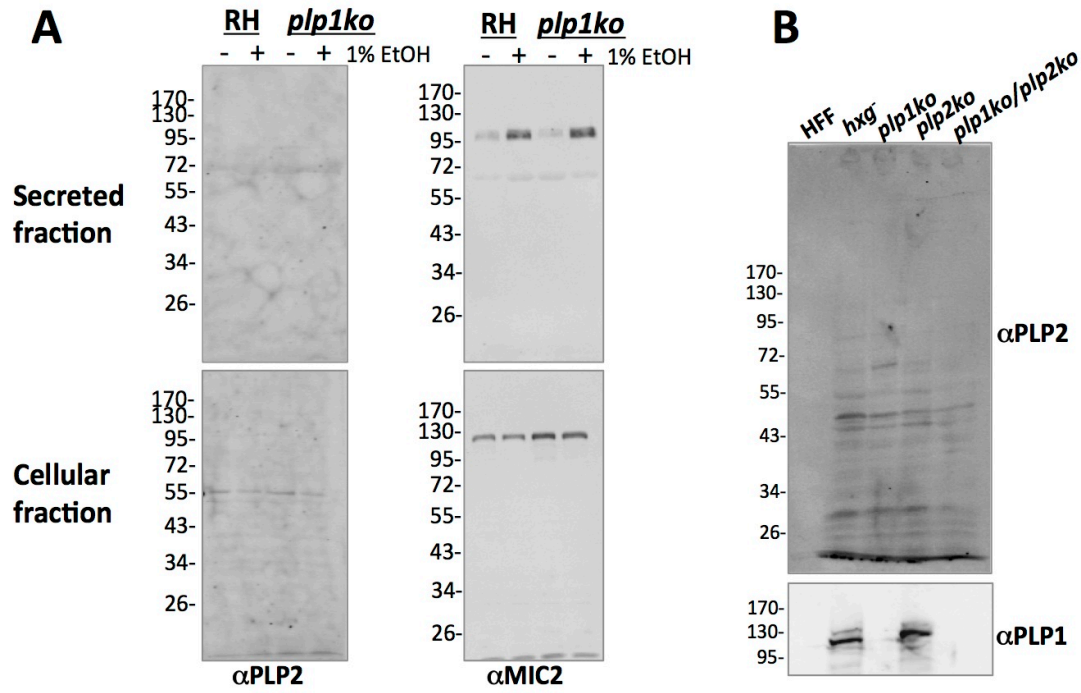

Figure 5. *PLP2 expression is not detected in tachyzoites lysate or secreted fraction.* **A)** RH and *plp1ko* parasite secreted fraction (secretion induced with 1% EtOH) and cellular fraction (lysate) were probed with PLP2 and MIC2 antibodies. **B)** Host cell lysate, parental, and single and double knockouts of *plp1* and *plp2* were probed for PLP1 and PLP2 expression.

PLP2 is not required for tachyzoite egress

The genome of *Toxoplasma gondii* has two MACPF-domain containing sequences called perforin-like protein 1 (PLP1), and perforin-like protein 2 (PLP2). While PLP1 has been shown to be essential for rapid host cell egress of tachyzoites, the function of PLP2 remains unknown. Here we further examined expression of PLP2 and its contribution to tachyzoite egress. We genetically ablated *plp2* from the

RH*Δku80Δhxg*, RH*Δku80Δplp1* strains, to generate a single and double knockout. By comparing these strains, we were unable to detect specific reactivity to PLP2 antisera in tachyzoite lysate or secreted fractions, using the anti-C-terminal domain antibody, which detected the recombinant PLP2 C-term (Figure 5, data not shown). Additionally, immunoprecipitation and pulse chase failed to detect PLP2 labeling (data not shown). These results suggest either the antibody has low specificity or the protein is not expressed to detectable levels.

We then tested for defects in parasite egress and egress-associated membrane permeabilization. By calcium ionophore induction, the *plp2ko* has no egress defect as compared to the parental strain, and the *plp1ko/plp2ko* is not more defective in parasite egress than the *plp1ko* (Figure 6A). Similar results were observed for PVM permeabilization by DsRed release from the vacuolar space to the host cytosol (Figure 6B). These results suggest that PLP2 does not contribute significantly to parasite egress or PLP1-mediated membrane permeabilization.

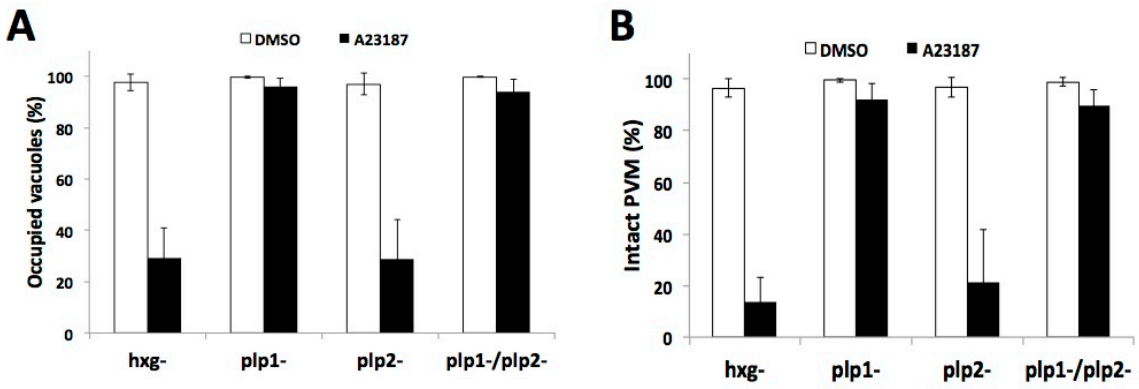

Figure 6. *Loss of plp2 does not reduce tachyzoite egress or affect egress-associated membrane permeabilization.* **A)** 30 hour vacuoles are treated with vehicle (DMSO, white bars) or ionophore (A23187, black bars) for 2 minutes and >100 vacuoles in >10 fields of view scored by IFA as occupied or unoccupied vacuoles. **B)** Intact PVM were observed by dsRed release from the vacuole space into the cytosol of infected, ionophore-treated cells.

Discussion:

Our findings indicate that loss of PLP1 leads to a secondary invasion defect based on showing that mechanical release of *plp1ko* parasites restored invasion to parental levels. Thus, PLP1 is likely dedicated to parasite egress and does not play a direct role in invasion. Since PLP1 is probably secreted from micronemes during both egress and invasion, how its activity is differentially regulated during these events remains unknown. PLP1 is expected to be active during egress to permeabilize the PVM but inactive during invasion to maintain membrane integrity for the formation of the nascent PV. While PLP1 is processed by a subtilisin, this proteolysis may play an inhibitory role rather than an activating one. The majority of PLP1 observed in native oligomers is unprocessed. Additionally, genetic ablation of SUB1, the protease responsible for processing PLP1 in the secretory fraction, does not affect egress [41], indicating that processing is not required for PLP1 activity. On the other hand, if SUB1 processing of PLP1 suppresses lytic activity, proteolysis may be down regulated during egress to promote PVM lysis and enhanced when parasites are extracellular to protect surrounding host cells or the parasite itself. SUB1 deficient parasites have an invasion defect due to the lack of processing several invasion related proteins. Whether the absence of PLP1 proteolysis in SUB1 deficient parasites and the proposed derepression of lytic activity play a role in this invasion defect is unknown. If SUB1 activity is suppressed during egress there may be functional differences in other SUB1 substrates during egress and invasion.

It is also interesting to note the substantial increase in parasite attachment and invasion for mechanically lysed vs. naturally egressed parasites. The enhanced invasion of mechanically liberated parasites suggests that such parasites are more fit for entry. While parasite inoculation and harvest occurred at the same time for both conditions, natural egress occurs somewhat asynchronously and this is likely a source of variation in invasion assays since parasites continuously secrete micronemes while extracellular.

Previous work demonstrated that PLP1 is necessary for PVM permeabilization [7]. Here we have shown that PLP1 is sufficient for membrane disruption. Though it is possible that other parasite or host factors regulate PLP1 activity, the results with recombinant protein PLP1 indicate they are not necessary for its function. The *Toxoplasma* genome contains another perforin-like protein, PLP2. We were unable to detect expression of PLP2 in tachyzoites by comparing western blots of parental and *plp2ko* lysates. Additionally, we observed no defect in parasite egress or PVM permeabilization for *plp2ko* and *plp1ko/plp2ko* strains. This suggests that PLP2 does not significantly contribute to rapid egress of tachyzoites or egress-associated PVM disruption. However, it is still possible that PLP2 promotes parasite egress at other stages of the life cycle, such as egress of bradyzoites or sporozoites.

While PLP1 is found to be sufficient for membrane damage, outstanding questions regarding its mechanism of pore-formation remain. For example, it has been demonstrated that perforin is capable of forming both small and large membrane lesions [42]. Perhaps consistent with this, we observe both larger and smaller oligomeric complexes of PLP1 on SDS-AGE. The formation of large oligomeric PLP1 complexes is consistent with the previous *in vivo* findings that PLP1 membrane lesions allow the exit of 120 kDa dsRed tetramers from the PV into the host cell [7]. Whether these oligomeric complexes are composed entirely of the pore-forming protein, or are made of incomplete rings with a free membrane edge, is yet to be determined. Structural studies are necessary to determine the arrangement of PLP1 in membrane lesions. If PLP1 forms incomplete rings, it is possible a phospholipase also contributes to membrane damage, as observed for *Listeria* [43-45]. The combination of a pore-forming protein and a phospholipase would maximize membrane damage, which would be beneficial for parasites during egress.

These results show that PLP1 is sufficient for membrane damage and follows a conserved mechanism of membrane lysis. PLP1 lytic activity is likely dedicated to

parasite egress and suppressed during host cell invasion. PLP1 does not appear to require other parasite factors, such as PLP2, or host cofactors for activity, though it remains possible some factors may negatively regulate PLP1 lysis.

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Chapter 3

Domain deletion analysis of PLP1 reveals a dual domain mode of membrane binding for efficient cytolysis and parasite egress

Introduction:

Predicted membrane-attack complex/perforin family (MACPF) domains are found in genomes of diverse organisms and are associated with a wide variety of adjacent domains. Only a few MACPF-associated domains, however, have been characterized. *Toxoplasma gondii's* perforin-like protein 1 (PLP1) has a highly conserved central MACPF domain, responsible for oligomerization and membrane permeabilization in other MACPF-domain containing proteins, along with unique N- and C-terminal extensions. The C-terminal domain is predicted to be β-sheet rich, a general feature that is conserved amongst C-terminal domains of other pore-forming proteins of the same superfamily [1]. Based on its position and structural features, the PLP1 C-terminal domain is predicted to contribute to membrane binding, but its role has not been addressed previously. The PLP1 N-terminal domain sequence contains no conserved domains and is found only in *Toxoplasma* and the related *Neospora* sequences. It may be more distantly related to the N-terminal extensions seen on some of the *Plasmodium* MACPF proteins [2]. N-terminal domains of other MACPF proteins include a low-density lipoprotein receptor A (LDLRA) domain and TSP domains in several complement proteins [3, 4]. Examples of N-terminal domains in CDCs include the lectin-binding domain of lectinolysin and an N-terminal sequence in LLO which drives translational

regulation [5-9]. Because of its unique characteristics, no obvious function for the PLP1 N-terminal domain has been proposed.

Here we investigated the role of each PLP1 domain by complementing the *plp1ko* with domain deletion mutants. We confirmed predicted roles for the PLP1 MACPF and C-terminal domains, and, unusually, membrane-binding activity in the N-terminal domain. These findings reveal a rare dual domain mode of membrane binding preceding pore formation and cytolysis. Loss of the N-terminal domain markedly reduces PLP1 function, leading to a delay in rapid egress; however, the reduced lytic activity does not significantly compromise parasite virulence. This suggests that the presence of lytic activity, rather than the absolute amount of activity, is sufficient for pathogenesis.

Materials and methods:

PLP1 knockout and complementation

PLP1 was genetically ablated in the RH*Δku80Δhxg* strain in a manner similar to that described previously [10]. The PLP1 complementation vector was constructed by replacing the 5' and 3' flanks of the M2AP complementation vector [11] with PLP1 5' and 3' UTRs amplified from genomic DNA. Primer sequences for these amplifications and other PLP1 experiments are shown in Table 3. The PLP1 signal anchor, amplified from cDNA, restriction enzyme cloning sites, and the epitope tag were generated by fusion PCR and cloned into the vector by restriction enzyme digestion and ligation. PLP1 domain boundaries were based on computational predictions of the conserved MACPF domain, delineating the protein into a central MACPF domain and an N- and C-terminal domain [10]. PLP1 domain deleted constructs were PCR amplified from cDNA and cloned into the complementation vector by restriction enzyme digest and ligation.

The RH*Δku80Δhxg*, RH*Δku80Δplp1* strains were transfected with 50µg of the dsRed expression plasmid, which contains a chloramphenicol resistance marker, [12], linearized within the tubulin promoter. DsRed expressing strains were cloned by

limiting dilution, confirmed by immunofluorescence, and maintained with chloramphenicol selection. The PLP1 complementation vector was digested within the 5' and 3' flanks and transfected into the RH*Δku80Δplp1dsRed* strain, maintained under 340 µg/ml 6-thioxanthine in DMEM/10 mM HEPES/ 1% dialyzed FBS and cloned by limiting dilution. Clones were confirmed by PCR of genomic DNA and by immunoblot.

Recombinant protein cloning, expression and purification

PLP1 sequences were PCR amplified from cDNA and cloned into the pET15b expression vector by restriction enzyme digestion and ligation. The expression vector was transformed into BL21 Codon+ *E. coli*. Expression was induced with 1 mM IPTG at OD 0.4-0.6 at room temperature for 4-10 h and confirmed by Coomassie stain and immunoblot. Soluble constructs (PLP1 Nterm and Cterm) were purified by nickel affinity column purification. Insoluble constructs (mature recombinant PLP1, recombinant MACPF domain) were isolated by extracting inclusion bodies as described by the manufacturer (Novagen). Briefly, inclusion bodies were denatured with 6M guanidine-hydrochloride, allowed to bind to the resin for 1 h at room temperature or 4°C overnight, washed and eluted with non-denaturing buffers. All recombinant protein samples were buffer-exchanged into 50 mM NaCl, 10 mM Tris-HCl, pH 8.0.

Antibody production

Recombinant PLP1 was used to generate antibodies in mice as described previously with 50µg recombinant protein per injection [13] and in rabbits by a commercial service (Cocalico). Recombinant N- and C-terminal domains were used to affinity purify domain-specific antibodies from rabbit sera using an amino-link kit according to the manufacturer's instructions (Pierce). Specificity and approximately optimal dilutions were determined by immunoblot and immunofluorescence.

PCR products	Primer Name	Sequence
Mature PLP1	NheI_PLP1_580.F	GCTAGCGCGCCAGATGACGATTTCGAT
	MfeI_PLP1_3454.R	CAATTGCAGGTCTAACAGCTTGAC
Nterm	NheI_PLP1_580.F	GCTAGCGCGCCAGATGACGATTTCGAT
	MfeI_PLP1_1372.R	CAATTGTGAGAGCGGCGCCGCCGC
Nterm+MACPF	NheI_PLP1_580.F	GCTAGCGCGCCAGATGACGATTTCGAT
	MfeI.PLP1.2406.R	CAATTGGAGATCCTGCGGTGTCAAC
Nterm+Cterm	NheI_PLP1_580.F	GCTAGCGCGCCAGATGACGATTTCGAT
fusion	PLP1.delMACPF.F	CAACAGGATGCGAGAACAGTGCTCTCAC GGG
fusion	PLP1.delMACPF.R	CCCGTGAGAGCACTGTTCTCGCATCCTG TTG
	MfeI_PLP1_3454.R	CAATTGCAGGTCTAACAGCTTGAC
MACPF	NheI.PLP1.1315.F	GCTAGCAACCGTCGCATCGCCGAAG
	MfeI.PLP1.2406.R	CAATTGGAGATCCTGCGGTGTCAAC
MACPF+Cterm	NheI.PLP1.1315.F	GCTAGCAACCGTCGCATCGCCGAAG
	MfeI_PLP1_3454.R	CAATTGCAGGTCTAACAGCTTGAC
Cterm	NheI_PLP1_2389.F	GCTAGCGTGGGGTTGACACCGCAGGAT
	MfeI_PLP1_3454.R	CAATTGCAGGTCTAACAGCTTGAC
Signal anchor/ multiple cloning site/ epitope tag	5.AvrII.PLP1.1.F	CCTAGGATGAGGTCACTCACAC
(fusion)	5.myc.SFTAP.1.F	GAACAAAAGCTGATCAGCGAAGAAGATC TGAACTGGTCTCATCCTCAG
(fusion)	3.MfeI.NheI.PLP1.189.R	GATCAGCTTTTGTTCCAATTGAGCTGCG CTAGCCAGACAAACGAACGTGATC
	3.PacI.SFTAP.135.R	TTAATTAAGTCGGCGCGCCTTTATC
Multiple cloning site quick change	pPLP1SFcomp.QC.MCS.F	CGACTGCGCCAGATGACGCGGCTAGCG GCTGCGC
	pPLP1SFcomp.QC.MCS.R	GCGCAGCCGCTAGCCGCGTCATCTGGC GCAGTCG
3' flank	5.TgPLP1.3'UTR.PacI.F	TTAATTAAAGAAGAGCCTCATAGAACCG
	3.TgPLP1.3'UTR.ApaI.R	GGGCCCGAAAAACTAGATGAAACACA
Mature recombinant PLP1	NdeI_PLP1_561.f	CATATGGCGCCAGATGACGATTTCGAT
Recombinant Nterm	NdeI_PLP1_561.f	CATATGAACCGTCGCATCGCCGAAG
	XhoI_PLP1_1372.r	CTCGAGTTATGAGAGCGGCGCCGCCGC
Recombinant MACPF	NdeI.PLP1.1315.f	CATATGCCGCTCTCAGCTGTATACACT
	XhoI.PLP1.2406.r	CTCGAGTCAGAGATCCTGCGGTGTCAAC
Recombinant Cterm	NdeI_PLP1_2389.f	CATATGGTGGGGTTGACACCGCAGGAT
	XhoI_PLP1_3165.r	CTCGAGTTACAGGTCTAACAGCTTGAC

Table 3: *Primers used for PLP1 cloning for parasite complementation and recombinant protein production.*

Membrane flotation

Membrane flotation was performed on host cell debris isolated from fully egressed cultures. Parasites were separated from host cell debris by centrifugation at 1000g for 10 min. Supernatant was collected and centrifuged again. The second supernatant was subjected to ultracentrifugation at 84,000g for 1 h in a Thermo Scientific Sorvall WX Ultra Series centrifuge. The HSP was washed once by resuspension in PBS and centrifugation as in the previous step. A sucrose density gradient was applied to the pellet. The pellet was resuspended in 750 µl of 85% sucrose and overlaid with 3 ml 65% sucrose and 1 ml 10% sucrose. Sucrose solutions were prepared in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4. Samples were centrifuged at 100,000g for 16 h at 4°C in a Thermo Scientific Sorvall WX Ultra Series centrifuge. Fractions (0.5 ml each) were collected from the top of the gradient and applied to SDS-PAGE. Blots were probed for PLP1, or GRA4 to confirm membrane-associated fractions.

