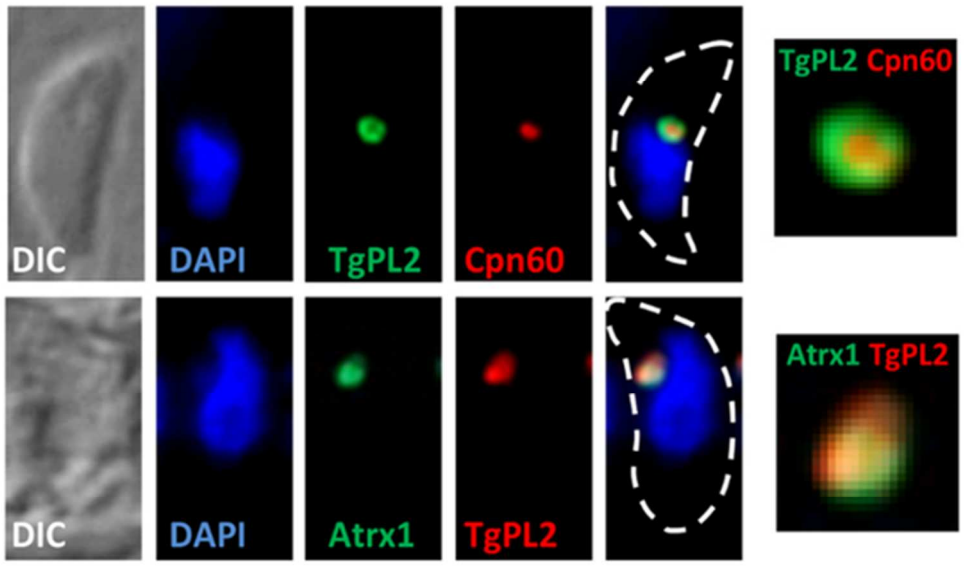

Graphical Abstract. Toxoplasma gondii is a parasitic protist containing an organelle named the apicoplast, a non-photosynthetic plastid harboring essential metabolic pathways. We are describing a novel phospholipase, localizing to the apicoplast and involved in maintaining lipids homeostasis in the organelle.

49x30mm (300 x 300 DPI)

TgPL2, a patatin-like phospholipase domain-containing protein, is involved in the maintenance of apicoplast lipids homeostasis in *Toxoplasma*

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Running title

A phospholipase important for apicoplast homeostasis

Keywords

Toxoplasma, Apicomplexa, apicoplast, phospholipase, patatin, lipids

Summary

Patatin-like phospholipases are involved in numerous cellular functions, including lipid metabolism and membranes remodeling. The patatin-like catalytic domain, whose phospholipase activity relies on a serine-aspartate dyad and an anion binding box, is widely spread among prokaryotes and eukaryotes. We describe TgPL2, a novel patatin-like phospholipase domain-containing protein from the parasitic protist *Toxoplasma gondii*. TgPL2 is a large protein, in which the key motifs for enzymatic activity are conserved in the patatin-like domain. Using immunofluorescent assays and immunoelectron microscopy analysis, we have shown that TgPL2 localizes to the apicoplast, a non-photosynthetic plastid found in most apicomplexan parasites. This plastid hosts several important biosynthetic pathways, which makes it an attractive organelle for identifying new potential drug targets. We thus addressed TgPL2 function by generating a conditional knockdown mutant and demonstrated it has an essential contribution for maintaining the integrity of the plastid. In absence of TgPL2, the organelle is rapidly lost and remaining apicoplasts appear enlarged, with an abnormal accumulation of membranous structures, suggesting a defect in lipids homeostasis. More precisely, analyses of lipid content upon TgPL2 depletion suggest this protein is important for maintaining levels of apicoplast-generated fatty acids, and also regulating phosphatidylcholine and lysophosphatidylcholine levels in the parasite.

Introduction

The phylum Apicomplexa comprises single-celled eukaryotic parasites of importance for human and animal health. These include *Toxoplasma gondii,* an opportunistic pathogen responsible for lethal complications in case of immune deficiencies or congenital toxoplasmosis, and *Plasmodium falciparum,* the deadly agent of malaria. The prevalence of these diseases and the emergence of parasitic drug resistances emphasize the need to identify novel chemotherapeutic targets. These protists were formerly a photosynthetic lineage that turned parasitic through a complex evolutionary history, shaped by tight symbiotic or parasitic interactions between different organisms (van Dooren and Striepen, 2013). One organelle, called the apicoplast, has been particularly intimately linked to Apicomplexa throughout the course of evolution. The apicoplast arose from secondary endosymbiosis: the eukaryotic ancestor of Apicomplexa engulfed and retained a eukaryotic alga that was already containing a plastid obtained by primary endosymbiosis of a cyanobacterium-like prokaryote (McFadden *et al.*, 1996). The organelle is surrounded by four membranes: the inner two are similar to those of the chloroplast, the next membrane (the periplastid membrane) likely arose from the plasma membrane of the algal cell, and the outermost membrane may have originated from the vacuole membrane formed during the last engulfment (van Dooren and Striepen, 2013). Establishment of the endosymbiont as an organelle has allowed bacterial and plastid genes transfer to the host nucleus, therefore many apicoplast proteins and pathways have prokaryote and plant-like characteristics, making them interesting potential drug targets (McFadden and Roos, 1999). Pharmacological and genetic manipulations in *T. gondii* and different *Plasmodium* life-stages have shown the importance of this non-photosynthetic organelle for long term viability of these parasites (Fichera and Roos, 1997). The organelle is a metabolic nexus (Sheiner *et al.*, 2013), as it hosts several vital pathways, including type II fatty acid (FA) (Vaughan *et al.*, 2009) and isoprenoid precursors biosynthesis (Yeh and DeRisi, 2011).

Tachyzoites are highly invasive forms of *T. gondii* that proliferate within a parasitophorous vacuole (PV), and are responsible for the acute form of toxoplasmosis. Parasite multiplication inside its host cells must coincide with a substantial biogenesis of membranes, which rely on both autonomous lipids synthesis and salvage mechanisms (Ramakrishnan *et al.*, 2013). Apicoplasts contain a type II fatty acid synthesis (FAS) complex whose products, elongated and incorporated into phospholipids mainly in the endoplasmic reticulum (ER) (Ramakrishnan *et al.*, 2012; Amiar *et al.*, 2016), are likely reimported for use in plastid membranes (Mazumdar *et al.*, 2006) through membrane interactions between the two organelles (Tomova *et al.*, 2009). The organelle possesses its own small genome (Wilson *et al.*, 1996), but the vast majority of apicoplast proteins are encoded by nuclear genes. Proteins localizing to the lumen of the organelle thus bear a N-terminal bipartite sequence required for import into the plastid (Waller *et al.*, 2000; DeRocher *et al.*, 2000). Vesicles enriched in lipids such as phosphatidylinositol 3-phosphate (Tawk *et al.*, 2011), mediate import of a subset of apicoplast membrane proteins lacking the canonical plastid-targeting motif (Bouchut *et al.*, 2014) and have been proposed to also provide lipid transport to the organelle. Several studies have highlighted the ability of intracellular tachyzoites to salvage from the host the essential lipids for which they lack the enzymatic machinery (Coppens, 2013) and lipidomic analysis of *P. falciparum* apicoplasts indicated the consistent use of host precursors for plastid biogenesis (Botté *et al.*, 2013). The scavenging of host lipids involves multiple pathways including the incorporation of low density lipoprotein-complexed cholesterol from host endo-lysosomes (Coppens *et al.*, 2000), interaction between host organelles and PV (Sinai *et al.*, 1997), acquisition through Rab vesicles (Robibaro *et al.*, 2002; Romano *et al.*, 2013), as well as compartmentalization into lipid bodies (Charron and Sibley, 2002).

In addition to the *de novo* synthesis and uptake of lipids, their recycling is also of crucial importance for membrane homeostasis. It requires the activity of lipid-hydrolyzing enzymes that promote the remodeling of membrane glycerolipids (e.g. phospholipids) (Renne *et al.*, 2015). Enzymes from the phospholipase A (PLA) superfamily specifically catalyze the hydrolysis at the sn-1 (PLA_1) and/or sn-2 (PLA_2) position of phospholipids to release free FAs and lysophospholipids (Burke and Dennis, 2009). Patatins are glycosylated proteins found in the tubers of potato (*Solanum tuberosum*) (Park *et al.*, 1983). These enzymes have a serine-aspartate catalytic dyad (Rydel *et al.*, 2003) and an active site that is structurally similar to cytosolic PLA_2; moreover, they have been shown to display a PLA_2-like activity (Senda *et al.*, 1996; Hirschberg *et al.*, 2001). However, they seem to have a broader substrate specificity: they can hydrolyze diverse substrates independently to the position of the cleaved ester bond, the headgroup of phospholipids, or the type of glycerolipid (Mansfeld, 2009). The patatin-like catalytic domain is widespread among prokaryotes and eukaryotes and is involved in numerous different cellular functions including fat metabolism and membrane remodeling (Kienesberger *et al.*, 2008; Scherer *et al.*, 2010). The genome of *T. gondii* codes for six proteins predicted to bear a patatin-like domain. TgPL1, the first patatin-like protein characterized in this parasite, does not seem to be an active phospholipase and localizes to cytosolic vesicles (Mordue *et al.*, 2007). However, it is secreted in the PV upon immune stresses and is important for parasite survival in activated macrophages (Mordue *et al.*, 2007; Tobin and Knoll, 2012). It has also been suggested to play a role in the maintenance of *T. gondii* chronic infection (Tobin Magle *et al.*, 2014).

In the present study, we report the characterization of a novel *Toxoplasma* patatin-like protein. It localizes to the periphery of the apicoplast, and its depletion showed it is an essential contributor to the lipid homeostasis of the organelle.

