Published in final edited form as:

Curr Opin Microbiol. 2013 August ; 16(4): 459–464. doi:10.1016/j.mib.2013.04.008.

Recent insights into apicomplexan parasite egress provide new views to a kill

Michael J. Blackman¹ and Vern B. Carruthers^{2,*}

¹Division of Parasitology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

²Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-5620

Abstract

A hallmark of apicomplexan pathogens such as *Plasmodium*, *Toxoplasma* and *Cryptosporidium* is that they invade, replicate within, and then egress from their host cells. Egress usually results in lysis of the host cell, with deleterious consequences for the host. In the case of malaria, for example, much of the disease pathology is associated with cyclical waves of host erythrocyte destruction. This review highlights recent advances in mapping the signaling pathways that lead to egress and the parasite molecules involved in responding to and transmitting those signals. The review also discusses new findings for effector molecules that mediate disruption of the bounding membranes that enclose the intracellular parasite and the manner in which membrane rupture occurs to finally release invasive forms of the parasite.

Introduction

Egress is a crucial step in infection by all intracellular pathogens. This event liberates the infectious agent from an expiring or nutritionally exhausted abode so that it can enter a fresh host cell and return to the business of expanding its brood. While various classes of intracellular agents use a range of emancipative strategies including ones that don't immediately kill the host cell (reviewed in [1], protozoa tend to use lethal cytolytic approaches that obliterate the cell. Cytolytic egress leads both to direct injury to tissues and also indirect collateral damage of the ensuing inflammatory and pyrogenic immune response leading to fever, a phenomenon exemplified by human malaria. Synchronous egress of malaria parasites from infected erythrocytes releases a bevy of pyrogens from the annihilated cells, causing the hallmark cyclical fevers of malaria. Malaria parasites, most notably *Plasmodium falciparum*, belong to the phylum Apicomplexa. This taxonomic group contains other important human pathogens including *Toxoplasma*, which causes opportunistic encephalitis and ocular disease, and *Cryptosporidium*, an agent of acute intestinal inflammation and diarrhea. Such parasites share an apical complex consisting of cytoskeletal structures and secretory organelles that are essential for motility, cell invasion and egress.

© 2013 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed: vcarruth@umich.edu; 734 763 2081; 1150 W. Medical Center Dr., 5740A Medical Science Building II, Ann Arbor MI 48109-5620.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

This review will focus on new developments in the mechanisms underlying egress by apicomplexan parasites, with special emphasis on cell exit by the pathogenic stages of the two most intensely studied genera, *Plasmodium* and *Toxoplasma*.

Kinases wire egress

Apicomplexan parasites harbor apical secretory organelles called micronemes that are crucial for parasite gliding motility, cell invasion and egress. Micronemes deploy transmembrane adhesive protein complexes that connect the substrate or host receptors with the parasite motor system (the glideosome) for propulsion and active invasion. For egress, micronemes discharge a perforin-like protein that, for *T. gondii* at least, disrupts host cell membranes to facilitate parasite escape and subsequent re-invasion of a new host cell [2,3]. While it has been known for some time that microneme secretion is promoted by elevation of cytosolic calcium within the parasite, only more recently have some of the molecular players in the calcium signaling pathway been identified.

Three simultaneously published studies provide new insight into the multiple modes of regulating cell egress by *T. gondii* [4–6]. Previous work by L. David Sibley's group demonstrated that TgCDPK1, a member of a calcium dependent protein kinase family, is crucial for protein secretion from the micronemes [7]. Following up on this, they newly show that a second member of the family, TgCDPK3, is only required for cell egress and is not necessary for microneme secretion except when secretion is triggered by certain stimuli [4]. In parallel, Chris Tonkin's group reported that TgCDPK3 is part of a calcium dependent signaling pathway that is triggered when the parasite senses a drop in environmental potassium occurring upon cell damage or permeabilization [5]. In a third parallel study, the Arrizabalaga and Boothroyd groups used whole genome sequencing of cell egress mutants to identify TgCDPK3 as an essential factor for calcium ionophore induced egress from host cells [6]. All three studies showed that TgCDPK3 is not required for gliding motility and cell invasion. The studies also established that TgCDPK3 localizes to the periphery of the parasite underlying the plasma membrane. The findings support a sensory hypothesis in which TgCDPK3 is part of a signaling pathway designed to sense changes in the environment that the parasite uses as cues for egress. If correct, this sensory role is most likely tied to situations where the parasite-infected cell has been damaged or is under direct assault from immune cells that recognize it as being infected.