Membrane flotation of recombinant N- and C-terminal domains was accomplished by preparing red blood cell ghosts (RBCg) as previously described [14]. 20 µM (final concentration) recombinant protein was mixed with or without $2x10^7$ RBCg in 100 µl PBS and incubated at 37°C for 15 min. A sucrose density gradient was applied by mixing the protein sample with 400 µl of 85% sucrose and then overlaying with 900 µl of 65% sucrose and 200 µl of 10% sucrose. Samples were centrifuged in a Thermo Scientific Sorvall MTX 150 micro ultracentrifuge at 462,000g for 3 h. Fractions (200 µl each) were collected and examined by SDS-PAGE and immunoblot for the 6xHis epitope tag.

SDS-Agarose gel electrophoresis

SDS-agarose gels were prepared by boiling 1% (w/v) agarose in SDS-running buffer (25 mM Tris base, pH 8, 250 mM glycine, 0.1% (w/v) SDS), poured into 15x17 cm vertical glass plates with a 1.5 mm gap and cooled to room temperature. Filtered parasite lysate or HSP were electrophoresed at 5-8 mA and transferred to PVDF

membrane in a semi-dry transfer machine at 20 V for 70 min. Titin from mouse muscle lysate was used as a size standard and membranes were briefly stained with Coomassie prior to immunoblotting for PLP1.

Hexafluoroisopropanol treatment

HSP was TCA-precipitated and resuspended in 500 µl of buffer (50 mM NaCl, 10 mM Tris, pH 7.4) or HFIP. Samples were allowed to dry in a speed-vac and pellets were resuspended in sample buffer prior to SDS-PAGE and immunoblot for PLP1.

Detection of parasite expression and secretion of domain deletion constructs

Parasites were filter-purified and attached to a glass slide coated with BD-CellTak (BD Biosciences) prior to fixation with ethanol. Immunofluorescence was performed for PLP1 and MIC2 and nuclei were stained with DAPI. Intracellular parasites inoculated for 24 h were also fixed with ethanol prior to staining for PLP1 and MIC2. Results are representative of 2 or more independent experiments.

For immunoblot detection, parasites were filter-purified in PBS, counted and lysed in hot sample buffer. Parasites ($1.5x10^7$) were loaded per lane and probed for PLP1 and MIC2. Microneme secretion with ethanol induction was performed as previously described [15], and samples were immunoblotted for PLP1 and MIC2.

Plaque assay

Parasites were filter-purified, counted, and 50 parasites were inoculated into each well of a 6-well plate. Plates were incubated for 7 days and stained with crystal violet. Plaque number was determined by visual examination. Plaque area was determined by measuring plaque diameter in 2 dimensions in bright field on a Zeiss Axio inverted microscope, averaging the diameters for 10 plaques/strain, and approximating a circle in area.

PVM permeabilization and lytic activity

PVM permeabilization was tested by inoculating parasites in HFF as described for the egress assay. Following 30 h incubation, wells were washed twice with warm PBS and parasites were immobilized by treating with 1 µM cytochalasin D (CytD) in EAB (120 µl/well) at 37°C for 3 min. Microneme secretion was induced by addition of 120 µl DMSO or 4 µM A23187 in the presence of 1 µM CytD for 3 min. Secretion was halted by addition of 240 µl of 2x fixative. Cells were stained with DAPI and imaged as above. Infected cells were determined by brightfield, while release of dsRed signal from the PV to the host cytosol indicated PVM permeabilization. Lytic activity was determined by normalizing PVM permeabilization to WT parasites.

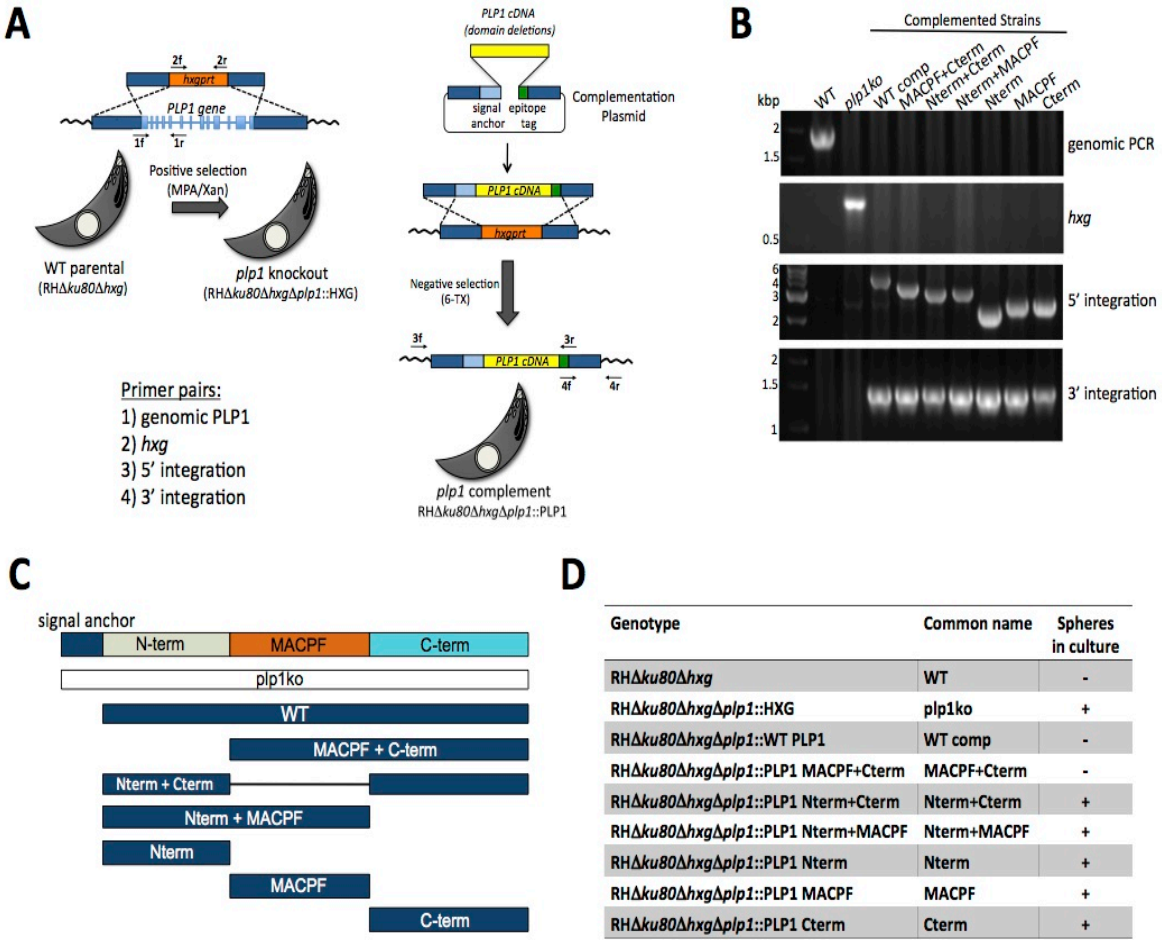
Mouse virulence

All animal experiments were conducted in accordance with the University of Michigan University Committee on the Use and Care of Animals. Ten 6-week old female Swiss-Webster mice were IP injected with 10, 100, or 1,000 tachyzoites. Mice that became moribund were humanely euthanized.

Results:

Precise generation of plp1ko domain deletion complementation strains

While PLP1 has a highly conserved MACPF domain, the N- and C-terminal domains flanking it are unique [2, 10]. We hypothesized the MACPF domain to be required for oligomerization and membrane permeabilization. The PLP1 C-terminal domain is predicted to be β-sheet rich, a feature of other pore-forming protein C-terminal domains involved in target cell membrane binding [16] [17] [18]. The PLP1 N-terminal domain has no conserved sequence, and most pore-forming proteins lack N-terminal domains. To identify functional domains of PLP1, we devised a novel complementation strategy involving regenerating the PLP1 knockout in the



Genotype	Common name	Spheres in culture
RHΔ*ku80Δhxg*	WT	-
RHΔ*ku80ΔhxgΔplp1*::HXG	plp1ko	+
RHΔ*ku80ΔhxgΔplp1*::WT PLP1	WT comp	-
RHΔ*ku80ΔhxgΔplp1*::PLP1 MACPF+Cterm	MACPF+Cterm	-
RHΔ*ku80ΔhxgΔplp1*::PLP1 Nterm+Cterm	Nterm+Cterm	+
RHΔ*ku80ΔhxgΔplp1*::PLP1 Nterm+MACPF	Nterm+MACPF	+
RHΔ*ku80ΔhxgΔplp1*::PLP1 Nterm	Nterm	+
RHΔ*ku80ΔhxgΔplp1*::PLP1 MACPF	MACPF	+
RHΔ*ku80ΔhxgΔplp1*::PLP1 Cterm	Cterm	+

Figure 7. *Generation of plp1 knockout and complementation strains for domain deletion analysis and immunofluorescence of complemented strains.* **A**) The *plp1* knockout was generated in the RH*Δku80Δhxg* strain by replacing the PLP1 locus with the hypoxanthine xanthine phosphoribosyl transferase (*HXG*) drug selection marker. The *plp1ko* was complemented at the endogenous locus by double homologous recombination and negative selection. **B**) PCR on parasite genomic DNA demonstrated *plp1* locus replacement with *HXG* in the knockout and *HXG* replacement with PLP1 cDNAs in complemented strains. **C**) PLP1 domain structure and domain deletions tested in this study. **D**) Table of parasite strains investigated in this study including the genotype, common name used throughout this paper, and the presence or absence of spheres in post-egressed cultures. Spheres were observed by bright-field examination of egressed cultures and images were acquired on brightfield at 200x magnification on a Zeiss Axio inverted microscope.

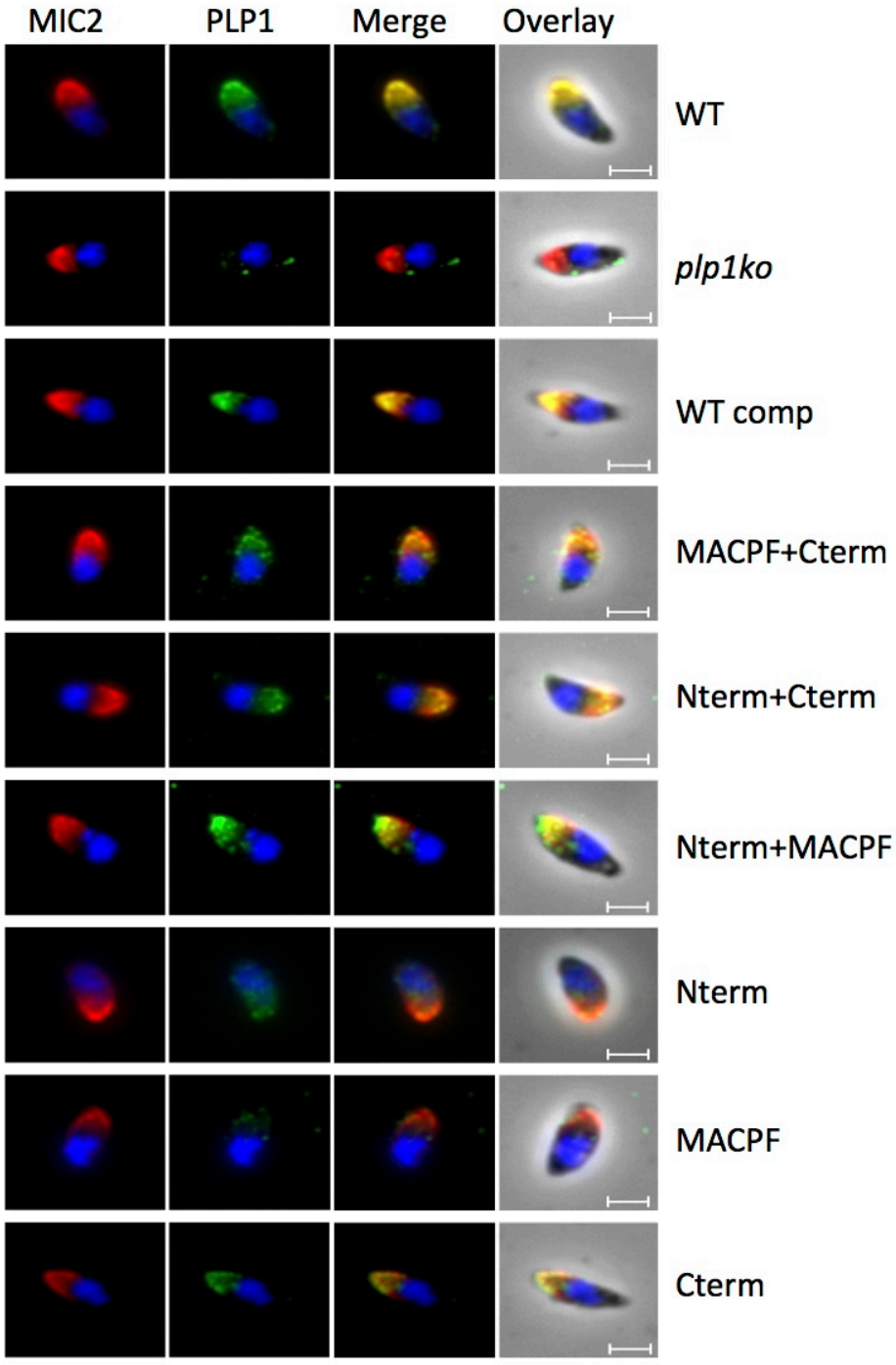


Figure 8: *PLP1 complement expression and localization*. Immunofluorescence with a polyclonal PLP1 antibody (green) showed some PLP1 complementation constructs co-localized with MIC2 (red), while other constructs were poorly detected. Nuclei were detected with DAPI (blue). Scale bar, 2 μm.

genetically tractable RH*Δku80* background and complementing the knockout at the PLP1 locus by double homologous recombination and negative selection against the hypoxanthine xanthine guanine phosphoribosyltransferase (HXG) drug selection marker (Figure 7A). The recreated knockout was confirmed by PCR and immunofluorescence (Figure 7B, Figure 8). A panel of domain deletion mutants was designed based on the computational prediction of the MACPF domain (Figure 7C). Replacement of *HXG* with *PLP1* cDNA at the *PLP1* locus was confirmed by PCR (Figure 7B). Figure 7D indicates the genotypes and hereafter used colloquial names for these complementation strains. Figure 7D also lists the presence or absence of spheres in egressed cultures, a characteristic of PLP1 deficiency and egress failure (see also Figure 9 for images of egressed cultures). The absence of spheres in the WT comp and MACPF+Cterm strains preliminarily suggests normal or near-normal egress whereas the presence of spheres in *plp1ko* and all of the other domain deletion strains implies defective egress.