Results

TGME49_231370 encodes for a putative patatin-like phospholipase

Homology searches for genes encoding proteins with patatin-like domains in the ToxoDB database (wwwToxoDB.org) reveal the presence of six paralogues in the genome of *T. gondii*. One of them, TGME49_231370, encodes for a 2904 amino acids-long protein that contains a patatin-like motif protein within residues 2032-2500 and a putative transmembrane domain at residues 212-234 (**Fig. 1A**). Searches revealed TGME49_231370 has potential homologues in other Apicomplexa, including *Plasmodium* species (**Fig. S1A, C, D**). Intriguingly, however, in these homologues, even for *Neospora caninum*, a close relative of *T. gondii*, the N-terminal part is shorter and the protein starts with a predicted signal peptide corresponding to the transmembrane domain of the *Toxoplasma* protein (**Fig. S1B**). On the other hand, all sequenced strains of *T. gondii*, as well as its closest extant coccidian relative, *Hammondia hammondi,* were predicted with the same internal transmembrane domain. To verify the predicted annotation, we thus performed cDNA amplification and sequencing around the region coding for the transmembrane domain, to look for the presence of a putative alternative initiation sites immediately upstream of this region. We confirmed no start codon exists in this region, suggesting the predicted ToxoDB annotation is correct.

Sequence alignment and biochemical analysis indicate that patatin-related proteins are similar to mammalian calcium-independent phospholipases ($iPLA_2$) (Balsinde and Balboa, 2005). Hydrolase activity of these enzymes relies on the catalytic center comprising a serine-aspartate catalytic dyad. These key residues for the enzymatic process are contained in an esterase box GXSXG and in a specific DXG/A sequence, respectively (Rydel *et al.*, 2003). In addition, an anion-binding element consisting of a glycine-rich motif XGGGXR/KG cooperates in the enzymatic activity and might be involved in the attachment of negatively-charged substrates such as phospholipids (Dessen *et al.*, 1999). We aligned the predicted

patatin-like domain of TGME49_231370 with those from the original *S. tuberosum* patatin and a human $iPLA_2$ (**Fig. 1B**). While the key catalytic serine was found missing in previously characterized TgPL1 (Mordue *et al.*, 2007), both the catalytic dyad and the anion binding box motifs are well conserved in TGME49_231370. Moreover, comparative modeling of TGME49_231370 patatin-like domain with *S. cardiophyllum* patatin 3D structure as a template (Rydel *et al.*, 2003), reveals it is likely to adopt a very similar fold (a modified α/β hydrolase fold with a central β-sheet sandwiched by α-helices), and that predicted active site residues are on conserved structural domains (**Fig. S2**). We tried different strategies to express the patatin domain of TGME49_231370 as a recombinant protein, but it was systematically insoluble, and our attempts to obtain a refolded soluble protein were unsuccessful, which prevented us from measuring an *in vitro* phospholipase activity. However, the conserved catalytic site residues and overall domain fold strongly suggest TGME49_231370 could be an active patatin. Therefore, we named this patatin domain-containing protein TgPL2.

TgPL2 is found primarily at the periphery of the apicoplast

The localization of TgPL2 was explored by epitope-tagging of the native protein. This was achieved in the TATi1-Ku80Δ cell line, which favors homologous recombination and would allow transactivation of a Tet-operator-modified promoter we would later use for generating a conditional mutant (Fox *et al.*, 2009; Sheiner *et al.*, 2011). A sequence coding for a C-terminal triple haemagglutinin (HA) epitope tag was inserted at the endogenous *TgPL2* locus by single homologous recombination (**Fig. 2A**). Transfected TATi1-Ku80Δ parasites were subjected to chloramphenicol selection and clones were isolated and checked for correct integration of the plasmid by PCR (**Fig. S3**). Anti-HA antibodies were used to detect tagged TgPL2 by immunoblot (**Fig. 2B**) and revealed a main product at about the expected molecular mass (315 kDa). It should be noted that smaller products, possibly resulting from degradation

or maturation processes, and at least two higher molecular mass products, which might reflect post-translational modifications, were also detected.

Immunofluorescence analyses (IFA) on intracellular TgPL2HA-expressing tachyzoites revealed an apicoplast localization for this protein (**Fig. 2C**). The staining pattern of TgPL2 encompasses the apicoplast luminal marker TgCpn60 (Agrawal *et al.*, 2009) and it co-localizes extensively with the plastid membrane thioredoxin TgAtrx1 (DeRocher *et al.*, 2008). This suggests TgPL2 is an apicoplast protein with a peripheral localization. In addition, TgPL2 was sometimes (in approximatively 10% of parasites) found to associate with punctate vesicles within the cytosol (**Fig. S4**). To get a better insight into TgPL2 localization, we used immunoelectron microscopy, which confirmed the association of the protein with the apicoplast periphery (although we could not determine which of the apicoplast membranes and/or inter-membrane spaces it is associated to, **Fig. 2D**).

To further determine if TgPL2 internal hydrophobic peptide is a transmembrane anchor, we used TX-114 phase partitioning analysis of C-terminally tagged TgPL2HA. We found most of the products detected by an anti-HA immunoblot are in the aqueous phase, suggesting these forms of TgPL2 do not contain a transmembrane domain (**Fig. S5B**). This indicates the internal hydrophobic domain of TgPL2 might be cleaved and is potentially acting as a signal peptide instead of a transmembrane domain.

Knock-down of TgPL2 leads to apicoplast loss and to a delayed death phenotype

To investigate the function of TgPL2, we generated an inducible knock-down mutant in the TgPL2HA-expressing TATi1-Ku80Δ background (Sheiner *et al.*, 2011). The strategy is based on the replacement of the endogenous promoter by an inducible-Tet07Sag4 promoter through a single homologous recombination at the *TgPL2* locus, to yield a conditional TgPL2 knock-down cell line (cKd-TgPL2HA) (**Fig. 3A**). In this cell line, the addition of anhydrotetracycline (ATc) can repress *TgPL2* transcription through inhibition of the TATi1

transactivator and subsequently block the Tet-operator (Meissner *et al.*, 2002). Several pyrimethamine-isolated clones were selected after diagnostic PCR for correct genomic integration (**Fig. 3B**). Subsequent analyses were performed on two independent clones, which were found to behave similarly, thus only one will be described here.

Transgenic parasites were grown for various periods of time in presence of ATc and protein down-regulation was evaluated. By IFA on cKd-TgPL2HA parasites, we could check that after promoter replacement TgPL2HA localizes to the apicoplast, like when expressed with its native promoter, and disappeared from the organelle after only one day of ATc treatment (**Fig. 3C**). Immunoblot analyses of cKd-TgPL2HA parasites (**Fig. 3D**) still showed a complex profile for HA-tagged TgPL2, and a slight overexpression of the protein after promoter replacement. Time course analysis confirmed TgPL2 is efficiently depleted after two days of drug treatment in cKd-TgPL2HA parasites (**Fig. 3D**).

The phenotypic consequences of TgPL2 depletion were first assessed by plaque assays. Confluent HFF monolayers were infected with the parental or the mutated cell lines in presence of ATc and the size of lysis plaques, resulting from multiple parasite lytic cycles, was measured after seven days (**Fig. 4A**). Non-treated cKd-TgPL2HA parasites formed plaques similar in size to those of the control, indicating that the promoter change did not alter TgPL2 function. ATc-dependent depletion of the protein, on the other hand, severely disturbed the lytic cycle. Because TgPL2 localizes to the apicoplast, we used IFA to investigate the fate of the organelle after depletion of the protein, using luminal marker TgCpn60, and we observed an almost complete loss of the plastid after four days of ATc incubation (**Fig. 4B**). Time course quantifications of the organelle loss (**Fig. 4C**), revealed a progressive disappearance of the plastid, with less than 10% of the parasites retaining the organelle after four days of treatment. Apicoplast dysfunctions are typically leading to a "delayed death" phenotype, by affecting *T. gondii* or *P. falciparum* intracellular growth only

in the next generation following the organelle loss (McFadden and Roos, 1999). Accordingly, we observed a replication phenotype (**Fig. 4D**), for reinvading cKd-TgPL2HA parasites pretreated for three days with ATc (time required for losing most of the apicoplasts). Noticeably, protein markers from other organelles such as micronemes, mitochondria, rhoptries, dense granules and the subpellicular inner membrane complex seemed to be normal in appearance and localization when checked by IFA upon TgPL2 depletion (**Fig. S6**), suggesting an essential and specific function of TgPL2 for maintaining the integrity of the apicoplast.

Active site serine is important for TgPL2 functions

In order to investigate the functional importance of TgPL2 putative phospholipase activity, we used CRISPR/Cas9 genome editing to mutate this amino acid in the native protein (Sidik *et al.*, 2014; Shen *et al.*, 2014). Parasites were transiently transfected with a plasmid to express a YFP-tagged Cas9 and a guide RNA, and a donor sequence targeting the codon corresponding to the predicted active site serine (serine 2071) to replace it either by an alanine, or one to induce a silent mutation (a negative control to assess for potential unspecific Cas9 effects) (**Fig. 5A**). 24 hours post-transfection, Cas9-YFP positive parasites were enriched using fluorescence-activated cell sorting and seeded onto new HFFs for an extra 24 hours of growth, before assessing apicoplast loss (**Fig. 5B**). All three independent transfections we performed showed a significant increase in apicoplast loss in the parasites that received the S20171A donor construct compared with the S2071S control (**Fig. 5C**). This indicates the putative active site serine is important for TgPL2 function at the apicoplast, and albeit it is not a direct proof TgPL2 is a phospholipase, it strongly suggests the enzymatic activity is important for this function.

