



# Understanding how the host nutrient environment influences the lipid metabolism and survival of Apicomplexa parasites

Serena Shunmugam

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## THÈSE

Pour obtenir le grade de

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## Etude pour comprendre comment l'environnement nutritionnel de l'hôte influence le métabolisme lipidique et la survie des parasites apicomplexes

## Understanding how the host nutrient environment influences the lipid metabolism and survival of Apicomplexa parasites

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*I dedicate this thesis to the two people who would have been  
so proud, my grandmother and father  
~For you Ma and Daddy~*

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---

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## ABSTRACT

---

Apicomplexa comprise of unicellular pathogens that cause major diseases such as toxoplasmosis and malaria. These obligate intracellular parasites heavily depend on lipid metabolism for their survival within their hosts. Their lipid synthesis relies on an essential combination of fatty acids (FAs), obtained from *de novo* synthesis and host scavenging. Current data, points at the parasites being able to channel a constant, and otherwise toxic, flux of host FA to lipid storage; only to be used when needed, during parasite division stages. This suggests that the biogenesis of parasite lipid storage, i.e., lipid droplets (LD), and their lipid/FA content mobilization is a crucial process for the parasite. Little is known about the generation/utilization of the parasite LDs. The processing and assembly of FA into major LD lipid class, triacylglycerol (TAG), are key processes for parasite survival.

My PhD work focused on answering questions surrounding the lipid metabolism of *Toxoplasma gondii* tachyzoite and *Plasmodium falciparum* blood stage parasites. I did this by focussing on three protein families namely, acyl-CoA synthetases (ACSs), phospholipases and acyltransferases (ATs). Using lipidomics and metabolite flux analysis, we show that these proteins have key roles in the mobilization or biogenesis of LDs. This work reveals that their metabolic activity depends on host nutrient availability.

I confirm the function of an ACS in activating FA for metabolic utilization by the parasite. Specifically, *TgACS1* functions as a TAG mobilizer and provides FAs to the parasite under low host nutrient environment, and more particularly during extracellular conditions. This protein is unique in that it contains a bubble-gum domain imperative for FA  $\beta$ -oxidation. Complementation studies now show insight into this previously understudied process in *Toxoplasma* parasites.

I then investigated another ACS, *TgACS3*, an essential protein that when absent leads to parasites death. This protein becomes even more crucial at a high host nutrient environment. Fluxomic analyses reveal that this protein has a preference in the metabolism of exogenous FFA that are usually redirected to TAG (LD) and PL synthesis. These ‘back-to-back’ papers illustrated the diverse functionality of the ACS protein family in *T. gondii* parasites and how their nutrient-dependent roles are central to TAG/LD metabolism.

Continuing with the theme of the nutrient adaptability, I studied a patatin-like phospholipase in schizont stage *P. falciparum* parasites. By lipidomic analyses I show this mitochondrial protein is responsible for the hydrolyses of phosphatidylglycerol to a lysobisphosphatidic acid. This balance seems to be important for schizont to ring conversion under lipid starvation. This work exemplifies that the lyso-phospholipid and phospholipid balance is crucial for the advancement of asexual parasites, but by changing their metabolic programme parasites can evade death but only in a nutrient rich environment.

The third and final study of my PhD, investigated the function of two acyltransferases; glycerol-3-phosphate acyltransferase (*TgGPAT*) and diacylglycerol acyltransferase 2 (*TgDGAT2*) that both localize to the endomembrane system and cytoplasm of the *Toxoplasma* parasite. *TgGPAT* and *TgDGAT2* are essential in different nutrient conditions, but both play a pivotal role in neutral lipid synthesis, specifically LD biogenesis (TAG formation). The loss of these proteins resulted in an accumulation of free FAs that cannot be ‘detoxified’ by incorporating them into lipid droplets, leading to parasite death.

My thesis work provides novel insights to better understand the parasites' metabolic reprogramming tactics, which in turn give us clues on developing therapeutic strategies in combating the diseases they cause.

# RESUME

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Le phylum des parasites apicomplexes comprend des pathogènes unicellulaires qui sont à l'origine de maladies telles que la toxoplasmose et le paludisme. La survie de ces parasites obligatoires unicellulaires est intimement liée à leur métabolisme lipidique. Leur anabolisme requiert une combinaison essentielle d'acides gras qui sont obtenus par néosynthèse ou détournés des ressources de son hôte. Les recherches actuelles indiquent que les parasites sont capables de canaliser les flux d'acides gras en provenance de l'hôte vers un mécanisme de stockage pour les utiliser lors du cycle reproductif et pour éviter les effets toxiques d'une accumulation. Cette observation suggère que le stockage de lipides sous forme de gouttelettes lipidiques, et leur mobilisation sous formes d'acides gras sont cruciales au métabolisme du parasite. Les processus de synthèses des classes majeures de lipides et triglycérols à partir des acides gras sont essentiels à la survie des apicomplexes.

Mon travail de thèse s'est concentré sur le métabolisme lipidique des parasites *Toxoplasma* et *Plasmodium falciparum* au stade tachyzoite de la phase sanguine de leur cycle de vie. J'ai travaillé sur trois familles de protéines ; les acyl-CoA synthétases (ACSs), les phospholipases et les acyltransférases (ATs). En utilisant des méthodes lipidomiques et d'analyse de flux métaboliques, nous montrons que ces protéines jouent un rôle clé dans la mobilisation ou la biogenèse des gouttelettes lipidiques. Ce travail indique que l'activité métabolique des parasites dépend de la disponibilité des nutriments pour l'hôte.

J'ai confirmé la fonction d'une ACS dans l'activation des acides gras pour permettre leur intégration aux voies métaboliques du parasite. La protéine *TgACS1* opère comme un mobilisateur de triglycérols pour fournir une source d'acides gras au parasite en conditions de stress nutritif, comme celles rencontrées lors de la phase extracellulaire du parasite. Cette protéine est unique puisqu'elle comporte

également un domaine « bubble-gum » qui est essentiel pour catalyser les réactions de  $\beta$ -oxydation des acides gras. Nos expériences de complémentation démontrent l'existence de ce mécanisme jusqu'alors méconnu chez les parasites *Toxoplasma*.

J'ai ensuite étudié une autre ACS, *TgACS3* qui est essentielle à la survie du parasite. La fonction de cette protéine est cruciale dans un environnement riche en nutriments pour l'hôte. L'analyse des flux métaboliques a révélé que cette protéine utilise préférentiellement les acides gras libres d'origine exogène, qui sont habituellement redirigés vers la synthèse de triglycérols pour former des gouttelettes lipidiques et la synthèse de phospholipides. Nos deux publications successives illustrent les fonctions diverses des ACS chez le parasite *T. gondii* et la dépendance nutritive de leurs fonctions dans le métabolisme des triglycérols/gouttelettes lipidiques

En continuant sur le thème de l'adaptabilité nutritive, j'ai étudié une phospholipase « patatin-like » présente lors du stade schizont chez le parasite *P. falciparum*. En utilisant des approches lipidomiques, nous avons démontré que cette protéine mitochondriale est responsable de l'hydrolyse du phosphatidylglycérol en acide lysobisphosphatidique. Cet équilibre semble important pour le passage du stade schizont au stade annulaire dans des conditions de carence lipidique. Ce travail illustre l'importance de la balance lyso-phospholipide/phospholipide pour la progression du développement asexuel du parasite ainsi que sa capacité à moduler son métabolisme pour survivre, mais seulement dans un environnement riche en nutriments.

La troisième et dernière étude conduite lors de ma thèse explore les fonctions de deux protéines : la glycérol-3-phosphate acyltransférases (*TgGPAT*) et la diacylglycérol acyltransférases 2 (*TgDGAT2*), qui sont toutes deux localisées dans le système endomembranaire et le cytoplasme des parasites *Toxoplasma*. *TgGPAT* et *TgDGAT2* sont essentiels, dans des conditions nutritives différentes, mais jouent toutes deux un

rôle primordial dans la synthèse des lipides neutre et notamment pour la synthèse de triglycérols. La déplétion de ces protéines entraîne une accumulation d'acides gras libres non-activés qui ne peuvent être intégrés aux gouttelettes lipidiques et deviennent toxiques, entraînant la mort des parasites.

