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DOI: 10.1016/j.chom.2016.02.006
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Graphical Abstract

Highlights
- Membrane phosphatidic acid (PA) regulation is linked to T. gondii microneme secretion
- Diacylglycerol kinase-1 (DGK1) underpins PA generation for microneme secretion
- The microneme surface protein APH detects PA at the parasite plasma membrane
- Both APH and DGK1 are critical for microneme secretion in T. gondii

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In Brief
Microneme secretion is essential for efficient propagation of Apicomplexan parasites. In this issue of Cell Host & Microbe, Bullen et al. (2016) demonstrate that this process is underpinned by phosphatidic acid regulation at the parasite plasma membrane, controlled by the essential enzyme DGK1 and sensed by the microneme protein APH.

Bullen et al., 2016, Cell Host & Microbe 19, 349–360
March 9, 2016 ©2016 Elsevier Inc.
http://dx.doi.org/10.1016/j.chom.2016.02.006
Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in Toxoplasma

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http://dx.doi.org/10.1016/j.chom.2016.02.006

SUMMARY

The obligate intracellular lifestyle of apicomplexan parasites necessitates an invasive phase underpinned by timely and spatially controlled secretion of apical organelles termed micronemes. In Toxoplasma gondii, extracellular potassium levels and other stimuli trigger a signaling cascade culminating in phosphoinositide-phospholipase C (PLC) activation, which generates the second messengers diacylglycerol (DAG) and IP3 and ultimately results in microneme secretion. Here we show that a delicate balance between DAG and its downstream product, phosphatidic acid (PA), is essential for controlling microneme release. Governing this balance is the apicomplexan-specific DAG-kinase-1, which interconverts PA and DAG, and whose depletion impairs egress and causes parasite death. Additionally, we identify an acylated pleckstrin-homology (PH) domain-containing protein (APH) on the microneme surface that senses PA during microneme secretion and is necessary for microneme exocytosis. As APH is conserved in Apicomplexa, these findings highlight a potentially widely used mechanism in which key lipid mediators regulate microneme exocytosis.

INTRODUCTION

Active host cell entry is an essential step in the propagation of obligate intracellular parasitism by apicomplexan parasites, members of which include the major etiologic agents of malaria (Plasmodium spp.) and toxoplasmosis (Toxoplasma gondii). Underpinning this process is the release of apical secretory organelles termed micronemes, secretion of which is a prerequisite for gliding motility, invasion, and egress from infected cells (reviewed in Sharma and Chitnis, 2013). Despite its central role in infectivity, our current understanding of the molecular mechanisms governing microneme secretion is sparse. Microneme exocytosis is known to follow changes in extracellular potassium levels (Singh et al., 2010), implicates cyclic GMP-dependent protein kinase G (PKG) (Brochet et al., 2014) and phosphoinositide regulation (Brochet et al., 2014), and responds to an increase in intracellular calcium concentration (Brochet et al., 2014; Garg et al., 2013; Singh et al., 2010; Singh and Chitnis, 2012; Wiersma et al., 2004). PKG activity promotes formation of the phosphoinositide-phospholipase C (PI-PLC) substrate P4,5P2 (Brochet et al., 2014), implicating PI-PLC as the downstream mediator of PKG activity. Concordantly, P. falciparum PI-PLC transcription is upregulated during late blood stages, and the P. berghei homolog is refractory to genetic deletion (Raabe et al., 2011). Inhibitor studies suggest that PI-PLC acts on P4,5P2 to generate the second messengers IP3 and DAG and stimulates Ca2+ release from the endoplasmic reticulum (ER) or other internal stores (Singh et al., 2010) through unidentified receptors (reviewed in Budu and Garcia, 2012). Ethanol stimulation of T. gondii microneme secretion also triggers an increase in calcium, likely as a result of PI-PLC-derived IP3 (Carruthers et al., 1999; Lovett et al., 2002). Members of the calcium-dependent protein kinase (CDPK) family are critically involved downstream of this signaling cascade (Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012; reviewed in (Holder et al., 2012)). Ultimately, a fusion event believed to involve SNARE-like proteins such as DOC2.1 (Farrell et al., 2012; Jean et al., 2014) enacts microneme exocytosis.

With a view to better deciphering microneme exocytosis, we have focused here on diacylglycerol (DAG) and phosphatidic acid (PA), downstream products of PI-PLC signaling at the parasite plasma membrane (PPM). DAG is interconverted to PA via DAG kinases (DGKs) and PA phosphatases (PAPs). In mammalian systems, PA is involved in signal transduction (Chasserot-Golaz et al., 2010), membrane dynamics (Kooijman et al., 2003), and exocytosis (reviewed in Ammar et al., 2013; Chasserot-Golaz et al., 2010), thus providing a precedent for PA to also play a role in microneme exocytosis in the Apicomplexa. We have identified and functionally characterized both the enzyme mediator of PA production and the corresponding PA sensor underpinning an essential mechanism of microneme exocytosis conserved across the Apicomplexa.

RESULTS AND DISCUSSION

Modulation of PA Levels Plays a Critical Role in T. gondii Microneme Secretion

The importance of PA signaling in microneme secretion was initially recognized by the putative increase in its precursor (DAG) during

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http://dx.doi.org/10.1016/j.chom.2016.02.006
PI-PLC signaling (Figure 1A). The localization of PA-generating enzymes precludes the involvement of phospholipase D (mitochondrial, http://www.toxodb.org/toxo/) or lysophosphatidic acid acyltransferase (ER) (Lindner et al., 2014), solely implicating DGKs as priority candidates for generation of PA at the PPM (Figures 1A and S1A–S1C).

We first utilized commercially available DGK (R59022) (de Chaffoy de Courcelles et al., 1985) and PAP inhibitors (propranolol) (Baron and Malhotra, 2002) to probe this pathway, hypothesizing that blocking PA production would hamper microneme secretion, while blocking PA conversion to DAG would enhance microneme secretion (Figure 1A). These inhibitors were used in parallel for egress (Figure 1B), invasion (Figure 1C), gliding (Figure 1D), and microneme secretion assays (Figures 1E–1L). As hypothesized, R59022 blocked parasite egress (Figure 1B), invasion (Figure 1C), gliding (Figure 1D), and microneme exocytosis (Figure 1E). Concordantly, promoting PA accumulation by blocking PAP-activity with propranolol stimulated parasite egress (Figure 1B), gliding motility (Figure 1D), and microneme secretion (Figures 1F–1L). Importantly, propranolol acts most significantly when used in a buffer mimicking extracellular potassium concentrations that enable basal microneme secretion in the absence of additional stimuli (Figures 1F/1H). These data suggest that for propranolol to effect microneme secretion, the parasite must already be “primed” whereby the remainder of the pathway (Figure 1A) is already underway; potassium stimulation leading to PI-PLC activity and the concurrent increase in DAG and IP₃ are necessary for propranolol functioning.

Ethanol has previously been linked to microneme secretion and has been proposed to act through stimulating PI-PLC activity (Carruthers et al., 1999; Lovett et al., 2002). Here we sought to compare the effects of microneme stimulation by propranolol versus ethanol by repeatedly and sequentially stimulating parasites with either 2% ethanol or 250 μM propranolol (Figure 1G). While parasites stimulated with ethanol underwent multiple rounds of secretion (Figure 1G, left panels), those stimulated with propranolol generated only one significant burst of secretion that appeared to completely deplete the micronemal contents (Figure 1G, right panels), implying that these two compounds have distinct mechanisms of action upon the microneme secretion pathway.

This was further validated through microneme secretion assays completed in buffer mimicking intracellular ionic conditions (Figure 1H). In such conditions, only ethanol stimulated microneme secretion, concordant with propranolol being effective only once the remainder of the pathway in Figure 1 is activated and thus PA is being produced. Furthermore, these data imply that unlike ethanol, propranolol cannot stimulate an increase in intracellular calcium levels to elicit microneme secretion. To confirm this, calcium-sensing GCaMP6-expressing tachyzoites (Borges-Pereira et al., 2015) were incubated in either intracellular or extracellular buffer; stimulated with calcium ionophore (A23187), ethanol, or propranolol; and calcium flux was subsequently monitored (Figure S1D; Movies S1, S2, S3, and S4). Under all conditions, both A23187 and ethanol stimulated an increase in intracellular (parasite) calcium, whereas propranolol had no effect under either condition (Figure S1D; Movies S1, S2, S3, and S4). These data confirm that unlike ethanol, propranolol does not elicit microneme secretion through increasing calcium levels.