TgPL2-deprived tachyzoites accumulate membranes surrounding the apicoplast

To gain insights into the function TgPL2, we sought to analyze apicoplasts morphology in parasites upon depletion of the protein, but before complete loss of the organelle. We thus treated cKd-TgPL2HA tachyzoites for two days with ATc and analyzed by IFA the staining pattern of peripheral apicoplast-associated TgAtrx1 protein and the luminal TgCpn60 marker. During the cell cycle, the shape of the apicoplast evolves from ellipsoid in G1/S phase, to elongated dumbbell or U-shaped forms prior to parasite division (Striepen *et al.*, 2000). In contrast with the TgPL2HA parental control cell line treated with ATc, few elongating apicoplasts were observed in the TgPL2-depleted parasites (**Fig. 6A**). Instead, not only the majority of the organelles had an ellipsoidal shape, but with a bulkier appearance compared with similarly-shaped organelles of the control cell line, as illustrated by the apparently enlarged peripheral TgAtrx1 labelling (**Fig. 6B**). Comparable results were obtained when using TgFtsH1, another peripheral apicoplast marker (Karnataki *et al.*, 2007) (**Fig. S7**).

We also analyzed by electron microscopy the ultrastructure of the apicoplasts found in cKd-TgPL2HA tachyzoites treated in the same conditions (**Fig. 7**). The luminal contents of the organelle appeared generally normal. On the other hand, the plastid, which is usually bound by four membranes, was surrounded by multiple membranous whorls, separated by large interspaces (**Fig. 7**). Overall, our results suggest there is an uncontrolled accumulation of membrane material in TgPL2 mutants, severely perturbing the homeostasis and the function of the organelle, which could subsequently lead to the delayed death phenotype. Thus, these data demonstrate TgPL2 has a specialized and important function for the apicoplast.

Specific lipid alterations in TgPL2-depleted parasites

TgPL2 is a putative phospholipase localizing to the apicoplast, which is known to be a major center for lipids biosynthesis. We thus performed a comprehensive lipidomic analysis, at different time points during TgPL2 depletion to evaluate the impact of TgPL2 loss on parasite lipids. cKd-TgPL2HA parasites were labelled with U-^{13}C glucose (uniformly-^{13}C labelled glucose), and analysis of mass isopotomer distribution was performed by gas chromatography-mass spectrometry (GC-MS), to evaluate *de novo* synthesis of FAs in the apicoplast. cKd-TgPL2HA tachyzoites treated for two days with ATc showed a small, yet significant, decrease of U-^{13}C glucose incorporation in short chain FAs (C14:0, C16:0, C16:1, C18:1) (**Fig. 8A**). This effect after two days of ATc treatment is rather limited, while U-^{13}C glucose incorporation into most FAs (C14:0, C16:0, C16:1, C18:1, C20:0, C20:1) was considerably decreased in parasites treated with ATc for three days (**Fig. 8B**). On the other hand, labelling with U-^{13}C acetate to detect its incorporation into FA chains by the ER-elongase machinery (Ramakrishnan *et al.*, 2015), showed a limited effect of TgPL2 depletion on elongation, with the parasites possibly relying on exogenous FA sources as a compensatory mechanism (**Fig. S8**). This confirms TgPL2 depletion affects primarily apicoplast function and has initially little impact on the synthesis of ER-derived FAs. Overall, these data are in accordance with the progressive loss of the apicoplast following TgPL2 depletion (**Fig. 4C**).

Tachyzoites treated with ATc for two days, which have lost TgPL2 but retain a fair proportion of organelles, and seem to experience rather limited consequences on their global FA synthesis profiles (**Fig. 8A**), were thus chosen for subsequent analyses to identify the effects of TgPL2 depletion on specific lipid classes. Upon TgPL2 depletion, the analysis and quantification of lipid species according to parasite cell numbers (nanomoles of lipids for 1.10^7 parasites/sample), showed an overall decrease in the amount most major phospholipid classes PC, PE and PI (**Fig. 9A**). This reduction in major phospholipid classes would be

consistent with a reduced fatty acid synthesis from FASII, subsequent to the loss of apicoplasts we previously observed and quantified after TgPL2 depletion (**Fig. 8A, B**), which is likely impacting phospholipid synthesis as previously described (Amiar *et al.*, 2016). Membrane homeostasis is characterized by its lipid composition, i.e. the relative ratio of each lipid class and molecular species (expressed in mol%), where the sum of each lipid class (in nanomoles) represents 100mol% of the total lipid composition. The overall membrane lipid composition should thus be similar in the case of an even decrease in the amount of phospholipids after TgPL2 depletion. Instead, we measured a specific decrease in lysophosphatidylcholine (LPC), while PC was found increased (**Fig. 9B**). These data suggest that PC is likely a substrate of TgPL2, which could use it to generate LPC, and subsequently free FAs. These could in turn be incorporated into other phospholipids classes such as PC, PE and PI, major components of the apicoplast membranes (Botté *et al.*, 2013) that seem to be the phospholipids which are most impacted by TgPL2 depletion. Overall, our lipidomic analyses provide evidence that TgPL2 seems to be an active phospholipase whose activity is important for phospholipids biosynthesis in the apicoplast.

Discussion

TgPL2 is the second patatin-related protein to be described in *Toxoplasma*. However, it is markedly different in function and localization from previously characterized TgPL1 (Mordue *et al.*, 2007). First, TgPL1 lacks the conserved serine that would make it a catalytically active enzyme (Mordue *et al.*, 2007). On the contrary, TgPL2 patatin-like domain is predicted to have conserved key determinants for functional enzymatic activity, although we did not manage to obtain a catalytically active recombinant domain. This domain was largely insoluble in a number of different experimental conditions and heterologous expression systems, and biochemical assays on immunoprecipitated TgPL2 from parasites extracts did not yield any measurable PLA_2-like activity either. Although we cannot exclude TgPL2 may not function as a phospholipase, the phenotype induced by the loss of TgPL2 at the cellular level fits well with a role in membrane homeostasis and lipid metabolism, which patatins and related active enzymes are known to be involved in (Kienesberger *et al.*, 2008; Scherer *et al.*, 2010). Moreover, targeted mutation of the putative active site serine residue recapitulated the phenotype induced by the depletion of the protein, indicating enzymatic activity is likely important for TgPL2 function. Finally, our lipidomic analyses, showing an accumulation of PC and reduction of LPC upon TgPL2 depletion, suggest the protein could be able to exert an efficient acyl hydrolase activity on this phospholipid *in vivo*.

Second, TgPL1 has been shown to be secreted to the PV and suggested to play a role in regulating the host immune response, both in the context of acute (Mordue *et al.*, 2007; Tobin and Knoll, 2012) and chronic infections (Tobin Magle *et al.*, 2014). In contrast, TgPL2 is clearly a resident of the apicoplast periphery, as demonstrated by our IFA and immuno-electron microscopy analyses. Most of the apicoplast-resident proteins are encoded in the nucleus, and therefore are translated in the cytosol from where they must be imported to the apicoplast lumen, to one of its four membranes, or to the inter-membrane spaces. To do so,

several apicoplast-resident proteins contain a topogenic bipartite sequence at their N-termini. It comprises a signal peptide, which allows the entry into the endoplasmic reticulum, followed by a transit peptide which is similar to the one found in proteins delivered to chloroplasts (Waller *et al.*, 1998). We have found TgPL2 is in the vicinity of several apicoplast membranes, despite the lack of a canonical bipartite targeting signal in its predicted amino acids sequence. However, it possesses a putative hydrophobic transmembrane region within its N-terminal part that might serve as an internal signal peptide. In *Toxoplasma*, several *bona fide* apicoplast membrane-associated proteins lacking canonical targeting signals have been identified, such as TgFtsH1 (Karnataki *et al.*, 2007) and TgAtrx1 (DeRocher *et al.*, 2008). Instead of a bipartite leader sequence, these contain an internal hydrophobic signal anchor presumably required for apicoplast targeting (Lim *et al.*, 2009). Interestingly, analysis of the C-terminally tagged TgPL2 profile by immunoblot reveals, besides the main ~300 kDa protein, several products of lower molecular mass which could reflect N-terminally processed products, and these were found to be essentially in the aqueous phase in Triton X-114 phase partitioning. Overall, this suggests the hydrophobic domain could serve as a signal peptide instead of a transmembrane anchor.

TgPL2 depletion led to an impairment of parasite growth *in vitro*, with the characteristic features of a delayed death, occurring only after parasite reinvasion. This was likely due to the progressive loss of the apicoplast observed in mutant parasites, and consistent with the localization of TgPL2 to the periphery of this organelle. Very strikingly, in parasites that were still retaining the organelle, the absence of TgPL2 led to an accumulation of multilamellar structures around the apicoplast, which seemed to contain otherwise normal luminal contents. There could be several explanations for this phenotype.

Lipids are commonly known for their structural role, but there are increasing evidences they are also used in a wide range of cellular signaling pathways. For instance in

plants, patatin phospholipases are known to modulate levels of oxylipin and jasmonic acid that act as second messengers for defense signaling pathways (Canonne *et al.*, 2011). Interestingly, patatin-related enzymes involved in plant signal transduction are localized in the cytosol, but also partially bound to endomembranes such as chloroplast (Holk *et al.*, 2002). It is thus also possible that depletion of TgPL2 has been impacting apicoplast membrane homeostasis through perturbation of regulatory lipids, which might govern vesicle trafficking to the organelle or local regulation of membranes. Interestingly, the phospholipids impacted by the disruption of TgPL2 are similar to those reduced by the disruption of TgATS1, an apicoplast acyltransferase that is involved in the bulk phospholipid synthesis in *T. gondii* (Amiar *et al.*, 2016), but also classically implicated in the biogenesis of lysophosphatidic acid and phosphatidic acid, which are well-known lipid precursors as well as strong signal transducers (Bullen and Soldati-Favre, 2016).