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## ABBREVIATIONS

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<b>3-PGA</b>	3- Phosphoglycerate
<b>ABCG</b>	ATP binding cassette G transporter proteins
<b>ACAT1</b>	acyl CoA: cholesterol acyltransferase1
<b>ACBP2</b>	Acyl CoA binding protein2
<b>ACC</b>	Acetyl CoA carboxylase
<b>AcCAT</b>	Acetyl CoA acetyltransferases
<b>ACD</b>	Acyl CoA dehydrogenase
<b>ACP</b>	Acyl carrier protein
<b>ACS</b>	Acetyl CoA synthetase
<b>ACT</b>	Artemisinin combination therapy
<b>AGPAT</b>	Acylglycerol-3-phosphate acyltransferase
<b>AIDS</b>	Acquired immuno-deficiency syndrome
<b>AMA1</b>	Apical membrane antigen
<b>APT</b>	Apicoplast phosphate transporter
<b>AT</b>	Acyltransferase
<b>ATP</b>	Adenosine triphosphate
<b>ATS1</b>	Plant-like glycerol-3-phosphate acyltransferase
<b>ATS2</b>	Plant-like acyl-glycerol-3-phosphate acyltransferase
<b>BCK</b>	DH Branched chain ketoacid dehydrogenase
<b>BCKDH</b>	Branched chain ketoacid dehydrogenase
<b>BMP</b>	Bis(Monoacylglycerol)Phosphate
<b>CCT</b>	CTP: phosphocholine cytidyltransferase
<b>CDP-DAG</b>	Cytidine diphosphate diacylglycerol
<b>CDS</b>	CDP synthases
<b>CK</b>	Choline kinase
<b>CL</b>	Cardiolipin
<b>CMP</b>	Cytidine monophosphate diacylglycerol
<b>CoA</b>	Co-enzyme A
<b>CPT</b>	Diacylglycerol choline phosphotransferase
<b>DAG</b>	sn-1,2-diacylglycerol

<b>DEH</b>	Dehydratase
<b>DGAT</b>	Diacylglycerol acyltransferase
<b>DGK</b>	Diacylglycerol kinase
<b>DHAP</b>	Dihydroxyacetone phosphate
<b>dHSM</b>	Dihydrosphingomyelin
<b>DOXP</b>	1-deoxy-D-xylulose 5-phosphate
<b>ECH</b>	enoyl-CoA hydratase
<b>ECR</b>	Enoyl reductase
<b>ECT</b>	Ethanolamine phosphate: cytidyltransferase
<b>EEF</b>	Exo-erythrocytic form
<b>EK</b>	Ethanolamine kinase
<b>EPC</b>	Ceramide phosphor ethanolamine
<b>EPT</b>	Diacylglycerol ethanolamine phosphotransferase
<b>ER</b>	Endoplasmic reticulum
<b>ETC</b>	Electron transport chain
<b>Etn</b>	Ethanolamine
<b>FA</b>	Fatty acid
<b>FabB</b>	$\beta$ -Ketoacyl-ACP synthase I/II
<b>FabD</b>	Malonyl CoA:ACP transacylase
<b>FabG</b>	$\beta$ -ketoacyl:ACP reductase
<b>FabH</b>	$\beta$ -Ketoacyl-ACP synthase III
<b>FabI</b>	Enoyl-ACP reductase
<b>FABP</b>	Fatty acid binding protein
<b>FabZ</b>	$\beta$ -Hydroxyacyl-ACP dehydratase
<b>Fas</b>	Fatty acid synthase
<b>G3P</b>	Glycerol-3-phosphate
<b>G3PDH</b>	Glycerol-3-phosphate dehydrogenase
<b>GABA</b>	$\gamma$ -Aminobutyric acid
<b>GAC</b>	Glideosome associated connector
<b>GC</b>	Guanylate cyclase
<b>GC-MS</b>	Gas chromatography coupled mass spectrometry
<b>GM3</b>	Monosialodihexosyl-ganglioside
<b>GPAT</b>	Glycerol-3-phosphate acyltransferase

<b>GpdA</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>GPI</b>	Glycosylphosphatidylinositol
<b>GRA</b>	Dense granule proteins
<b>GTP</b>	Guanosine-triphosphate
<b>HCD</b>	Hydroxy acyl-CoA dehydrogenase
<b>HDL</b>	High-density lipoproteins
<b>IMC</b>	Inner Membrane Complex
<b>IPC</b>	Inositol phosphorylceramide
<b>IPP</b>	Isopentenyl pyrophosphate
<b>IVN</b>	Intravacuolar network
<b>LBPA</b>	Lysobisphosphatidic acid
<b>LCAT</b>	Lecithin:cholesterol acyltransferase
<b>LCFA</b>	Long chain fatty acid
<b>LC-MS</b>	Liquid Chromatography Mass spectrometry
<b>LD</b>	Lipid droplets
<b>LDL</b>	Low density lipoprotein
<b>LPA</b>	Lysophosphatidic acid
<b>LPAAT</b>	Lysophosphatidic acid acyltransferase
<b>LPG</b>	Lysophosphatidylglycerol
<b>LPI</b>	Lysophosphatidylinositol
<b>LPL</b>	Lysophospholipase
<b>LPC</b>	Lysophosphatidylcholine
<b>MAF1</b>	Mitochondrial association factor1
<b>MEF</b>	Murine embryonic fibroblasts
<b>MEP</b>	2-C-methyl-D-erythritol 4-phosphate
<b>MIC2</b>	Microneme protein 2
<b>MSP</b>	Merozoite surface protein
<b>NADH</b>	Nicotinamide adenine dinucleotide
<b>NCR1</b>	Niemann-Pick Type C1-Related protein
<b>NO</b>	Nitric oxide
<b>PA</b>	Phosphatidic acid
<b>PAP</b>	Phosphatidic acid phosphatase
<b>PC</b>	Phosphatidylcholine

<b>PDH</b>	Pyruvate dehydrogenase
<b>PDHC</b>	pyruvate dehydrogenase complex
<b>PE</b>	Phosphatidylethanolamine
<b>PEMT</b>	Phosphatidylethanolamine N-methyltransferase
<b>PEP</b>	Phosphoenol pyruvate
<b>PG</b>	Phosphatidylglycerol
<b>PH</b>	Pleckstrin homology
<b>PI</b>	Phosphatidylinositol
<b>PI3P</b>	Phosphatidylinositol-3-monophosphate
<b>PIP2</b>	Phosphatidylinositol 4,5-bisphosphate
<b>PI-PLC</b>	Phosphatidylinositide-phospholipase C
<b>PK</b>	Pyruvate kinase
<b>PL</b>	Phospholipid
<b>PLA</b>	Phospholipase A
<b>PLC</b>	Phospholipase C
<b>PLD</b>	Phospholipase D
<b>PMT</b>	Phosphatidylethanolamine methyltransferase
<b>PPM</b>	Parasite plasma membrane
<b>PS</b>	Phosphatidylserine
<b>PSD</b>	Phosphatidylserine decarboxylase
<b>PSS</b>	Phosphatidylserine synthase
<b>PT</b>	Phosphatidylthreonine
<b>PTS</b>	Phosphatidylthreonine synthase
<b>PV</b>	Parasitophorous vacuole
<b>PVM</b>	Parasitophorous vacuole membrane
<b>RBC</b>	Red blood cell
<b>Rh</b>	Rhoptry proteins
<b>RMVs</b>	RBC-derived microvesicles
<b>RONs</b>	Rhoptry Neck Proteins
<b>ROPs</b>	Rhoptry bulbous body proteins
<b>SAM</b>	S-adenosylmethionine
<b>SCD</b>	Stearoyl CoA desaturase
<b>SCP</b>	Sterol carrier protein

<b>SD</b>	Serine decarboxylase
<b>SDPM</b>	Serine Decarboxylase-Phosphoethanolamine Methyltransferase
<b>Ser</b>	Serine
<b>SLS</b>	Sphingolipid synthase
<b>SMS</b>	Sphingomyelin synthase
<b>SUB</b>	Subtilisin-like serine proteases
<b>Suf</b>	Sulphur mobilization
<b>TAG</b>	Triacylglycerol
<b>TCA</b>	Tricarboxylic acid cycle
<b>TPT</b>	Triose phosphate transporter
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>TVM</b>	Tubovesicular membranes
<b>VLCFA</b>	Very long chain fatty acid
<b>WHO</b>	World health organization

# CHAPTER 1: APICOMPLEXAN PARASITES

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## ***An introduction to the phylum of Apicomplexa***

The 'super-phylum' alveolates is host to three eukaryotic phyla; Dinoflagellata, Ciliophora (Ciliata), and Apicomplexa (Sporozoa) (Adl *et al.*, 2012). The feature unifying these organisms is the presence of a unique membrane structure forming flattened vesicles, called 'cortical alveoli', tightly apposed and maintained underneath the plasma membrane through a network of underlying sub-pellicular microtubules (Gould, Waller and McFadden, 2008). This structure allows for the organism's motility and is termed in inner membrane complex (IMC) in apicomplexa parasites.