Despite the significant effect of propranolol on microneme secretion, invasion efficiency of treated parasites remained unchanged (Figure 1C, right panel), which is not surprising in light of the gliding assays (Figure 1D). Parasite gliding utilizes proteins secreted from the micronemes and assays reveal that in the presence of propranolol, parasites are indeed stimulated to glide; however, gliding activity is altered in a propranolol-concentration-dependent manner. At the maximal concentration of propranolol used in this study (500 μM), gliding trails are predominantly single circles, suggesting that the parasites secrete their micronemes in one burst and can then no longer glide as their micronemal contents are depleted/exhausted, concordant with data shown in Figure 1G. Importantly, as the concentration of propranolol is decreased, parasites increase the amount and type of trails that they deposit (multiple overlapping circles/random trails) indicating that while microneme secretion is still occurring, these lower concentrations are not sufficient to fully deplete/exhaust the micronemal contents and instead revert the gliding phenotype back to that seen for parasites stimulated...
with ethanol (Figure 1D). In light of these data, it is not surprising that the invasion efficiency of propranolol treated parasites is not increased compared to untreated parasites.

Confirmation that propranolol impacts upon the pool of PA generated through the PI-PLC pathway (Figure 1A) was sought through use of various inhibitors including the DGK inhibitor (R59022, Figure 1E), PI-PLC inhibitor (U73122, Figure 1I), its inactive analog U73343 (Figure 1J), and PKG inhibitor Compound 2 (Figure 1K). These data revealed that blocking production of PA at any point along the pathway outlined in Figure 1A precludes propranolol activity and that it therefore acts upon a pool of PA upregulated during the microneme secretion-signaling pathway. Furthermore, pre-treatment of parasites with a chelator of intracellular calcium (BAPTA-AM, Figure 1L) blocked ethanol, zaprinast (a phosphodiesterase inhibitor), and propranolol-stimulated microneme secretion, confirming that the production of PA alone cannot override the requirement for a rise in intracellular calcium during this process (Figure 1L). These data are in complete agreement with the primary propranolol secretion assays (Figures 1F/1H) and highlight the importance of both arms of the pathway (Figure 1A) running concurrently to enact efficient microneme secretion. Overall, these data highlight the importance of a delicate balance between DAG and PA levels during microneme exocytosis.

![Figure 2](image)

**Figure 2. TgPI-PLC Is Crucial for Parasite Survival**

(A and C) Endogenously C-terminally tagged TgPI-PLC (A) or TgPI-PLC cKD (C) localize to apical puncta and peripheral accumulations within intracellular parasites and cluster apically in extracellular parasites. GAP45: parasite periphery. Scale bar 2 μm.

(B) TgPI-PLC-ty migrates at the expected size on western blot (125kDa). GRA3: loading control.

(D) PI-PLC-ty cKD parasites are regulated by ATc in 48 hr. GRA1: loading control.

(E) PI-PLC-ty cKD but not parental ΔKU80 parasites display a severe growth defect in the absence of PI-PLC-ty (PI-PLC-ty cKD +ATc).

(F) IFA of PI-PLC-ty cKD parasites ± ATc (72hr). Parasite morphology is grossly affected following depletion of PI-PLC-ty. GAP45: parasite periphery. MIC2: micronemes. GRA3: dense granules. ROP1: rhoptries.

(G) Electron microscopy reveals gross morphological defects in PI-PLC-ty cKD parasites +ATc (bottom two panels) while PI-PLC-ty cKD parasites (−ATc) appear morphologically normal. P: parasite

**PI-PLC Is Essential for Lytic Stage Growth**

The signaling pathway outlined in Figure 1A strongly implicates the importance of PI-PLC in generating both IP$_3$ and DAG to underpin efficient microneme exocytosis. As such, we appended 3ty-tags to the endogenous C terminus of Tgpiriplc (Figures 2A–2D and S2A/S2B) to confirm its localization by indirect immunofluorescence assay (IFA). Previous reports utilized a peptide antibody to localize PI-PLC to the parasite periphery (Fang et al., 2006); however, we detected endogenous PI-PLC both in the parasite cytosol and in peripheral and apical accumulations (Figures 2A/2C). Importantly, PI-PLC-ty appeared to cluster at the parasite apex in extracellular parasites (Figures 2A/2C, bottom panels), concordant with it playing a role in microneme exocytosis. The dual localization of PI-PLC-ty does however suggest that PI-PLC likely plays multiple roles in lipid regulation and does not act exclusively for PPM lipid regulation/microneme exocytosis.

Attempts to knockout Tgpiriplc were unsuccessful, and we therefore generated a Tet-inducible knockdown (PI-PLC-ty cKD) that efficiently regulated PI-PLC-ty expression upon addition of anhydrotetracycline (ATc) (Figures 2C–2G and S2C/S2D). Plaque assays clearly demonstrated a significant impact on lytic stage growth in the absence of PI-PLC (Figure 2E), concordant with PI-PLC playing an essential role within the parasite. Importantly, PI-PLC depletion caused significant morphological abnormalities whereby rosette formation was perturbed and nuclear material was seen within the parasitophorous vacuole (Figures 2F/2G). This severe phenotype precluded the completion of assays specifically investigating the role of PI-PLC in microneme exocytosis, yet implicated PI-PLC in a vast and yet incompletely understood signaling cascade.
**TgDGK1 Is Essential for Lytic Stage Growth**

The effects of both R59022 and propranolol on microneme secretion, combined with the severe deleterious phenotype we observed upon PI-PLC depletion, strongly implicated the presence of a pool of PA at the parasite periphery. Consequently, to confirm that these inhibitors were indeed acting upon a pool of PPM-associated PA, we utilized a yeast-derived molecular probe that binds PA (Spo20pGFP<sub>wt</sub>) or its inactive form that does not bind PA (Spo20pGFP<sub>mut</sub>) (Nakanishi et al., 2004) by IFA (Figures 3A/3B). Spo20pGFP<sub>wt</sub> but not Spo20pGFP<sub>mut</sub> specifically labels the parasite periphery (Figure 3A); however, this peripheral localization is abolished by addition of R59022, whereupon Spo20pGFP<sub>wt</sub> re-localizes to the cytosol (Figure 3B, bottom panels) confirming both the specificity of Spo20pGFP<sub>wt</sub> in vivo and the activity of R59022 toward a plasma membrane DGK to block PA production.

Three genes were found to encode putative DGKs in the *T. gondii* genome (*Tgdgk1*, ToxoDB: TGM4E9_202460; *Tgdgk2*, ToxoDB: TGM4E9_259830; and *Tgdgk3*, ToxoDB: TGM4E9_239250), but only *Tgdgk1* and *Tgdgk3* are conserved across the Apicomplexan phylum (Figure S1A). Only *Tgdgk1* localizes to the parasite periphery (Figures 3C and S1A), concordant with a role in peripheral PA metabolism. Furthermore, *Tgdgk2* (dense granule/PV localization, Figure S1A) and *Tgdgk3* (microneme localization, Figure S1A) were found to be dispensable for parasite survival (data not shown) while *Tgdgk1* was refractory to genetic deletion, and conditional knockdown strategies were implemented. A Tet-inducible knockdown (myc-DGK1cKD) efficiently depleted *Tgdgk1* in vivo and the activity of R59022 toward a plasma membrane DGK is involved in PA metabolism at the parasite periphery (Figure S1A). Only Tgdgk1 localizes to the parasite periphery (Figures 3C and S1A), concordant with a role in peripheral PA metabolism. Furthermore, Tgdgk2 (dense granule/PV localization, Figure S1A) and Tgdgk3 (microneme localization, Figure S1A) were found to be dispensable for parasite survival (data not shown) while Tgdgk1 was refractory to genetic deletion, and conditional knockdown strategies were implemented. A Tet-inducible knockdown (myc-DGK1cKD) efficiently depleted Tgdgk1 in vivo and the activity of R59022 toward a plasma membrane DGK is involved in PA metabolism.