Assuming the apicoplast-localized TgPL2 is an active phospholipase, its primary function might also be to regulate the membrane homeostasis of the organelle by directly participating in the recycling/reshuffling of local lipids that are trafficked from and towards the apicoplast. During parasite division, the apicoplast replicates first by elongating, and then by dividing (Striepen *et al.*, 2000). Although this process likely necessitates a considerable amount of membranes, very little is known about the source of the material required. The apicoplast being a major hub for the production of lipids building blocks (Mazumdar *et al.*, 2006; Amiar *et al.*, 2016), *de novo* biosynthesis from the organelle itself would be one obvious possibility. Other potential membrane sources are the vesicles trafficking to the apicoplast to bring nucleus-encoded proteins (Bouchut *et al.*, 2014), which will subsequently fuse with the outermost membrane to deliver their content. In any case, the amount of lipids required for building the four-membrane bound compartment has to be tightly regulated, especially during its biogenesis.

PLA_2-like phospholipases are usually cleaving phospholipids at the sn-2 position to generate lysophospholipids and FAs. By doing so, they participate in lipid turnover and membrane remodeling. The membrane whorls around the apicoplast are reminiscent of what can be seen in the context of phospholipidosis, a disorder observed in animals and humans, which is characterized by an accumulation of concentric lamellar bodies within cells and an intralysosomal accumulation of phospholipids (Anderson and Borlak, 2006). Interestingly, impairment of a lysosomal PLA_2 activity has been shown to lead to phospholipidosis in mouse (Hiraoka *et al.*, 2006) and illustrates the fact that a default in phospholipids turnover by a single phospholipase can lead to a considerable accumulation of membranes.

Our lipidomic analyses highlighted a specific increase in the relative accumulation of PC upon TgPL2 depletion, and a concomitant decrease in LPC. No such decrease in the LPC:PC ratio has ever been reported in the previous studies on mutants affected for apicoplast lipid synthesis (Bisanz *et al.*, 2006; Mazumdar *et al.*, 2006; Ramakrishnan *et al.*, 2012; Botté *et al.*, 2013; Lindner *et al.*, 2014; Ramakrishnan *et al.*, 2015; Amiar *et al.*, 2016; Shears *et al.*, 2017), though it should be noted only a few measured LPC levels in the apicoplast. This suggests that this effect is specific to TgPL2 knockdown leading to a decrease of LPC production, rather than general defect in apicoplast biogenesis. Because the LPC and free FAs generated from PC by TgPL2 would then in turn be available for incorporation into newly generated phospholipids, this would participate in the general dynamics of phospholipids biosynthesis. Indeed, the global parasite lipid composition reflects a combination of both scavenged and *de novo* synthesized FAs (Coppens, 2013). With this in mind, TgPL2 could thus participate to the shuffling/reassembly of those FAs arising from both sources by providing free FAs from existing lipids and lysophospholipids as acceptors of FAs from different sources. The molecular composition of LPC reflects this possibility, as after two days of TgPL2 depletion, it seems to contain longer FAs that are more likely to be scavenged

than synthesized such as C24:0, whilst typical apicoplast-generated FAs were lacking (**Fig. S9**).

Although TgPL2 depletion impacted the general production of PC, the detrimental effects we observed manifested primarily on the apicoplast, and not on other membrane-bound organelles. Besides the striking accumulation of membranes around the plastid revealed by our ultrastructural studies, the paucity of elongated apicoplasts in remaining organelles after TgPL2 depletion suggested their division was compromised. One might speculate this apicoplast-related TgPL2 function could be conserved in other Apicomplexa possessing homologues of this enzyme. Consistent with this, no TgPL2 homologue can be found in *Cryptosporidium* and gregarine species, which are apicoplast-lacking apicomplexan lineages (McFadden *et al.*, 1996).

Lipids are essential to cellular architecture and function. Although the contribution of the apicoplast to lipid metabolism has been extensively studied since the organelle was identified twenty years ago, there are still major conundrums that remain. Among them are, for example, the identification of all possible sources of FAs within the organelle, and also how lipids would be transported across membranes, especially in the context of cooperation between the apicoplast and the ER for phospholipids synthesis. It appears lipid metabolism in apicomplexan parasites is quite complex and dynamic, with a variety of potentially redundant pathways. In this context, the specific and essential contribution of TgPL2 for maintaining apicoplast lipid homeostasis might illustrate this flexibility, while also providing interesting new possibilities for interfering with these parasites.

Experimental procedures

Host cells and T. gondii culture

T. gondii tachyzoites were cultured in confluent human foreskin fibroblasts (HFF, American Type Culture Collection, CRL 1634) grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 5% (v/v) decomplemented fetal calf serum, 2 mM glutamine and a cocktail of 100 µg/ml penicillin-streptomycin. Conditional knock-down of generated constructs was performed with 1 µg/ml ATc.

cDNA sequencing

RNA from *T. gondii* RH strain was isolated using NucleoSpin RNA II kit (Macherey-Nagel) and cDNA was obtained and randomly amplified by RT-PCR with Superscript III First-Strand Synthesis kit (Invitrogen). Resulting cDNAs were amplified with the Taq Polymerase (New England BioLabs) using primers ML2504 (sequences of primers used in this study are listed in Table S1) and ML2505 to amplify a region on the 5' of *TGME49_231370* encompassing the fragment coding for the putative transmembrane domain. Subsequent semi-nested PCRs were performed using primers ML2410 and ML2505. Fragments were cloned in the TOPO-TA vector (Invitrogen) and sent for sequencing using SP6 primer (Eurofins MWG).

Phylogenetic analysis

Sequence alignments were performed using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm of the Geneious software suite (http://www.geneious.com). The phylogenetic tree was built using the Neighbor-Joining method included in the same software suite.

Modelling of the patatin domain

The patatin-like domain of TGME49_231370 (amino acids 2028-2524) was modelled using the UCSF Chimera software (https://www.cgl.ucsf.edu/chimera/), using the crystal structure of *S. cardiophyllum* patatin (PDB entry 4PK9) as a template.

Molecular cloning

The ligation independent strategy (Huynh and Carruthers, 2009) was used for the C-terminal HA_3-tagging at the endogenous locus of *TgPL2*. A 1.7 kb genomic fragment of *TgPL2* 3' locus was amplified by PCR with the Phusion polymerase (New England BioLabs) using primers ML1987 and ML1988 and inserted in frame with the HA_3 tag-coding sequence present in the pLIC-HA_3-CAT plasmid. The resulting vector, pLIC-TgPL2-HA_3-CAT, was linearized with MluI and 40 µg of DNA were transfected into the TATi1-Ku80Δ cell line to allow single homologous recombination. Primers ML1476 and ML2939 were used to check for correct integration by PCR.

The dihydrofolate reductase (DHFR)-TetO7Sag4-TgPL2 plasmid was designed to generate the cKd-TgPL2 cell line. A 1.6 kb genomic fragment of *TgPL2* 5' locus, starting from the ATG, was amplified by PCR with the Phusion polymerase from genomic DNA using primers ML2028 and ML2029. The resulting fragment was cloned with BglII/NotI into the DHFR-TetO7Sag4 vector (Morlon-Guyot *et al.*, 2014), downstream of the ATc-inducible TetO7Sag4 promoter. The TgPL2HA-expressing TATi1-Ku80Δ cell line was transfected with 50 µg of the NcoI-linearized DHFR-TetO7Sag4-TgPL2 plasmid to allow single homologous recombination. Positive clones were verified by PCR after genomic DNA extraction using the Wizard SV genomic DNA purification system (Promega). The native locus was detected with primers ML2076 and ML2077 and the recombined locus with primers ML2076 and ML1774, and ML1771 and ML2077.

Generation of transgenic parasites

Freshly egressed tachyzoites were resuspended in 800µL of cytomix (120 mM KCL, 10 mM K_2HPO4 pH 7.6, 5 mM MgCl2, 25 mM Hepes, 2 mM EDTA, 5 mM MgCl2) complemented with 0.15 mM CaCl2, 3 mM glutathione and 3 mM ATP prior to electroporation (50Ω, 25µF, 2.02 kV, on a BTX ECM 630 electroporator) with generated DNA constructs. Transgenic parasites were selected with 20 µM chloramphenicol, for those transfected with plasmids bearing chloramphenicol acetyltransferase (CAT), and with 1 µM pyrimethamine, for those transfected with plasmids bearing DHFR cassettes. Clones were isolated by limiting dilution in 96-well plates.

CRISPR-mediated site-directed mutatagenesis of TgPL2 putative active serine

The guide sequence was designed against the proximal region to the highly conserved active serine of TgPL2. It was synthesized as oligonucleotides ML2923 and ML2924, which were annealed and then introduced into BsaI site of the pSS013-CAS9NLS-YFP plasmid (kind gift from Boris Striepen and Michael Cipriano, University of Georgia, USA), resulting the plasmid pU6-sgTgPL2. This plasmid allows the expression of the guide RNA under the control of a U6 promoter, and also expresses a nuclear localized Cas9-YFP. This allows the isolation of transfected parasites by Fluorescence-activated cell sorting. Template sequences corresponding to the alanine conversion (S2071A) or the silent mutation version (S2071S) of the TgPL2 active site serine were synthesized by IDT (Iowa, U.S). This template sequence was then subcloned into the TOPO blunt vector (Thermo Fisher), and amplified by PCR with primers ML2921/ML2922. 40 µg of pU6-sgTgPL2 were co-transfected with 5 µg of each template sequence into RHKu80Δ parasites. 24 hours post-transfection, the fluorescent parasites were sorted with an ARIA IIIu flow cytometer (Becton Dickinson), and then seeded

onto a HFF monolayer cultivated for another 24 hours before analyzing the presence of the apicoplast by IFA, using an antibody against TgCpn60.

Triton X-114 phase partitioning

To assess TgPL2 membrane association, Triton X-114 partitioning was performed on protein extracts from 10^8 parasites as described previously (Seeber *et al.*, 1998).

Immunoblot analysis

Protein extracts from 10^7 freshly egressed tachyzoites kept with or without ATc for 1, 2 or 3 days were resuspended in SDS sample buffer and separated on a NuPAGE Novex 3-8% acrylamide gradient tris-acetate gel (Invitrogen). HA-tagged TgPL2 was detected with a rat anti-HA (Roche, clone 3F10) antibody (1/1000). A mouse anti-ROP5 (Leriche and Dubremetz, 1991) antibody (1/1,000) was used for loading control.