Apicomplexa (with over 6000 species) are unlike their related alveolates since they have embraced an obligate intracellular parasitic lifestyle. Apicomplexa are comprised of five dominant groups of morphologically and ecologically diverse protists including several pathogenic protists (Leander *et al.*, 2017);

a) **Gregarinida**, which inhabit invertebrate marine freshwater and terrestrial intestines, coelems and reproductive vesicles. They are potentially threatening to insect farms and laboratory colonies (Leander *et al.*, 2017).

b) **Piroplasmida**, which are named after their pear-shaped blood stages. They include organisms which are responsible for cattle-affecting diseases such as babesiosis (*Babesia bovis*) and theileriosis (*Theileria parva/annulata*). These parasites require a tick (Ixodidae) vector to facilitate transmission of the diseases (Jalovecka *et al.*, 2018).

c) **Cryptosporidida** has come into much-needed limelight in recent years as the second most common cause of infant diarrhoea in developing countries (Kotloff *et al.*, 2013). These parasites have a unique epicellular parasitic lifestyle, developing their

parasitophorous vacuole (i.e. the intracellular niche within which apicomplexan develop in the host cell) just on the surface of the invaded host cell.

d) **Haemosporidia** is comprised of three genera: *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. The later genus is responsible for malaria infection that can be considered one of the oldest pandemics.

e) **Coccidia**, this is the largest group of apicomplexa protozoa which houses parasites such as *Neospora caninum*, *Sarcocytis sp.* and *Eimeria sp.* which cause infection in dogs, cattle, poultry, and some mammals, leading to ruin for the livestock industry and other domesticated animals. One of the most infectious *Coccidia* species that accounts for 30% of global population and can infect virtually any mammalian/bird species is *Toxoplasma gondii*.

Apicomplexa are unicellular eukaryotes responsible for major human and veterinary diseases such as malaria and toxoplasmosis, which pose massive social and economic burdens. These parasites are defined by a unique polar end known as the apical complex. This complex contains specific secretory machinery required for the invasion of the parasite into host cells (Michael J. Blackman and Bannister, 2001). They also have a complex lifestyle in which they alternate between sexual and asexual stages in two hosts (dormant vs lytic cycles). These parasites are also identifiable by a non-photosynthetic plastid called the 'apicoplast' which is essential for metabolic processes. Additionally, their mitochondrion genome (mitosome in the case of *Cryptosporidida*) is highly reduced due to massive gene migrations to the nucleus (Berná, Rego and Francia, 2021).

### ***Plasmodium falciparum***

Malaria is arguably one of the most overwhelming infectious diseases throughout the world's existence. It has been documented as early as 2700 BC (Tuteja, 2007). This

disease is highly prevalent in poverty-stricken regions of the world such as sub-Saharan Africa, South Asia and parts of South America. According to the World Malaria Report (2021), nearly half of the world's population is at risk of being infected with malaria (World Health Organization, 2021). Since 2015, the baseline of WHO's global malaria strategy, 24 nations have registered increases in malaria mortality. Due to service disruptions caused by the COVID-19 pandemic, emergence of drug resistance and vaccine inefficiency; critical 2020 milestones of WHO's global malaria strategy have been missed, and without immediate and dramatic action, the 2030 targets will not be met.

Malaria is caused by five species of Plasmodium parasites namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The female *Anopheles* mosquito is responsible for the transmission of the parasite between human hosts (Tuteja, 2007). *P. falciparum* causes the most severe form of the disease and is the dominant species found in Africa. In rural communities in Africa where proper healthcare is limited the effects of malaria are most noticeable. Around 95% of malaria deaths occur in Africa (World Health Organization, 2021). The spread of drug (artemisinin combine therapy, Nobel 2015 (Youyou, Ōmura and Campbell, 2015)) resistant parasites, the lack of an efficient vaccine, and the inconsistency of malaria control programmes have increased the need for new innovations in Plasmodium research (Phillips *et al.*, 2017).

### ***Toxoplasma gondii***

*T. gondii* is a unicellular eukaryote that infects the cells of warm-blooded animals (mammals and birds), including humans, and causes toxoplasmosis. Toxoplasmosis is a disease considered as asymptomatic in most cases. However, it can be fatal for the foetus in primary-infected pregnant women and for immunocompromised individuals such as AIDS and leukaemia patients (Aparecida Müller De Barros *et al.*,

2022). Despite being well studied, no efficient vaccine exists in preventing transmission of toxoplasmosis.

*T. gondii* is an excellent model organism for the study of Apicomplexa parasites and intracellular parasitism. The first characteristic that deems this parasite a model organism, is that it can rapidly divide during tachyzoite stages (every 6 to 8 hours) resulting in a high parasite yield within an easily controllably *in vitro* laboratory system. This propagation is achieved using several cell-types from warm-blooded animals employing the standard tools of culture cellular. The size of the parasite (five times the size of *Plasmodium*) makes it easier to observe in optical and electronic microscopy which allows visualization and study of organelles, mechanisms of invasions and the complex structure of the parasite (Kim and Weiss, 2004).

### ***The complex life cycle of apicomplexa parasites***

The life cycle of this phylum of parasites is extremely complex and normally consists of two separate host types. This thesis is centred on the two parasites listed above. The life cycle of haemosporidians and coccidians compose of a primary vector (responsible for the sexual development, gamogony) and a secondary vector (merogony) in which the continuous replication of the parasite will occur.

### **Plasmodial life cycle**

The multistage life cycle of Plasmodial parasites can be separated into three distinct cycles:

The sporogonic cycle (in the mosquito), the exo-erythrocytic cycle and the intra-erythrocytic developmental cycle (both in the human host), shown in **Figure 1**. A human plasmodial infection is initiated when a feeding *Anopheles* mosquito injects mature sporozoites of the parasite into the bloodstream of the host dermis. This is the

beginning of the exo-erythrocytic stage. The sporozoites, transmitted via the saliva of the mosquito, make their way in the bloodstream to the liver of the new host (Silvie *et al.*, 2008). The initial movement from the dermis to a blood vessel is random and not every sporozoite will reach the hepatocytes in the liver. Those that reach the liver will penetrate the hepatocytes where they remain for 9–16 days and undergo asexual replication known as exo-erythrocytic schizogony (Tuteja, 2007). During this stage a schizont forms within this hepatocyte that is made up of multiple haploid merozoites. The infected hepatocyte ruptures to release thousands of merozoites into the bloodstream to infect erythrocytes. Asexual division begins inside the erythrocyte, which is also the site for the formation of the other morphological stages of the parasites in the human host. After invasion merozoites develop into ring-stage parasites that will mature to form trophozoites (Phillips *et al.*, 2017). At the end of the trophic phase, multiple rounds of nuclear division occur through asexual reproduction (erythrocytic schizogony). Schizonts are produced in the red blood cells (RBCs) and consist of up to 32 individual merozoites (Leete and Rubin, 1996). The infected RBCs eventually rupture, releasing the merozoites into the bloodstream where they invade new RBCs. The erythrocytic schizogony process is repeated every 48 hours, (known as the intra-erythrocytic developmental cycle- **Figure 1**) and coincides with the clinical symptoms of malaria (Tuteja, 2007; Phillips *et al.*, 2017).