**Given that these data confirm that DGK1 is involved in PA synthesis at the parasite periphery, we developed an alternative and more synchronized way to deplete Tgdgk1 via a fusion with a destabilization domain (DD) (Herm-Go¨tz et al., 2007). To ensure that the N-terminal DD fusion did not impact upon DGK1 function, we first complemented the myc-DGK1cKD strain with an N-terminally DD/HA-tagged copy of Tgdgk1 regulated by Shld-1 (myc-DGK1cKD (Comp. DD/HA-DGK1cKD); Figures S2N–S2P). Expression of DD/HA-DGK1 in the absence of myc-DGK1 (+Shld-1/−ATc) rescued parasite fitness (Figure S2N), demonstrating that the phenotype described for myc-DGK1cKD was indeed due to myc-DGK1 depletion and also that the DD fusion does not interfere with DGK1 function. As expected, DD/HA-DGK1 localized to the parasite periphery (Figure S2O), and western blot analyses confirmed that myc-DGK1cKD was still regulated by ATc in the complemented strain (Figure S2P).

Next we generated a parasite line expressing endogenously N-terminally tagged DDmyc-DGK1cKD stabilized by Shld-1 (Sidik et al., 2014). Plaque assays of DDmyc-DGK1cKD minus Shld-1 reconfirmed the importance of Tgdgk1 for parasite survival (Figure 3K), and IFAs showed that destabilization of the protein recapitulated the phenotype observed for the myc-DGK1cKD strain +ATc (Figure 3L). Rather unexpectedly, the loss of parasite integrity also took time to manifest following shld-1 removal, suggesting that the phenotype, likely due to a detrimental imbalance of PA/DAG, takes time to build up (Figure 3L, −Shld-1). In parasites with only a marginal loss in membrane integrity, a gradual and significant impact of DGK1 destabilization on induced egress was noted (Figure 3M), and microneme secretion at these time points was also impaired (Figure 3N). Overall, these data clearly demonstrate the importance of PA generation by DGK1 to enact microneme secretion in *T. gondii*.

**APH Acts as a PA Sensor and Is Essential for Microneme Secretion**

Given that PA levels clearly impact microneme exocytosis, we sought to identify the protein sensor of PA at the parasite membrane. We completed bioinformatics searches in ToxoDB and PlasmoDB for pleckstrin homology (PH)-domain-containing proteins, as these domains are known to bind phosphoinositides. We found 14 genes containing predicted PH-domains in *T. gondii* and seven genes in *Plasmodium* (Table S1); however, only one gene was conserved across all Apicomplexa
Figure 3. TgDGK1 Is Crucial for Parasite Survival

(A) Marker of PA (Spo20pGFP WT) but not its inactive form (Spo20pGFP mut) labels PA at the parasite periphery alongside GAP45.

(B) Treatment of Spo20pGFP WT parasites with DGK-inhibitor R59022 ablates membrane localization.

(C) myc-DGK1cKD (-ATc) and myc-DGK1cKD (+ATc).

(D) ATc: - +

100- 70-

60-

30-

20-

myc-DGK1cKD

(E) -ATc +ATc

ΔK180

myc-DGK1cKD

(F) GAP45

GAP45/MIC2

GAP45

GAP45/MIC2

myc-DGK1cKD (-ATc)

myc-DGK1cKD (+ATc)

(G) myc-DGK1cKD

(H) myc-DGK1cKD

(I) Relative PA fatty acid content (%)

-ATc +ATc

0 1 2 3 4

Relative PA fatty acid content (%)

-ATc +ATc

0 1 2 3 4

-ATc +ATc

0 1 2 3 4

-ATc +ATc

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(ToxoDB: TGME49_249970), and it is this gene that formed the basis of our analysis (Figure S3). In addition to a PH domain, the protein encoded by TGME49_249970 possesses an N-terminal stretch of 21 highly conserved residues with strong predictions for the presence of myristoylation (G2) and palmitoylation (C7) motifs (Figures 4A–4C, S3A, and S3B). We termed this protein “APH” (acylated pleckstrin-homology domain) and revealed its localization to the micronemes with specific anti-APH antibodies (Figure 4A, top panel). This localization was confirmed by generation of transgenic parasites expressing a ty-tagged second-copy (TgAPH-ty) (Figure 4A middle panel, Figures S3C and S3D). In addition, a fusion of the conserved N-terminal 21 residues of TgAPH to mCherry (N21-APAm-ch) localized to the micronemes (Figure 4A bottom panel), and mutation of the putative acylation motifs (G2A or C7A) caused APAm to redistribute throughout the parasite cytosol (Figure 4B). Overall, these data imply that APAm is trafficked to the micronemes via palmitoylation/myristoylation within the first N-terminal 21 residues. Concordantly and as expected for a myristoylated/palmitoylated protein, TgAPH-ty was found to be insoluble in PBS and fully soluble in 1% Triton X-100 (Figure 4C, left panel). In contrast, TgAPAm myristoylation (G2A) and palmitoylation (C7A) mutants were found to be readily soluble in PBS (Figure 4C, right panels). Lastly and consistent with acylation, protease protection assays confirmed that APAm is localized to the outer micronemal surface (Figure 4D). It should be noted that all apicomplexan parasites possess micronemes and contain a highly conserved aph gene (Figure S3B) and thus are all likely to utilize this protein for the same role.

Confirmation that TgAPAm binds PA was sought through both phosphoinositide-strip (PIP-strip) and liposome-binding assays (Figures 4E–4G and S3E). PIP-strips established that bacterially produced TgAPAm and the PH-domain alone, the orthogonal Plasmodium falciparum protein (PIAPH) and the yeast Spo20pWT/Mut proteins bind strikingly to PA and not as intensely to any other phosphoinositides (Figure S3-E). In these assays an excess of protein is overlaid upon an excess of lipid (100 pmol/spot); thus, we were unable to distinguish binding of the Spo20pWT/Mut to PA in vitro despite its clear specificity in vivo. We were however able to validate these results for APAm via liposome binding assays, wherein the specificity of TgAPAm, the PH-domain, and PIAPH binding to PA was confirmed and shown to be statistically significant (Figures 4F and 4G). To further strengthen this data and assess directly how APAm responds to changes in PA levels, we expressed a non-acylated, soluble form of APAm by N-terminal fusion with DD (DDmyc-APAm) and investigated its localization upon propranolol treatment (Figures 4H/4I). DDmyc-APAm was found to re-localize to the parasite apex upon propranolol treatment, concordant with it binding to PA. This effect was ablated in the presence of DGK-inhibitor R50922 (Figure 4H), indicating that DDmyc-APAm responds to the pool of PA produced specifically by DGK1 and enhanced by propranolol treatment.

Being refractory to genetic deletion, Tgaph was instead deleted by a gene excision strategy utilizing the rapamycin-dependent, dimerable Cre-recombinase (Andenmatten et al., 2013) (Figures S3F–S3H). Following addition of rapamycin, loss of Tgaph expression was seen in 10%–20% of the population (Figure 5A, top panel). We were unable to clone any excised parasites, thus formally confirming that the Tgaph gene is essential. Within this system, removal of TgAPAm did not alter microneme biogenesis, localization (Figure 5A, bottom panel), or intracellular growth (Figure S3H); however, concordant with a role in microneme secretion, Tgaph-excised parasites (ty-APAm-cKO) displayed a marked block in invasion (Figure 5B) and egress (Figure 5C). The low excision rate precluded quantitative assessment of microneme secretion; therefore, we generated an additional conditional knockdown line both for completion of microneme secretion assays and also to confirm the data from the DiCre strain. As such, we generated an ATc-regulatable conditional knockdown of Tgaph (ty-APAm-cKD) (Figures S3I–S3K).
Figure 4. APH Localizes to the Micronemes and Binds PA

(A) Endogenous TgAPH (top) and TgAPH-ty (middle) localize to the micronemes. The N-terminal 21 residues of TgAPH are sufficient for microneme targeting (N21-APH-mCh), MIC2/4/6: microneme markers.

(B) Mutating the putative myristoylation (TgAPH-ty_G2A, top panel) or palmitoylation motif (TgAPH-ty_C7A, bottom panels) ablates microneme localization. GAP45: parasite periphery.