Expression and localization of the domain deletions was tested by immunofluorescence, immunoblotting and a microneme secretion assay. Although the complementation plasmid included a C-terminal epitope tag, the epitope tag was not detected by immunofluorescence and only a faint proform of PLP1 was observed by immunoblot (data not shown). These findings suggest that the tag is removed after the protein is synthesized, possibly by proteolysis while the protein traffics to the micronemes. Immunofluorescence with an antibody to the entire mature PLP1 (αPLP1) showed absence of signal in the *plp1ko* and mostly apical signal in the complemented strains, which co-localized with MIC2 in most parasites (Figure 8). Immunoblotting parasite lysate with αPLP1 showed that the WT complement is expressed at a slightly lower level than the WT parental PLP1, despite expression from the endogenous locus (Figure 10A). This lower expression could be due to the absence of introns and mRNA splicing or the transient presence of the epitope tag. As confirmed by affinity purified domain specific antibodies (αNterm, αCterm), the domain deletions were expressed at

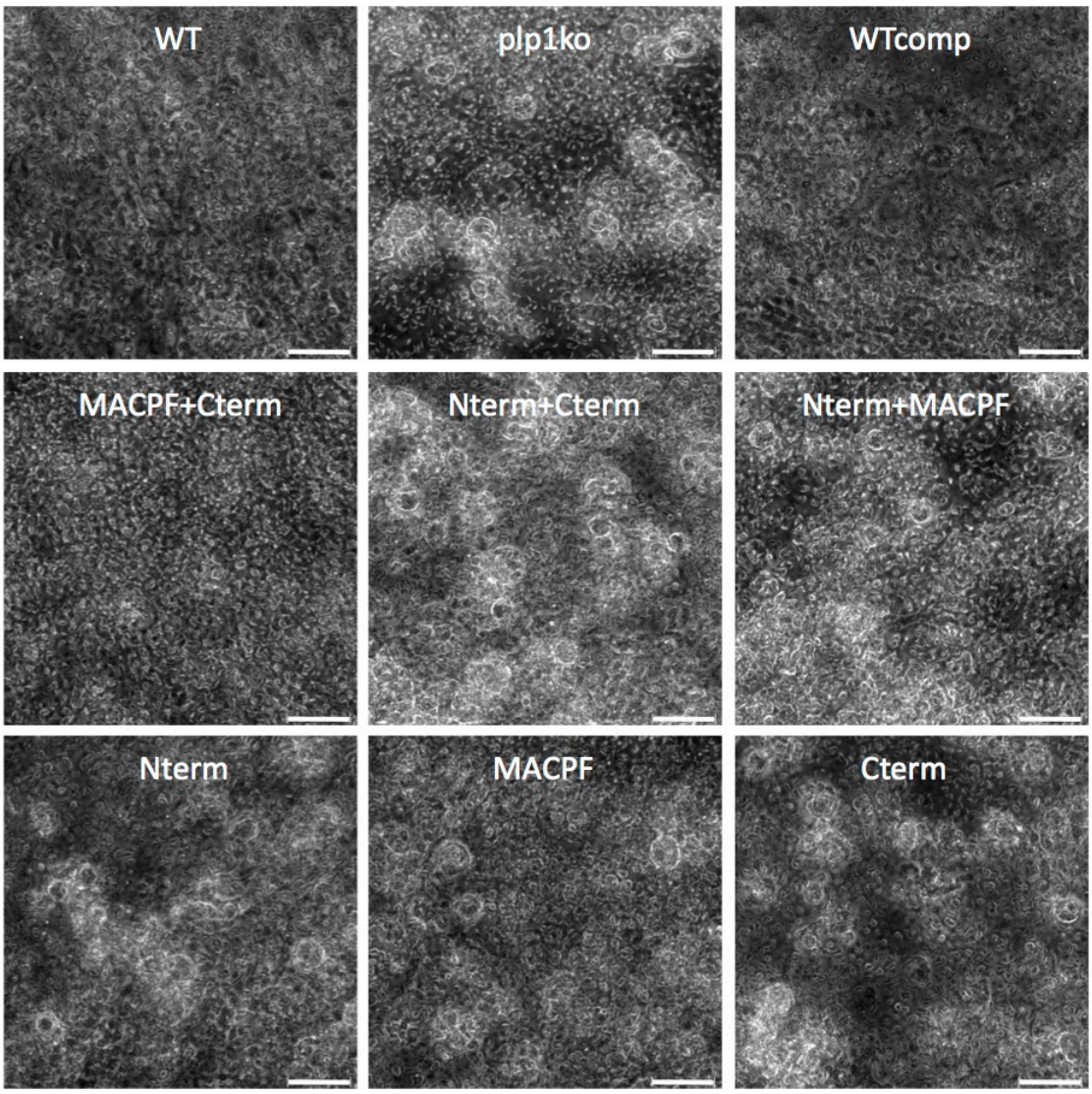


Figure 9: *Spheres observed in egressed cultures*. Images from naturally egressed WT, *plp1ko*, and complemented strain tissue culture flasks. Scale bar indicates 50 µm. Spheres were observed by bright-field examination of egressed cultures and images were acquired on brightfield at 200x magnification on a Zeiss Axio inverted microscope.

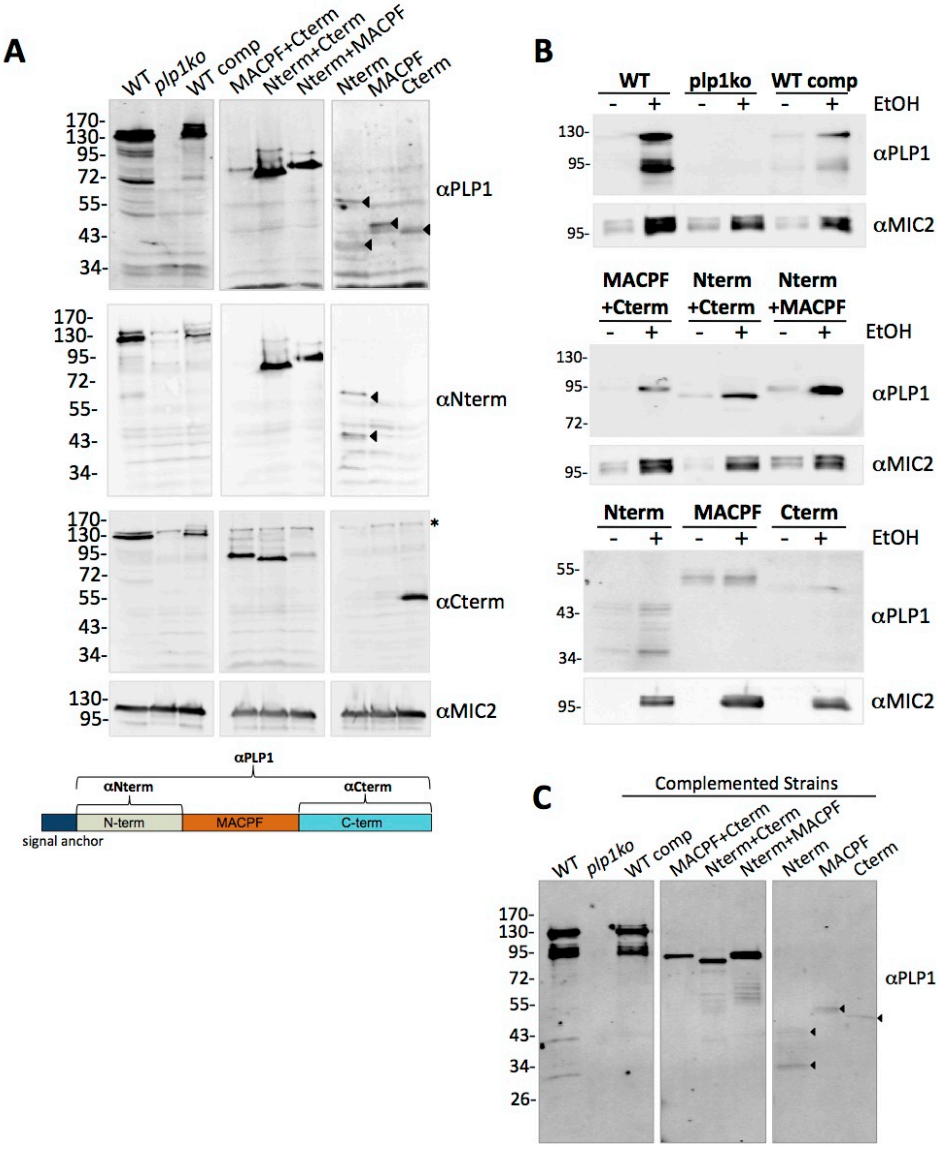


Figure 10. *PLP1 domain deletion constructs are expressed by the parasites and secreted from the micronemes.* **A**) Parasite lysate immunoblotted with a polyclonal (aPLP1) or affinity purified (αNterm, αCterm) PLP1 antibodies. Arrowheads indicate faint bands specifically detected by the antibodies. Asterisk indicates non-specific band. The schematic below indicates domains recognized by the antibodies. **B**) Microneme secretion was induced with 1% ethanol and the secreted fraction was immunoblotted for PLP1 and MIC2. **C**) Immunoblot of secreted fractions demonstrated that PLP1 is processed in the N-terminal domain, indicated by the presence of multiple bands detected by the PLP1 antibody in constructs with the N-terminal domain and absent in the *plp1ko*. Arrowheads indicate specific bands for single domain constructs. Molecular mass markers are indicated in kDa.

similar levels to the WT comp except for the N-terminal domain and the MACPF domain, which were poorly detected (middle and lower panels of Figure 10A). Low detection may be due to poor antigenicity or from protein instability. Ethanol-induced secretion was observed for all of the domain deletions, further supporting the localization of these constructs to the micronemes (Figure 10B). Since the PLP1 signal anchor is the only translated element common to all of the constructs, our findings are consistent with it being sufficient to target the constructs to the micronemes. In secreted samples, we detected proteolytic processing of constructs containing the N-terminal domain, which mirrors the previously reported processing of PLP1 by SUB1, a micronemal subtilisin-like protease (Figure 10B,C) [19].

PLP1 domain deletion leads to intermediate or complete loss of function

Having established expression and secretion of the mutant constructs, we next tested for defects in the lytic cycle by plaque assay. Equal numbers of parasites for each strain were allowed to form plaques in HFF cell monolayers for one week. We observed no significant differences in plaque numbers between WT, *plp1ko* and complemented strains (Figure 11A), suggesting equal viability and that none of the deleted PLP1 domains are required for suppressing PLP1 lytic activity during membrane transport or storage in the micronemes. Accordingly, we experienced no obvious problems in complementing the *plp1ko* with any of the domain deletions as might be expected for expression of a toxic mutant.

While plaque numbers were similar between strains, we observed a conspicuous difference in plaque size between WT and *plp1ko* parasites. WT parasites formed large plaques and *plp1ko* parasites formed much smaller plaques. This decrease in plaque size is likely due to both the pronounced egress defect and the moderate invasion defect. Interestingly, plaque size of the complemented strains mirrored WT or the *plp1ko* for the majority of the strains; however, the MACPF+Cterm (i.e., deletion of the N-terminal domain) strain had an intermediate plaque size (Figure 11B).

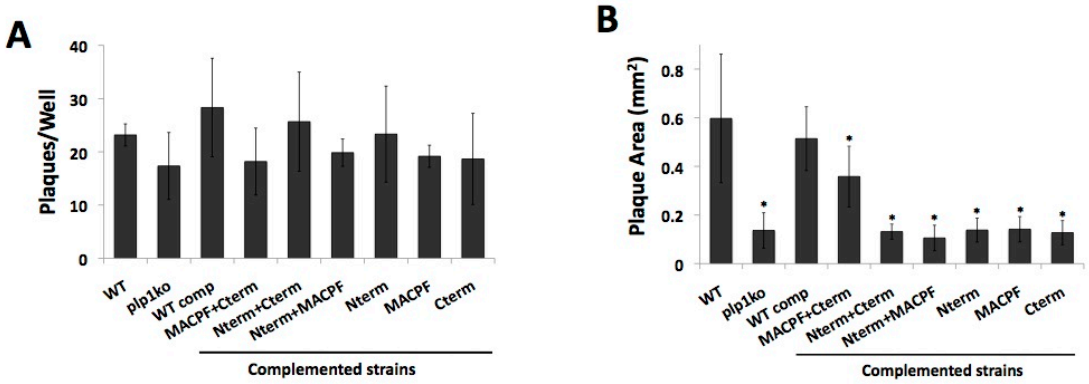


Figure 11. *Plaque assay demonstrates viability of complemented strains, with defects observed in plaque size.* **A**) 50 parasites per well were allowed to form plaques for 7 days. **B**) Plaque area was measured by determining the average diameter in two dimensions and approximating a circle. (*$p < 0.05$ by one-tailed student's test)

In an induced egress assay, the majority of WT parasites rapidly leave the vacuole while *plp1ko* parasites remain associated with the vacuole over the course of several minutes. In the complemented strains, the WT comp is similar to WT in egress and most other strains resemble the knockout, indicating non-functional complementation. Again, the MACPF+Cterm strain showed an intermediate phenotype with the majority of parasites still in vacuoles, though more parasites egress than the *plp1ko* (Figure 12A). We then compared the kinetics of egress for the WT comp, MACPF+Cterm and *plp1ko* strains. The egress time course showed that the MACPF+Cterm parasites have a notable delay in egress, but do not remain trapped as long as the *plp1ko* parasites (Figure 12B).

To determine if the differences in egress were due to differences in PLP1 lytic activity, we tested the complemented strains for their ability to permeabilize the PVM by immobilizing intracellular parasites with cytochalasin D prior to ionophore treatment. PVM permeabilization was observed by release of the fluorescent vacuolar marker dsRed into the host cytosol. We found that most WT parasites exhibited a damaged PVM, whereas *plp1ko* parasites showed an intact PVM (Figure 12C). The WT comp strain showed somewhat less PVM damage, likely due to the

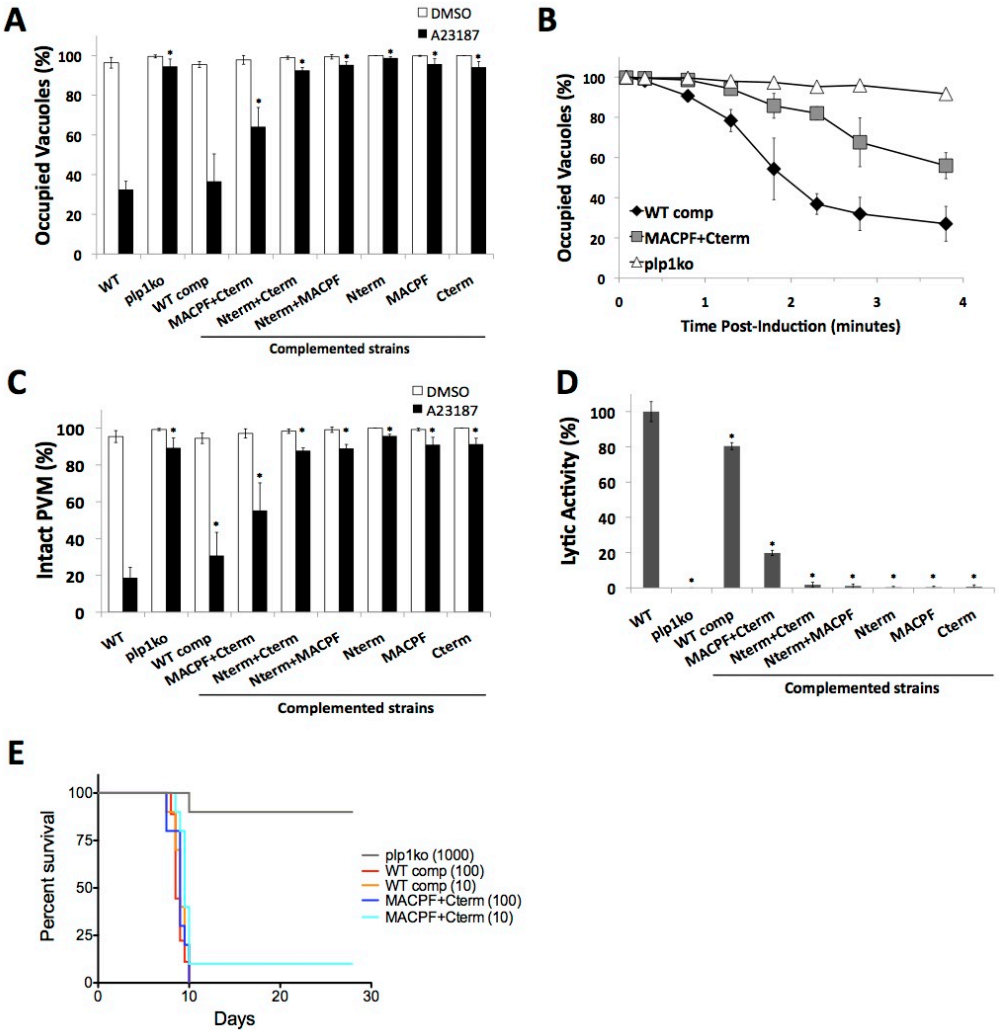


Figure 12. *Phenotypic analysis of PLP1 domain deletion strains reveals intermediate or non-functional complementation.* **A**) Egress was tested following ionophore (A23187) treatment by immunostaining vacuoles for dense granule protein 7 (GRA7) and parasites for surface antigen 1 (SAG1) and quantifying vacuoles containing parasites (occupied) and parasite-free vacuoles (unoccupied). **B**) Time course of parasite egress as in (**A**) with indicated times for each strain. **C**) PVM permeabilization was determined by treating infected cells with cytochalasin D prior to ionophore (A23187) treatment and fixation; infected cells were identified by brightfield and PVM permeabilization was indicated by release of the PV-targeted fluorescent protein dsRed into the host cytosol. **D**) Lytic activity determined from PVM permeabilization was normalized to WT parasites. Graphs depict the average and standard deviation of 3 independent experiments. (*$p < 0.05$ by one-tailed student's t-test.) **E**) Mouse survival after infection. Ten Swiss Webster mice per strain were injected intraperitoneally with the indicated numbers of parasites and monitored for morbidity and mortality.

lower level of PLP1 expression. Most other strains had no significant PVM damage; however, the MACPF+Cterm strain showed more PVM damage than the *plp1ko*. By normalizing PVM permeabilization to WT, we observed 80% of WT activity in the WT comp and 20% of WT activity in the MACPF+Cterm (Figure 12D). Together, these results support a crucial role for the MACPF and C-terminal domains for PLP1 activity since complemented strains lacking one of these domains had no PLP1 activity. Loss of the N-terminal domain does not abolish PLP1 lysis activity, but reduces it substantially. The absence of spheres from naturally egressed cultures of MACPF+Cterm parasites is consistent with lytic egress occurring in this strain without the failure events seen in non-functional strains.

Although *plp1ko* parasites can be propagated normally *in vitro*, previous work demonstrated a substantial virulence defect in the mouse infection model [10], implying that rapid parasite egress is crucial for pathogenesis. The MACPF+Cterm complemented strain provided another tool with which to investigate the role of rapid egress in pathogenesis since this strain had an egress defect that was intermediate between WT and *plp1ko*. To examine this further, we infected mice with 10 or 100 WT comp or MACPF+Cterm parasites, or 1,000 *plp1ko* parasites, and observed the outcome of infection with respect to mouse survival. As expected, *plp1ko* showed an attenuation of virulence, which was reversed in the WT comp. Notably, MACPF+Cterm parasites were also highly virulent and not significantly different (p = 0.09 for 10 tachyzoites/mouse) from the WT comp (Figure 12E), despite the survival of one mouse infected with MACPF+Cterm parasites, confirmed by serology. These results suggest that even low levels of lytic activity are sufficient for virulence and the moderate egress delay does not have a significant impact on infection under the conditions used.