Plaque assays

Confluent monolayers of HFFs grown in 24-well plates were infected with freshly egressed tachyzoites. Cells were incubated with or without ATc for 7 days, fixed in cold methanol for 5 min and stained by Giemsa. Images were acquired with an Olympus MVX10 macro zoom microscope equipped with an Olympus XC50 camera. Plaque area measurements were performed with AxioVision software (Zeiss). Independent experiments were conducted three times and at least 20 plaques were measured per condition in each experiment.

Intracellular growth assays

HFFs grown on coverslips in 24-well plates were infected with freshly egressed tachyzoites pretreated for 72 h with ATc. Remaining extracellular parasites were washed out 2 h post-

infection. Infected cells were kept in presence of ATc and fixed 24 h later for 20 min with 4% (w/v) paraformaldehyde in PBS. The number of parasites per vacuole was scored. Independent experiments were conducted three times, and 200 vacuoles were counted for each condition.

Immunofluorescence microscopy

Intracellular tachyzoites grown on a monolayer of HFFs and incubated in the presence or absence of ATc for various periods of time were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS, permeabilized for 10 min with 0.3% (v/v) Triton X-100 in PBS, and blocked with 0.1% (w/v) bovine serum albumin (BSA) in PBS. The primary antibodies used for detection were rat anti-HA (1/500, Roche), mouse anti-V5 (1/500, Invitrogen), mouse anti-TgAtrx1 (1/1,000) and mouse anti-mitochondrial F1βATPase (1/1,000) (P. Bradley, unpublished data), anti-TgCPN60 (1/2,000) (Agrawal *et al.*, 2009), anti-AMA1 (Lamarque *et al.*, 2014) (1/10,000), anti-TgIMC1 (Mann and Beckers, 2001) (1/1,000), anti-GRA3 (Achbarou *et al.*, 1991) (1/500), and anti-RON4 (Besteiro *et al.*, 2009) (1/500) antibodies. For DNA staining, fixed cells were incubated for 5 min in a 4',6-diamidino-2-phenylindole (DAPI) solution at 1µg/ml. All images were acquired on a Zeiss Axio Imager Z2 epifluorescence microscope equipped with an ORCA-flash 4.0 camera (Hamamatsu) and driven by the ZEN software (Zeiss). For quantification, at least three independent replicates were performed and 200 parasites were counted for each condition.

Electron microscopy

For immunoelectron microscopy imaging of HA-tagged TgPL2, samples were treated as described previously (Lévêque *et al.*, 2016). Infected fibroblast monolayers were grown in

cell culture Petri dishes, fixed 1h at room temperature in PBS containing 4% (w/v) paraformaldehyde and 0,005% (w/v) glutaraldehyde, and then extracted with 1% (v/v) Triton X100 in PBS for 10 min. After washing, fresh fixative was applied and samples kept at 4°C. Samples were incubated 15 min with PBS 0,05M glycine, rinsed in PBS and scraped with a piece of Teflon in PBS containing 2% (w/v) gelatin. Cells were pelleted and embedded in 12% (w/v) gelatin, cut in small blocks (< 1mm) and infused 24h in 2.3M sucrose on a rotating wheel; blocks were mounted on specimen pins and frozen in liquid nitrogen. Cryo-sectioning was performed on a Leica UCT cryo-ultramicrotome, 80 nm cryosections were picked-up in a 1:1 mixture of 2.3M sucrose and 2% (w/v) methylcellulose in water and stored at 4°C. For on-grids immunodetection, grids were floated twice 2 min on water to remove methylcellulose/sucrose mixture, then blocked with 2% (w/v) skin-fish gelatin (SFG, Sigma) in PBS for 5 min. Successive incubation steps were performed on drops as follows : 1) rat monoclonal anti-HA (clone 3F10, Roche) in 2% (w/v) SFG, 2) rabbit polyclonal anti-rat IgG antibody (Sigma) in PBS 0.1% (w/v) BSA, 3) Protein A-gold (UMC) in PBS 0.1% (w/v) BSA. Four 2 min washes in PBS 0.1% (w/v) BSA were performed between steps. After Protein A, grids were washed 4 times 2 min. with PBS, fixed 5 min in 1% (w/v) glutaraldehyde in water then washed 6 times 2 min with water. Grids were then incubated with 2% (w/v) methylcellulose: 4% (w/v) uranyl acetate 9:1 for 15 min on ice in the dark, picked-up on a wire loop and air-dried.

Lipid extraction and analysis of T. gondii tachyzoites

Lipid extraction and analysis of tachyzoites was performed as follows: intracellular tachyzoites (4×10^8 cell equivalents) were extracted in chloroform/methanol/water (1:3:1, v/v/v containing 50 nmol laurate (C12:0) as internal standard) for 1 h at 4°C, with periodic sonication. For MS analysis, polar and apolar metabolites were separated by phase

partitioning. Total FA analysis of the lipophilic fraction was analyzed by GC-MS. In all cases, lipids were extracted from the same cell numbers and experiments were repeated for at least *n*=3 biological replicates.

Stable isotope labelling of T. gondii fatty acids and phospholipids

Stable isotope labelling using U-^{13}C-glucose or U-^{13}C-acetate (Cambridge Isotope Laboratories, USA), lipid extraction, and GC-MS analysis was performed as previously described (Ramakrishnan *et al.*, 2013). Briefly, freshly infected HFF were incubated in the presence or absence of ATc (0.5 μM, Sigma-Aldrich) in either glucose-free medium supplemented with 8 mM U-^{13}C- glucose or low-glucose DMEM, supplemented with 8 mM U-^{13}C- acetate. In TgPL2 repression experiments, the ^{13}C-carbon source was added simultaneously with ATc. Other supplements (glutamine, sodium bicarbonate, and foetal bovine serum) were added according to normal culture conditions. Parasites were harvested at indicated time points and metabolites extracted and partitioned as above. An aliquot of the lipid extract was derivatized on-line using MethPrep II (Alltech) and the resulting FA methyl esters were analyzed by GC-MS as previously described (Ramakrishnan *et al.*, 2013). All FAs were identified by comparison of retention time and mass spectra with authentic chemical standards and label incorporation was calculated as the percent of the metabolite pool containing one or more ^{13}C atoms, after correction for natural abundance and the amount of ^{13}C-carbon source in the culture medium (as determined by GC-MS analysis). Statistical differences were determined by measuring standard deviation and confirmed by Student's *t*-test ($p<0.05$).

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Author Contribution

S.B. and C.Y.B. conceived and designed the studies. M.F.L., L.B., Y.Y.B., H.M.N., M.G., C.Y.B. and S.B. performed the experiments and analyzed and interpreted the data. M.F.L. and S.B. drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. TGME49_231370 contains a patatin-like phospholipase domain.

(**A**) Schematic representation of TGME49_231370 showing transmembrane (TM) and patatin-like domains (amino acids numbering is shown on top). (**B**) Alignment of amino acid sequences from the phospholipase domains of *Solanum tuberosum* patatin (Genbank accession number: CAA81735), human calcium-independent phospholipase A2-γ isoform 1 (Genbank accession number: NP_056538) and *T. gondii* TgPL2 (ToxoDB accession number: TGME49_231370) using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm. Amino acids of the catalytic dyad (S, D) are marked by an arrowhead. Bars denote conserved motifs among patatin-like phospholipases. The degree of conservation for similar amino acids at each position is indicated by the key.

Figure 2. HA-tagged TgPL2 localizes to the periphery of the apicoplast.

(**A**) Schematic representation of the strategy to generate transgenic parasites expressing a C-terminal HA_3-tagged version of the endogenous TgPL2 in a TATi1-Ku80Δ background. Black arrows represent primers used for the diagnostic PCR presented in **Fig. S3** to confirm the integration of the plasmid. (**B**) Immunoblot analysis of HA_3-tagged TgPL2 expression in protein extracts from the TgPL2HA and TATi1-Ku80Δ (negative control) cell lines. Parasite lysates were probed with an anti-HA antibody. The arrowhead designates the major form of TgPL2HA at about 300 kDa. Probable post-translational modifications or cleavages and degradation products are shown by the star and the bar, respectively. TgROP5 was used as a loading control. (**C**) IFA performed on intracellular TgPL2HA transgenic parasites using anti-TgCpn60 and anti-TgAtrx1 as markers of the apicoplast lumen and membranes, respectively, and anti-HA antibodies. Parasite shape is outlined in the merged pictures. DNA was stained

with DAPI. DIC: differential interference contrast. Scale bar represents 1 µm. (**D**) Localization of HA_3-tagged TgPL2 at the apicoplast by immuno-electron microscopy.

Figure 3. Genetic knockdown of *TgPL2*.

(**A**) Schematic representation of the approach used to generate, in the TgPL2HA-expressing TATi1-Ku80Δ parental cell line, a *TgPL2* conditional mutant by replacement of the endogenous promoter with an ATc-regulated promoter. Black arrows represent primers used for PCR amplifications presented in panel **B**) to verify plasmid integration. The plasmid contains the *TgPL2* 5'coding sequence, the *tet* operator TetO7, the dihydrofolate reductase (DHFR) selection marker and the TgSAG4 minimal promoter p*TgSag4*. (**B**) PCR analysis with primer pairs depicted in panel **A**) to confirm the correct integration of the plasmid by homologous recombination. The parental TgPL2HA cell line was used as control for detection of the endogenous locus. (**C**) IFA performed with an anti-HA antibody on intracellular cKd-TgPL2HA transgenic parasites, incubated in absence or presence of ATc for 1 day. DIC: differential interference contrast. Scale bare represents 5 µm. (**D**) Immunoblot carried out on lysates from the TATi1-Ku80Δ, TgPL2HA and cKd-TgPL2HA cell lines grown or not in the presence of ATc for up to 2 days. Parasite lysates were probed with anti-HA and anti-TgROP5 (used as a loading control) antibodies.