(C) Parasites were solubilized in either PBS or 1% Triton X-100 (TX100) and split into soluble (S) and pellet (P) fractions. APH is PBS insoluble, while G2A and C7A mutants are PBS soluble.

(D) Digitonin (Dig.) permeabilization facilitates TgAPH digestion by proteinase K (PK). TgARO; rhoptry surface protein. MIC2 band shift (*) represents cleavage of small external portion.


(F) Immunoblots of recombinant TgAPH, the PH domain alone, or PIAPH-associated with liposomes. Recombinant proteins were incubated with liposomes containing phosphatidylcholine (PC), PC:phosphatidylserine (PS) (1:1 molar ratio) or phosphaticid acid (PA):PC (1:1 molar ratio). Fractions 1 and 2 represent
Upon ATc treatment, ty-APH-cKD was efficiently depleted (Figure 5D) and exhibited abrogated lysis plaque formation (Figure 5E), impaired invasion (Figure S3K), and a block in egress (Figure 5F), thus confirming the ty-APH-cKO phenotype (Figures 5A–5C). Importantly, ATc-treated ty-APH-cKD parasites (Figure 5G, right panels) but not untreated (Figure 5G, left panels) or TATi-1 parental parasites (Figure S3L) failed to secrete their micronemes when stimulated with A23187, ethanol, or propranolol, demonstrating that TgAPH is indeed essential for microneme exocytosis.

CONCLUSIONS

Previous studies have indirectly shown the importance of the PI-PLC pathway during microneme secretion signaling by focusing on the production of Ca²⁺ (Carruthers et al., 1999; Lovett et al., 2002; Raabe et al., 2011) and the subsequent CDPK-dependent signaling cascade culminating in microneme exocytosis (Lourido et al., 2012; McCoy et al., 2012; reviewed in Holder et al., 2012; Garrison et al., 2012). Here we have delved into the importance of DAG and PA generated by PI-PLC during this signaling cascade. Importantly, we have directly linked these specific signaling events to the microneme surface protein: APH, which is essential for microneme secretion, invasion, and egress. This function is likely conserved across the phylum, since APH is found in all apicomplexan parasites characterized to date (Figure S3B), including the less well studied Theileria spp. and Babesia spp., which also contain micronemes (Rudzinska et al., 1976; Schein et al., 1978). We propose that following specific external stimuli such as changing potassium levels, PI-PLC activity is upregulated to produce more DAG, which is in turn converted to PA by DGK1. Concurrently, PI-PLC-generated IP₃ liposome bound proteins; fractions 3–5 contain unbound proteins. Immunoblots were probed with rabbit anti-APH or myc antibodies. Blots are representative of three replicate experiments.

Figure 5. TgAPH Is Crucial for Microneme Secretion, Egress and Invasion

(A) Top: IFAs probed with TgAPH-specific antibodies (red) confirm APH loss following 48 hr rapamycin treatment and concurrent gain of YFP in ty-APH-LoxP parasites. Bottom: In ty-APH-cKO parasites, ablation of TgAPH (indicated by the presence of YFP) had no effect on the localization of other micronemal proteins as indicated by correct MIC4 localization. Scale bar 2 μm unless otherwise stated. (B and C) Parasites lacking TgAPH (ty-APH-cKO) are significantly impaired in (B) invasion and (C) egress. Red (GAP45): total parasites. YFP: parasites lacking TgAPH. Error bars represent ±SD for 50 vacuoles/cells counted in triplicate from six biological replicates. (D) Western blot analyses demonstrate the absence of TgAPH protein in the ty-APH-cKD line in the presence of ATc (48 hr). Profilin (PRF); loading control. (E) Plaque formation was impaired for TgAPH-depleted parasites (ty-APH-cKD +ATc) but not parental lines (ty-APH-cKD -ATc/TATi-1 ± ATc). (F) Tachyzoites depleted in TgAPH (ty-APH-cKD +ATc), but not wild-type ΔKU80 parasites, were impaired in egress. Results represent mean ± SD of 100 vacuoles counted in triplicate from three biological replicates. (G) ty-APH-cKD parasites are unable to secrete their micronemes as indicated by the absence of signal when ESA was probed for various MICs. ESA: excreted secretory antigens/microneme secreted fraction. Red arrowhead: secreted MIC. →: full-length protein. GRA1; control for parasite viability. Catalase; control for parasite lysis.
stimulates Ca\(^{2+}\) release, leading to CDPK activation. These events culminate in microneme fusion at the PPM, which is presumably enacted initially by APH binding to PA, ultimately facilitating the close apposition of the microneme to the PPM, DOC2.1 fusion, and eventually microneme exocytosis (Figure 1A).

While these data reveal the importance of APH and PA during microneme secretion, they also reveal the importance of DAG/PA regulation during membrane signaling and homeostasis. Given the constitutive presence of low-level PA at the PPM, as indicated by Spo20p\(_{\text{wt}}\) labeling, it is not surprising that conditional depletion of DGK1 or PI-PLC has a significant effect on overall parasite fitness. In other systems, DGK knockdown has been linked to apoptosis via lipid deregulation (Huang and Freter, 2015; Tsuchiya et al., 2015); thus, such an extreme phenotype upon PI-PLC or DGK1 depletion is to be expected and suggests that both PI-PLC and DGK1 functioning is important for maintaining diverse membrane signaling events not limited to microneme exocytosis. Such a role fits with the slight changes seen in PA levels detected upon DGK1 depletion. Furthermore, the conservation of DGK1 across the Apicomplexa (Figure S1) and its relatively weak homology with its mammalian counterpart make it an ideal candidate for anti-parasitic intervention strategies.

DAG/PA generation is a reversible process, and we propose that the rapid conversion of PA back to DAG acts as the “off switch” during microneme secretion-dependent events such as gliding. This is supported by the effect of propranolol that indicates that blocking PAP activity with this compound prevents multiple independent rounds of secretion and promotes one single, large burst of microneme exocytosis (Figure 1G), thus overriding the “off switch.” The putative enzyme underpinning this step in T. gondii (PAP2, ToxoDB: TGME49_247360) (Figure S4) is not conserved across the Apicomplexa and is only present within the coccidial subgroup of the phylum (http://toxodb.org/toxo/; Figure S4A); thus, additional PAPs may be involved in PA/DAG conversion throughout the phylum. Additionally, knockdown of TgPAP2 using the recently developed 3’ UTR excision strategy (Pieperhoff et al., 2015) revealed no significant lytic cycle defect, suggesting that this protein is either not essential or only a small amount (such as that remaining following knockdown) is required for maintaining PA/DAG homeostasis (Figures S4B–S4H). Other putative candidates for PAP functioning do however also exist within the Apicomplexa (Figure S4), and one of these other candidates could instead fulfill the role; however, this remains to be experimentally validated. In addition, other members of the Apicomplexa, such as Plasmodium merozoites, might not necessarily need to “turn off” microneme secretion while moving between host cells, and thus PAP activity is not necessarily an absolute requirement across the entire phylum. The significant phenotype generated by conditional depletion of Tgdgk1, and its conservation across the phylum, does however position it as a far more central target in the development of intervention strategies and thus of significantly greater importance.

In conclusion, the conservation of both DGK1 and APH, combined with data indicating that both PIAPH and TgAPH specifically bind PA and TgAPH localizes to the micronemes, strongly suggests that the pathway identified here is likely conserved across the phylum. These findings therefore greatly enhance our understanding of the signaling events underpinning apicomplexan microneme exocytosis and provide data on not solely the signaling events but also the protein sensors involved. Importantly, DGK1 and PI-PLC have been linked here to broader roles than purely enacting microneme exocytosis and ultimately highlighting DGK1 as an apicomplexan-specific drug target underpinning essential lipid signaling-related events in this important group of pathogens.