To identify the basis for non-functionality of the domain deletions, we assessed PLP1 membrane binding and oligomerization in the complemented strains. Membrane flotation of host cell debris from naturally egressed, complemented cultures showed membrane association of high molecular weight complexes for

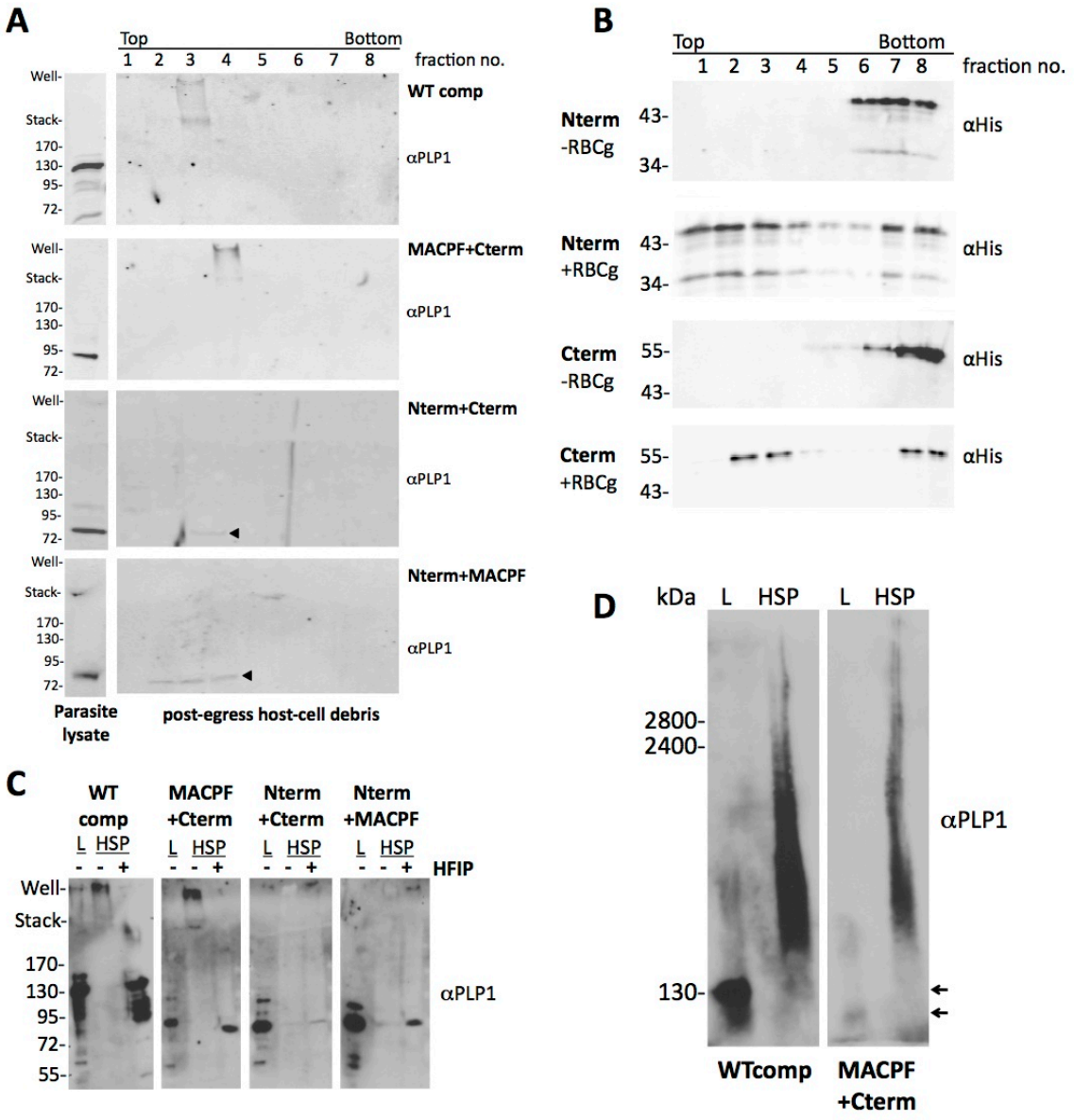


Figure 13. *Functional analysis of PLP1 domain deletions reveals differences in membrane binding and oligomerization*. **A**) Membrane flotation of host cell debris from complemented strains immunoblotted for PLP1. Arrowheads indicate specific bands. **B**) Membrane flotation of recombinant N- and C-terminal domains using red blood cell ghosts (RBCg) and immunoblotted for the 6xHis epitope tag. **C)** Host cell debris from complemented strains was TCA precipitated, treated with HFIP or buffer and immunoblotted for PLP1. **D**) SDS-Agarose gel electrophoresis and PLP1 immunoblot of WTcomp and MACPF+Cterm lysate (L) and high-speed pellet (HSP). Marker is Coomassie stained mouse muscle lysate; the indicated sizes are in kDa. Arrows indicate monomers in lysate.

both WT comp and MACPF+Cterm. This suggests the Nterm is not required for membrane binding or oligomerization. We also observed membrane binding for the Nterm+Cterm and Nterm+MACPF constructs, but no significant signal was seen in the well or stacking gel for these two constructs (Figure 13A), indicating the absence of oligomerization. We were unable to observe any specific signal upon membrane flotation for other constructs due to either low expression levels, minimal membrane binding activity, or both.

To determine if the N- or C-terminal domains were sufficient for membrane binding, we produced the corresponding recombinant, His-tagged proteins in *E. coli*. The N-terminal domain was completely soluble and the C-terminal domain was partially soluble. Recombinant Nterm was processed by bacterial proteases in a pattern similar to endogenous, secreted Nterm, whereas the Cterm was unprocessed by parasitic or bacterial proteases (Figure 10C, 13B). This suggests that similar protease-sensitive sites are exposed on recombinant and endogenous Nterm and that recombinant Nterm is structurally similar to the endogenously expressed Nterm. We attempted to generate recombinant MACPF domain but this construct was poorly expressed, largely insoluble, and unstable in solution upon refolding (data not shown). Recombinant Nterm and Cterm were incubated with red blood cell ghosts and subjected to membrane flotation. Both processed and unprocessed Nterm as well as the Cterm constructs were detected in lighter density sucrose fractions in the presence of membranes (Figure 13B). This is consistent with each of these domains being sufficient for membrane association, suggesting that PLP1 uses two distinct domains for binding membranes.

We next examined the domain deletion strains with detectable PLP1 membrane association for the ability to form PLP1 oligomers. We observed PLP1 signal in the well and stacking gel for both WT comp and MACPF+Cterm as previously noted, and no significant high molecular weight signal for the Nterm+Cterm and Nterm+MACPF (Figure 13C). Treatment of the HSP with HFIP resulted in mature and processed PLP1 bands observed in the WT comp and a single

band in the MACPF+Cterm sample. Bands equivalent to the lysate form were observed in both buffer and HFIP treated samples of the Nterm+Cterm and Nterm+MACPF samples. Together these results indicate an essential role for the MACPF and Cterm in PLP1 oligomerization. Though membrane binding was observed for the Nterm+Cterm, the absence of the MACPF domain in this construct precluded oligomerization. For the Nterm+MACPF domain, the membrane binding observed did not lead to detectable amounts of oligomerization, suggesting that the protein is not positioned to oligomerize when membrane-bound by only the N-terminal domain.

To determine if WT comp and MACPF+Cterm oligomers differed in their high molecular weight complexes, we applied lysate and HSP from both samples to SDS-AGE and immunoblotted for PLP1. We observed similar high molecular weigh smears for both WT comp and MACPF+Cterm samples, confirming that the N-terminal domain is not required for formation of large molecular weight complexes (Figure 13D).

Discussion:

To examine the role of each PLP1 domain in lytic activity, we complemented the knockout parasites with domain deletion constructs. The enhanced homologous recombination of the RHΔ*ku80* strain permitted targeting the constructs to the endogenous locus, resulting in expression under the native promoter. We found the PLP1 signal anchor is sufficient for microneme targeting of all tested PLP1 domains as we observed apical localization by immunofluorescence and ethanol-induced secretion. Similar to the previously generated randomly integrated PLP1-complemented strain [10], we observed less PLP1 expression in the WT-complemented parasites than the parental strain. This suggests that intronic sequences promote expression or the epitope tag interferes with expression, or a combination of the two scenarios. Importantly, we observe similar levels of expression for most of the domain deletion constructs using affinity purified

domain-specific antibodies. The lower levels of expression seen for the Nterm and MACPF domains expressed individually may be due to proteolytic processing of the Nterm and a lack of epitopes in the MACPF domain, and/or construct instability and degradation. Regardless, the domain deletion complementation strategy was useful for determining the functional role for each domain in pore-formation *in vivo*, a first of its kind for a MACPF protein.

Complementation with the domain deletion constructs did not affect parasite viability, suggesting that the domain deletion constructs are not toxic to the parasite. A toxic construct might result from loss of a domain necessary for preventing premature pore-formation. Instead, parasites may be protected from autolysis during PLP1 trafficking and storage in the micronemes by the signal anchor and environmental factors, respectively. These modes of intracellular regulation are akin to those of perforin, which is initially made as an inactive precursor form, which traffics through the early secretory system (ER and Golgi) before encountering a protease that removes a C-terminal propeptide [20]. Propeptide removal creates mature perforin, but the protein remains inactive because of the acidic environment of the lytic granules in which it resides [20]. We failed to detect proteolytic processing of the C-terminal domain in parasite lysate or the secreted fraction, which suggests the absence of a C-terminal propeptide for PLP1. However, the PLP1 proform, which includes an N-terminal presumptive trans-membrane signal anchor, might suppress PLP1 activity by keeping it in an inactive conformation or by orienting it away from the membrane. Although the luminal pH of micronemes is not known, this or other environmental factors might prevent premature pore formation.

The finding of predicted functions for the MACPF and β-sheet rich C-terminal domain highlights the conservation of this domain family. The MACPF domain is necessary for oligomerization and membrane permeabilization whereas the C-terminal domain provides membrane-binding activity necessary for pore-formation. We also identified a novel activity for a MACPF-associated N-terminal domain in

membrane binding. We speculate that binding through the N-terminal domain provides another means of enhancing lytic activity by allowing recognition of more than one membrane receptor. An N-terminal lectin-binding domain in the CDC lectinolysin binds to Lewis antigen and is thought to enhance pore formation by concentrating the toxin at fucose-rich sites on target membranes [7, 9]. Although C8γ has lectin-binding activity, it is a lipocalin protein associated with C8α and C8β and does not contain a MACPF-domain [21]. Thus, it will be important to consider the possibility that Nterm membrane binding is mediated by recognition of carbohydrates. To more precisely determine if the defect in the ΔN-term complemented strain is due to the loss of the N-term membrane binding activity, it should be overexpressed in the parasitophorous vacuole (PV) space. This would saturate the N-term binding sites and prevent the N-term of full-length PLP1 from binding membranes during egress. This strain is expected to display a similar defect in membrane permeabilization and egress as observed for the ΔN-term complemented strain. Overexpressing the C-terminal domain, which is the principal membrane binding domain, in the PV, should also result in an egress and membrane permeabilization defect; in fact, this strain should have a more profound defect, as the C-terminal domain is essential for activity.

While the protein sequence of the Nterm is not highly conserved, other MACPF proteins in apicomplexan genomes also contain N-terminal domains of varying lengths. Apicomplexan parasites complete their lifecycle in a wide variety of hosts and tissues. These domains may provide unique regulatory elements necessary for augmenting lytic activity at each stage of the life cycle. Future work on parasite pore-forming proteins is expected to lead to further insights into the molecular mechanisms by which these pathogens progress through their hosts and cause disease.

Although the loss of PLP1 results in an egress delay and profound virulence attenuation, we did not detect a significant virulence defect for the MACPF+Cterm strain, which also has a significant delay in egress. This suggests that the presence

of lytic activity, rather than the absolute rate of parasite egress, is the deciding factor in pathogenesis. The basis of virulence attenuation for the *plp1ko* parasites is still an open question. What factors lead to host control of *plp1ko* infection yet susceptibility to WT parasites? One deciding factor may be indicated in the different plaque sizes of WT and *plp1ko* parasites. WT parasites form large plaques in a monolayer and *plp1ko* parasites form much smaller plaques, though with similar plating efficiency. Plaque size is dependent on multiple factors including invasion, egress, and motility, and is an indication of parasite viability and capacity to complete the lytic cycle. The small plaque size of PLP1-deficient parasites is likely due to the combined egress and secondary invasion defects. *plp1ko* parasites might expend more resources for egress, thus compromising motility during intercellular migration. The combined invasion and egress defects may render extracellular *plp1ko* parasites more susceptible to phagocytosis than WT parasites, which are able to rapidly egress and invade a neighboring cell, spending a limited time in the extracellular environment. Additionally, the small plaques in the *plp1ko* may indicate a limited amount of lytic damage occurring in *plp1ko* infections. High levels of tissue damage from multiple rounds of lytic replication in WT parasite infections may exacerbate the immune response while reduced tissue damage by *plp1ko* parasites may be more efficiently repaired by the host, resulting in a dampened immune response and recovery from infection. Verification of these hypotheses will require more careful examination of the differences in host immune responses and the lytic damage caused by WT and *plp1ko* parasites in the mouse infection model.

The field of pore-forming protein biology has been stimulated with breakthroughs in structural and biochemical techniques. Here we have demonstrated the utility of investigating the function of a pore-forming protein in a parasite system. Future studies will contribute knowledge into the diverse ways pore-forming proteins function and to the molecular mechanisms of virulence in these important pathogens.

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Chapter 4
An environmental pH switch regulates PLP1 lytic activity during egress and invasion

Introduction:

Pore-forming proteins (PFPs) are important virulence factors and immune mediators. The lytic activity of pore-forming proteins (PFPs) must be tightly regulated to promote targeted lysis and suppress membrane damage where activity would be detrimental. There are multiple steps at which pore-formation may be controlled. First, PFP expression may occur in response to specific cues, such that the PFP is only produced when and where lytic activity is needed. Second, PFP activity should be inhibited while the newly synthesized PFP traffics to its destination; otherwise, the PFP would result in autolysis of the producing cell. Third, PFP secretion should be timely, to conserve resources and concentrate the PFP when and where activity is advantageous. Finally, the PFP should be discriminatory as to which cellular membranes are targeted for attack upon secretion. While restraints are likely to occur at all of these stages, few PFPs have been thoroughly examined for regulatory mechanism.

PLP1 is transcribed throughout cell division and the lytic cycle, which suggests its activity is not transcriptionally regulated. We hypothesize the PLP1 signal anchor and membrane receptor availability limit autolysis during trafficking. PLP1 is secreted from the micronemes in conjunction with parasite egress, which occurs in response to various signals described in the introductory chapter. However, as microneme secretion is required for egress and invasion, PLP1 should be inactive during invasion to protect the prospective host cell and

promote formation of an intact parasitophorous vacuole membrane. Thus, it is unclear how lytic activity is controlled during the continuous process of intercellular migration. Potential factors influencing activity during the course of egress and invasion include membrane receptor availability, proteolytic processing, and environmental conditions.

The role of environmental conditions in regulating pore-forming activity has been previously described for the CDC, Listeriolysin O (LLO), and the MACPF protein, perforin. LLO is highly active in the acidic environment of the phagosome, from which it mediates *Listeria* escape into the host cytosol [1, 2]. In the host cytosol at neutral pH, LLO is less active, which maintains an intact host cell for bacterial replication. The importance of regulating LLO activity is demonstrated by the avirulence of perfringolysin O-expressing *Listeria*, which escape from the phagosome, but are unable to replicate to high levels in the host cytosol because of host cell lysis [3]. LLO activity is further augmented by chloride, which is transported into the *Listeria* containing phagosome by the cystic fibrosis transmembrane conductance regulator (CFTR) [4].

Conversely, perforin is inactive in the acidified secretory granules of perforin-producing cells, and has optimal activity in the neutral-pH environment of the immunological synapse [5, 6]. Interestingly, perforin membrane binding is pH-independent, though membrane permeabilization only occurs at neutral pH, which suggests neutral pH promotes conformational changes or monomer-monomer interactions required for oligomerization or transmembrane insertion [5]. However, perforin membrane binding is calcium dependent regardless of pH. Since intracellular calcium concentrations are low, this prevents perforin binding to intracellular membranes beyond the ER, and promotes perforin binding to target cells upon secretion into the extracellular space, where calcium is more abundant [7, 8].

Thus, PFP have evolved to function in the specific environments in which their activity is essential. Herein we describe the identification of an environmental pH-switch, which regulates PLP1 pore formation during egress

and invasion. We initially observed that recombinant PLP1 activity is optimal at an acidic pH and minimal at neutral pH. Additionally, pH-neutralization reduced parasite egress and egress-associated PLP1-dependent membrane permeabilization. We detected a reduction in vacuolar pH late in the parasite replication cycle and evidence for membrane disruption upon invasion at low pH. These results suggest that a pH-switch regulates PLP1 activity to promote membrane permeabilization during egress by acidic conditions and suppress lytic activity with neutral pH during host cell invasion. It is unlikely that vacuolar acidification occurs solely to enhance PLP1 activity. Instead, we find that low pH stimulates microneme secretion and parasite motility and thus overcomes the high potassium inhibition of these processes. Therefore, we hypothesize that vacuolar acidification is an endogenous cue, which promotes parasite egress. As a result, exploration of TgPLP1 activity has broadened our understanding of the environmental factors that not only influence PLP1 lytic activity, but that also regulate other processes essential to the parasite lytic cycle.

Materials and methods:

Recombinant protein expression and assays:

Recombinant PLP1 and LLO were generated as previously described [9]. Streptolysin O (SLO) was purchased from Murex and prepared according to the manufacturer's instructions. pH-dependent hemolysis was assessed by washing red blood cells in PBS (pH 7.4), pelleting the RBC, and resuspending in PBS of indicated pH (prepared by mixing sodium mono- and diphosphate in different amounts and adjusting pH with HCl or NaOH) with 100 nM recombinant protein. RBC and recombinant protein were incubated at 37°C for 1 h, pelleted, and hemolysis was measured by absorbance at 540 nm of the supernatant.

pH-dependent binding was tested by incubating red blood cell ghosts, prepared as previously reported, with recombinant protein in PBS of the indicated pH [9]. RBC ghosts and recombinant protein was incubated at 37°C for

30 min; cells were pelleted and washed three times with cold PBS at neutral pH and bound samples were analyzed by SDS-PAGE and immunoblot.