The Sibley study also showed that activation of a third component of the egress-signaling cascade, cGMP-dependent protein kinase G (TgPKG), triggers egress [4]. Previous work using a selective apicomplexan PKG inhibitor termed compound 1 established that TgPKG was necessary for microneme secretion, but the new work revealed that activating TgPKG is also sufficient to induce microneme secretion and egress. TgPKG appears to function independent of calcium and it is proposed to act upon the same substrate(s) as TgCDPK3. Collectively, the findings illustrate the complex signaling pathways regulating *T. gondii* egress and suggest a model in which the parasite can respond to multiple signals to escape cells.

The TgCDPK3 findings generally mirror those of PfCDPK5, which was shown to be crucial for *Plasmodium* merozoite egress but not invasion [8]. Activation of PfCDPK5 may be dependent on release of calcium from internal parasite stores, since a gradual increase in cytoplasmic Ca^{2+} was observed in the hours leading up to egress in *P. falciparum* [9]. However, unlike TgCDPK3, PfCDPK5 appears to function downstream of PfPKG and it is crucial for natural egress. PfPKG is nonetheless required for egress, since both compound 1 and another PKG inhibitor called compound 2 potently block egress in *P. falciparum* blood-stages [10]. The identification of kinase substrates, such as those reported recently for

TgCDPK1 [11], should provide important new insight into the signaling events accompanying apicomplexan egress. Progress in refining selective inhibitors [12] will also help realize the potential impact of extinguishing kinase activity on infection.

Do micronemes DOC2 the apical membrane?

While the identification of CDPKs revealed insight into upstream aspects of the calcium regulated signaling pathway governing microneme secretion, the extent to which fusion of micronemes with the parasite apical membrane depends on calcium regulation remained unknown. Recent work by the Gubbel and Duraisingh labs used whole genome sequencing to identify a putative double C2 domain protein (DOC2.1) as an essential factor for *T. gondii* microneme secretion, egress and invasion [13]. Similar proteins in other systems use the C2-domains for calcium dependent membrane association, thus it was proposed that DOC2.1 facilitates microneme fusion with the apical membrane, likely in association with other membrane fusion components including SNARES. In this model, DOC2.1 is predicted to redistribute to the apical membrane in response to elevated calcium, though low expression levels precluded testing this. Conditional knockdown of the *P. falciparum* ortholog also impaired microneme secretion and invasion. The authors concluded that the invasion defect was sufficient to explain the observed decrease in parasite propagation without involving an effect on egress. However, recent findings strongly suggest that calcium signaling regulates *Plasmodium* merozoite egress by triggering the secretion of exonemes, which contain an essential egress factor called SUB1 [14]. Thus, it will be interesting to address in future work whether DOC2.1 or another DOC2 protein is involved in exoneme secretion.

Rhoptry secretion is not required for egress

Rhoptries are club shaped secretory organelles that are discharged during invasion [15] and egress [16]. Although their roles in invasion and intracellular survival are well documented, the contribution of rhoptry contents to egress remained unknown until the recent characterization of *T. gondii* rhoptry secretion mutants [17,18]. The mutants, which fail to secrete the rhoptry contents due to a defect in rhoptry positioning, are invasion incompetent but show normal motility and they efficiently egress from host cells. While these findings suggest that rhoptry contents do not contribute to *Toxoplasma* egress, it remains to be determined if this is a general feature of other apicomplexans.

A new checkpoint for initiation of motility and egress

Micronemes are thought to fuse with the apical membrane at a site located within a thimble-like cytoskeletal structure termed the conoid (or apical prominence in *Plasmodium* merozoites). Recent work in *T. gondii* suggests that the conoid is also a resting site for at least one additional egress factor, a protein methyltransferase called TgAKMT required for the initiation of motility [19]. That TgAKMT null parasites are substantially defective in induced egress and invasion is consistent with the role of motility in these events. Interestingly, AKMT null parasites show normal microneme secretion, which likely explains the ability of such parasites to permeabilize the parasitophorous vacuole membrane (PVM) based on the release of TgPLP1 from the micronemes [2]. TgAKTM strikingly relocates from the conoid to the parasite cytoplasm upon elevation of intracellular calcium. Precisely how this redistribution affects the initiation of motility and the methylation of substrates awaits further study. Nonetheless, this study along with the DOC2.1 findings suggests that the redistribution of egress factors from and to the apical region likely contributes to the calcium dependent activation of the parasite glideosome and microneme secretion, respectively. It should be noted that, whilst activation of the glideosome must be required for invasion by *Plasmodium* merozoites, whether it is a prerequisite for egress of

Plasmodium merozoites from hepatocytes or erythrocytes remains unclear, in part because active motility of these small zoites is difficult to visualize and quantify.