### EXPERIMENTAL PROCEDURES

#### Antibodies

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#### Proteinase K Protection Assay

Freshly egressed T. gondii tachyzoites were resuspended in 1.5 ml cold SoTE (0.6 M sorbitol, 20 mM Tris–HCl [pH 7.5], and 2 mM EDTA) and split into three tubes (0.5 ml each). Cold SoTE was added to tube 1 as a control. Tubes 2 and 3 were permeabilized with 0.5 ml cold 0.01% digitonin (Sigma)/SoTE. Samples were carefully mixed by inversion and incubated on ice/10 min prior to centrifugation (2,000 x g/4 C/10 min). Supernatant was discarded, and 0.5 ml cold SoTE was added to tube 1, while 2.25 µl of cold Proteinase K (Sigma, 20 mg/ml)/SoTE was added to tube 3. All tubes were gently inverted and incubated on ice/30 min. Proteinase K was inactivated by addition of cold tri-chloroacetic acid to a final concentration of 10% on ice/30 min. Samples were centrifuged (14,000 rpm/20 min), washed 2 x with acetone, air dried, and resuspended in TE prior to SDS-PAGE.

#### IFA

HFF monolayers grown on coverslips were inoculated with tachyzoites and grown at 37°C. Cells were subsequently fixed with 4% paraformaldehyde (PFA) or 4% PFA/0.05% glutaraldehyde (PFA/Glu) for 10 min, then quenched with 0.1 M glycine/PBS. Cells were permeabilized (0.2% Triton X-100/PBS), blocked (2% BSA/0.2%Triton/PBS), and probed with primary antibodies (PFA or 4% PFA/0.05% glutaraldehyde (PFA/Glu) for 10 min, then quenched with 0.1 M glycine/PBS. Cells were permeabilized (0.2% Triton X-100/PBS), blocked (2% BSA/0.2% Triton/PBS), and probed with primary antibodies (60 min) prior to washing (0.2% Triton/PBS) and incubation with secondary antibodies (Alexa488- or Alexa594-conjugated goat anti-mouse/rabbit). Nuclei were stained with DAPI, and coverslips were mounted in FluoromountG. Image analysis was done using an LSM 700 confocal scanning microscope (Zeiss).

#### Microneme Secretion Assay

Freshly egressed parasites were resuspended in equal volume intracellular (IC) buffer (5 mM NaCl, 142 mM KCl, 1 mM MgCl\(_2\), 2 mM EGTA, 25 mM HEPES, pH 7.2 with KOH) prior to pelleting (1,050 rpm/10 min). Pellets were subsequently washed in IC buffer and re-pelleted before resuspension in serum-free media with listed pre-treatment compounds for 30 min/room temperature (RT). After pre-treatment, parasites were pelleted and resuspended in serum-free media and DSMO, 500 µM propanol, 2% ethanol, 500 µM zaprinast, or 3 µM A23187 (37°C/5–30 min). Parasites were pelleted (1,000 x g/5 min/4°C), and supernatant was transferred to new Eppendorf tubes and re-pelleted (2,000 x g/5 min/4°C). Final supernatant (excreted secreted antigens [ESA}s) and pellet fractions were resuspended in sample buffer (50 mM Tris–HCl [pH 6.8], 10% glycerol, 2 mM EDTA, 2% SDS, 0.05% bromophenol blue, 100 mM DTT) and boiled prior to analysis by immunoblotting.

For Figure 1F, parasites were resuspended in 100 µl IC buffer ± 500 µM propanol (37°C/30 min). Samples were spun as above, and supernatant was collected (Intra. Figure 1F). Pellets were resuspended in 100 µl serum-free media ± 500 µM propanol (37°C/30 min), pelleted as before, and supernatant was collected (Extra. Figure 1F). Final pellet was resuspended in 100 µl serum-free medium ± 500 µM propanol +2% ethanol, pelleted, and supernatant was collected (Extra +EtOH, Figure 1F).
**Protein-Lipid Overlay Assay**

PITP-strip (Echelon Biosciences) assays were carried out according to manufacturer’s instructions. Briefly, strips were incubated (1 hr) in PBS/BSA (PBS, 0.1% Tween 20, 3% BSA) prior to incubation with 0.5 μg/ml of each protein for 1 hr. Strips were washed 3 x 10 min with PBS/0.1% Tween before incubation with specific antibodies for 1 hr/RT. PITP-strips were subsequently washed and probed with specific secondary antibodies.

**Liposome Binding and Membrane Flotation Assay**

Lipids (Avanti Polar Lipids Inc.); 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-phosphate (POPA) were solubilized in chloroform, mixed, and vortexed in tubes at specific ratios (PC = 100 mol% PC; PS/PC = 50 mol% PC and 50 mol% PS; PA/PC = 50 mol% PC and 50 mol% PA); Chloroform was evaporated under N₂. Dried lipids (2 μmol) were hydrated for 30 min in 1 ml of lipid rehydration buffer (100 mM NaCl, 1 mM CaCl₂, and 50 mM Tris-Cl [pH 7.4]) prior to three freeze/thaw cycles and extrusion through 4 μm pore-size filters using a mini-extruder (Avanti Polar Lipids) to produce liposomes.

For binding assays, liposomes (200 nmol) were incubated with 500 pmol recombiant protein (37°C/1 h) in a final volume of 100 μl. Reaction mixture was subsequently diluted to 200 μl with 85% sucrose, then layered with 2.8 ml 65% sucrose and 1 ml 10% sucrose, and centrifuged (115,000 g x 4°C/16 hr). Sucrose solutions were prepared in buffer containing 100 mM NaCl, 50 mM Tris-Cl (pH 7.4). Five 1 ml fractions were collected from the top of each tube and were analyzed by immunoblot. Band intensity was quantified using an Odyssey imaging system (Li-Cor Inc.). Liposome binding efficiency was calculated as % of bound = bound/(bound + unbound) * 100.

**PA Extraction and Quantification**

Total lipid spiked with 25 nmol C13:0 fatty acid was extracted by chloroform:methanol:2.5 ml (v/v/v) in the presence of 0.1 M HCl. The pooled organic phase was subjected to biphasic separation by adding 1.0 M HCl and was then dried under N₂ gas flux prior to being dissolved in 1-butanol. Total lipid was separated by 2D HPTLC with 1-butanol. Total lipid was separated by 2D HPTLC with 1-butanol. SUPL INFORMATION

**REFERENCES**


Supplemental Information

Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in Toxoplasma

Hayley E. Bullen, Yonggen Jia, Yoshiki Yamaryo-Botté, Hugo Bisio, Ou Zhang, Natacha Klages Jemelin, Jean-Baptiste Marq, Vern Carruthers, Cyrille Y. Botté, and Dominique Soldati-Favre
Figure S1

Supplemental figures

A

TgDGK1 (TGME49_202460, Plasma membrane, 73 kDa), NcDGK1 (NCLIV_022470), PdDGK1 (PF14_0681), BbDGK1 (XP_001611607.1), CgDGK1 (cgd4_4340)

B

TgDGK2 (TGME49_259830, 69 kDa), NcDGK2 (NCLIV_027060)

C

HsDGK1 (NP_003638.1, 64 kDa)

D

lower
**Figure S4**

### A

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<td>Lipin</td>
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<td>PAP2-like protein</td>
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### B

- **pap2 genomic locus**
- **PAP2 ty KI plasmid**
- **Recombinant pap2 locus**

### C

- **ΔKu80**
- **PAP2-ty**

### D

- **PAP2-ty KI**
- **GAP45**
- **Merge**
- **PAP2-ty KI**
- **MIC4**
- **Merge**
- **PAP2-ty KI**
- **GRA1**
- **Merge**

### E

- **Pap2 genomic locus**
- **PAP2 ty KI plasmid**
- **Recombinant pap2 locus**

### F

- **ΔKu80**
- **PAP2 ty LoxP**
- **PAP2 ty KD**

### G

- **ΔKu80**
- **PAP2 ty KI**
- **PAP2 ty LoxP**
- **PAP2 ty KD**

### H

- **PAP2-ty KI**
- **PAP2-ty LoxP**
- **PAP2-ty KD**

### Notes

1. Six-pass transmembrane proteins of PAP2 superfamily. Most likely to perform the function of converting PtdOH to DAG at membranes.
2. Soluble proteins commonly involved in converting PtdOH to DAG for signal transduction, often translocated to the nucleus.
3. Annotated in Plasmodb.org as potentially a PAP of the apicoplast, not likely to have PAP activity.
4. PAP2 family carboxy terminal protein. Likely incorrectly annotated in ToxoDB.org as listed sequence is unattainable by PCR amplification (data not shown).
Supplemental figure legends