Egress assays and egress-associated PVM permeabilization:

Egress assays were conducted as previously described with the following treatments: NH_4Cl, bafilomycin, dicyclohexylcarbodiimide (DCCD), omeprazole, and sodium orthovanadate; inhibitors were purchased from Sigma and tested at the indicated concentrations.

Egress-associated membrane permeabilization was tested by treating infected cells with 1 µM cytochalasin D (CytD) for 3 min at 37°C, and then adding 4 µM A23187/DMSO, 1 µM CytD, 12.5 µg/ml propidium iodide (PI) with the indicated final concentrations of NH_4Cl and DCCD and incubated for 3 min. Following the incubation, cells were washed twice with warm PBS and fixed with formaldehyde. Membrane permeabilization was quantified by the number of infected cells with PI+ nuclei.

Cell-wounding assays:

Cell wounding was tested by pre-loading host cells with 1 µM calcein-AM in phenol-red free DMEM and incubating for 1 h at 37°C, followed by two washes with warm PBS. Parasites were filter-purified in Endo buffer [10] (44.7 mM K_2SO_4, 106 mM sucrose, 10 mM $MgSO_4$, 20 mM Tris-H_2SO_4 (pH 8.2), 5 mM glucose, 3.5 mg/ml BSA) and applied to host cells in a 96-well plate by centrifuging at 500g for 5 min. Supernatant was removed and replaced with 100 µl/well "invasion buffer" of the following composition: 110 mM NaCl, 0.9 mM NaH_2PO_4, 44 mM $NaHCO_3$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.4 or 5.4. Plates were incubated for 10 min at 37°C and centrifuged as above. Fifty µl of supernatant was transferred to another plate and calcein fluorescence was read in a 96-well plate reader.

Microneme secretion assays:

Microneme secretion was tested with A23187 induced secretion as described previously [11] in the presence of vehicle, 10, 40 mM NH_4Cl, 10, 40 μM DCCD, and 100, 400 μM omeprazole [12]. Low-pH induced secretion was tested with parasites purified in Endo buffer, and resuspending in invasion buffer or Endo buffer of the indicated pH at 37°C for 2 min. Calcium-dependent secretion was tested by pre-incubation with BAPTA-AM as described previously [11].

Gliding assays:

Gliding experiments were conducted with a heated stage on a Zeiss Axio inverted microscope. For pH-dependent gliding, parasites were filter purified in control buffer containing 145 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 15 mM MES, 15 mM HEPES, pH 8.4, and allowed to settle in a glass-bottom petri dish. After parasites had settled, images were collected every 100 msec for 6 min. Then buffer was exchanged for pH 7.4 or 5.4 and images collected every 100 msec for 30 min. For inhibitor gliding experiments, parasites were filter-purified in PBS, resuspended and allowed to settle in HBSSC. After initial images were collected, buffer was exchanged for 40 mM NH_4Cl or 40 μM DCCD in HBSSC and parasite motility was observed as above. Maximum projection images and videos were examined for motile parasites and the percent of motile parasites graphed over time.

pHluorin assays:

Superecliptic and ratiometric pHluorin vectors were kindly provided by Gero Miesenbock by material transfer agreement (University of Michigan, SSP no. 13477; Memorial Sloan-Kettering Institute, SK# 19367) [13]. The genes were subcloned into the DsRed vacuolar expression vector [14]. Parasites were transfected with plasmid, transformed parasites were selected for with chloramphenicol and cloned by limiting dilution. Superecliptic pHluorin was highly expressed by parasites in the parasitophorous vacuole. Ratiometric

pHluorin was transcribed but not translated by parasites as demonstrated by production of mRNA by RT-PCR and lack of detection by fluorescence microscopy, immunoblot or pulse chase ^{35}S-methionine/cysteine metabolic labeling and immunoprecipitation. Ratiometric pHluorin was subsequently codon-optimized, chemically synthesized (GenScript) and subcloned into the DsRed vacuolar expression vector. Codon-optimized ratiometric pHluorin was expressed by the parasites, and fluorescent parasites recovered upon drug selection and cloning by limited dilution. However, fluorescence was lost upon prolonged passage despite continuous drug selection.

Results:

Environmental pH has been shown to effectively compartmentalize the lytic activity of other pore-forming proteins. Thus, we tested whether PLP1 activity is pH-dependent or independent. Upon incubating recombinant protein with erythrocytes at different pH values, we observed increased hemolysis at low pH (Figure 14A). The effect of pH on PLP1 lytic activity was similar to that of LLO, with more lytic activity at low pH and less activity at neutral pH. These results were in contrast to SLO, which had a similar amount of lytic activity across a broad range of pH values. We next tested the effect of pH on PLP1 membrane binding using red blood cell ghosts as a model membrane. We observed that PLP1 membrane binding activity mirrors the lytic activity with more binding at acidic pH than neutral pH. These results suggest PLP1 cytolysis is pH-dependent at the membrane-binding stage.

We then reasoned if PLP1 activity is optimal at acidic pH, the pH of the parasitophorous vacuole, where PLP1 functions, might be acidic during parasite egress. To test if parasite egress is sensitive to pH-neutralization, we performed induced egress assays with NH_4Cl, a weak base that accumulates in acidic compartments until a neutral pH is reached. We found parasite egress is suppressed by NH_4Cl treatment with both ionophore and dithiothreitol (DTT) induced egress (Figure 15A,B).

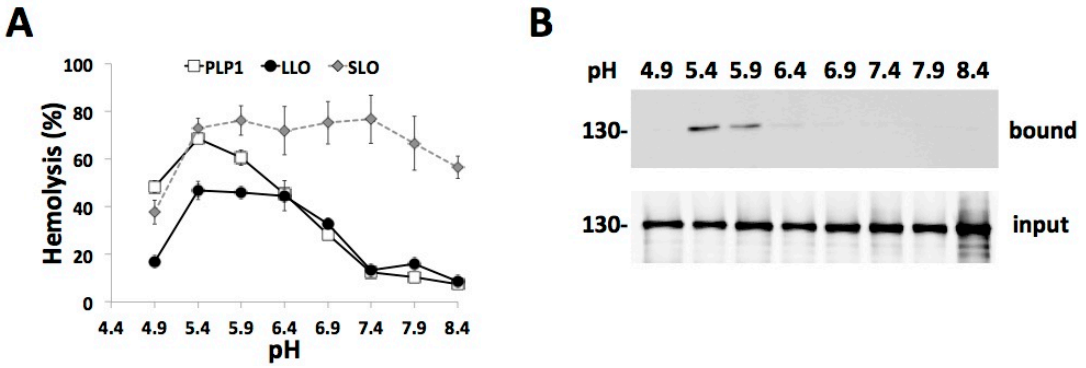

Figure 14: *PLP1 activity is pH-dependent*. **A)** Hemolytic activity was compared by incubating erythrocytes with 100 nM recombinant protein in PBS of the indicated pH. Results are representative of 3 independent experiments each with triplicate wells. **B)** pH-dependent binding was compared by incubating red blood cell ghosts with recombinant protein at varying pH and immunoblotting with αHis (bound) and αPLP1 (input). The experiment was repeated twice with similar results. Molecular mass is indicated (kDa).

Parasitophorous vacuolar acidification may occur through passive accumulation of metabolic wastes, or active delivery of protons by a proton-pump. We tested the effect of H^+-ATPase inhibitors on induced egress to determine if the acidification occurred passively or actively. Parasite egress was sensitive to a non-specific ATPase inhibitor, sodium orthovanadate, indicating that active proton pumping may occur during induced egress. We further found that parasite egress was specifically sensitive to the P-type ATPase inhibitor, DCCD, while the V-type ATPase inhibitor, bafilomycin, and omeprazole, a H+/K+ exchange inhibitor, had no effect on induced egress (Figure 15C-F). Although these results suggest an active acidification process, we cannot rule out the possibility that passive accumulation of metabolic wastes also contributes to an acidified vacuole.

To determine whether the reduced parasite egress was due to an inhibition of PLP1 activity occurring with pH-neutralization, we tested the effect of these treatments on egress-associated membrane permeabilization. By paralyzing parasites with cytochalasin D, we were able to observe membrane permeabilization of infected cells by propidium iodide (PI), which stains nuclei

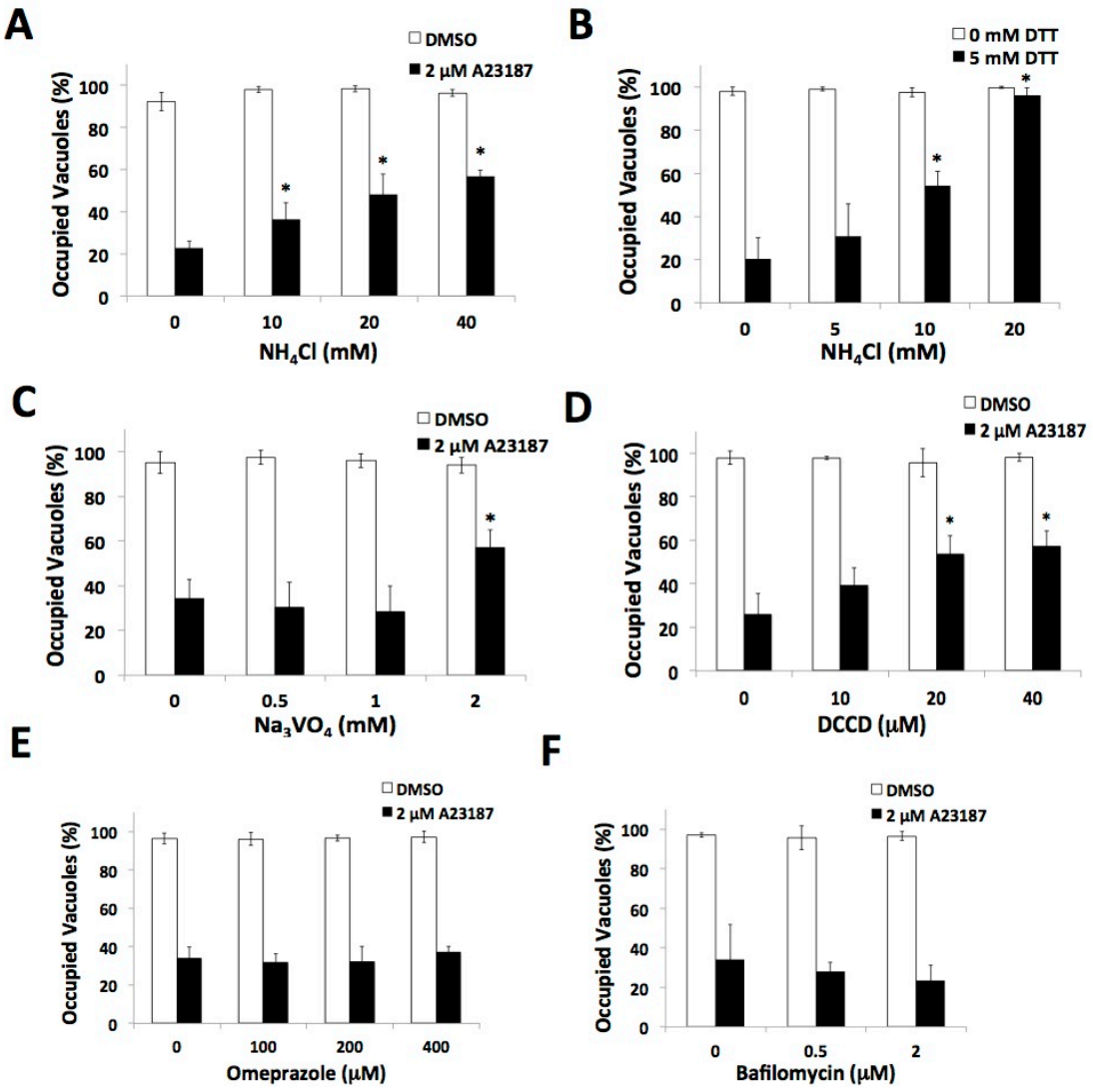

Figure 15: *pH neutralization suppresses parasite egress.* **A-F)** Parasites were allowed to replicate for 30 h (A23187) or 35 h (DTT) prior to treatment with vehicle (DMSO, buffer) or egress inducer (2 μM A23187, 5 mM DTT) for 2 min in the presence of the indicated compound. Immunofluorescence was performed for parasites (SAG1) and parasitophorous vacuole (GRA7) and occupied vacuoles were quantified. Results are the average of 3 independent experiments. (*p < 0.05, student's t-test compared to A23187 alone)

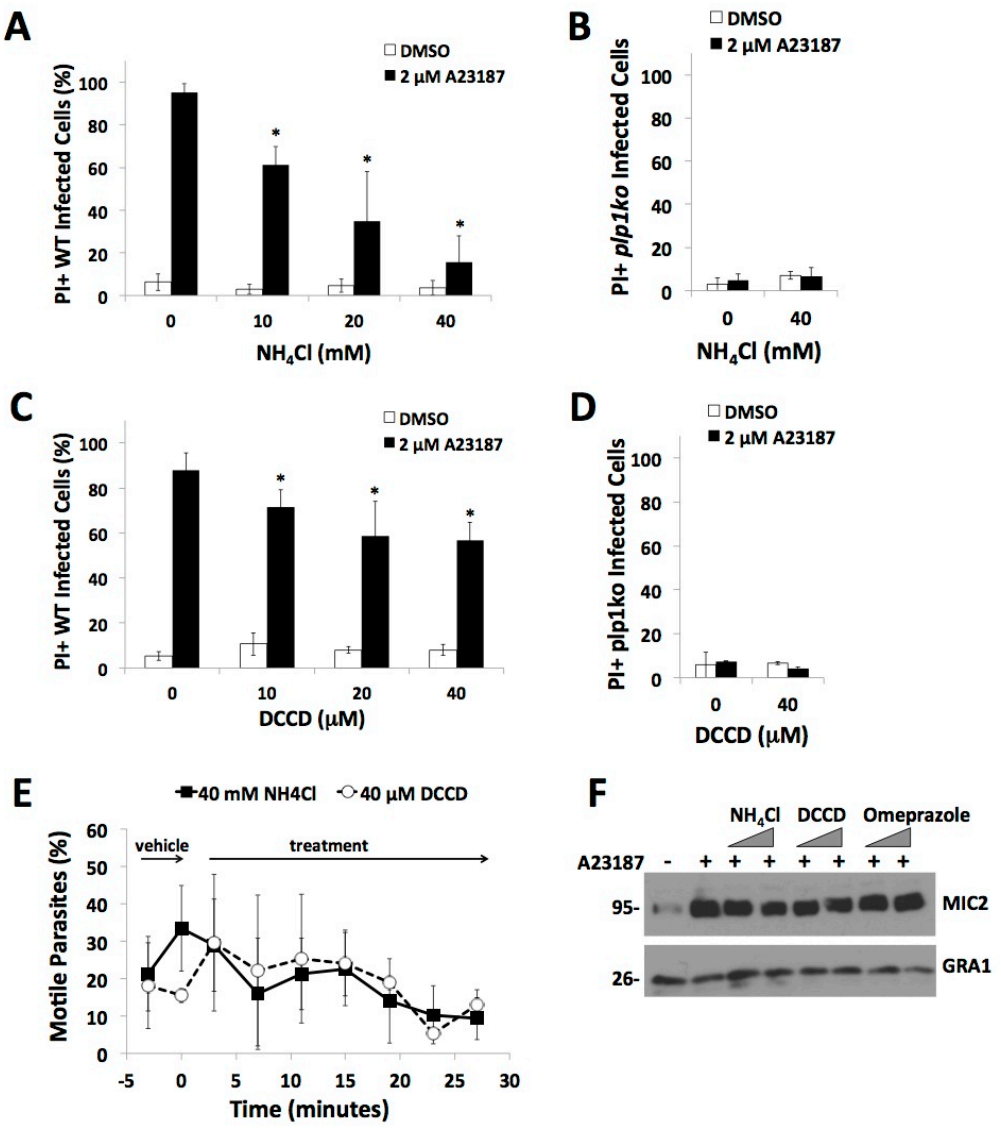

Figure 16: *pH-neutralizing agents reduce egress-associated, PLP1-dependent membrane permeabilization*. **A-D)** CytD-treated infected cells were incubated with vehicle (DMSO) or ionophore (A23187) plus propidium iodide (PI) for 3 min. PI+ infected cells were quantified by microscopy. Results indicate average and standard deviation of 3 independent experiments for WT and 2 independent experiments for *plp1ko*. **E)** NH_4Cl and DCCD do not affect parasite motility. Curves indicate average and standard deviation for 3 (NH_4Cl) and 2 (DCCD) independent replicates. **F)** Microneme secretion was tested with 10 or 40 mM NH_4Cl, 10 or 40 µM DCCD, and 100 or 400 µM omeprazole and the secreted fraction was probed for MIC2 and dense granule protein 1 (GRA1). Results are representative of 2 independent experiments.

of cells with membrane damage. While vehicle-treated cells maintain intact membranes, ionophore treatment leads to the permeabilization of the majority of infected cells. This activity is PLP1-dependent since no permeabilization was observed in ionophore-treated *plp1ko*-infected cells (Figure 16B,D). Ionophore-induced membrane permeabilization was sensitive to both NH_4Cl and DCCD, suggesting that pH-neutralization suppresses PLP1 activity (Figure 16A,C). NH_4Cl and DCCD did not inhibit parasite gliding motility or microneme secretion, ruling these out as possible off-targets (Figure 16E,F).