Action in the host cytoplasm

Whilst a multitude of signaling events in the parasite controlling egress are emerging, recent work suggests that an equally elaborate series of events unfolds in the cytoplasm of the infected host cell before egress. The finding that host calpain-1 is required for efficient egress of both *T. gondii* and *P. falciparum* suggested a role for the active enzyme in disrupting the host cytoskeleton [20]. Supporting this, a follow up study showed that inhibition or depletion of calpain-1 in malaria infected erythrocytes diminished proteolysis of several key cytoskeletal proteins including α/β -spectrins and ankyrin-1 during egress [21]. This work revealed evidence for perturbation of the host cytoskeleton beginning ~35 h post-infection and involving the loss of α -adducin from the cytoskeleton. Because α -adducin connects actin and spectrin filaments to the cytosolic face of the plasma membrane, its loss is expected to create lesions in the cytoskeleton. Consistent with this, atomic force microscopy revealed discontinuities in the cytoskeleton of infected erythrocytes [21]. Further work based on an RNAi interference screen of host signaling factors revealed a calcium-based signaling cascade required for egress of *T. gondii* and *P. falciparum* [22]. The pathway is proposed to involve a heterotrimeric G protein (including a G α_q component), which activates phospholipase C to generate diacylglycerol as an activator of protein kinase C. Protein kinase C phosphorylation inactivates α -adducin, causing cytoskeletal lesions. It was further suggested that these cytoskeletal perturbations trigger the opening of a plasma membrane mechanosensitive cation channel, TRPC6, thus activating calpain-1 via an influx of calcium from the extracellular medium. While a role for TRPC6 is well supported, other studies have shown depletion of extracellular calcium has little or no effect on egress [9,23], arguing against the calcium influx theory. Recent work by Glushakova and colleagues proposed that the PV is a key source of calcium that could be mobilized to the host cell during egress [9], and Garg et al. [24] also observed an increase in intracellular calcium levels in *P. falciparum* schizonts just prior to egress. Together, these studies raise the possibility that TRPC6 acts at the PVM to mobilize calcium to the host cytoplasm for activation of calpain-1 and possibly other calcium-responsive egress factors. In this model, the observed swelling of the PV that occurs several minutes before egress would activate TRPC6 for calcium release. Future work examining the subcellular distribution of TRPC6 and the use of genetically encoded calcium sensors in the PV could test this hypothesis.

Breaking down the barriers

Egress requires disruption of the PMV and host cell plasma membrane, as well as its underlying cytoskeleton. Parasite effector molecules directly responsible for rupture of these barriers at egress remain only partially defined. The *T. gondii* micronemal perforin-like protein TgPLP1 likely acts by punching holes in the PVM, a model supported by a recent detailed genetic and *in vitro* analysis [3] showing that TgPLP1 is sufficient for membrane disruption, with an essential requirement for the central MACPF domain and the β -sheet rich C-terminal domain, which has membrane-binding activity. Like many pore-forming proteins, TgPLP1 appears to oligomerize upon membrane binding to form large, multimeric membrane-embedded complexes. Whether the primary function of TgPLP1 is to weaken the PVM or to render the membrane permeable to components of the PV (e.g., ions, proteins) that subsequently activate downstream effector steps in egress, remains unknown.

There are 5 perforin-like genes in the *Plasmodium* genome (PPLP1-5) and so, in view of the TgPLP1 functional data, it is interesting to speculate on a possible equivalent role for a perforin-like protein in *Plasmodium* egress. Very recent work [24] has suggested a role for

PPLP1 and/or PPLP2 in *P. falciparum* asexual blood-stage egress, showing that both proteins are expressed and that PPLP1 localizes to micronemes in merozoites. Secretion of PPLP1 appeared to be calcium-dependent, as was its subsequent binding to membranes of the parasitized host cell. Binding of PPLP1 monomers to erythrocyte membranes led to PPLP1 oligomerization and lytic activity. Curiously however, *Plasmodium berghei* parasites null for PPLP1 (Ishino et al 2005) or PPLP2 [25] displayed no phenotype in asexual blood stages, with PPLP2 disruptants showing only a block in gametocyte egress from their host erythrocytes. This suggests a different role for these perforin-like molecules in different *Plasmodium* species, or the existence of some functional redundancy. Ultimately, the importance of perforin-like proteins in *Plasmodium* asexual blood-stage egress may need to be confirmed in additional genetic studies.