Supplemental figure 1 (related to figure 1). DGK conservation across the Apicomplexa, as well as the closest H. sapien orthologue. A. Localisation and molecular weight of each of the TgDGKs is indicated. Tg: Toxoplasma gondii. Nc: Neospora caninum. Pf: Plasmodium falciparum. Bb: Babesia bovis. Cp: Cryptosporidium parvum. Hs: Homo sapien. SP: Signal peptide. TMD: transmembrane domain. C1: C1 domain (DAG binding domain). Accession numbers shown in brackets. Catalytic and accessory domains contain catalytic residues important for DGK activity. TgDGK2 localises to the dense granules/PV as shown by colocalisation with dense granule marker GRA1. TgDGK3 localises to the micronemes as shown by colocalisation with micronemal marker MIC4. GAP45 is a marker of the parasite periphery. Scale bar; 2µm. B. Multiple alignment of the catalytic and accessory domains of each of the DGK1 sequences using Multalin. Catalytic domain alignment is shown in blue box. Accessory domain alignment is shown in red box. Red residues: conserved. Blue residues: similar. C. Western blots of myc-DGK1cKD and DGK2-ty expressing parasites treated with either DMSO, 30µM R59022 or 500µM propranolol reveal that DGK protein levels are unchanged following inhibitor treatment. GRA3 represents a loading control. D. Cytochalasin-D treated GCaMP6-expressing tachyzoites in buffer mimicking either intracellular (red) or extracellular (blue) conditions were stimulated with Calcium ionophore (A23187), ethanol, propranolol (Prop.) or DMSO and corrected total fluorescence was measured. An increase in calcium was seen in parasites treated with A23187 and ethanol, but treatment with propranolol or DMSO did not stimulate and increase in calcium.

Supplemental figure 2 (related to figure 3). Analysis of PI-PLC and TgDGK1. A. Cloning strategy for C-terminal 3ty knock-in at the endogenous Tgpiplc locus. Primers listed in Table S2. B. PCR showing correct integration of (A). Expected band sizes: a/b, 1435bp. a/c, 1231bp. d/e, 1197bp. C. Cloning strategy for generation of PI-PLC-ty cKD strain. Primers listed in Table S2. Gold star represents guide binding/cleavage site. Red and pink regions represent homology sequences used for recombination. D. PCR showing correct integration of (C). Expected band sizes: A/B, 908bp. C/B, 455bp. E. Cloning strategy for C-terminal 3ty knock-in at the endogenous TgDGK1 locus. Primers listed in Table S2. F. PCR showing correct

H. PCR showing correct integration of (G). Expected band sizes: A/B, 2171bp. E/B, 2202bp. C/D, 2238bp. C/F, 3219bp. * represents non-specific band. I-K. Immunofluorescence assays of mycDGK1cKD parasites treated +/-ATc (72hr) and probed for makers of the rhoptries (ARO, I), apicoplast (HSP60, J) or the mitochondria (HSP70, K) reveal that these organelles localise correctly in the presence (-ATc) and absence (+ATc, 72hr) of mycDGK1 when the parasites remain intact. SAG1; marker of the parasite plasma membrane. Scale bar; 2µm. L. Electron microscopy of mycDGK1cKD parasites treated for 72hr with ATc reveal significant morphological defects. P: parasite. PVM: parasitophorous vacuole membrane. TVN: tubulovesicular network. PM: parasite plasma membrane. M. Parental wildtype parasites (ΔKU80) treated with ATc for up to 96hr show normal morphology for markers of the micronemes (MIC2), plasma membrane (SAG1), IMC (GAP45) and parasitophorous vacuole (GRA3). Scale bar; 2µm. N. Plaque assay demonstrating DD/HA-DGK1 complements myc-DGK1 in the presence of Shld-1/ATc. O. IFA of myc-DGK1cKD (comp. DD/HA-DGK1cKD) parasites reveals DD/HA-DGK1 localises to the periphery. GAP45: parasite periphery. Scale bar: 1µm. P. Western blot of myc-DGK1cKD (comp. DD/HA-DGK1cKD) parasites +/-Shld-1, +/-ATc reveals that both myc-DGK1 and DD/HA-DGK1 are correctly regulated by Shld-1 or ATc. Profilin; loading control.

APH antibodies (left panel) and ty antibodies recognising the same band in TgAPH-ty samples. E. Coomassie stained gels revealing proteins utilised for PIP-strip assays and liposome binding assays. Expected sizes: GST-TgPH, 39.7kDa; GST-PfAPH, 56 kDa; GST-TgAPH, 52 kDa; GST-Spo20pWT/Mut, 32.3kDa. F. Cloning strategy for generation of ty-APH-Lox and ty-APH-cKO strains. G. PCR confirmation of bands of the expected sizes following integration (recombinant ty-TgAPH-Lox locus). H. Standard intracellular growth assays revealed that intracellular growth is not impaired in the absence of TgAPH (ty-APH-cKO) when compared to the parental strain (ty-APH-Lox). Error bars represent +/-s.d for 100 vacuoles counted in triplicate. I. Cloning strategy for generation of ty-APH-cKD strain. J. PCR confirmation of bands of the expected sizes following integration of (D) (recombinant ty-TgAPH-Lox locus). K. Parasites pre-treated with ATc for 48hrs to remove TgAPH were inoculated on HFF monolayers and allowed to invade for 1hr prior to analysis via red/green invasion assay. Error bars represent +/-s.d for 100 vacuoles counted in triplicate. L. Microneme secretion assays of TATi-1 strain parasites treated +/-ATc reveal no defect in microneme secretion under any stimuli condition.

Supplemental figure 4 (related to figure 1-3): PAP2 is not essential for lytic stage growth. A. Table indicating all identified phosphatidic acid phosphatases and lipins in the Apicomplexa. Immunofluorescence assay (IFA) reveals lipin localises to the parasite cytosol. Lipin is labelled with ty antibodies. GAP45 represents the parasite periphery. Scale bar; 2µm. 1: Carman and Han (2009), 2: Siniossoglou (2013), 3; toxodb.org. B. Cloning strategy for C-terminal 3ty knock-in at the endogenous Tgpap2 locus. Primers listed in Table S2. C. PCR showing correct integration of (B). Expected sizes: A/B-972bp, A/C- 1124bp, D/B- 993bp. D. IFA reveals PAP2-ty as distinct puncta throughout the parasite. GAP45; parasite periphery. MIC4; micronemes. GRA1; dense granules. Scale bar; 2µm. E. Cloning strategy for generation of PAP2-ty KD through use of the 3’UTR U1 excision strategy. F. PCR showing correct integration of (E). Expected sizes: A/B- 972bp, D/B- 1074bp, A/C-1206bp. G. Western blot showing correct size of PAP2-ty on western blot (35kDa) and absence of PAP2-ty signal in PAP2-ty KD strain. GRA3 represents a loading control. H. Plaque assays reveal no dramatic phenotype for PAP2-ty KD parasites compared to parental strains.
Supplemental movies

**Supplemental movie 1 (related to figure 1):** GCaMP6-expressing tachyzoites in intracellular buffer treated with DMSO do not display any increase in calcium as measured by corrected total fluorescence.

**Supplemental movie 2 (related to figure 1):** GCaMP6-expressing tachyzoites in intracellular buffer treated with calcium ionophore display an increase in calcium as measured by corrected total fluorescence.

**Supplemental movie 3 (related to figure 1):** GCaMP6-expressing tachyzoites in intracellular buffer treated with ethanol display an increase in calcium as measured by corrected total fluorescence.