To test if the neutral pH environment the parasite normally experiences during invasion suppresses PLP1 activity to avoid membrane damage, we examined the effect of pH on invasion-associated membrane permeabilization. Host cells were pre-loaded with calcein-AM, and WT and *plp1ko* cells were allowed to settle on host cells in Endo buffer (pH 8.2), which blocks invasion. Endo buffer was subsequently removed and replaced with buffer of the same ionic composition as media at pH 7.4 and 5.4. After a 10-minute incubation period, supernatant was collected and calcein release from host cells was quantified in a 96-well plate reader. We observe a similar amount of background release for *plp1ko* cells at neutral and acidic pH, and for WT parasites at neutral pH. WT parasites incubated at low pH, however, had a significant increase in calcein release from host cells, which suggests PLP1 activity was induced under these conditions (Figure 17).

To observe changes in vacuolar pH, we overexpressed a GFP variant, superecliptic pHluorin in the vacuolar space. Superecliptic pHluorin is highly fluorescent at neutral pH, and fluorescence is quenched at low pH [13]. We verified the pH-dependence of fluorescence by measuring the signal in live, infected cells, and infected cells lysed at low and neutral pH. Fluorescence was completely quenched in cells lysed at low pH (Figure 18A). We detected a modest but significant drop in fluorescence upon treatment with ionophore, which is reversed with NH_4Cl, supporting the hypothesis that a decrease in

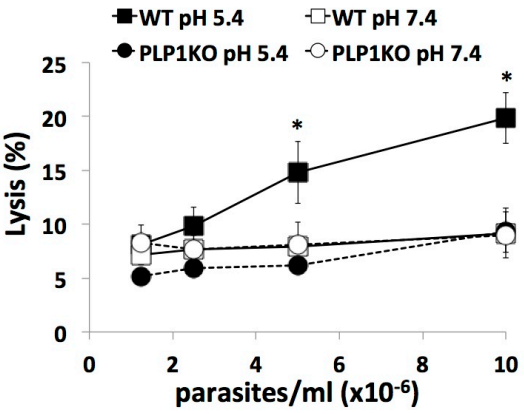

Figure 17: *Low pH promotes PLP1-mediated membrane permeabilization during cell invasion.* Host cells were pre-loaded with calcein-AM and WT and *plp1ko* parasites were applied to host cells in low or neutral pH buffer. Calcein release from host cells was quantified by 96-well plate reader following 10 min of exposure to parasites. Graph indicates average and standard deviation of triplicate wells and graph is representative of 3 independent experiments. (*p < 0.05 by student's t-test)

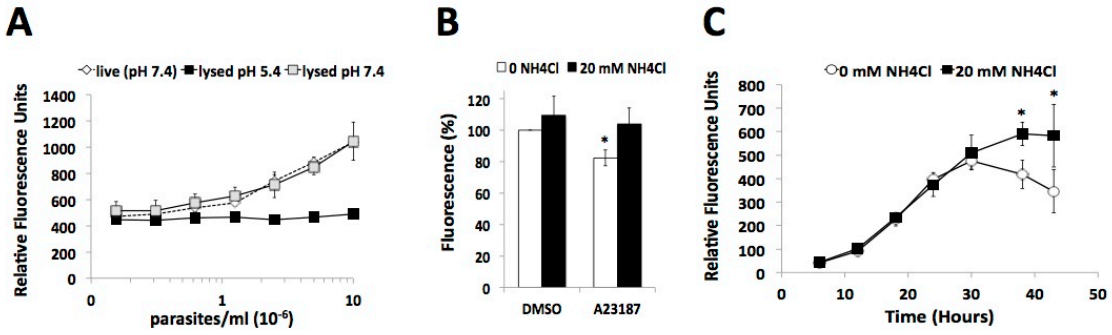

Figure 18: *Detection of egress-associated vacuolar pH changes with super-ecliptic pHluorin*. **A)** Super-ecliptic pHluorin fluorescence is similar between live parasites and parasites lysed in neutral pH buffer yet fluorescence is quenched when parasites are lysed in low pH. **B)** Ionophore (A23187) addition leads to a modest but significant (*p < 0.05 student's t-test) reduction in fluorescence, which is reversed with addition of NH_4Cl. **C)** Fluorescence is similar between buffer and NH_4Cl-treated wells up to 30 h post-inoculation, after which the two curves diverge, suggesting a decrease in parasitophorous vacuolar pH.

vacuolar pH occurs upon egress induction (Figure 18B). To observe changes in natural egress, we followed the fluorescent signal over the course of intracellular replication, normalized to NH_4Cl treatment. If the parasitophorous vacuolar pH is neutral, NH_4Cl treated cells are expected to have a similar amount of fluorescence as cells in buffer alone. Conversely, if vacuolar pH is acidic, NH_4Cl treated cells should show a stronger signal than untreated cells due to the pH neutralizing effect of treatment. Super-ecliptic pHluorin signals increased identically during parasite replication until ~30 h post-inoculation. At this time point, however, the curves began to diverge, with a substantial suppression of fluorescence that was reversed by NH_4Cl treatment. These findings suggest that a population-scale decrease in vacuolar pH occurs late in the replication cycle, prior to parasite egress (Figure 18C).

Having established that PLP1 activity is pH-dependent, and that low pH stimulates activity during egress, we then tested the effect of pH on parasite microneme secretion and gliding. Previous work has shown that parasite gliding is inhibited by alkaline pH [10]. We tested the effect of pH on gliding by purifying parasites in high potassium, alkaline buffer (pH 8.4), switching to low or neutral pH buffers containing high potassium and observing motility over time. Low pH stimulated motility in >90% of observed parasites and motility was sustained in a majority of parasites for 15 minutes, confirming previous findings [10]. Switching from alkaline to neutral pH led to no significant change in motility (Figure 19A). The effect of pH on microneme secretion was tested by incubating parasites in high potassium buffer of varying pH and probing the secreted fraction by immunoblot. More microneme secretion was detected at low pH compared to neutral and alkaline pH (Figure 19 B-D). These findings suggest that extracellular pH regulates microneme secretion, which is required for motility. Dense granule secretion was not substantially affected by pH. Low-pH induced microneme secretion was sensitive to the calcium chelator, BAPTA, indicating dependence on intracellular calcium (Figure 19C). Interestingly, SUB1 proteolysis of secreted microneme substrates is inhibited by low pH, suggesting

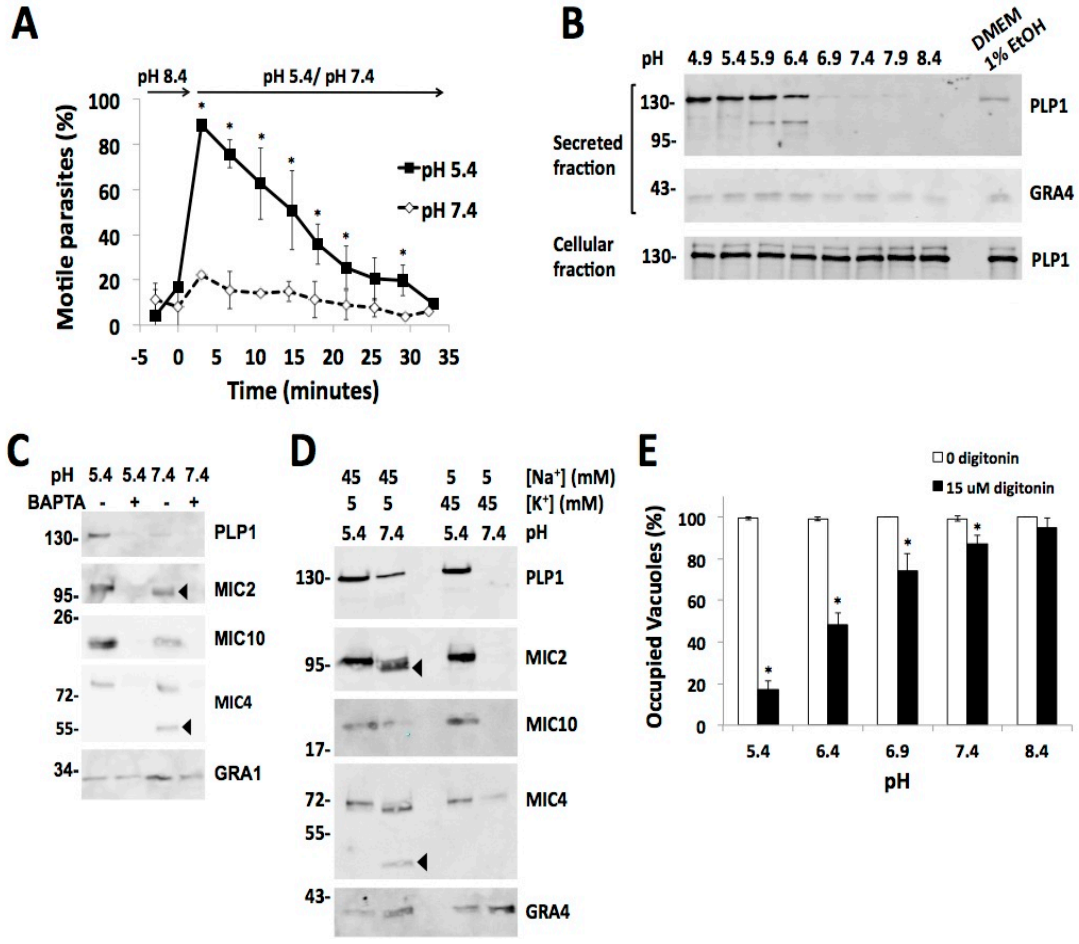

Figure 19: *Low pH stimulates microneme secretion independent of potassium*. **A)** The number of motile parasites was measured over time in low and neutral pH buffer; graph reflects the average and standard deviation of 3 independent experiments. (**p* < 0.05 comparison of 5.4 vs. pH 7.4 curves by student's *t*-test pH.) **B)** PLP1 secretion was tested across a range of pH values and compared to GRA4 secretion. **C)** Low-pH induced microneme secretion was blocked by addition of the calcium chelator, BAPTA-AM. **D)** Secretion of microneme proteins was tested in low and neutral pH with low and high potassium buffers. Arrowheads indicate SUB1 processed products. **E)** Infected cells were permeabilized with digitonin in the presence of high potassium buffer of indicated pH values. Parasite egress was quantified as previously described. (**p* < 0.05 by student's *t*-test, comparing with vs. without digitonin.)

the protease functions optimally at neutral pH (Figure 19B-D). Acidic pH stimulated microneme secretion even in the presence of high potassium (Figure 19D). As intracellular potassium concentrations are higher than extracellular, we reasoned low pH might induce microneme secretion, promoting parasite egress. We tested the effect of low pH on egress by permeabilizing infected cells with digitonin in high potassium buffer with varying pH values. Acidic pH induced parasite egress in a dose-dependent manner. This finding is consistent with a model in which low pH stimulates microneme secretion to initiate motility and deliver PLP1 into the parasitophorous vacuole where its activity is further promoted by low pH (Figure 19E, Figure 20).

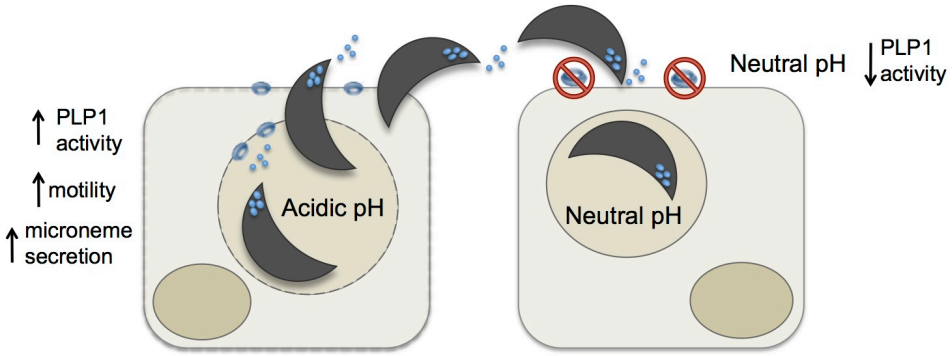

Figure 20: *Proposed model for pH-switch regulation PLP1 activity during egress and invasion*. Acidic pH at egress stimulates microneme secretion, parasite motility, and PLP1 lytic activity. As parasites migrate to a neighboring cell, the neutral pH in the extracellular space suppresses PLP1 membrane binding and lytic activity and allows parasites to invade the host cell with an intact parasitophorous vacuole membrane.

Discussion:

PLP1 is a crucial egress and virulence factor of *Toxoplasma gondii* [14]. PLP1 is stored in and secreted from micronemes. Since micronemes are discharged during both host cell invasion and egress, it remained a mystery as to how PLP1 activity is regulated during egress and invasion. The regulation of activity was apparent as membrane disruption is detected associated with *Toxoplasma* egress yet no overt membrane damage has been found to occur

during invasion [14, 15], which is as expected as the parasite requires an intact membrane to form the parasitophorous vacuole. Here we provide evidence that a pH-switch regulates PLP1 activity during egress and invasion.

Recombinantly produced PLP1 displayed increased hemolysis and membrane binding at acidic pH, with minimal activity at neutral pH. Additionally, pH-neutralization suppressed PLP1 activity during parasite egress, leading to a reduction in the ability of the parasite to rapidly egress from host cells. We found that induced egress is sensitive to a specific type of proton-pump inhibitor, the P-type ATPase inhibitor, DCCD. These data suggest active proton pumping may occur to promote parasite egress. Other mechanisms might also contribute to vacuolar acidification including build-up of metabolic waste products, or delivery of acidic organelles to the vacuole.

P-type ATPases, also called E1-E2 ATPases, are an evolutionarily conserved group of cation or lipid transporters. The parasite genome contains two putative P-type ATPases (TGGT1_001710, TGGT1_036530) of the Type IIIA subfamily, which includes H^+-ATPases of plants and fungi [16]. Both of these genes are upregulated in bradyzoites, which form the latent tissue cyst [17, 18].

One of these genes, TgPMA1, is localized to the parasite plasma membrane in the bradyzoite, and loss of TgPMA1 led to a reduction in *in vitro* stage conversion [18]. *pma1ko* parasites apparently had no growth defect as a tachyzoite in the type II strain, though whether the parasites have an egress defect was not determined. TgPMA2 expression is detected in tachyzoites, though its function remains to be characterized. To determine if either of these ATPases are the DCCD-sensitive factor, single and double knockouts of both P-type ATPases should be performed in a type I strain and tested for defects in egress and vacuolar homeostasis. Interestingly, *Plasmodium* exports a V-type H^+-ATPase to the erythrocyte membrane, indicating that related parasites actively manipulate the pH of the cells in which they reside [19]. It also remains possible that a host H^+-ATPase may be the DCCD-sensitive protein functioning parasite egress.

Using the pH-sensitive probe super-ecliptic pHluorin, we detected a reduction in parasitophorous vacuolar pH late in the replication cycle. Earlier investigations determined that upon host cell entry, the parasitophorous vacuole avoids fusion with endosomes, preventing acidification and degradation of the invaded parasite. Previous efforts to examine vacuolar homeostasis also found a free-flow of small molecules (<1,300 Da) between the parasitophorous vacuole and host cytosol [20], implying an equivalent neutral pH in both sites. However, these studies were carried out at 24 h or less post-inoculation. Our results concur with the vacuolar pH being neutral at this time point during replication (Figure 18C), but suggest that acidification occurs later during the replicative cycle, perhaps just prior to egress. Further efforts to more precisely determine vacuolar pH during parasite egress are currently underway.

Previous work has established that the high intracellular potassium concentration of the host cell inhibits parasite microneme secretion and motility to prevent egress while parasites are replicating [21, 22]. It was proposed that egress-associated host cell permeabilization leads to a drop in potassium concentrations, which promotes microneme secretion and exit from the host cell [21, 23]. However, we find no evidence for egress-associated host cell permeabilization in the absence of PLP1 (Figure 16B,D). It is possible that late events during intracellular replication lead to a disturbance in host cell ion homeostasis. However, the finding that low pH stimulates parasite microneme secretion suggests a mechanism by which the high-potassium inhibition of microneme secretion may be overcome. We propose that the parasitophorous vacuole becomes acidified late during intracellular replication. Our findings suggest that even a relatively modest decrease in pH to 5.9-6.4 is sufficient to substantially promote microneme secretion, augment PLP1 membrane binding and cytolytic activity, and enhance egress. This pH regulatory mechanism likely works in parallel with other sensory and signaling pathways to coordinate egress under different circumstances. The neutral pH parasites encounter on invasion suppresses PLP1 membrane binding and lytic activity (Figure 20).

The increased microneme secretion at low pH complicates the interpretation of the invasion-associated membrane permeabilization assay. It is difficult to distinguish how much increased microneme secretion vs. an increase in PLP1 activity contributes to the membrane damage observed. The effect is probably due to a combination of the two factors. What remains clear, however, is that membrane permeabilization is PLP1-dependent since it is not detected in the PLP1-deficient strain at either pH.

It is also apparent from assays with the recombinant protein that PLP1 activity itself is indeed heightened at acidic pH. The molecular mechanisms by which this occurs remain to be determined. LLO pH-sensitivity is mediated by three acidic amino acids in the transmembrane helices, which are proposed to lead to denaturation at neutral pH and temperatures above 30°C through charge repulsion [24, 25]. Although the amino acid sequences of PLP1 and LLO are not conserved, predictions may be made based on position of similar residues when a crystal structure of PLP1 is available. One potential feature contributing to PLP1's pH-dependent membrane binding is the C-terminal basic patch, which is enriched in basic amino acids. These residues would be positively charged at acidic pH and charge attraction may contribute to binding negatively charged phospholipids. Interestingly, PLP1 activity is pH-dependent at least at the stage of membrane binding, while perforin activity is pH-dependent after membrane binding. This demonstrates that membrane attack may be regulated by different environmental conditions at multiple steps of pore-formation. Comparison of PLP1 and perforin structures will also illuminate divergent features in the conserved MACPF structure, which mediate the activity of these proteins in the varied environments in which they function.

In summary, these findings demonstrate the importance of external pH in *Toxoplasma* lytic replication cycle. Acidic pH promotes parasite microneme secretion and gliding motility, and enhances the membrane-permeabilizing activity of the crucial egress factor, PLP1, which promotes rapid parasite egress. Further investigations into mechanisms of vacuolar pH homeostasis, pH sensing,

and the function of other parasite and host proteins at low and neutral pH will likely identify other key players and processes governing parasite pathogenesis.