In *Plasmodium*, pharmacological or genetic strategies have implicated the two classes of endoproteases: subtilisin-like serine protease SUB1 and the cysteine protease-like SERA proteins. *Plasmodium* SUB1 is stored in exonemes until its discharge into the PV lumen shortly before egress. A diverse range of parasite-derived substrates has been identified for SUB1, including merozoite surface proteins and PV-resident members of the SERA family [26–28]. Of the nine *P. falciparum* SERA genes, only SERA5 (serine-type active site) and SERA6 (cysteine-type active site) are essential in asexual blood stages [29]. Although SERA5 has not been shown to possess enzyme activity a recombinant protein corresponding to its presumed prodomain, or a short peptide thereof, showed egress-inhibitory activity in *P. falciparum* cultures [30]. Intriguingly, given the apparent essentiality of SERA5 in *P. falciparum*, disruption of the only two Ser-type genes in *P. berghei* had no phenotypic consequences throughout the parasite life cycle [31], though it resulted in increased expression levels of the Cys-type PbSERA3 (the orthologue of *P. falciparum* SERA6). To date, attempts to disrupt PbSERA3 have been unsuccessful, suggesting it is indispensable in blood stages. A genetic analysis of *P. falciparum* SERA6 [32] indicated an essential role for its putative active site Cys residue, further supporting suspicions that it is a protease. Mutations that block SERA6 cleavage by SUB1 were lethal, and cleavage by *P. falciparum* SUB1 of a recombinant form of the *P. berghei* orthologue activated an E64-sensitive autocatalytic processing activity. Collectively these observations suggest a model in which SUB1 processing activates SERA6 protease activity. However, a direct role for SERA6 or any other blood-stage SERA in egress has yet to be demonstrated, and – if these proteins are indeed proteases – their physiological substrates are unknown.

P. falciparum dipeptidyl peptidase 3 (DPAP3) emerged as a putative egress regulator through use of selective small molecule inhibitors and activity-based probes [33,34]. DPAP3 inhibition reduced expression of mature SUB1 and also reduced SERA5 processing. A proteomic analysis designed to identify proteolytic events associated with egress, combined with the use of a selective DPAP3 inhibitor, revealed that 64% of the proteolytic events occur downstream of DPAP3 activation [35]. However, as with the SERA family, direct substrates of DPAP3 have yet to be identified.

There is still no satisfactory mechanistic explanation for how proteases might mediate membrane disruption at egress, but evidence for their crucial role in the pathway continues to accumulate. An electron microscopic study of *P. falciparum* gametocyte egress [36] confirmed the ‘inside-out’ model implied by several previous studies (including the PPLP2 knockout study mentioned above), showing that the PVM ruptures at several sites within seconds of activation of egress, followed by eventual erythrocyte membrane rupture at a single point. Rupture of the two membranes was differentially sensitive to protease inhibitors, whilst PVM rupture was induced by the potassium ionophore nigericin suggesting that, as in *T. gondii* [37], *Plasmodium* senses a drop in potassium within the expiring host cell as a trigger for egress. It is unlikely that this is the only trigger for malaria

parasites though since it was recently shown that egress of asexual blood-stage parasites is not affected by growing the parasite in medium containing potassium levels equivalent to those inside the host cell [38]. Finally, some caution should be exercised in comparisons of egress of *Plasmodium* asexual and sexual stages, since distinctions in the regulation of egress between different developmental stages likely exist.

There is now a general consensus that PVM rupture occurs before host cell membrane rupture. The PV is a relatively Ca^{2+} -rich compartment, so it is likely that PMV rupture (and/or its prior permeabilization by pore-forming proteins) enables rapid upregulation of $[\text{Ca}^{2+}]$ in the host cell cytosol, which might facilitate activation of calpain-1. It is worth noting that SUB1 enzymatic activity is calcium-dependent, so high Ca^{2+} levels in the PVM are likely important for its role in egress.