**Supplemental movie 4 (related to figure 1):** GCaMP6-expressing tachyzoites in intracellular buffer treated with propranolol do not display any increase in calcium as measured by corrected total fluorescence.
**Supplemental tables**

**Supplemental table 1 (related to figures 4&5): PH-domain containing proteins in the Apicomplexa**

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**Supplemental table 2 (related to figures 1-5):** primers used in this study

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Supplemental methods

Parasite culture:
*T. gondii* tachyzoites (RH hxgprt-ko, ΔKU80, TATi-1 (Meissner, Schlüter, & Soldati, 2002) or ΔKU80::DiCre strains (Andenmann et al., 2013; Huynh & Carruthers, 2009)) were grown in human foreskin fibroblast (HFF) monolayers in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine and 25 µg/ml gentamicin (37°C, 5% CO₂). Parasite transfections were performed by electroporation as described previously (Soldati & Boothroyd, 1993). The hypoxanthine-xanthine-guanine phosphoribosyl transferase (*hxgprt*) (Donald, Carter, Ullman, & Roos, 1996) and dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) genes (Donald & Roos, 1993) were used as positively selectable markers as described previously.

*T. gondii* cloning strategies

TgPI-PLC-ty
To generate endogenously ty-tagged *Tgpiplc* (TgPIPLC-ty) fragments were generated with Q5 (NEB) DNA polymerase using primers outlined in Table S2. Fragments were cloned into *KpnI/NsiI* sites of the pTUB8MIC13-3Ty-HX vector (11) and linearized with *NdeI* prior to transfection (20µg). Parasites were selected by Mycophenolic acid (MPA)/Xanthine and clones were obtained by limiting dilution. Correct integration was confirmed by PCR of genomic DNA using primers listed in Table S2.

TgPI-PLC-ty cKD
To generate the TgPI-PLC-ty cKD strain, a PCR product of 5’COR-pT8TATi1 HXtet07S1mycNtCOR plasmid (12) with approximately 30bp homology to *Tgpiplc* was generated with KOD polymerase (Novagen) using primers listed in Table S2. A Cas9/CRISPR guide targeting the N-terminus of *Tgpiplc* was generated using Q5 mutatgenesis (NEB) and primers listed in Table S2. Following transfection, green fluorescent parasites were cloned into 96 well plates. Regulatable clones were subsequently ty-tagged at the endogenous locus as outlined above. Correct integration was confirmed by PCR of genomic DNA using primers listed in Table S2.

TgDGK1-ty
To generate endogenously ty-tagged *Tgdgk1* (*TgDGK1-ty*) fragments were generated with Phusion (NEB) DNA polymerase using primers outlined in Table S2. Fragments were cloned into *KpnI/NsiI* sites of the pTUB8MIC13-3Ty-HX vector (Friedrich et al., 2010) and linearized with *StuI* prior to transfection (20µg). Parasites were selected by MPA/Xanthine and clones were obtained by limiting dilution. Correct integration at both sites was confirmed by PCR of genomic DNA using primers listed in Table S2.

**myc-DGK1cKD**

To generate the myc-DGK1cKD plasmid, 5’flanking and N-terminal homology regions were amplified from ΔKU80 strain parasites using primers indicated in Table S2. Briefly, a 1930bp region of genomic DNA corresponding to the 5’ flanking region upstream of the *Tgdgk1* start codon and a 667bp region of genomic DNA corresponding to the N-terminal *Tgdgk1* region from the codon 3’ to the ATG were cloned into *NcoI/BamHI* and *BglII/NotI* sites in the 5’COR-pT8TATi1-HX-tet07S1mycNtCOR plasmid (Salamun, Kallio, Daher, Soldati-Favre, & Kursula, 2014), respectively. Prior to transfection (100µg) into ΔKU80 parasites, the vector was linearized with *ShI1*. Parasites were selected with MPA/Xanthine and clones were obtained by limiting dilution. Correct integration at both sites was confirmed by PCR of genomic DNA using primers listed in Table S2.

**DD/HA-DGK1cKD**

To generate the DDHA-DGK1 plasmid, *Tgdgk1* cDNA was amplified by PCR with Phusion DNA polymerase using primers indicated in Table S1. PCR product was inserted into the pTub8DDHAGFP-HX construct between *Nsil/PacI* sites. This construct was transfected into both RH (DD/HA-DGK1cKD) and myc-DGK1cKD (myc-DGK1cKD (comp. DD/HA-DGK1cKD)) strains where it was maintained as a second copy. Expression of DD/HA-DGK1 was controlled by addition or removal of Shld-1.

**DDmyc-DGK1cKD**

The Cas9/CRISPR system combined with a PCR product generated using primers listed in Table S2 was utilized to generate endogenously N-terminally DDmyc-tagged
$d_{gk}1$ (DDmyc-DGK1cKD) and positive clones were generated. Expression of DDmyc-DGK1 was subsequently regulated by addition or removal of Shld-1.

$Spo20pGFP_{wt/mut}$

The PA-binding region of the yeast SNARE protein Spo20p (Spo20p$^{WT}$, amino acids 51-91) and mutated Spo20p (Spo20p$^{mut}$, amino acids 51-91, S69P and L67P mutations) coding sequences were amplified by PCR with Phusion DNA polymerase using the vectors GFP-Spo20p$^{WT}$ and GFP-Spo20p$^{mut}$ (kindly provided by N. Vitale (Zeniou-Meyer et al., 2007)) respectively, as templates. PCR products were inserted into the pTub8DDmycGFP-HX construct between PstI and PacI sites. Expression was regulated by addition of Shield for 4hrs.

$TgAPH$-ty

Primers for $Tgaph$ were based on the $Tgaph$ (TGME49_293470) sequence obtained from http://toxodb.org/ and are listed in Supplemental Table 2. The vector pTub8TgAPH-Ty-HX was obtained by PCR amplification of $Tgaph$ cDNA cloned into the EcoRI/Shfl (Nsil) sites in pTub8mycGFPfmyoATail-ty1-HX (Herm-Götz et al., 2002) to generate APH-ty parasites.

$TgAPH$-ty (G2A, C7A)

Mutation of the putative acylation sites (G2A, C7A) in the pTub8TgAPH-ty-HX strain were obtained by PCR amplification. Mutants were verified by DNA sequencing and subsequently referred to as TgAPH-ty-G2A or TgAPH-ty-C7A.

$N21$-APH-mCherry

The TgAPH-N21-mCherry-ty-HX fusion construct was generated by first amplifying the sequence encoding the first N-terminal 21 amino acids of $Tgaph$ and subsequently inserting this fragment into the pTub8TgPRF-mCherry-ty-HX construct between EcoRI/NsiI sites to generate N21-APH-mCherry parasites.

$DDmyc$-APH

To generate the DDmyc-APH plasmid, $aph$ cDNA was PCR amplified with Phusion DNA polymerase using primers indicated in Table S2. PCR product was inserted into the pTub8DDmycGFP-HX construct between NsiI/PacI sites. Clonal DDmyc-APH
strain was obtained by HXGPRT drug selection and cloning by limiting dilution. Expression was regulated by the presence of Shld-1 for 4 hrs. For DDmyc-APH relocalisation studies, freshly egressed DDmyc-APH parasites were treated with either DMSO or 30 µM R59022 for 30 mins, followed by treatment with Propranolol (100 µM) for 10 mins.

ty-APH-Lox/ty-APH-cKO

*Tgaph* cDNA (with LoxP sequence added to its 5’) was subcloned from pTub8ty-APH-HX and inserted into the Tub8-loxP-KillerRed-loxp-YFP-HX plasmid (Andenmatten et al., 2013) between EcoRI/Pacl. Five prime and 3’ fragments (~2 kb each) of *Tgaph* were amplified by PCR and subsequently cloned into of the Tub8-loxP-ty-APH-loxp-YFP between KpnI/EcoRI and SacI sites respectively. The final construct 5’APH-loxP-ty-APH-loxP-YFP-3’APH-HX was linearized with BglII/NcoI prior to transfection to generate the ty-APH-Lox strain. To generate ty-APH-cKO, ty-APH-Lox parasites were treated with rapamycin overnight.

ty-APH-cKD

To make the ty-APH-cKD (ATc-inducible) strain, ty sequence was inserted into the *Tgaph* coding sequence after the N-terminal 21 amino acids. This was sub-cloned into TetO7Sag4YFP between EcoRI/Pacl sites. The DHFR-TS selection cassette was then cloned between SacI sites to generate the final ty-APH-cKD. To delete the endogenous *Tgaph*, 5’ and 3’ *TgAPH* fragments (~2.5 kb each) were PCR amplified using primers listed in Table S2 and subsequently cloned into of the pTub5CATSag1 plasmid using NatI/SpeI and KpnI/XhoI sites respectively. The final construct 5’APH-pTub5CATSag1-3’APH was linearized with SbfI/SmaI prior to transfection to generate ty-APH-cKD parasites.