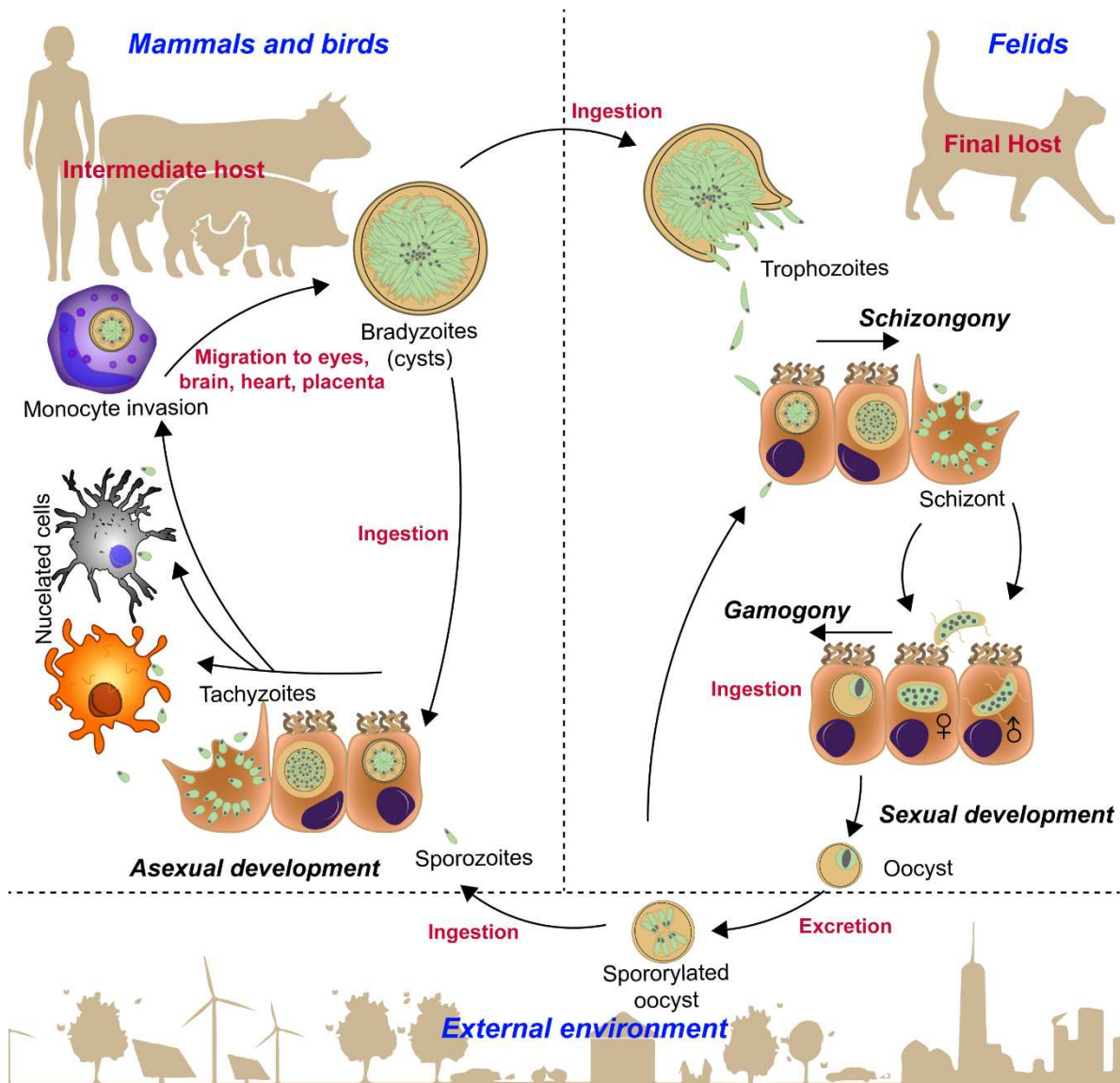


A few of the intra-erythrocytic parasites form male and female gametocytes. These are ingested by the mosquito where the sexual stage of the life cycle takes place. The gametocytes develop into gametes which fuse to form a zygote within the insect's gut. The zygote develops into an oocyst, where sporozoites are formed and released. These enter the salivary glands, allowing the parasite to be spread among new human hosts when the mosquito feeds (Miller et al., 2013).

### ***Toxoplasma* life cycle**

*T. gondii* parasites are unique in that they can invade almost any nucleated cell in any warm-blooded host. This contributes to the complexity of their life cycle which is split between two host (intermediate host and definitive host, Figure 2) and three stages depending on their environmental context (Dubey, 2009) :

- a) Bradyzoite
- b) Sporozoite
- c) Tachyzoite



**Figure 2: Lifecycle of *Toxoplasma gondii* parasites.**

The definitive host of *T. gondii* parasite are felids, where sexual development occurs. The oral ingestion of a bradyzoite cyst equally leads to its rupture once entering the intestinal tract of the host. Individual trophozoites from this cyst will enter epithelial cells. The parasite then enters the first round of schizogony (asexual) that results in the development of schizonts which will lyse the intestinal cell that will commence gamogony through the development of female and male gametes. The fusion of these two cells will lead to the formation of an oocyst that is eventually excreted out of the felid as sporulated oocysts in the environment that can last months to years. The ingestion by other mammals or birds will then release the sporozoites of the sporulated oocysts in the intestinal tract of the intermediate host. Asexual development will occur first in the epithelial cells that will release hundreds of tachyzoites that can invade any nucleated cell. Monocyte invasion will facilitate the migration of the tachyzoite parasite to the eye, brain, placental or heart tissue where bradyzoite conversion will occur. Cysts that form

here can stay viable for multiple days after the death of the host, ingestion by an intermediate or final host will re-start the life cycle of this parasite.

### **Final host:**

The sexual developmental (Di Genova *et al.*, 2019) stages of this parasite only occur in felines (the definitive host who lacks a desaturase responsible for the formation of linoleic acid). The main source of infection in felines is ingestion of prey containing tissue cysts (bradyzoites) or oocyst containing sporozoites (from other cat faeces). After ingestion the walls of the cysts are disintegrated and releases bradyzoites or sporozoites after which they invade the epithelial cell making up the wall of the digestion tract. Schizogony of these parasites will give rise to thousands of merozoites that will infect new erythrocytes continuing this lytic component of the cycle. Some may start to differentiate into micro/macrogametes. The fusion of these two sexes leads to the formation of an immature oocysts. As they pass through the intestinal tract, they can be release out of the cat through the cat faeces. The external aerated environment triggers the maturation of the oocysts which are extremely resistant to severe environmental conditions and survive here for months. For further transmission sporozoites ingested by a sequential host to complete its highly lytic-form (tachyzoites) responsible for the spread of the parasite in an intermediate host (Attias *et al.*, 2020).

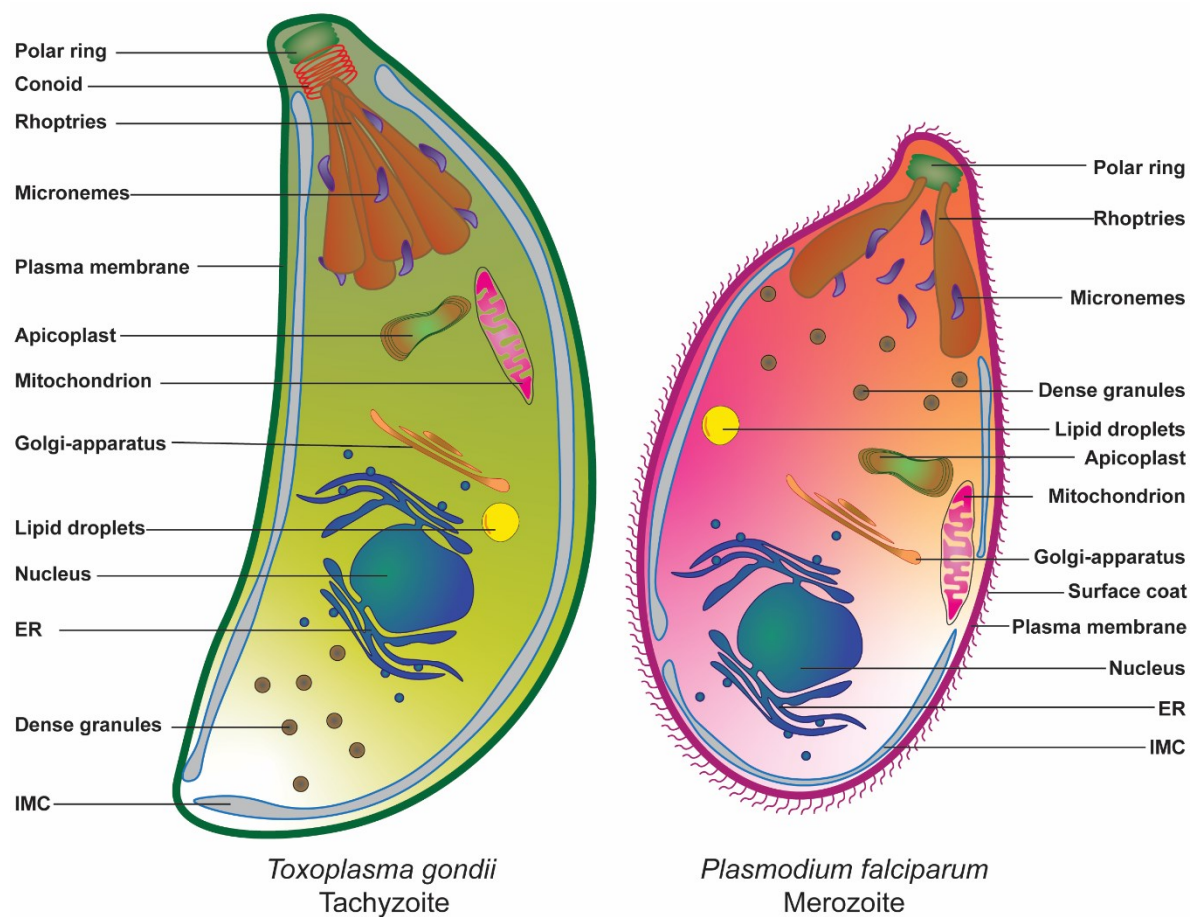
### **Intermediate hosts:**

Intermediate hosts such as humans and cattle ingest sporozoites and bradyzoites from prey/food. Once entering the intestinal tract, these cells can differentiate into tachyzoites. This is a highly proliferative asexual form of the parasite. These stages are characterized by rapid division and dissemination in the host responsible for the symptomatic form of the disease (Dubey, 2009; Attias *et al.*, 2020). Following rupture

of invaded enterocytes, tachyzoites invade monocytes and are spread through to the other cells/organs via the circulatory system (blood). Under certain environmental changes in different tissue (eye, brain, muscle) and immune pressure, tachyzoites can convert to the slow-growing and less-metabolically active bradyzoites stages (as tissue cysts) which are responsible for the chronic phase of toxoplasmosis. These tissue cysts can stay dormant in a host for their entire lifetime and can be re-converted back into tachyzoites under favourable environmental conditions (Francia and Striepen, 2014). This is frequently observed in immuno-deficient patients, chemotherapy and organ recipients which may be fatal in some cases. If a pregnant woman is primo-infected with *T. gondii* parasites the lack of a memory immune response will allow the transversal of the parasite across the placental membrane and infection of the foetus can lead to congenital conditions or fatality (NHS, 2020).