The big event

Several groups have taken biophysical approaches to dissect the rapid dynamics of *P. falciparum* merozoite egress from the infected erythrocyte and the associated mechanical alterations to the host cell. A study combining diffraction phase microscopy, atomic force microscopy and optical tweezers showed an increased membrane stiffness of the infected erythrocytes blocked for egress at a stage following PVM rupture [39]. Elevated perturbations of the erythrocyte membrane following PVM rupture were interpreted as being due to the trapped mobile merozoites striking the membrane surface. It was concluded that the inhibitors prevented the cytoskeletal destabilization required for normal egress. Time-lapse microscopy has revealed that egress is preceded by a transient swelling of the PV and shrinkage of the residual host cell cytoplasmic compartment accompanied by overall swelling of the entire schizont, suggestive of a build-up of osmotic pressure. Increased mobility or disaggregation of the intracellular merozoites then occurs, presumed to be due to their release from the central residual body and breakdown of the PVM [40–42]. Shortly before final schizont rupture – which is an almost explosive event in this species – ‘poration’ (permeabilization) of the erythrocyte membrane occurs, elegantly shown by the accessibility of erythrocyte cytoskeleton to externally-provided fluorescent phalloidin in the moments leading up to rupture, as well as the loss of a GFP-tagged transgene product from the erythrocyte cytosol [24,40,41]. This mirrors analogous findings in *T. gondii* [43]. Intriguingly, poration of *P. falciparum* schizonts was not blocked by the cysteine protease inhibitor E64, indicating that it is independent of calpain-1 activity.

Host erythrocyte rupture itself is rapid, taking place in ~400 milliseconds. High-speed video microscopy was used to show that egress comprises three main steps: (1) the appearance of a single ~1 μm pore allowing the osmotically-driven release of 1–2 merozoites; (2) an outward curling of the erythrocyte membrane from the pore epicentre; and (3) finally a rapid inversion (‘buckling’) of the entire erythrocyte membrane, acting to physically eject and disperse the remaining parasites [42,44]. The latter 2 steps were considered to rely on elastic instability of the infected erythrocyte membrane, perhaps aided by prior modification and/or degradation of the underlying erythrocyte cytoskeleton.

In contrast to the explosive nature of *Plasmodium* egress from the erythrocyte, video and electron microscopic examination of induced *T. gondii* egress has revealed a process that much more resembles invasion, with some distinctions. Upon induction of egress with calcium ionophore the intracellular tachyzoite rosettes disassemble, the parasites extend their conoids and begin to move within the PV, eventually rupturing it to move through the host cell cytoplasm and breach the plasma membrane [45]. The role of gliding motility in induced egress was very evident, although motility is not absolutely required for natural egress [46]. Clearly the major challenge now in both *Plasmodium* and *Toxoplasma* is to

consolidate all these observations with an improved understanding of the molecular events leading up to egress.

Conclusions

Egress of apicomplexan pathogens is fundamental to their life cycle and important for pathogenesis. As this review illustrates, increased attention has produced substantial advances in our understanding of egress over recent years. Despite this, important questions remain unanswered. What is the natural trigger for egress in *Plasmodium* blood stages? How are the calcium-dependent and cGMP-dependent signals that lead to egress sensed and orchestrated? Are secreted microneme/exoneme proteins involved in egress (e.g., TgPLP1, SUB1) selectively discharged prior to those involved in invasion? Why does the malaria parasite possess apparently abundant PV-resident endoproteases for egress when *T. gondii* appears to lack these? How do proteases and pore-forming proteins rupture the membrane that enclose the intracellular parasite, and are additional effector components involved? Answers to these and other central questions will allow full exploitation of the resulting knowledge for disease control.

Acknowledgments

We thank My-Hang Huynh for proof reading the manuscript. Work in the authors' labs is supported by grants from the UK Medical Research Council (U117532063 to MJB), the European Union Framework Programme 7-funded EVIMalaR network (contract no.242095 to MJB), the US National Institutes of Health (AI063263, AI46675, AI097099 to VBC) and the Stanley Medical Research Institute (04R796 to VBC)