Figure 4. Depletion of TgPL2 leads to a delayed death phenotype.

(**A**) Plaque assays on HFF monolayers infected with TgPL2HA or cKd-TgPL2HA cell lines treated or not with ATc during 7 days. Quantification of the lysis plaques size is presented on the graph. AU: arbitrary units. Values are the mean ± standard error of the mean plaque area (20 plaques were measured in each condition) from one representative experiment out of three. (**B**) IFA carried out on TgPL2HA and cKd-TgPL2HA cell lines grown for 4 days with

or without ATc using antibodies against TgCpn60 to detect the apicoplast, and against the HA tag to detect TgPL2. DIC: differential interference contrast. Scale bar represents 5 µm. (**C**) Quantification of the number of parasites from the mutant or the parental cell line harboring TgCpn60-labelled apicoplasts, when grown or not with ATc for up to 4 days. Values are the mean ± standard error of the mean of three independent experiments, where 200 parasites were counted for each condition. (**D**) Intracellular growth of TgPL2HA and cKd-TgPL2HA parasites cultivated with ATc for 3 days and allowed to invade new HFF cells. ATc treatment was maintained and the number of parasites per vacuole was counted 24h after inoculation. Values are the mean ± standard error of the mean of three independent experiments, where 200 vacuoles were counted for each condition.

Figure 5. The putative active site serine is important for TgPL2 function at the apicoplast. (**A**) CRISPR/Cas9 genome editing tool was used to directly mutate the codon corresponding to active site serine at position 2071. The position of the guide ARN is shown, as well as sequences of the two different donor DNAs that were used to edit the genome to replace the serine by an alanine (S2071A), or simply create a silent mutation (S2071S). The region to be edited has been recodonized to in the donor DNA to prevent further processing of the sequence. (**B**) 24 hors post-transfection, parasites were selected for the presence of Cas9-YFP by fluorescence-activated cell sorting, and seeded onto a new HFF monolayer for an additional 24 hours before labelling and counting the apicoplasts by IFA using TgCpn60 labelling. DNA was stained with DAPI. DIC: differential interference contrast. Scale bar represents 5 µm. (**C**) Three independent experiments were performed and all showed an increased proportion of parasites missing their apicoplast upon transfection with the S2071A template, suggesting the active site serine is important for TgPL2 function at the apicoplast.

Figure 6. Apicoplast morphology is affected upon TgPL2 depletion.

(**A**) TgPL2HA and cKd-TgPL2HA intracellular tachyzoites were treated for 2 days with ATc and the shape of remaining apicoplasts was classified based on TgAtrx1 labelling (compact or elongated, red signal on top images, DAPI-stained DNA is shown in blue), and quantification was performed on at least 100 parasites. Values are the mean ± standard error from *n*=3 independent experiments. (**B**) IFA performed on parental and mutant cell lines, incubated for 2 days with ATc using an anti-TgCpn60 antibody for apicoplast lumen detection. TgAtrx1 was used as marker of apicoplast membranes. Parasite shape is outlined in the merged pictures. DNA was stained with DAPI. DIC: differential interference contrast. White and red scale bars represent 5 and 1 µm, respectively.

Figure 7. TgPL2 depletion leads to an accumulation of membranes surrounding the apicoplast.

Ultrastructural analysis by electron microscopy of cKd-TgPL2HA tachyzoites treated for 2 days with ATc, showing examples of abnormal apicoplasts, with an accumulation of membranes in their periphery and enlarged intermembrane space. Numbered pictures on the right represent magnification of the corresponding squared parts of the left image.

Figure 8. Stable isotope labelling combined to GC-MS analysis of total lipids shows long term TgPL2 depletion affects apicoplast fatty acid synthesis.

Apicoplast FA synthesis was determined by ^{13}C glucose labelling in cKd-TgPL2HA parasites treated or not with ATc for two (**A**) or three (**B**) days. Results are mean of *n*= 3 biological replicates ± standard deviation. Stars indicate significant statistical differences confirmed by Student's t-test ($p<0.05$).

Figure 9. TgPL2 depletion leads to a noticeable alteration of the PC/LPC balance.

Lipids from cKd-TgPL2HA parasites treated or not for two days with ATc were analyzed by HPTLC 2D combined to GC-MS analysis. (**A**) Each phospholipid class was extracted, quantified and normalized to an internal standard. Overall, phospholipids are significantly reduced under ATc treatment. (**B**) Relative abundance of lipid species in the total lipid composition (mol%). LPC is significantly reduced upon ATc treatment (light grey bars) when compared to samples cultured in the absence of ATc (grey bars), where relative PC is significantly increased. Exact quantification was normalized by parasites number and the addition of a C13:0 internal standard before lipid extraction. Results are mean from n=3 biological replicates ± standard deviation. Stars indicate significant statistical differences confirmed by Student's t-test ($p<0.05$). CL, cardiolipin ; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PE-Cer, phosphatidylethanolamine-Ceramide.

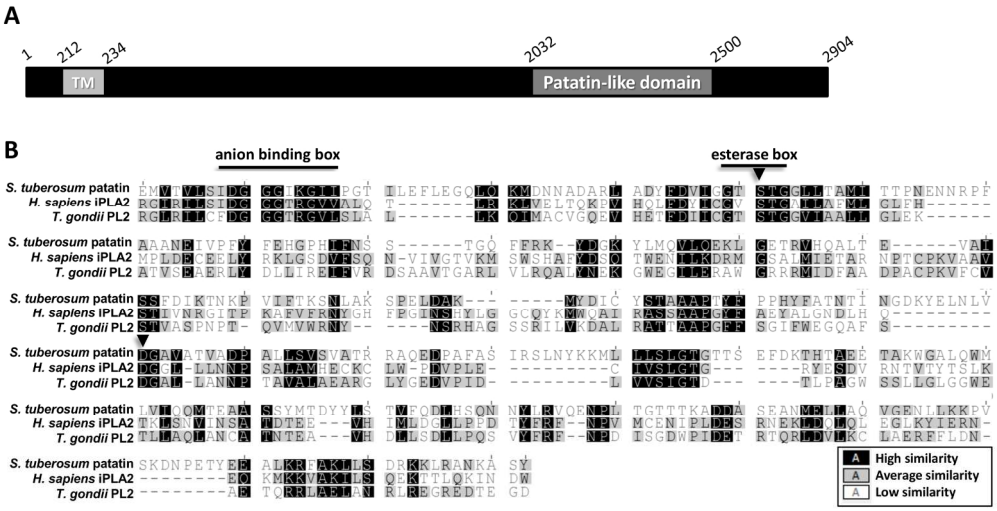

Figure 1. TGME49_231370 contains a patatin-like phospholipase domain

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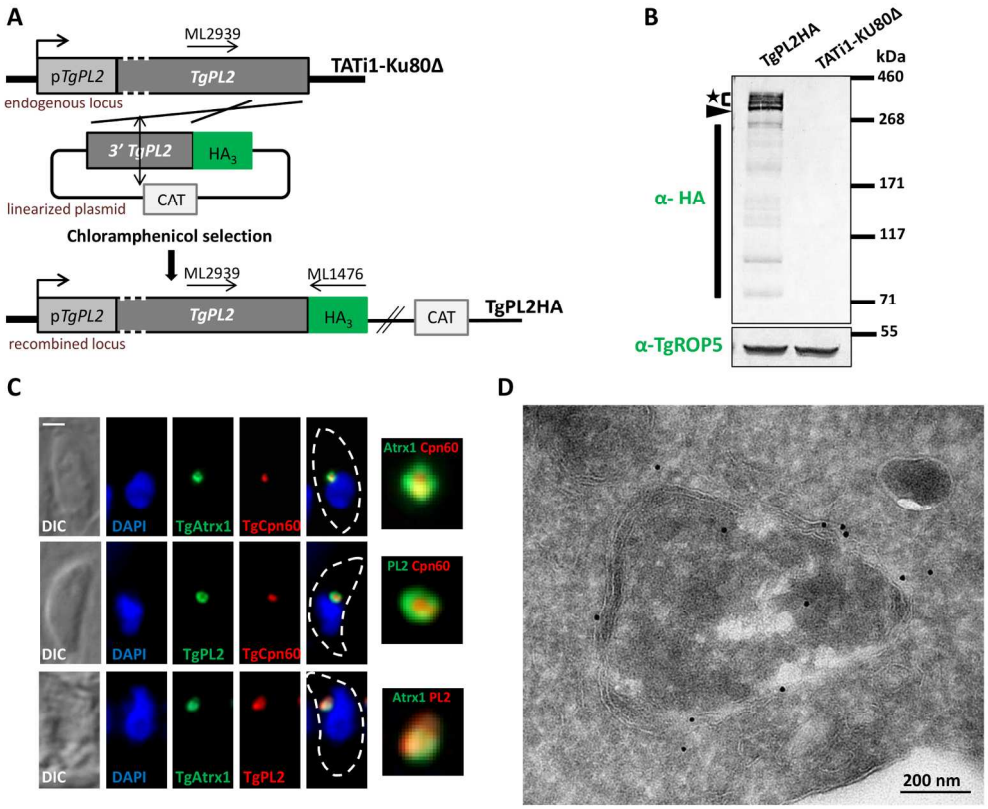