### ***The cellular structure of Apicomplexan parasites***

The multi-faceted life cycle of apicomplexan parasites consists of drastic changes to the overall structure of the parasite. This thesis mainly focuses of the rapidly dividing stages namely the merozoite and tachyzoite stages in *P. falciparum* and *T. gondii*, respectively. There are some organelles that are shared amongst these forms and contribute to their metabolic similarity.



**Figure 3: Structure of Apicomplexa parasites.**

The structure of *T. gondii* tachyzoites (left) and *P. falciparum* merozoite (right). ER: endoplasmic reticulum, IMC: inner membrane complex.

### The general structure:

Like with all eukaryotic cells, this unicellular parasite (Figure 3) has a nucleus, mitochondria, endoplasmic reticulum (ER, with/without ribosomes) and Golgi-apparatus. The ER is largely found at the basal end of the parasite, whereas the Golgi is normally adjacent to the apical end of the parasite nucleus. Many lipid pathways reside within the ER and Golgi, predominantly making up the eukaryotic-type pathway for glycerolipid synthesis (Shunmugam, Arnold, *et al.*, 2022; Walsh *et al.*, 2022). A

single mitochondrion exists whose biogenesis is correlated to the cell-cycle (Nishi *et al.*, 2008). This intracellular parasite is unique in that it also contains additional membranes once inside the host namely, parasitophorous vacuole membrane (PVM), parasitophorous vacuole (PV), parasite plasma membrane (PM).

### **The pellicle:**

The pellicle is a trilaminar structure which is made up of the outer membrane and the inner membrane complex (IMC, Figure 3). Two continuous membranes make up this cytoskeletal organelle consisting of: sacs of flattened vesicles just beneath the parasite plasma membrane or alveoli and a rigid network of filaments. The IMC is crucial during the development of the parasite and is used as a scaffold in mother parasites during endogeny by daughter cells, which recycling maternal IMC membranes. In *T. gondii* tachyzoites and bradyzoites, the IMC is composed of three rows of fused rectangular vesicles encircling the parasite with openings at the apical and basal ends (Porchet and Torpier, 1977; Dzierzinski *et al.*, 2004; Del Carmen *et al.*, 2009). A similar arrangement is seen in *Plasmodium* gametocytes where the IMC is formed from 9 to 15 ring-like plates (Meszoely *et al.*, 1982; Kono *et al.*, 2012). However, in all other *Plasmodium* life stages, the IMC is formed from a single fused vesicle (Dubremetz *et al.*, 1979; Bannister and Dlugewski, 1990; Raibaud *et al.*, 2001; Kono *et al.*, 2012).

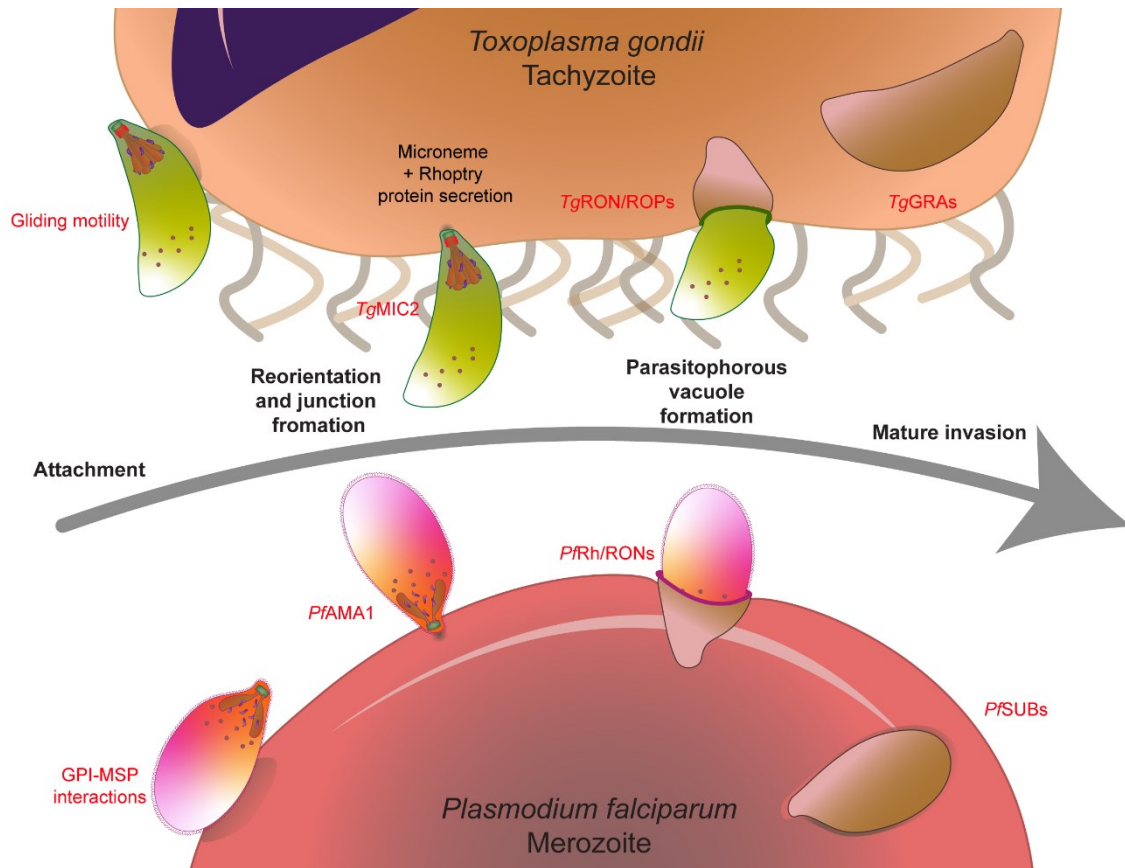
### **The apical complex:**

The Apicomplexan phylum is defined by the presence of specialised secretory organelles called the apical complex (Cowman, Berry and Baum, 2012) which facilitates secretion and invasion. These organelles include the a) rhoptries (two in *P. falciparum* and 8 or more in *T. gondii*); club shaped structures which are rich in proteins and lipids, b) micronemes; rod-shaped electron dense structures and c) dense granules; which are all central to parasite invasion and proliferation in its host. The

apical organelles are enclosed by an intricate structure consisting of a) the conoid which is an assembly of spirally arranged fibres, b) the polar ring, c) two intraconoidal microtubules, and the d) the preconoidal rings. The structures described here are visibly apparent during the merozoite and tachyzoite stages in *P. falciparum* and *T. gondii* (Michael J Blackman and Bannister, 2001).

### **The invasion machinery:**

Erythrocytic invasion for *Plasmodial* is a complex process requiring a sequence of interactions and events to take place (Weiss et al., 2015, Figure 4). The initial contact between the host RBC and merozoite is facilitated by the reversible interaction of the transmembrane erythrocyte band 3 receptor with multiple glycosyl-phosphatidyl inositol (GPI)-anchored merozoite surface proteins (Li *et al.*, 2004). As with *Toxoplasma* parasites as well, 'gliding motility' facilitates this contact with the new host cells and the apically located organelles (micronemes and rhoptries) act as the main drivers through the secretion of essential proteins. Microneme proteins are initially secreted for irreversible parasite attachment, reorientation, and junction formation such as MIC2 in *Toxoplasma* and AMA1 in *Plasmodium* (Narum and Thomas, 1994; Carruthers and Sibley, 1997; Cowman, Berry and Baum, 2012). Thereafter, rhoptries empty their contents (*Tg*RON/ROPs and *Pf*Rh/RONs) to enable surface shedding, actin-myosin motility, and eventual vacuole formation to allow the cell to be internalized by its new host (Cowman, Berry and Baum, 2012). Finally, post-invasion after PV formation, the dense granule proteins, called GRA proteins are released into the PV and further excreted out into the host nucleus (Hakimi and Bougdour, 2015; Mercier and Cesbron-Delauw, 2015). GRA proteins play a critical role in the interaction with the host as they can adapt the host cell gene expression depending on the parasite cell requirements.