References and recommended Reading

1. Friedrich N, Hagedorn M, Soldati-Favre D, Soldati T. Prison break: pathogens' strategies to egress from host cells. *Microbiol Mol Biol Rev.* 2012; 76:707–720. [PubMed: 23204363]
2. Kafsack BF, Pena JD, Coppens I, Ravindran S, Boothroyd JC, Carruthers VB. Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science.* 2009; 323:530–533. [PubMed: 19095897]
- 3*. Roiko MS, Carruthers VB. 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.* 2013; 288:8712–8725. Detailed examination of TgPLP1, using both genetic and *in vitro* approaches to show it is a pore-forming protein and to dissect functional domains. [PubMed: 23376275]
4. Lourido S, Tang K, Sibley LD. Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. *EMBO J.* 2012; 31:4524–4534. [PubMed: 23149386]
- 5*. McCoy JM, Whitehead L, van Dooren GG, Tonkin CJ. TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog.* 2012; 8:e1003066. Identified a novel calcium dependent kinase involved in sensing changes in extracellular ions as a cue for *Toxoplasma* egress. [PubMed: 23226109]
- 6*. Garrison E, Treeck M, Ehret E, Butz H, Garbuz T, Oswald BP, Settles M, Boothroyd J, Arrizabalaga G. A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in *Toxoplasma*. *PLoS Pathog.* 2012; 8:e1003049. Used whole genome sequencing of *Toxoplasma* mutants to identify a novel calcium dependent kinase that is necessary for calcium ionophore induced egress and for the establishment of chronic infection.
7. Lourido S, Shuman J, Zhang C, Shokat KM, Hui R, Sibley LD. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. *Nature.* 2010; 465:359–362. [PubMed: 20485436]
8. Dvorin JD, Martyn DC, Patel SD, Grimley JS, Collins CR, Hopp CS, Bright AT, Westenberger S, Winzeler E, Blackman MJ, et al. A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science.* 2010; 328:910–912. [PubMed: 20466936]

- 9*. Glushakova S, Lizunov V, Blank PS, Melikov K, Humphrey G, Zimmerberg J. Cytoplasmic free Ca²⁺ is essential for multiple steps in malaria parasite egress from infected erythrocytes. *Malar J*. 2013; 12:41-2875-12-41. Showed that calcium elevation within the malaria parasite and within the infected host cell are important for parasite egress. [PubMed: 23363708]
10. Taylor HM, McRobert L, Grainger M, Sicard A, Dluzewski AR, Hopp CS, Holder AA, Baker DA. The malaria parasite cyclic GMP-dependent protein kinase plays a central role in blood-stage schizogony. *Eukaryot Cell*. 2010; 9:37–45. [PubMed: 19915077]
11. Lourido S, Jeschke GR, Turk BE, Sibley LD. Exploiting the unique ATP-binding pocket of *Toxoplasma* Calcium-Dependent Protein Kinase 1 to identify its substrates. *ACS Chem Biol*. 2013 (in press).
12. Lourido S, Zhang C, Lopez MS, Tang K, Barks J, Wang Q, Wildman SA, Shokat KM, Sibley LD. Optimizing small molecule inhibitors of Calcium-Dependent Protein Kinase 1 to prevent infection by *Toxoplasma gondii*. *J Med Chem*. 2013; 56:3068–3077. [PubMed: 23470217]
- 13*. Farrell A, Thirugnanam S, Lorestani A, Dvorin JD, Eidell KP, Ferguson DJ, Anderson-White BR, Duraisingh MT, Marth GT, Gubbels MJ. A DOC2 protein identified by mutational profiling is essential for apicomplexan parasite exocytosis. *Science*. 2012; 335:218–221. Used whole genome sequencing of a *Toxoplasma* conditional egress mutant to identify a putative calcium regulated membrane protein required for microneme secretion in both *Toxoplasma* and *Plasmodium*. [PubMed: 22246776]
14. Agarwal S, Singh MK, Garg S, Chitnis CE, Singh S. Ca(2+) -mediated exocytosis of subtilisin-like protease 1: a key step in egress of *Plasmodium falciparum* merozoites. *Cell Microbiol*. 2012 (in press).
15. Besteiro S, Dubremetz JF, Lebrun M. The moving junction of apicomplexan parasites: a key structure for invasion. *Cell Microbiol*. 2011; 13:797–805. [PubMed: 21535344]
16. Alexander DL, Mital J, Ward GE, Bradley P, Boothroyd JC. Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. *PLoS Pathog*. 2005; 1:e17. [PubMed: 16244709]
17. Beck JR, Fung C, Straub KW, Coppens I, Vashisht AA, Wohlschlegel JA, Bradley PJ. A *Toxoplasma* palmitoyl acyl transferase and the palmitoylated Armadillo Repeat protein TgARO govern apical rhoptry tethering and reveal a critical role for the rhoptries in host cell invasion but not egress. *PLoS Pathog*. 2013; 9:e1003162. [PubMed: 23408890]
18. Mueller C, Klages N, Jacot D, Santos JM, Cabrera A, Gilberger TW, Dubremetz JF, Soldati-Favre D. The *Toxoplasma* protein ARO mediates the apical positioning of rhoptry organelles, a prerequisite for host cell invasion. *Cell Host Microbe*. 2013; 13:289–301. [PubMed: 23498954]
19. Heaslip AT, Leung JM, Carey KL, Catti F, Warshaw DM, Westwood NJ, Ballif BA, Ward GE. A small-molecule inhibitor of *T. gondii* motility induces the posttranslational modification of myosin light chain-1 and inhibits myosin motor activity. *PLoS Pathog*. 2010; 6:e1000720. [PubMed: 20084115]
20. Chandramohanadas R, Davis PH, Beiting DP, Harbut MB, Darling C, Velmourougane G, Lee MY, Greer PA, Roos DS, Greenbaum DC. Apicomplexan parasites co-Opt host calpains to facilitate their escape from infected cells. *Science*. 2009; 324:794–797. [PubMed: 19342550]
21. Millholland MG, Chandramohanadas R, Pizzarro A, Wehr A, Shi H, Darling C, Lim CT, Greenbaum DC. The malaria parasite progressively dismantles the host erythrocyte cytoskeleton for efficient egress. *Mol Cell Proteomics*. 2011; 10:M111.010678. [PubMed: 21903871]
- 22*. Millholland MG, Mishra S, Dupont CD, Love MS, Patel B, Shilling D, Kazanietz MG, Foscett JK, Hunter CA, Sinnis P, Greenbaum DC. A host GPCR signaling network required for the cytolysis of infected cells facilitates release of apicomplexan parasites. *Cell Host Microbe*. 2013; 13:15–28. Identified a calcium dependent signaling pathway within *Plasmodium* and *Toxoplasma* infected cells involved in disrupting the host cytoskeleton prior to egress. [PubMed: 23332153]
23. Wasserman M, Alarcon C, Mendoza PM. Effects of Ca⁺⁺ depletion on the asexual cell cycle of *Plasmodium falciparum*. *Am J Trop Med Hygiene*. 1982; 31:711–717.
24. Garg S, Agarwal S, Kumar S, Shams Yazdani S, Chitnis CE, Singh S. Calcium-dependent permeabilization of erythrocytes by a perforin-like protein during egress of malaria parasites. *Nat Commun*. 2013; 4:1736. [PubMed: 23591903]