Acknowledgments:

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Chapter 5
Discussion

The molecular mechanisms of host cell egress by intracellular pathogens are emerging. Prior assumptions of this process occurring passively have been laid aside as key pathogen and host egress effectors are discovered. The importance of investigating egress has been highlighted by the dramatic virulence defect of the egress-defective *plp1*-deficient strain of the protozoan parasite, *Toxoplasma gondii*. My thesis studies aimed to further dissect the phenotype of the *plp1ko*, the molecular mechanism of PLP1 pore-formation, and the regulation of PLP1 lytic activity. This work led to the identification of conserved and novel features of PLP1 pore-formation and an environmental pH switch that regulates PLP1 activity during egress and invasion. These findings open multiple pathways for future investigation, a few of which I discuss below.

While PLP1 appears to function through a conserved mechanism of pore-formation, we are still unaware of the structural nature of the pore and the precise role of pore-formation during egress. Our collaborators are currently seeking to detect PLP1 pores in native and synthetic membranes using cryoelectron microscopy (EM). CryoEM will help determine the size of PLP1 pores and the average number of monomers per lesion. The size of PLP1 pores may provide clues as to its function during egress. For example, if PLP1 forms large pores, as suggested by oligomeric size on SDS-AGE, it is possible that other proteins pass through the PLP1 pore to exert functions on host cytosolic components during egress. If PLP1 pores are small, the function of pore-formation may serve to allow the passage of small molecules, such as ions or second messengers, leading to a

signaling cascade of downstream events in the host cytosol. Other functions associated with pore size should also be considered. For example, do large pores lead to a greater degree of membrane destabilization than small pores? How many pores must be formed to facilitate parasite egress? Could other methods of membrane disruption replace pore-formation?

CryoEM might also discern the arrangement of PLP1 domains in the pore. The MACPF and C-terminal domains are predicted to form similar structures as observed for other MACPF pores [1, 2]. However, it will be interesting to determine where the unique N-terminal domain lies in relation to the membrane-spanning MACPF domain. Since the N-terminal domain has membrane binding activity, does it sit adjacent to the C-terminal domain on the membrane, or is it released from the membrane and occupying space above, in, or around the transmembrane channel? If it resides within or near the channel, does the N-terminal domain also play a role in discriminating between factors that might transit the pore? If the N-terminal domain has functions beyond membrane binding during parasite egress, this could also contribute to the defect in membrane permeabilization and egress observed in the ΔN-term complemented strain.

Crystal structures are now available for multiple CDCs and MACPF proteins including Plu-MACPF, perforin and complement protein C8 [1, 3, 4]. A crystal structure of the PLP1 monomer would be beneficial for distinguishing unique structural features of PLP1. Structural comparison of PLP1 with other pore-forming proteins could identify features and residues suggesting the basis for pH-sensitivity and membrane binding. For example, the PLP1 computational model of the MACPF domain contains an inserted loop with pairs of acidic and basic residues that may contribute to oligomerization via a mechanism similar to perforin [5]. A crystal structure would determine the shape of this feature and its orientation to the rest of the MACPF domain, which may promote oligomerization, and also the arrangement of potential disulfide bonds, which may stabilize the MACPF domain.

Biochemical experiments with recombinant PLP1 have provided invaluable information regarding its function. Unfortunately, the current recombinant protein system with *E. coli*-produced, solubilized and refolded protein is not reliable because the lytic specific activity is variable. We have pursued and continue to pursue alternative expression systems. For example, PLP1 expression in insect cells produced soluble protein, but the purified product showed no membrane binding or lytic activity. One possible reason for the variable activity observed with the *E. coli* derived protein is that recombinant PLP1 is partially processed by bacterial proteases. We note this processing occurs in the N-terminal domain, similar to the processing observed in the parasite-secreted fraction, and we have shown that loss of the N-terminal domain reduces lytic activity. While the mature form of PLP1 is the major band upon purification, processed forms are always present to varying degrees. Thus, it is possible these processed forms inhibit lytic activity of the recombinant protein via a dominant negative mechanism. To block this proteolysis, one could attempt expression in a system deficient in a subtilisin protease [6-8]. Or, using a construct with a different epitope tag at each terminus would promote isolation of unprocessed, mature recombinant PLP1. Another limitation with regards to the recombinant protein system is our ignorance of the PLP1 membrane receptor(s). Knowledge of the preferred membrane receptor would facilitate the design of a more optimal *in vitro* system for testing activity of recombinant protein, or purified endogenous protein.

Little is known about the phospholipid composition of the parasitophorous vacuole membrane (PVM). Although the PVM is derived from the host cell plasma membrane, host membrane proteins are mostly excluded from the PVM during its formation, which is extensively modified as the parasite replicates [9, 10]. The relationship between the number of parasites per vacuole and the rate of parasite egress has not been carefully examined. However, parasite replication is not a prerequisite for egress, since egress may be induced from vacuoles that contain single parasites [11]. This suggests the PLP1 membrane receptor is present from an

early stage of parasite intracellular residence. This also demonstrates that a single parasite secretes sufficient quantities of PLP1 to permeabilize membranes and, as parasites invade host cells singly, validates the necessity of controlling PLP1 activity during invasion and egress.

Identifying the PLP1 membrane receptor may be accomplished using recombinant N- and C-terminal domains, which we have shown to have membrane-binding activity. These single domains are readily produced by *E. coli* and can be purified from the soluble fraction. We predict that these domains bind unique receptors to facilitate targeted and rapid pore-formation. If both domains bound the same receptor, this would result in competition between the domains and potentially lead to a jumble of orientations, which would inhibit oligomerization and pore-formation. Evidence suggesting the domains may have different binding specificity includes the differences in primary sequence, predicted secondary structure, and proteolytic processing. The N-terminal domain is enriched in acidic amino acids (16% acidic, 11% basic) compared to the MACPF (10% acidic and basic) and C-terminal domain (11% acidic, 10% basic), which would lead to the N-terminal domain being highly negatively charged at a neutral pH. The C-terminal domain is predicted to be β-sheet rich, and mass spectrometry analysis of partial trypsin digested C-term supports the secondary structure predictions (Figure A1). The N-terminal domain has no strong secondary structure predictions and may be largely disordered. Furthermore, the micronemal protease subtilisin 1 (SUB1) processes PLP1 in the N-terminal domain, while the C-terminal domain is apparently protected from proteolysis. However, SUB1 is active at neutral pH and inactive at acidic pH below pH 5.9, so it is possible that SUB1 activity is limited during parasite egress. In support of this, the *sub1ko* has no egress defect, and we observe minimal PLP1 proteolysis associated with PLP1 secreted during egress. We hypothesize that SUB1 processing of PLP1 may inhibit lytic activity during invasion, as described in Chapter 2.

Preliminary studies using recombinant constructs ascertained that recombinant PLP1 preferentially bound to phosphatidylinositol-4-phosphate (PI4P) on a lipid strip array (Figure A2). We found the C-terminal stretch of basic residues, termed the basic patch, was sufficient to confer PI4P binding when fused to GST, an otherwise soluble and non-membrane binding protein (Figure A2). To determine if PI4P is an important membrane receptor for PLP1 during parasite egress, a PI4P binding protein, such as GOLPH3, or GFP fused to the PLP1 basic patch, could be overexpressed in the parasitophorous vacuole [12]. This could demonstrate the occurrence of PI4P on the PVM and saturate the available PI4P, potentially preventing PLP1 from binding it upon secretion during egress. Alternatively, one could overexpress a PI4P phosphatase, such as Sac1, to reduce PI4P levels on the PVM and result in a similar phenotype [13, 14]. Additionally, it would be interesting to complement the *plp1ko* with a construct lacking the basic patch, or with a mutated basic patch. These mutant strains would be expected to have a delay in egress-associated membrane permeabilization and parasite egress. As PI4P is more abundant on the cytosolic leaflet of mammalian plasma membranes, PI4P selectivity may also be a mechanism controlling PLP1 activity during egress and invasion. This would promote PLP1 membrane binding during egress and prevent it during invasion.

With recombinant PLP1 we observed cholesterol-dependent membrane binding activity (Figure A3). The C-terminal domain, which is the required membrane-binding domain, is sufficient for this activity. While many bacterial toxins exhibit cholesterol-dependent membrane binding, this is the first known MACPF protein to have cholesterol-dependent membrane binding. This may have implications with regard to the mechanism of parasite protection from autolysis. Since PLP1 is secreted in the confines of the PV, multiple membrane surfaces are in close proximity including the parasite plasma membrane, the PVM and the intravacuolar network (IVN) [15, 16]. We have preliminary data indicating the parasite plasma membrane is resistant to lysis with cholesterol-dependent

detergents compared with host plasma membranes, suggesting that the parasite plasma membrane is low in cholesterol (Figure A4). Cholesterol-dependent membrane binding might allow PLP1 to discriminate between the parasite plasma membrane and surrounding vacuolar membranes to allow the parasite to selectively lyse the PVM during egress. It should be noted that PLP1 may not bind cholesterol directly as rPLP1 was not observed to bind to cholesterol on lipid strips. Thus, PLP1 may prefer lipids found in cholesterol-enriched microdomains, rather than directly binding to cholesterol, like the cholesterol-dependent cytolysins. The importance of cholesterol-dependent membrane binding for PLP1 activity during egress could be tested in a similar over-expression approach as previously described by over-expressing the cholesterol-binding domain of a CDC in the PV.

The role of low membrane cholesterol in protection from autolysis might be tested with parasites that have higher plasma membrane concentrations. Potential strains to test include those deficient in acyl-CoA:cholesterol acyltransferase enzymes (ACAT). *Acat1* and *acat*2 knockout parasites have a deficiency in the conversion of cholesterol to cholesterol-esters, which are cholesterol storage bodies [17, 18]. Subsequently, the *acat1ko* and *acat2ko* strains are predicted to have an accumulation of cholesterol in their plasma membranes. A buildup of cholesterol in the parasite plasma membrane may be demonstrated by increased sensitivity to cholesterol-dependent detergents, compared to wildtype controls. Then, heightened sensitivity to PLP1 autolysis could be tested through multiple methods involving induced microneme secretion and observation of membrane disruption through LDH release, propidium iodide staining, or loss of viability. As parasites lacking *acat1* or *acat2* display growth defects and differences in cell and PV morphology, confounding factors such as inefficient microneme secretion should be carefully considered in interpretation of results. To control for this, microneme secretion of the *acatko* strain should be blocked and the parasites should be exposed to the secreted fraction from a wildtype or *plp1ko* strain. These

experiments would highlight the importance of cholesterol sequestration as a mechanism of protection from PLP1-inflicted autolysis.

While the identity of the PLP1 membrane receptor and its potential role in controlling lytic activity remain to be fully elucidated, we have demonstrated an important role for pH in the regulation of PLP1 membrane binding and pore-formation. These results have opened multiple questions for future exploration. Our results to date demonstrating an acidification using super-ecliptic pHluorin were performed in a 96-well plate format, so it is unclear if the modest decrease in signal is due to a modest decrease in pH at the population level, or dramatic decreases in individual vacuoles, which occur transiently. As already mentioned, we are in the process of measuring vacuolar pH during replication and egress with the use of a second pH-sensitive probe, ratiometric pHluorin [19]. Ratiometric pHluorin is fluorescent at two wavelengths, one of which is pH-sensitive, while fluorescence from the other wavelength is constant over a wide range of pH values. Although multiple technical hurdles have been overcome, and the feasibility of the system has been demonstrated, our current challenges are associated with using live cell imaging to capture natural egress, which occurs randomly and rapidly in the cell culture population. We also hope to test the effect of pH-neutralizing treatments on vacuolar pH during natural and induced parasite egress. Due to the rapidity of wildtype egress, we may not have sufficient temporal resolving power to capture rapid changes in pH. In this case, we will utilize the *plp1ko*, which has a substantial egress delay, and we can monitor parasite motility as an indicator of egress initiation.

While the actual pH of the vacuole remains to be determined, the finding that microneme secretion is pH-dependent suggests a new paradigm for the signaling events surrounding parasite egress. We propose that an egress-associated vacuolar acidification overcomes the high-potassium inhibition of microneme secretion during intracellular replication. During animal infection, parasites are induced to egress when the host cell becomes permeabilized, such as from attack by host

immune cells, and immune attack has been demonstrated to be an important factor leading to rapid turnover of peritoneal macrophages [20]. However, the parasite is capable of replicating in multiple cell types, and intraperitoneal injection, though a convenient model for testing virulence, is not a natural infection route. Thus, it is unclear to what extent externally triggered egress occurs during infection by oral inoculation or reactivation of the latent tissue cyst. Our model suggests an endogenous method of triggering parasite egress in the absence of external stimulation.

Parasitophorous vacuolar homeostasis is a neglected area of investigation. Previous results indicating free, bidirectional flow of small molecules between the host cytosol and parasitophorous vacuole (PV) were conducted between 18 and 24 h of inoculation. These data support a neutral vacuolar pH during parasite replication, which is consistent with our results to date. However, our results suggest the possibility that the PV is acidified late in the replication cycle. Vacuolar acidification might occur through build-up of metabolic waste, which may signal to the parasite that the host cell in which it resides is no longer a hospitable environment capable of supporting further replication. Other mechanisms of acidification include active pumping of protons, which is supported by the inhibition of egress and PLP1 activity during egress with DCCD, which blocks P-type H^+-ATPases. Another possible mechanism of acidification includes the delivery of acidic organelles to the vacuole. The source of protons could be either the host cell or the parasite cell, or a combination of the two. At this point, we cannot rule out the possibility that the infected cell cytosol becomes acidified, leading to an acidified vacuole. While we are currently focused on testing the pH of the PV, it would also be interesting to follow the pH of the host cytosol during parasite replication by expressing ratiometric pHluorin in the host cytosol. Interestingly, alkaline treatment is a commonly used *in vitro* method of inducing parasites to transform into a tissue-cyst-like state [21]. This suggests that blocking vacuolar acidification

may block normal signals leading to parasite egress and this leads the parasite to switch to the slowly replicating bradyzoite stage instead.

If the PV is indeed acidified while the host cytosol remains at a neutral pH, this pH-differential may lead to other important changes in physiology. Acidic conditions may modify membrane phospholipids, and protein structure and functionality. For example, a local pH-gradient was shown to drive membrane deformation in a protein-free system containing a specific phospholipid [22]. Additionally, a proton-electrochemical force drives phospholipid flipping in yeast [23]. It would be interesting to determine if a similar mechanism serves to populate the internal leaflet of the PVM with the proper phospholipids for PLP1 activity. A pH gradient may also provide a physical measure promoting PVM disruption by promoting swelling of the PV. Previous investigators noted that hypotonic conditions promote parasite egress, whereas hypertonic conditions delay egress [24]. In response to environmental changes, alterations in membrane physiology and protein function may synergize to promote membrane destabilization and permeabilization during pore-forming protein attack.

While PLP1 is a major egress effector, it is likely that other parasite proteins also function during egress. Many pore-forming proteins work in conjunction with a phospholipase to maximize membrane destruction [25-28]. Thus, it is possible PLP1 also partners with a phospholipase to promote parasite egress. If so, however, it appears the phospholipase requires PLP1 to initiate membrane permeabilization, since we detect no evidence of membrane damage during induced egress in the absence of PLP1. Other studies in the lab have used the quantitative egress assay described herein to identify additional parasite gene knockout strains with partial defects in rapid egress. However, further research is required to determine the function of these proteins during egress.

Although roles for host proteins in egress have been demonstrated, their precise functions require further investigation. *Toxoplasma* is still able to egress to a certain degree from calpain-knockout cells and in cells with siRNA knockdown of

other egress-associated host genes (calpain, calmodulin, calmodulin-dependent kinases, Gα subunits, phospholipase C, protein kinase C) [29, 30]. Indeed, the authors identified their effect on egress by the presence of enlarged parasitophorous vacuoles beyond the time point at which parasites normally egress. Thus, it is unclear whether the loss of these host proteins blocks egress signaling or inhibits attempted egress. This could be tested by comparing the ability of parasites to complete induced egress in control and knockdown host cells. If the host genes involved mediate upstream signals of egress, there would be a minimal effect of knockdown on induced egress. However, if the host factor-induced cytoskeletal disassembly is required for parasite egress, there should still be an inhibition of parasite egress under induced egress conditions.

Finally, the mechanisms of egress should be compared between different strains of *Toxoplasma gondii* and phylogenetically related organisms. As parasite strains are observed to have different rates of replication and virulence, and egress is an important step of the lytic cycle contributing to pathogenesis, differences in egress between strains may also contribute to differences in pathogenesis. Is PLP1 a crucial egress factor for other strains and do these parasites respond to similar egress signals? Another point to be addressed is the role of PLP1 in egress during different stages of the parasite life cycle. The latent tissue cyst and the environmentally hardy oocyst are surrounded by a cyst wall, which protects the parasites and yet poses a more challenging barrier during egress. It is likely parasites use degradative enzymes to remove these barriers rather than pore-forming proteins. However, the tissue cyst resides inside a host cell, so perhaps a pore-forming protein facilitates host cell permeabilization following breakdown of the tissue cyst wall.

Comparing mechanisms of egress between closely and distantly related species, such as *Neospora* and *Plasmodium*, will reveal conserved and novel aspects of parasite egress. As apicomplexans are obligate intracellular parasites, some features are likely to be conserved such as the protein secretion and activation of

motility, along with the associated signaling pathways, though the signaling cues are likely to vary between species. Apicomplexan egress studies have been conducted in *Plasmodium* and *Toxoplasma*. *Plasmodium* merozoite egress from erythrocytes is dependent on parasite and host proteases, and roles for pore-forming proteins have been recently identified[29, 30, 32-35]. *Plasmodium* egress from liver cells involves release of a merosome, or a membrane-encased structure containing merozoites, from the liver into circulation[36, 37]. Merosomes are thought to rupture in capillaries, releasing merozoites into the pulmonary microvasculature blood stream. Mechanisms contributing to parasite egress in the mosquito have not been investigated.