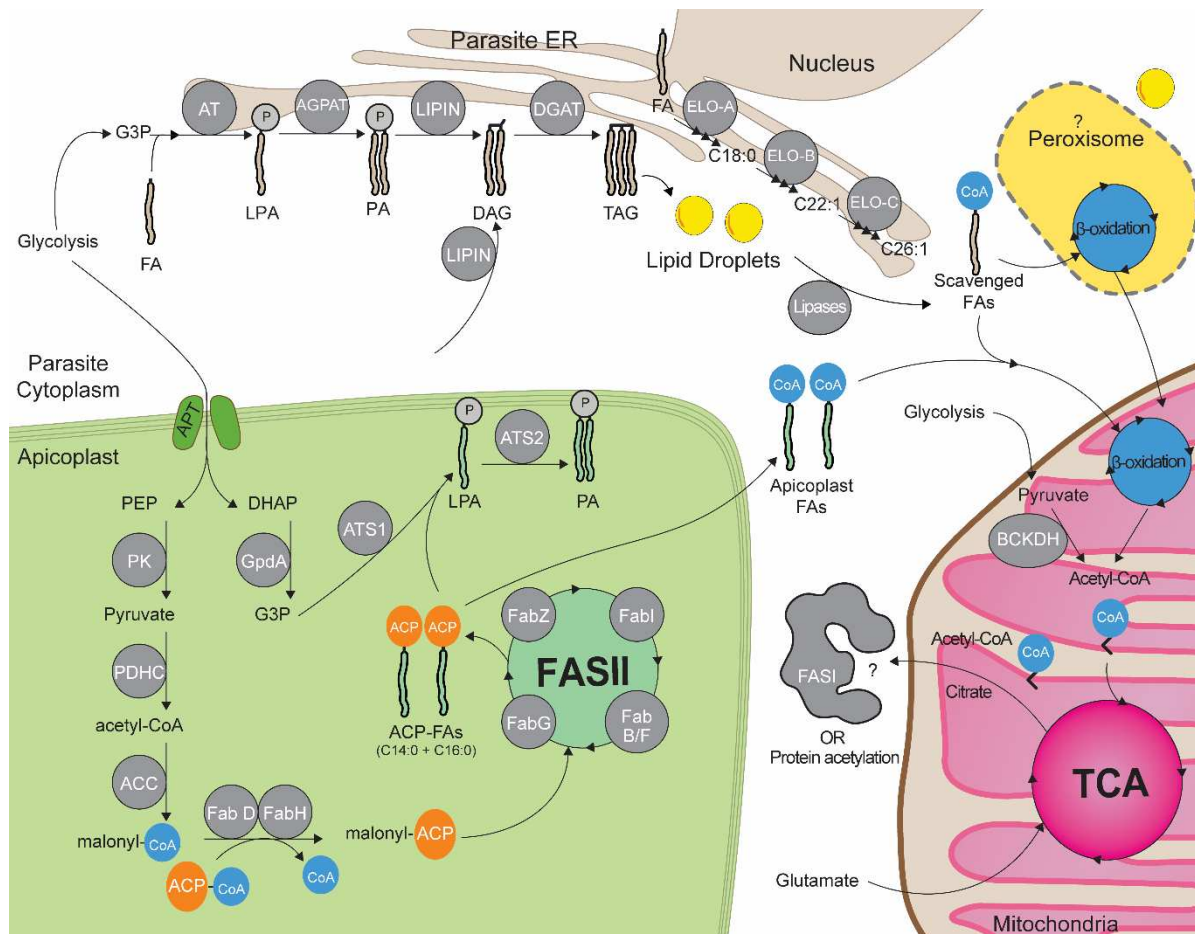


**Figure 4: Schematic representation of active invasion process for *T. gondii* and *P. falciparum* parasites.**

The invasion process (from left to right) of apicomplexa parasites consists of parasite attachment, reorientation/junction formation, parasitophorous vacuole formation and mature invasion. The proteins involved for each process for *T. gondii* tachyzoites (top) and *P. falciparum* merozoites (bottom) are indicated in red. GPI-MSP: glycosylphosphatidylinositol-merozoite surface proteins, MIC2: microneme protein 2, AMA1: apical merozoite antigen 1, RON: rhoptry neck proteins, ROP: rhoptry bulbous body proteins, Rh: rhoptry proteins, GRA: dense granule proteins, SUB: subtilisin-like serine proteases.

## The metabolic organelles:

Apicomplexan parasites possess a single apicoplast and mitochondrion like their algal ancestors (**Figure 5**).



**Figure 5: Lipid pathways of the metabolic organelles found in Apicomplexa parasites.**

The lipid metabolic pathways can be found in the ER (endoplasmic reticulum), the apicoplast, the mitochondrion, the peroxisome, and the lipid droplets (LD) of the parasite. Phosphoenolpyruvate (PEP) and DHAP (dihydroxyacetone phosphate) generated from glycolysis occurring in the cytosol of the parasites enter the apicoplast via the apicoplast phosphate transporter (APT). They are then converted to pyruvate and glycerol-3-phosphate (G3P) by a PK (pyruvate kinase) and GpdA (glycerol-3-phosphate dehydrogenase), respectively. A PDHC (pyruvate dehydrogenase complex, only present in the apicoplast) is responsible for the formation of acetyl-coenzyme A (CoA) from pyruvate. Acetyl-CoA carboxylase (ACC) converts this to malonyl-CoA. FabD/H complex leads to the attachment of an acyl carrier protein (ACP) and the loss of CoA resulting in ACP-malonyl. The fatty acid synthase II (FASII) complex (comprised of FabG/ B/F/I/Z) are responsible for the formation of

short fatty acid (FA) chains, mainly C14:0 and C16:0 FA-ACP-chains. Esterification of FA-ACP from FASII or FA-CoAs from scavenged FAs on a G3P backbone (i.e., acylation on position sn-1) can occur in both the apicoplast by a plant-like acyltransferase *TgATS1* (plant-like glycerol 3-phosphate acyltransferase), and/or at the ER by an acyltransferase (AT). It is the first step for the de novo synthesis of all glycerolipids and results in the synthesis of lysophosphatidic acid (LPA). LPA can be further acylated with another FA chain resulting in PA (phosphatidic acid) by ATS2- in the apicoplast or acyl-G3P-acyltransferase (AGPAT)-based in ER [6]. LIPIN is responsible for the dephosphorylation of PA into diacylglycerol (DAG). DAG can be further acylated into triacylglycerol (TAG) by diacylglycerol acyltransferases (DGATs). Elongation of FA chains can occur in the elongation pathway in the ER via elongases-A/B/C (ELO-A/B/C). FASI is present in *T. gondii* parasite and not essential during tachyzoite stages and has not been further characterized. Host-scavenged FAs, FA hydrolysed from TAG/DAG in LD or other lipids, and apicoplast-derived FAs can enter  $\beta$ -oxidation in the mitochondria/peroxisome. This process has not been characterized in apicomplexa parasites, but current evidence points towards the existence of a similar process. This will result in the production of Acetyl-CoA that can be incorporated into the tricarboxylic acid cycle (TCA), the main source of electrons for energy generation. Adapted from Shunmugam et al., 2022.

### **Apicoplast:**

A primary endosymbiotic event which, involved the engulfment of *Cyanobacterium* by a eukaryotic heterotroph gave rise to the chloroplast we find in plants and algae. This organelle was acquired by several apicomplexan in an event involving secondary endosymbiosis where plastids of Chromalveolata were derived from red algae (Lim and McFadden, 2010; van Dooren and Striepen, 2013). Their coevolution during the ancestral acquisition of this relict plastid and subsequent loss of photosynthesis resulted in divergent metabolic pathways compared to mammals and plants. Due to its evolutionary origins the apicoplast is made up of four membranes enriched in phospholipids and not galactolipids like their algal ancestors (Botté *et al.*, 2013). This organelle houses 4 principle metabolic processes (**Figure 6**) which are crucial to the development of the parasite and can be considered the parasites' kryptonite:

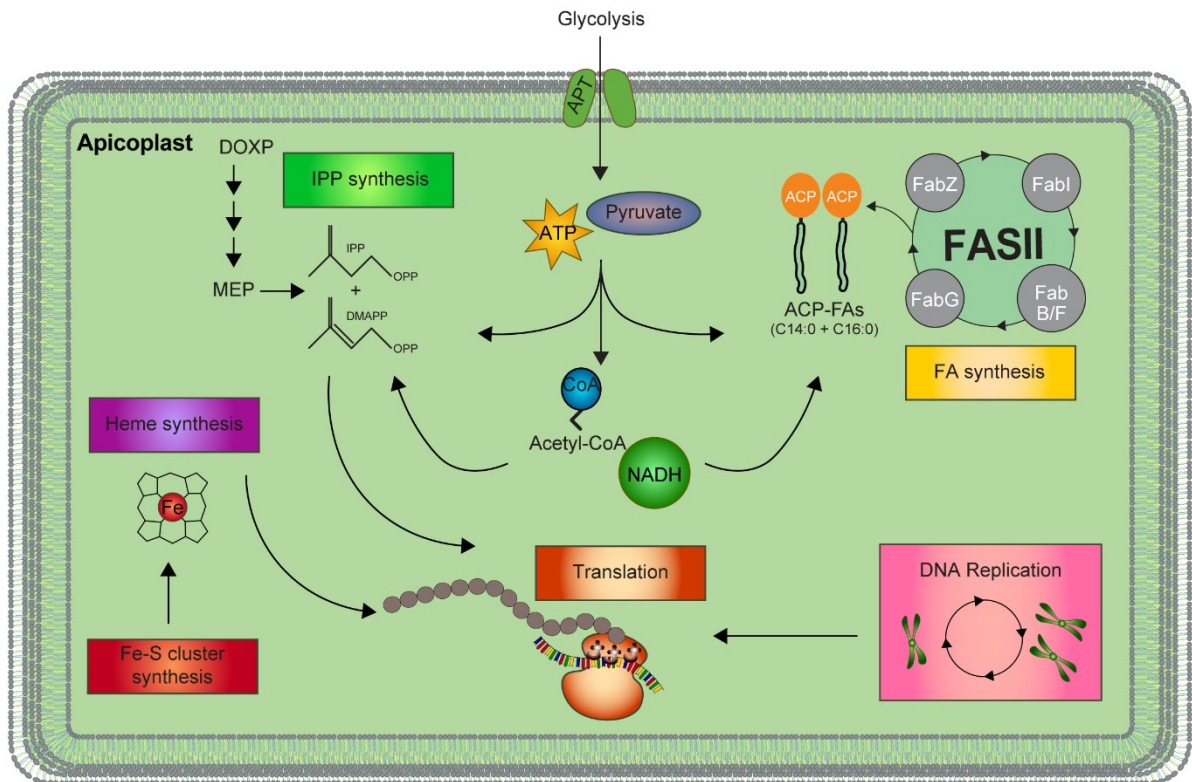
a) FASII pathway: An apicoplast-located fatty acid synthesis is dispensable in erythrocyte stages of the malaria parasite, which instead predominantly salvage fatty acids. However, the indispensability of the FASII pathway has been proven in *Plasmodium* liver stages and *Toxoplasma* tachyzoites. The total contribution of short chain fatty acids (palmitate, myristate) required for membrane biogenesis is attributed to the apicoplast FASII. This pathway aids in parasite division and survival within the host (Mazumdar and Striepen, 2007; Ramakrishnan, Melissa D Docampo, *et al.*, 2012a; Amiar *et al.*, 2016; Amiar, Nicholas J Katris, *et al.*, 2020a).

b) Isoprenoid precursor assembly pathway: Isopentenyl pyrophosphate (IPP) is an essential metabolic output of the apicoplast organelle and are important lipid components involved in tRNA modification, protein prenylation, ubiquitination etc. There are two pathways that contribute to their synthesis: a eukaryotic mevalonate pathway and a prokaryotic MEP/DOXP (2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate) pathway. Isoprenoid precursor synthesis is the only essential role of the apicoplast in *Plasmodium* erythrocytic stages (Baumeister *et al.*, 2011; Yeh and Derisi, 2011). Inhibiting IPP synthesis blocks apicoplast elongation and inheritance by daughter merozoites, and apicoplast biogenesis is rescued by exogenous IPP and polyprenols (Okada *et al.*, 2022). The DOXP pathway is also essential in *Toxoplasma* but a lack of sensitivity to drugs targeting this pathway in tachyzoites suggests the pathway cannot be exploited due to delivery across host and parasite membranes (Nair *et al.*, 2011; Sheiner, Vaidya and Mcfadden, 2013).

c) Iron (Fe)-sulphur cluster biosynthesis: These clusters are a common prosthetic group essential for protein function by mediating electron binding and transport, deeming them critical for redox reaction in the cell. Of the 4 Fe-S cluster synthesis pathways only one gene of the Suf pathway is encoded in the apicoplast genome

(Waller *et al.*, 1998; Salomaki and Kolisko, 2019). This pathway supplies Fe-S clusters for enzymes producing isopentenyl pyrophosphate (IPP), which is required for the protein/pathway preventing the loss of the apicoplast (Ralph, van Dooren, *et al.*, 2004). Retention of the apicoplast genome seems to be strongly correlated to the Suf pathway.

d) Heme biosynthesis pathway: Heme is a prosthetic group added to many proteins to allow them to carry small molecules/electrons for many redox reactions. This process takes place in the apicoplast, mitochondria and the cytosol. Only one homologue of the process has been retained in the apicoplast of these parasites. The other steps have been transferred to the other cellular compartments. Heme synthesis is essential for liver and mosquito stages of *Plasmodium* parasites. In RBC stages, the parasite is in an environment of excessive heme levels and instead it is processed and stored in the form of hemozoin to prevent toxicity (Kořeny *et al.*, 2011; Salomaki and Kolisko, 2019). Recent work has shown the evidence of a functional heme biosynthesis pathway in the *Toxoplasma* apicoplast (Bergmann *et al.*, 2020).



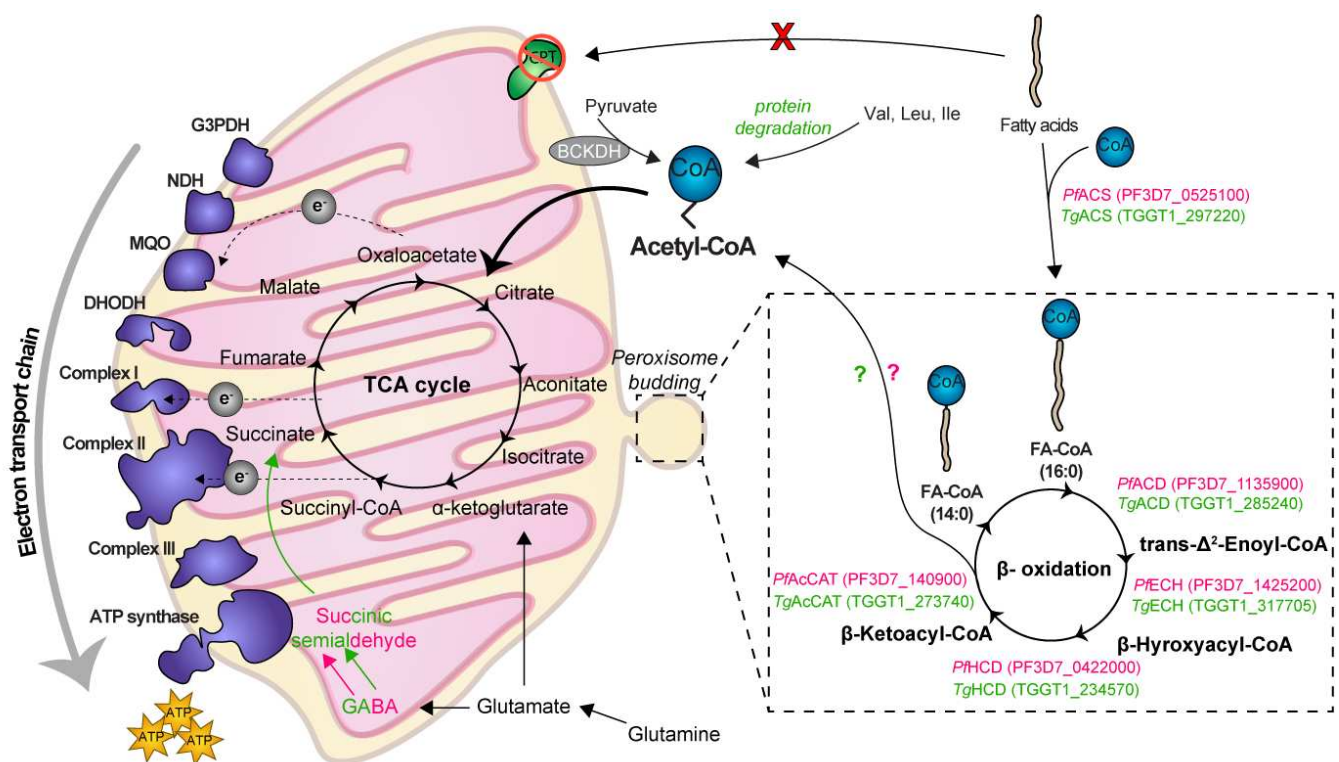
**Figure 6: Metabolic pathways in the apicoplast of apicomplexan parasites.**

Four metabolic processes exist within the apicoplast, isoprenoid (IPP) synthesis, fatty acid (FA) synthesis, Heme synthesis and iron-sulphur (Fe-S). DOXP: 1-deoxy-D-xylulose 5-phosphate, MEP: mevalonate/methylerythritol phosphate (MEP), NADH: nicotinamide adenine dinucleotide, ACP: acyl carrier protein, CoA: co-enzyme A. Adapted from (Ralph et al., 2004)).