- 25*. Deligianni E, Morgan RN, Bertuccini L, Wirth CC, Silmon de Monerri NC, Spanos L, Blackman MJ, Louis C, Pradel G, Siden-Kiamos I. A perforin-like protein mediates disruption of the erythrocyte membrane during egress of *Plasmodium berghei* male gametocytes. Cell Microbiol. 2013 (in press) Phenotypic characterization showing that knockout of PPLP2 results in a selective defect in erythrocyte membrane rupture following male gametocyte activation.
26. Silmon de Monerri NC, Flynn HR, Campos MG, Hackett F, Koussis K, Withers-Martinez C, Skehel JM, Blackman MJ. Global identification of multiple substrates for *Plasmodium falciparum* SUB1, an essential malarial processing protease. Infect Immun. 2011; 79:1086–1097. [PubMed: 21220481]
27. Withers-Martinez C, Suarez C, Fulle S, Kher S, Penzo M, Ebejer JP, Koussis K, Hackett F, Jirgensons A, Finn P, Blackman MJ. *Plasmodium* subtilisin-like protease 1 (SUB1): insights into the active-site structure, specificity and function of a pan-malaria drug target. Int J Parasitol. 2012; 42:597–612. [PubMed: 22543039]
28. Fulle S, Withers-Martinez C, Blackman MJ, Morris GM, Finn PW. Molecular determinants of binding to the *Plasmodium* Subtilisin-like Protease 1. J Chem Inf Model. 2013 (in press).
29. McCoubrie JE, Miller SK, Sargeant T, Good RT, Hodder AN, Speed TP, de Koning-Ward TF, Crabb BS. Evidence for a common role for the serine-type *Plasmodium falciparum* serine repeat antigen proteases: implications for vaccine and drug design. Infect Immun. 2007; 75:5565–5574. [PubMed: 17893128]
30. Alam A, Chauhan VS. Inhibitory potential of prodomain of *Plasmodium falciparum* protease serine repeat antigen 5 for asexual blood stages of parasite. PLoS One. 2012; 7:e30452. [PubMed: 22291957]
31. Putrianti ED, Schmidt-Christensen A, Arnold I, Heussler VT, Matuschewski K, Silvie O. The *Plasmodium* serine-type SERA proteases display distinct expression patterns and non-essential in vivo roles during life cycle progression of the malaria parasite. Cell Microbiol. 2010; 12:725–739. [PubMed: 20039882]
- 32*. Ruecker A, Shea M, Hackett F, Suarez C, Hirst EM, Milutinovic K, Withers-Martinez C, Blackman MJ. Proteolytic activation of the essential parasitophorous vacuole cysteine protease SERA6 accompanies malaria parasite egress from its host erythrocyte. J Biol Chem. 2012; 287:37949–37963. Biochemical and genetic examination of SERA6 in *P. falciparum*, presenting evidence that it is an indispensable cysteine protease that is activated through cleavage by PfSUB1. [PubMed: 22984267]
33. Arastu-Kapur S, Ponder EL, Fonovic UP, Yeoh S, Yuan F, Fonovic M, Grainger M, Phillips CI, Powers JC, Bogoy M. Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. Nat Chem Biol. 2008; 4:203–213. [PubMed: 18246061]
34. Deu E, Leyva MJ, Albrow VE, Rice MJ, Ellman JA, Bogoy M. Functional studies of *Plasmodium falciparum* dipeptidyl aminopeptidase I using small molecule inhibitors and active site probes. Chem Biol. 2010; 17:808–819. [PubMed: 20797610]
35. Bowyer PW, Simon GM, Cravatt BF, Bogoy M. Global profiling of proteolysis during rupture of *Plasmodium falciparum* from the host erythrocyte. Mol Cell Proteomics. 2011; 10:M110.001636. [PubMed: 20943600]
- 36*. Sologub L, Kuehn A, Kern S, Przyborski J, Schillig R, Pradel G. Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito. Cell Microbiol. 2011; 13:897–912. Electron microscopic study showing conclusively that PVM rupture occurs at a number of sites, before eventual rupture of the bounding host cell membrane at a single point, with both steps differentially sensitive to protease inhibitors. [PubMed: 21501358]
37. Fruth IA, Arrizabalaga G. *Toxoplasma gondii*: induction of egress by the potassium ionophore nigericin. Int J Parasitol. 2007; 37:1559–1567. [PubMed: 17618633]
38. Pillai AD, Addo R, Sharma P, Nguitragool W, Srinivasan P, Desai SA. Malaria parasites tolerate a broad range of ionic environments and do not require host cation remodelling. Mol Microbiol. 2013; 88:20–34. [PubMed: 23347042]
39. Chandramohanadas R, Park Y, Lui L, Li A, Quinn D, Liew K, Diez-Silva M, Sung Y, Dao M, Lim CT, et al. Biophysics of malarial parasite exit from infected erythrocytes. PLoS One. 2011; 6:e20869. [PubMed: 21698115]