Figure 2. HA-tagged TgPL2 localizes to the periphery of the apicoplast

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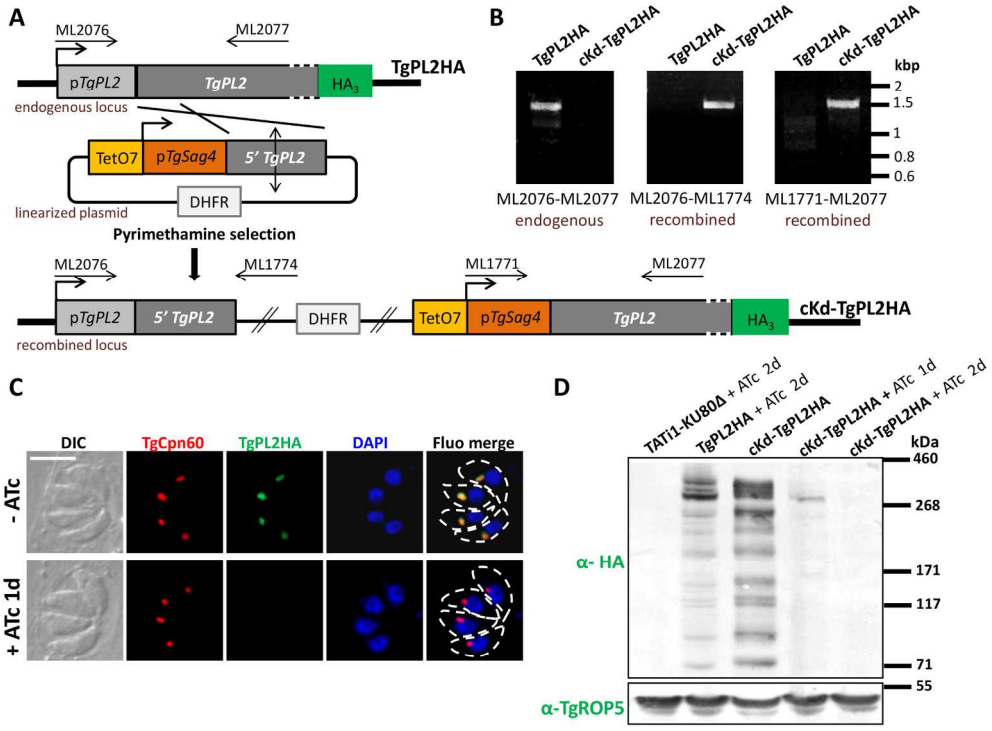

Figure 3. Genetic knockdown of TgPL2

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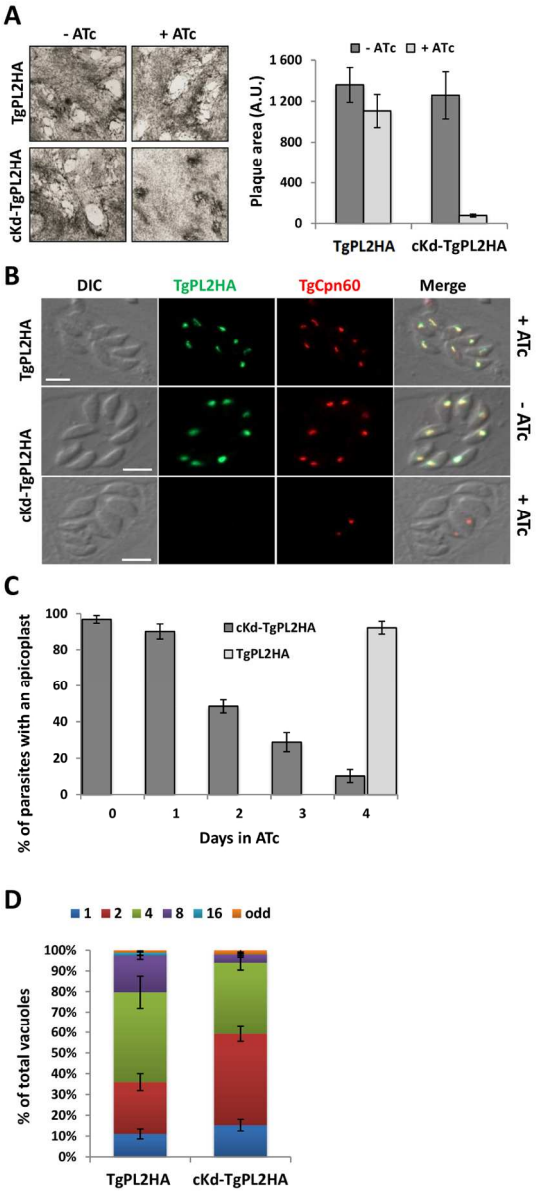

Figure 4. Depletion of TgPL2 leads to a delayed death phenotype

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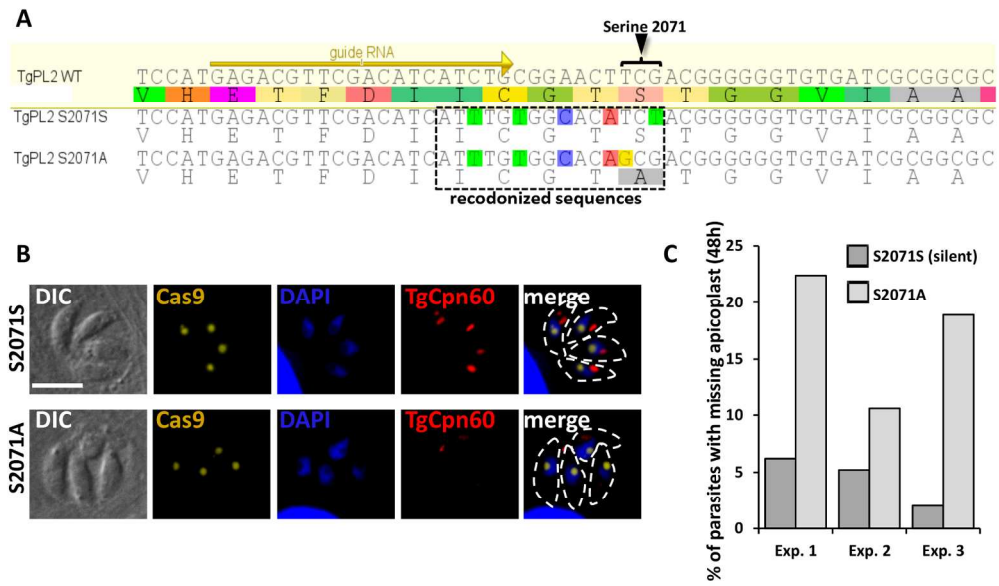

The putative active site serine is important for TgPL2 function at the apicoplast

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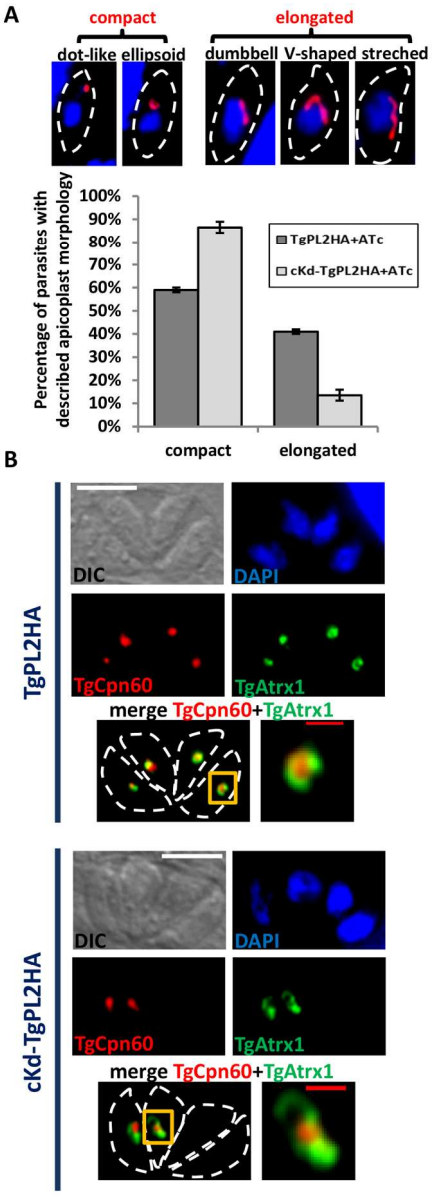

Figure 6. Apicoplast morphology is affected upon TgPL2 depletion

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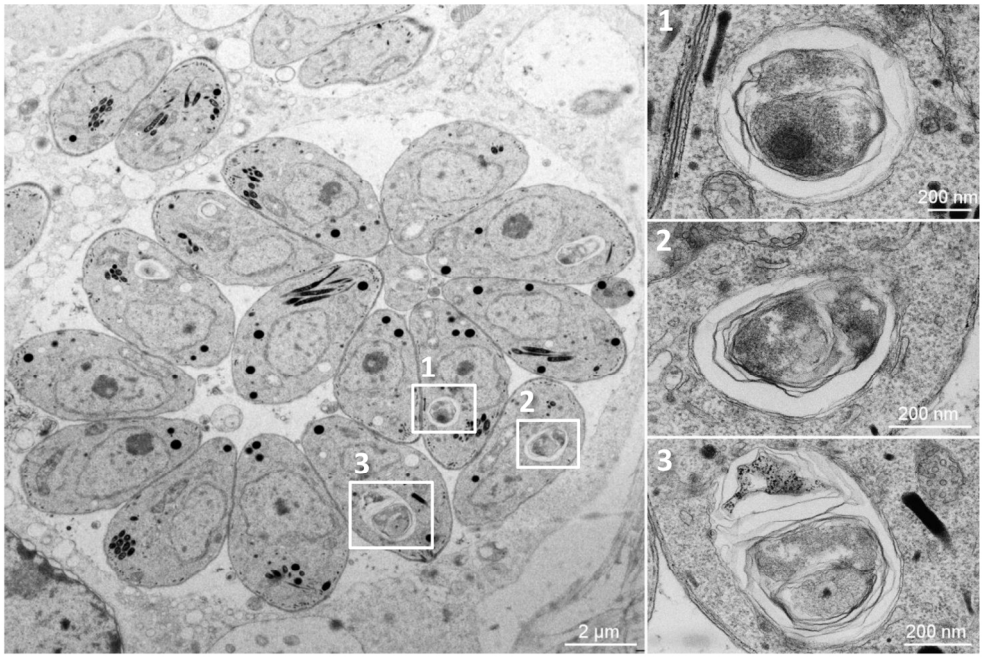