*TgPAP2*-ty **KI**

To generate endogenously ty-tagged *Tgpap2* (TgPAP2-ty) fragments were generated with Phusion (NEB) DNA polymerase using primers outlined in Table S2. Fragments were cloned into KpnI/NsiI sites of the pTUB8MIC13-3Ty-HX vector (11) and linearized with PstI prior to transfection (20µg). Parasites were selected by MPA/Xanthine and clones were obtained by limiting dilution. Correct integration was confirmed by PCR of genomic DNA using primers listed in Table S2.
To generate the TgPAP2ty cKD plasmid, fragments were generated with Phusion (NEB) DNA polymerase using primers outlined in Table S2. Fragments were cloned between KpnI/NsiI sites of pLIC-HA-FLAG-flox (3’UTR-SAG1-HXGPRT)-4xU1 for C-terminal knock-in of three ty tags and U1-mediated silencing (Pieperhoff et al., 2015; Rugarabamu, Marq, Guérin, Lebrun, & Soldati-Favre, 2015). The resultant plasmid was termed PAP-ty_3’UTR-SAG1-HXGPRT. Plasmid DNA (80µg) was digested with PstI prior to transfection (80µg) in DiCre expressing parasites, prior to cloning by limiting dilution. Correct integration was confirmed by PCR of genomic DNA using primers listed in Table S2. Parasite clones were subsequently referred to as PAP2ty LoxP. To generate TgPAP2-ty cKD strain parasites, extracellular PAP2-ty LoxP parasites were treated for 4hrs with 50nM rapamycin prior to cloning by limiting dilution. Correct integration was confirmed by PCR of genomic DNA using primers listed in Table S2. Resultant parasite strain is referred to as PAP2ty KD.

Electron microscopy

myc-DGK1cKD or PI-PLC-ty cKD parasites were grown in ATc for 48hrs and upon egress were transferred to fresh HFF monolayers in the presence of ATc. 24hrs later cells were fixed for electron microscopy using standard procedures (total 72hrs ATc treatment). Briefly, infected HFF monolayers were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, post-fixed in osmium tetroxide, dehydrated in ethanol and treated with propylene oxide prior to embedding in Spurr’s epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examination using a Technai 20 electron microscope (FEI Company).

Intracellular growth assay

HFF monolayers were inoculated with freshly egressed parasites and incubated for 24hrs before fixation with PAF/Glu. IFAs were completed using α-GAP45 to detect individual parasites. Results represent mean +/- standard deviation of 100 parasites counted in triplicate from three biological replicates.

Induced egress assay
Freshly egressed parasites were inoculated on HFF monolayers and incubated for 30-32hrs. For R59022 experiments, cells were incubated with specified concentrations of R59022 or DMSO at room temperature for 30mins prior to stimulation of egress. For ty-APH-cKO assays, extracellular parasites were treated with rapamycin (50 mM) for 4hrs and then allowed to grow for forty-eight hours to ensure excision of ty-APH. Freshly egressed parasites were grown for 30hrs on fresh HFF monolayers. Egress was stimulated by serum free media containing 3µM A23187, 500µM propranolol or 0.06% DMSO as a control at 37°C/8mins prior to fixation. Cells were labelled with GAP45 and the proportion of egressed versus non-egressed vacuoles was calculated by counting 100 vacuoles in triplicate for three independent experiments. For the ty-APH-cKO the average number of vacuoles with parasites expressing YFP and non-YFP which had egressed was determined by counting 100 vacuoles in duplicate for three independent experiments.

**Invasion assay**

R59022 and propranolol invasion assays were completed by pre-incubating extracellular parasites with inhibitors at room temperature for 30mins at concentrations consistent with other R59022 (Nobe et al., 2004) and propranolol (Baron & Malhotra, 2002) inhibition studies. Parasites were subsequently inoculated on HFF monolayers and allowed to invade for 1hr at 37°C. Monolayers were subsequently fixed and invasion was monitored by standard red/green invasion assays (Rugarabamu et al., 2015).

For the ty-APH-Lox and ty-APH-cKO strains, invasion assays were performed using the non-YFP expressing parasites as an internal control. Extracellular ty-APH-Lox parasites were treated with 50 mM rapamycin for 4hrs prior to their inoculation on HFF monolayers. Forty-eight hours later, freshly egressed parasites were passed on HFF monolayers and allowed to invade for 60mins at 37°C, prior to washing of monolayers. Cells were subsequently grown for a further 24hrs to determine the ratio of YFP (ty-APH-cKO): non-YFP (ty-APH-Lox) parasites. Parasites were stained with α-GAP45 antibodies and the ratio between YFP and non-YFP parasite-containing vacuoles was calculated. Results represent mean +/- standard deviation of 50 parasites counted in triplicate from six biological replicates.

**Gliding assays**
Freshly egressed parasites were washed in serum free media and inoculated onto gelatin-coated coverslips. 2% ethanol, 500/250/200 µM propranolol, or 30 µM R59022 was added to cells prior to incubation at 37°C/30mins. Media was removed and parasites were fixed with PAF/Glu prior to non-permeabilising IFA and staining with anti-SAG1 antibodies.

Plaque assays
Freshly egressed parasites were inoculated on HFF monolayers +/- anhydrotetracycline (ATc). Parasites were grown for seven days prior to fixation with PAF/Glu (as outlined previously) and subsequent staining with crystal violet.

Recombinant protein purification
N-terminal GST-fusion proteins of full length TgAPH and the TgAPH PH domain, as well as Spo20pWT/Mut were cloned using BamHI/XhoI sites of the pGEX-4T plasmid using primers outlined in Table S2. Polyclonal antibodies to TgAPH were obtained by immunizing rabbits with the recombinant full-length protein purified from E. coli. For recombinant PfAPH expression, the gene was first codon optimised by Genescript for expression in E. coli with a myc-tag added after the N-terminal 23 residues, before cloning into the pGEX-4T3 plasmid as outlined above. Codon optimised coding sequence is shown in upper case and the myc-tag in bold:

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gaattcgetaacaaaATGGGCAAACACGTGCCGTATAACTGCTATGAACGCTGGGG
CAAACGCACCCTGGATGGCCTGCTGATGCATGAGCAGAAAGCTCATC
TCCGAGGAGGACCTGAACTATGATCCGCAGATGCCAACAGCGGCACCC
TGAGCGTGTGCTGCGAAAACGTATTATGAAAGCACCAGAAGGCAACAC
AACAAATATGATTATATTATTAACAAAGAACCAGTTGAAAAAACACAAAC
CATGAACCGAAAGTGTGATTGCAACCGGCCTGCCCAGCTACCTGATCC
CGACCCCGCGCAAATTTAATTTGAGCAGATGAAGAAGAGATGA
AGATGATGATGAAAAACTGAGCACCAGATGATGACGATAAAAAACTTAGC
GCACAAAGATATTAAAGATGAAAAATTCAGCAGTATCGCAAAAACCCTGA
CCAAAAATTGTGAAAAATTTAACCGCGATTCTTTCATGAAAACCGTGAAAGTG
ACCTGCAGCAAAAGATGCGAAAATGCTGGAAGTATGATGAAAACCTAGC
ATAGCGATGCAAATTTGAGCAGTATCGCAAAAATACCCGAG
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Calcium flux assay

Freshly egressed GCaMP6-expressing tachyzoites (Borges-Pereira et al., 2015) were collected, pretreated with 0.2µM Cytochalasin D in serum-free media for 30 mins and attached by centrifugation to poly-l-lysine coated Fluorodish plates (World Precision Instruments). Parasites were imaged using an Eclipse Ti Inverted microscope and acquisition was performed at room temperature. Fluorescence intensity was recorded after the addition of DMSO, 500µM propranolol, 2% ethanol or 3µM calcium ionophore A23187 and the corrected total fluorescence (CTF) was calculated.

CTF = Integrated Density – (Area of selected field * Mean fluorescence of background read).
Supplemental references