40. Gilson PR, Crabb BS. Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *Int J Parasitol.* 2009; 39:91–96. [PubMed: 18952091]
41. Glushakova S, Humphrey G, Leikina E, Balaban A, Miller J, Zimmerberg J. New stages in the program of malaria parasite egress imaged in normal and sickle erythrocytes. *Curr Biol.* 2010; 20:1117–1121. [PubMed: 20537541]
- 42**. Abkarian M, Massiera G, Berry L, Roques M, Braun-Breton C. A novel mechanism for egress of malarial parasites from red blood cells. *Blood.* 2011; 117:4118–4124. Used high-speed video microscopy to capture steps in egress, showing that initial rupture of the host cell membrane at a single point is following by an elasticity-dependent process that ejects the remaining merozoites from the infected cell. [PubMed: 21297002]
43. Black MW, Arrizabalaga G, Boothroyd JC. Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Mol Cell Biol.* 2000; 20:9399–9408. [PubMed: 11094090]
44. Callan-Jones A, Albarran Arriagada OE, Massiera G, Lorman V, Abkarian M. Red blood cell membrane dynamics during malaria parasite egress. *Biophys J.* 2012; 103:2475–2483. [PubMed: 23260049]
45. Caldas LA, de Souza W, Attias M. Calcium ionophore-induced egress of *Toxoplasma gondii* shortly after host cell invasion. *Vet Parasitol.* 2007; 147:210–220. [PubMed: 17560036]
46. Lavine MD, Arrizabalaga G. Exit from host cells by the pathogenic parasite *Toxoplasma gondii* does not require motility. *Eukaryot Cell.* 2008; 7:131–140. [PubMed: 17993573]

Highlights

Egress is a fundamental event in the biology of apicomplexan parasites

Kinases, a methyl transferase and a putative vesicular fusion protein regulate egress

Signal transduction in the host cytosol aid in dismantling the cytoskeleton for parasite exit

A protease cascade in the malaria parasitophorous vacuole is required for egress

Egress occurs via rupturing membranes from the inside out