Comparing the molecular mechanisms of vacuole escape used by different microorganisms demonstrates a few emerging themes. Pathogens deploy membrane-permeabilizing agents such as pore-forming proteins and phospholipases to breach the vacuolar membrane, and enlist host proteins, such as calpain, which may facilitate vacuolar escape by dismantling cytoskeletal elements [29] [31]. Though vacuolar escape occurs at different points in the life cycle of each microbe, similar questions may be posed: How is lytic activity controlled inside vs. outside the vacuole? What role does the cytoskeleton play in vacuole stability? How are host proteins recruited and how is their activity regulated? What is the fate of vacuolar contents upon lysis? Crosstalk between the branches of parasitology, microbiology, and immunology, has been a boon for investigators of pore-forming protein function. Continuing synergy will promote future forays to answer these questions and others related to diverse pathogens and the pore-forming proteins they employ for pathogenesis.

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APPENDICES

APPENDIX 1

PLP1 C-terminal domain partial trypsin digest

Materials and methods:

Recombinant PLP1 C-terminal domain was purified from the soluble fraction or inclusion bodies prepared as previously described[1]. A time course of trypsin (Promega) digest was performed for C-term purified from soluble and insoluble fractions with equivalent results (Figure A1-A). C-term from the insoluble fraction had fewer non-specific bands upon purification and was used for N-terminal sequencing. For N-terminal sequencing, 10 μg of recombinant protein was incubated with or without 0.02 μg trypsin in trypsin digest buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol) at 37°C for 10 min. Following trypsin digest, protein samples were applied to 15% SDS-PAGE and transferred to PVDF by wet transfer in glycine-free buffer (CAPS buffer: 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10.5) at 50V for 90 min at 4°C. Western blots with αHis and αPLP1 were used to identify trypsin products downstream of the N-terminal 6xHis tag (Figure A1-B). The membrane was stained with Coomassie Blue-R-250 in 40% MeOH/1% acetic acid, destained with 50% MeOH, and rinsed with water (Figure A1-C). Bands of interest were indicated with a pencil-drawn arrow on the membrane and N-terminal sequencing of bands was performed by MidWest Analytical, Inc. (St Louis, MO). N-terminal sequences were compared against potential trypsin digest sites and predicted secondary structural features (Figure A1-D).

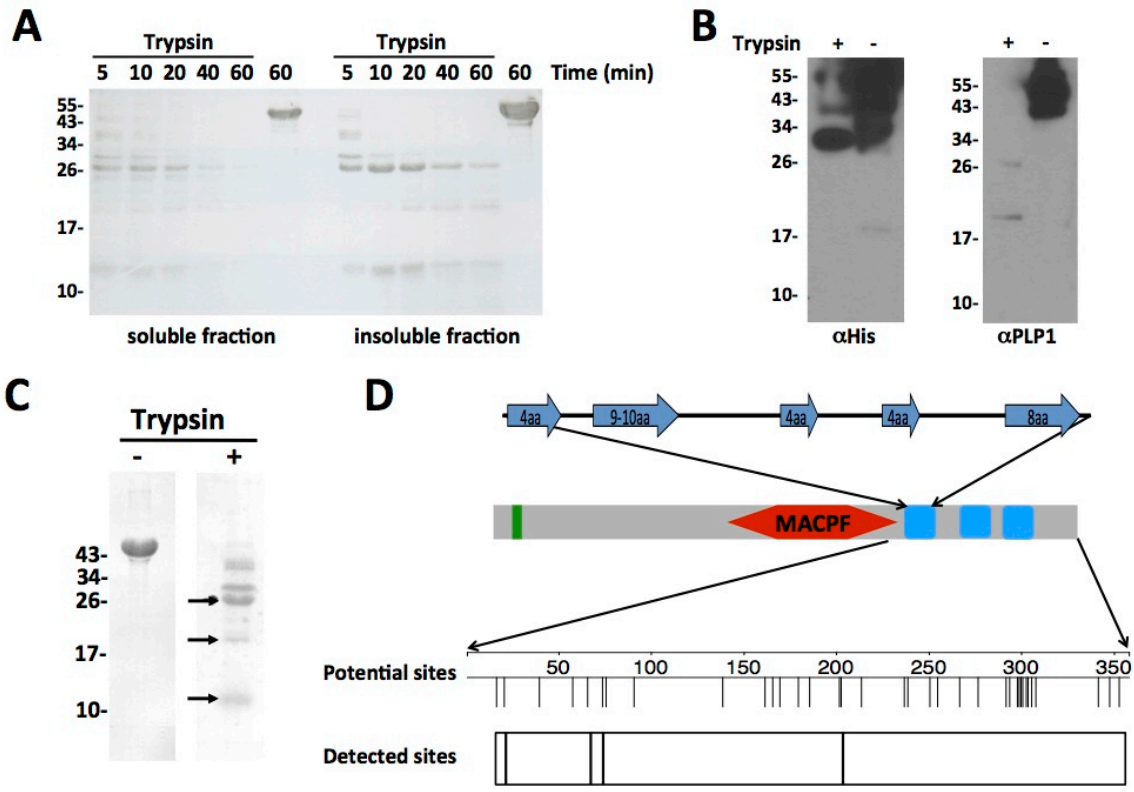

Figure A1: *Partial trypsin digest of C-terminal domain supports secondary structure prediction.* A) Partial trypsin digest patterns were compared for recombinant protein purified from the soluble and insoluble fraction. Similar digest results indicate refolded protein is structurally similar to soluble protein. B) Immunoblot of digested protein to identify bands lacking the 6xHis tag. C) Partial trypsin digest bands indicated with arrows were sent for N-terminal sequencing (Midwest Analytical, Inc.). D) TgPLP1 C-terminal domain contains three repeats of 5 β-sheets[2]. PLP1 C-terminal domain contains 36 potential trypsin digest sites, only 5 of which were experimentally detected, indicating exposure of these sites to trypsin activity and potential protection of other sites by secondary structure.

[1] Roiko MS, Carruthers VB. (2013) Functional dissection of *Toxoplasma gondii* perforin-like protein 1 reveals a dual domain mode of membrane binding for cytolysis and parasite egress. J Biol Chem 288(12): 8712-25.

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APPENDIX 2

PLP1 basic patch is sufficient for phosphatidylinositol (4) phosphate (PI4P) binding

Materials and methods:

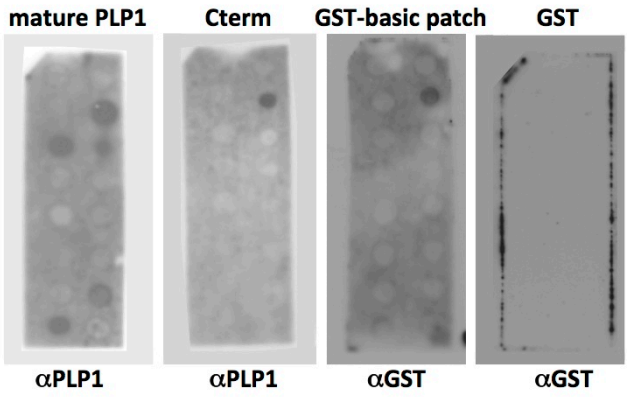
Mature PLP1 and C-term recombinant protein were produced as previously described[1]. PLP1 basic patch cDNA was generated by ligating the following primers with SalI/XhoI overhangs with 5' phosphorylation: 5.SalI.PLP1.3250-3303.XhoI.f (TCGACGGCAAGGCGAAGGGGAACGGCAAAAAGAAAAAGGGAAGGAAGG GTAAGAATAAGTAAC), 3.XhoI.PLP1.3303-3250.SalI.r (TCGAGTTACTTATTCTTACCC TTCCTTCCCTTTTTCTTTTTGCCGTTCCCCTTCGCCTTGCCG). Primers were dissolved in water to a concentration of 500 µM, and 1 µl of each primer was mixed with 1 µl Promega Buffer B (final salt concentration = 25 mM NaCl, 3 mM $MgCl_2$, 3 mM Tris-HCl, pH 7.5) and 17 µl H_2O. The reaction mix was incubated in a beaker of boiling water for 5 minutes; the beaker was removed from the heat source and allowed to cool to room temperature for 3h, cooled further on ice, and stored at -20°C. Prior to ligation, annealed primers were precipitated with ethanol and resuspended in water. Expression vector pGEX4T3 was digested with SalI, XhoI, and treated with shrimp alkaline phosphatase. Annealed primers and digested pGEX4T3 were ligated with T4 Quick Ligase. Ligation products were confirmed by sequencing. pGEX4T3 and pGEX4T3-basic patch were transformed into Origami *E. coli*, and protein induction and purification were performed per manufacturer protocol (Novagen).

Lipid strip blots were performed per manufacturer protocol (Echelon Biosciences). Mature PLP1, GST, and GST-basic patch concentrations were 0.5

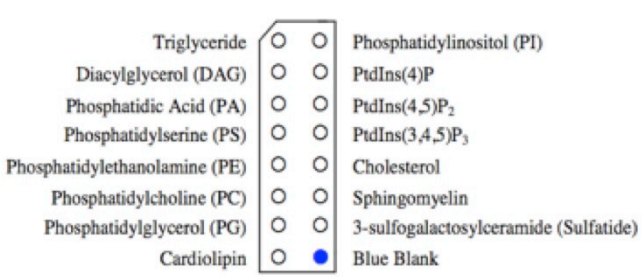
μg/ml and C-term was 0.2 μg/ml. Bound mature PLP1 and C-term were detected with αPLP1 and bound GST and GST-basic patch were detected with αGST.

[1] Roiko MS, Carruthers VB. (2013) Functional dissection of *Toxoplasma gondii* perforin-like protein 1 reveals a dual domain mode of membrane binding for cytolysis and parasite egress. J Biol Chem 288(12): 8712-25.

A



B



Phospholipids bound	Mature PLP1	Cterm	GST-basic patch	GST
phosphotidylinositol (4) phosphate	+++	++	++	-
phosphotidylinositol (4,5) phosphate	+	-	-	-
3-sulfogalactosylceramide	++	-	-	-
phosphatidic acid	++	-	-	-
cardiolipin	++	-	-	-

Figure A2: *PLP1 basic patch is sufficient for binding phosphatidyl inositol-4-phosphate.* A) Recombinant protein was tested for specific lipid binding on a lipid strip and detected with the indicated antibody. Mature PLP1 (0.5 µg) exposure time was 1 second; Cterm (0.2 µg) and GST-basic patch (0.5 µg) were exposed for 1 minute. GST (0.5 µg) was exposed overnight. B) Lipid strip diagram. C) Table of phospholipids recognized by each protein tested and the relative amount bound.

APPENDIX 3

PLP1 C-terminal domain is sufficient for cholesterol-dependent membrane binding

Materials and methods:

Recombinant protein was produced as previously described[1]. Parasite lysate was generated by sonicating filter-purified parasites in egress assay buffer (EAB) (HBSS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES) and pelleting the insoluble fraction. Cholesterol was extracted from host cells by treating HFF in a 6-well plate with 1 ml of the indicated concentration of mβCD in egress assay buffer EAB for 30 min at 37°C. Following initial incubation, the solution was replaced with recombinant protein (100 nM) or RH lysate ($1x10^8$/ml) in the equivalent buffer with or without mβCD and cells were incubated for an additional 10 min at 37°. Wells were washed 4 times with warm PBS and cells were scraped into 200 µl EAB. 50 µl of 5x SDS sample buffer was added to the cell samples and samples were boiled. Bound protein was detected by immunoblot with αPLP1, αHis, or αMIC4 and equivalent cell loading was confirmed with αtubulin.

[1] Roiko MS, Carruthers VB. (2013) Functional dissection of *Toxoplasma gondii* perforin-like protein 1 reveals a dual domain mode of membrane binding for cytolysis and parasite egress. J Biol Chem 288(12): 8712-25.

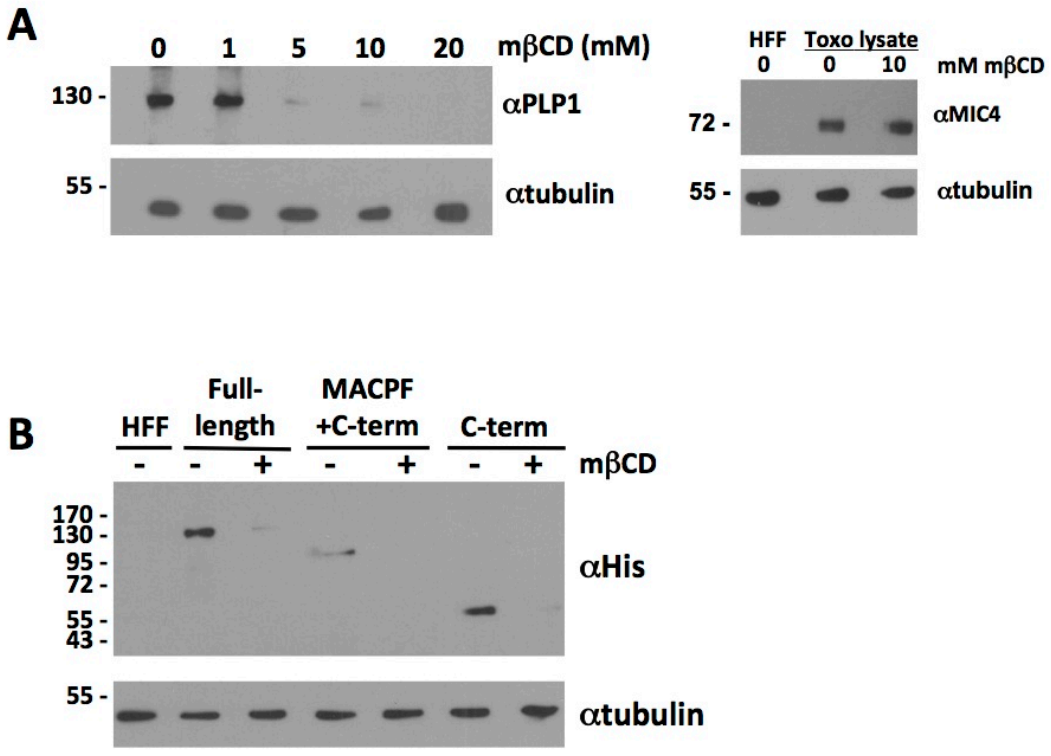


Figure A3: *PLP1 exhibits cholesterol-dependent membrane binding through the C-terminal domain.* A) Cholesterol was extracted from host cells with mβCD prior to addition of recombinant mature PLP1. Bound protein was detected with αPLP1 and tubulin was used as a loading control. Cholesterol-dependent binding of MIC4 was tested from soluble parasite lysate. PLP1 membrane binding was reduced with mβCD treatment and MIC4 membrane binding was not affected by cholesterol extraction. B) Host cells were treated with buffer or 10 mM mβCD and incubated with the indicated recombinant PLP1 constructs. Bound recombinant protein was detected with αHis and αtubulin serves as a loading control for host cells.

APPENDIX 4

Parasites are resistant to lysis with cholesterol-dependent detergent

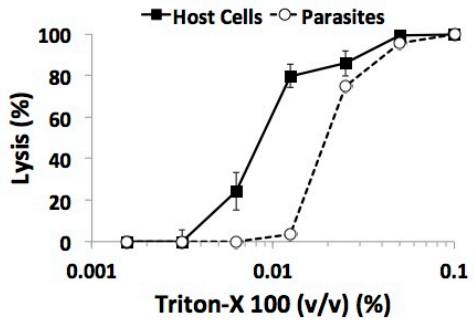
Materials and methods:

Parasites were filter-purified and resuspended in egress assay buffer (EAB) (HBSS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES) to $3.66x10^5$ parasites/ml to equal the approximate amount of membrane surface area per well in a 96-well plate. A 1:2 serial dilution of Triton-X-100 or saponin was performed in EAB in a 96-well plate in triplicate wells with a final volume of 100 µl/well. 50 µl of parasites was added to each well of a 96-well plate and 50 µl of the detergent serial dilution was added and the plate was incubated at 37°C for 30 min. Following incubation, the plate was centrifuged at 500 rpm for 5 min. 10 µl of supernatant was used for LDH release assay according to the manufacturer's protocol (BioVision LDH-Cytotoxicity Assay Kit II). Absorbance was read at 450 nm and normalized to sonicated parasites.

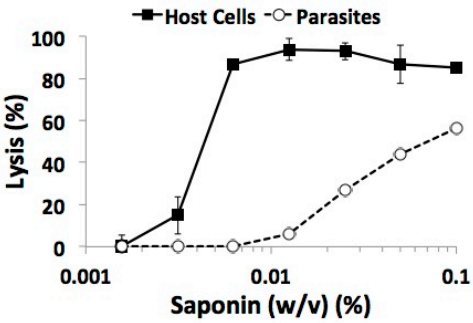
HFF in a 96-well plate were washed twice with warm PBS and 100 µl of a Triton-X-100 or saponin (serial dilution in EAB performed as above) was applied to host cells in triplicate wells. Incubation, centrifugation, and LDH assay were performed as for parasites. A450 nm was normalized to mechanically lysed cells.

The approximate concentration to achieve 50% lysis was determined for each detergent and cell type. The high ratio of parasite/host cell for saponin lysis suggests parasites are highly resistant to lysis with cholesterol-dependent detergents.

A



B



C

50% Lysis	Triton-X 100 (%)	Saponin (%)
Host cells	0.009	0.005
Parasites	0.02	0.08
Parasite/Host	2.2	16

Figure A4: *Tachyzoites are resistant to lysis with cholesterol-dependent detergent compared with host cells.* A) Parasite and host cell Triton-X-100 sensitivity by LDH release assay normalized to mechanically lysed cells. B) Parasite and host cell saponin sensitivity by LDH release assay normalized to mechanically lysed cells. C) The approximate detergent concentration required to achieve 50% lysis was determined for both cell types and the ratio of parasite/host concentration was calculated.