Figure 7. TgPL2 depletion leads to an accumulation of membranes surrounding the apicoplast

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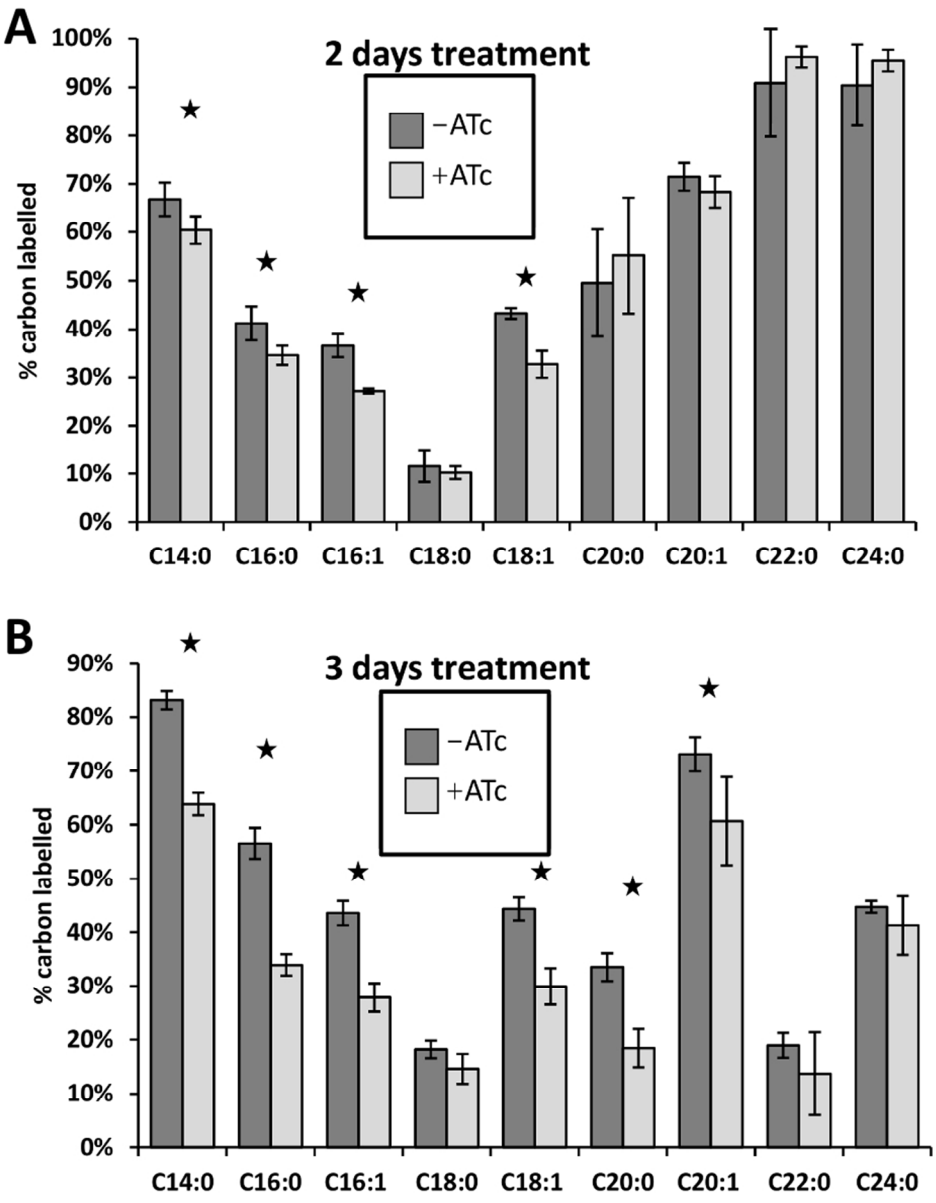

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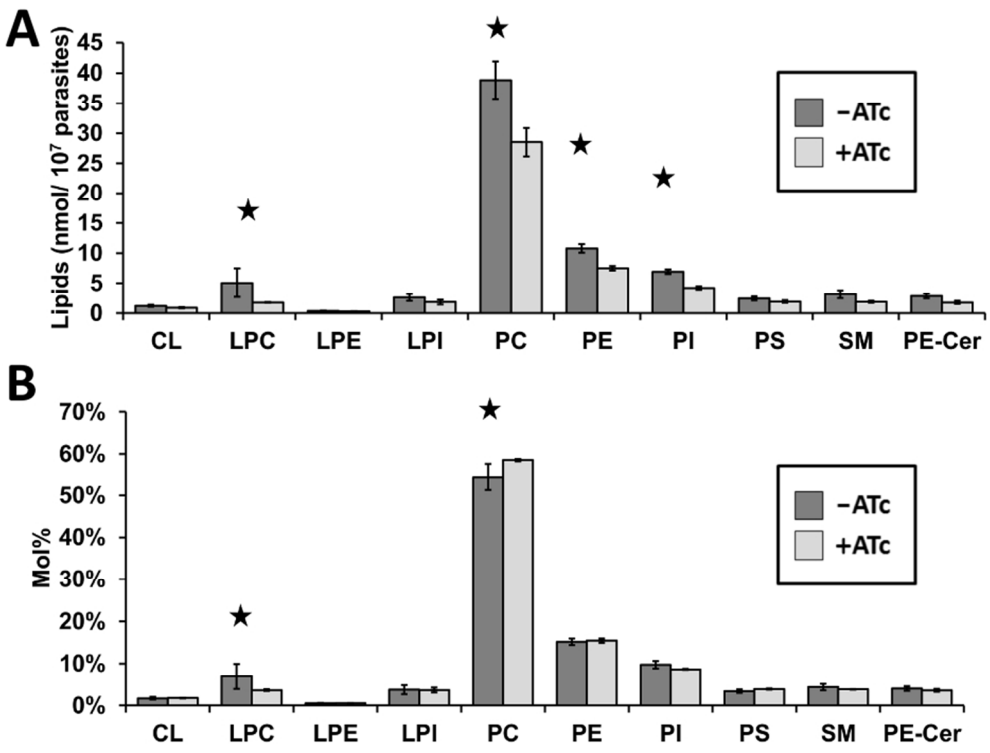

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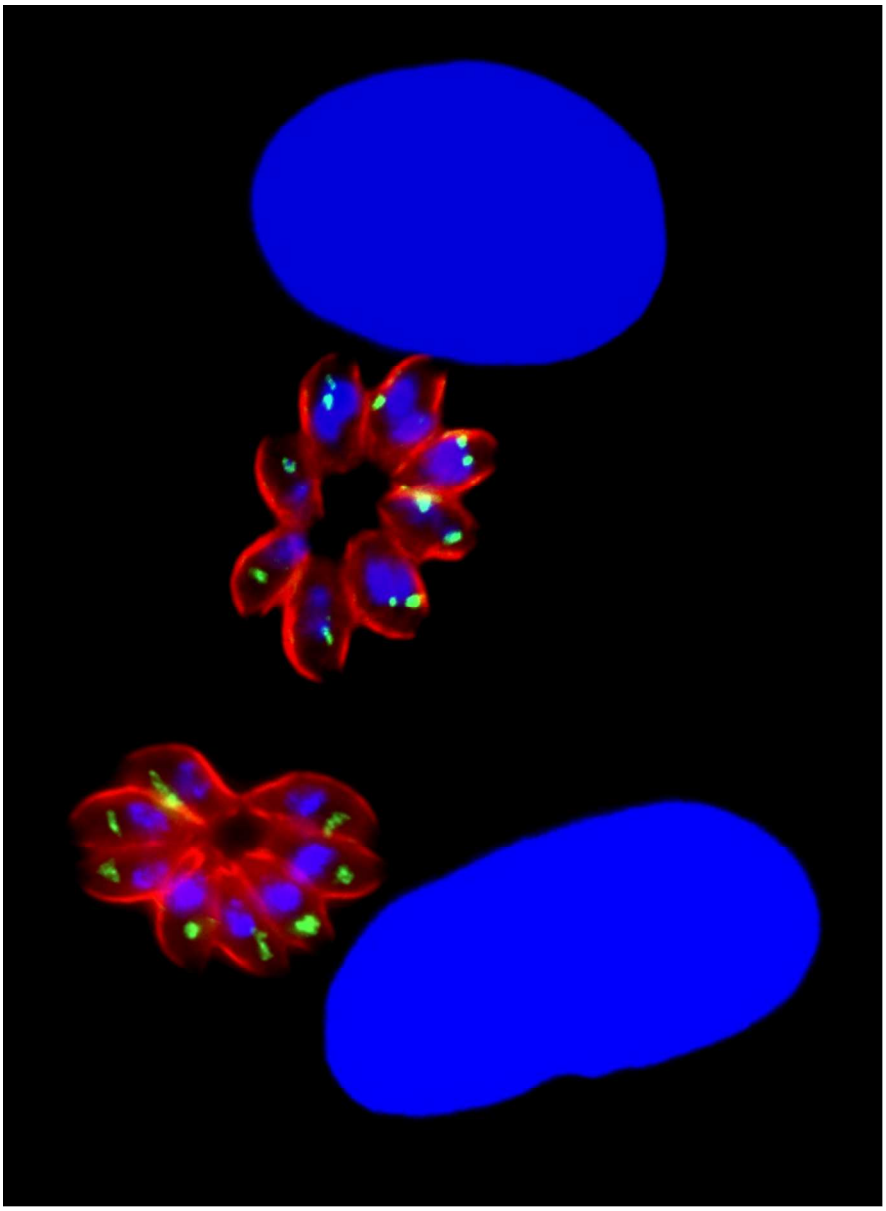

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