### **Mitochondria:**

The mitochondrial genome only encodes for 3 proteins and instead mitochondrial genes are transferred to the nucleus to allow for nuclear control. This organelle is in close physical proximity to the apicoplast and essential for both *Toxoplasma* and *Plasmodium* spp., being the synthesis 'factory' for several metabolites. The Tricarboxylic acid cycle (TCA, **Figure 7**) is central to mitochondrial metabolism and contributes to biosynthetic and catabolic functions as being the primary source of electrons that fuel, the mitochondrial electron chain generating ATP. The 'textbook' TCA cycle is fed acetyl-CoA from the production of pyruvate from glycolysis, the breakdown of branched amino acids or the  $\beta$ -oxidation of fatty acids (FA). Acetyl-CoA

is combined with oxaloacetate to form citrate, which then enters a multi-stepped process which results in CO<sub>2</sub>, NADH, FADH<sub>2</sub> and GTP, and ultimately regenerates oxaloacetate (van Dooren, Stimmler and Mcfadden, 2006; Seeber, Limenitakis and Soldati-Favre, 2008; Sheiner, Vaidya and Mcfadden, 2013). The exact processes contributing to the acetyl-CoA pool for the TCA cycle is not entirely known. Only one pyruvate dehydrogenase (PDH) complex is present in the apicoplast which seems not to appreciably contribute to acetyl-CoA pool (Foth *et al.*, 2005; Crawford *et al.*, 2006; Fleige *et al.*, 2007). Instead, evidence points towards a branched-chain keto acid dehydrogenase (BCKDH) complex being the missing link required for fuelling the TCA cycle (MacRae *et al.*, 2013; Oppenheim *et al.*, 2014; Kloehn *et al.*, 2020) in all apicomplexa (except *Cryptosporidium*).



**Figure 7: The metabolic pathways of the mitochondrion of apicomplexa parasites.**

Fatty acids (FA) in other eukaryotic models can contribute to acetyl-CoA production via  $\beta$ -oxidation in the mitochondria or peroxisomes which is hypothesized to be similar for apicomplexan parasites. A carnitine palmitoyl transferase I (CPT) to allow for the transport of FA (namely C16:0) into the matrix does not exist in *T. gondii* and *P. falciparum* parasites.

Instead, it is believed that  $\beta$ -oxidation may exist in the cytoplasm or another organelle, like the peroxisome to overcome this dilemma. Putative protein homologues (bioinformatical interpretation, bottom right) for the traditional  $\beta$ -oxidation cycle exist in malaria (gene ID in pink) and *Toxoplasma* (gene ID in green) parasites, namely acyl-CoA synthetase (ACS), acyl-CoA dehydrogenase (ACD), enoyl-CoA hydratase (ECH), hydroxy acyl-CoA dehydrogenase (HCD), and acetyl-CoA acetyltransferases (AcCAT). Metabolites proving this pathway are yet to be fully identified. The acetyl-CoA from this process, protein degradation and pyruvate via a branched chain ketoacid dehydrogenase (BCKDH) feed the tricarboxylic acid cycle (TCA), the main source of electrons for energy production via the electron transport chain found in the mitochondrial inner membrane. Glutamate can also enter the TCA cycle and contribute to electron production. In *T. gondii* parasites, a  $\gamma$ -aminobutyric acid (GABA) shunt can also contribute to electron production from glutamate (in green). Adapted from MacRae et al., 2012; Sheiner, Vaidya and Mcfadden, 2013; Demas, 2016; Moog, Przyborski and Maier, 2017, Oppenheim et al., 2014 and Nugraha, Jeelani and Nozaki, 2022.

### **Peroxisome:**

Peroxisomes are multifunctional organelles present in virtually all eukaryotic cells. They are extremely plastic and can transform their shape, size (0.1- 0.5 $\mu$ M diameter) number and function strictly depending on their external environment (Lodhi and Semenkovich, 2014). These single membrane organelles normally house several oxidases and catalases involved in hydrogen peroxide metabolism and are tightly associated to fatty acid oxidation. This organelle is unique in that it is not essential under standard nutritional conditions, i.e. in the presence of favoured carbon sources such as glucose. In yeast, it is known that excessive oleic acid induces peroxisome formation and the upregulation of peroxisomal proteins (Kohlwein, Veenhuis and van der Klei, 2013). Interestingly, in yeast  $\beta$ -oxidation solely occurs in the peroxisomes and is completely absent of mitochondrial  $\beta$ -oxidation which is preferred in most other eukaryotes (**Figure 7**).

Peroxisomal biology has only recently been investigated in the field of apicomplexan parasites and now bioinformatical and wet-lab studies suggest (with some debate) the

existence of a peroxisome (Kaasch and Joiner, 2000; Ludewig-Klingner *et al.*, 2017; Moog, Przyborski and Maier, 2017). Microscopy studies have hinted at single-membrane peroxisomes in *P. falciparum* parasites (Mcintosh, Elliott and Joiner, 2005) but it seems they lack some proteins traditionally involved in  $\beta$ -oxidation. Under starvation conditions, FA oxidation metabolism has been demonstrated in *P. falciparum* parasites despite not being previously annotated (Demas, 2016).

No structural or metabolic proof illustrates the presence of peroxisomes in *Toxoplasma* parasites. Genomic and proteomic studies now show the existence of peroxisomes in this parasite (Moog, Przyborski and Maier, 2017). For this thesis, it is of interest to acknowledge the loss of a carnitine palmitoyl acyltransferase in *Toxoplasma*, required for the transport of acyl-CoA across the mitochondrial membrane for fatty acid  $\beta$ -oxidation (Danne *et al.*, 2012). It is likely that instead a peroxisomal-like organelles exists as all fatty-acid oxidation are present in *Toxoplasma*, but in *Plasmodium* spp., the functions of this organelle are fulfilled by scavenging by-products from the host (Ding, Clayton and Soldati, 2000; Kaasch and Joiner, 2000; Mbekeani *et al.*, 2018).

### **Lipid droplets:**

Lipid droplets (LDs) also known as lipid bodies/lipid particles/oil bodies, like peroxisomes, are considered highly dynamic organelles heavily dependent on the cell nutrient status. This single-membraned organelle is known as the inert lipid storage depots/banks primarily comprised of triacylglycerol (TAG) or sterol esters used under sub-optimal conditions (Kohlwein, Veenhuis and Van Der Klei, 2013; Vallochi *et al.*, 2018). LDs have been observed in asexual trophozoite stage *P. falciparum* parasites (one per parasite) but seem to be absent in gametocyte stages (Jackson *et al.*, 2004; Gulati *et al.*, 2015). LDs are closely associated to the food vacuole where heme is converted to hemozoin as it is believed that the internalized host phospholipids (PLs)

are converted to neutral lipids (TAG sterol esters) and redirected to LDs (Jackson *et al.*, 2004). Gulati *et al.*, (2015) showed that inhibition of TAG-mobilization from LD prompted gametocyte conversion.

Intracellular *Toxoplasma* tachyzoites recruit host LDs and mitochondria to facilitate the scavenging of certain biomolecules that are not *de novo* synthesized (i.e. Cholesterol) (Charron and Sibley, 2002; Nolan, Romano and Coppens, 2017a) or deficient (low DAG/TAG levels) (Shunmugam, Arnold, *et al.*, 2022). The knockdown of a phosphatase (*Tg*LIPIN) illustrates that the channelling between active membrane biogenesis and the storage of lipid in the form of LDs is crucial to the survival of the parasite and prevents lipotoxic death (Shunmugam *et al.*, 2021).

In other eukaryotic organisms LDs and peroxisomes are in proximity within the cell (Lodhi and Semenkovich, 2014) and is thought to be metabolically driven depending on the external environment. Whether there is intimate crosstalk between these two organelles in Apicomplexa parasites is yet to be fully investigated.

## CHAPTER 2: LIPID METABOLISM OF APICOMPLEXAN PARASITES

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Lipids constitute one of the fundamental building blocks of life and apicomplexan parasites are no different in demanding this crucial resource through *de novo* synthesis within the apicoplast (FASII) and host/environment scavenging. Lipid metabolism is critical to signalling, energy storage and membrane biogenesis. Their importance in several cellular functions renders their pathways as potential drug targets (Coppens and Botté, 2020). The enzymes of the apicoplast-derived fatty acid synthesis pathway have already been successfully exploited as a target against *Toxoplasmosis* infections and heavily studied in *P. falciparum* (McLeod *et al.*, 2001; Perozzo *et al.*, 2002; Surolia *et al.*, 2004; Botte *et al.*, 2011). There are multiple classes of lipids that must be considered (Shunmugam *et al.*, 2022).

### **Fatty acids (FA) and activation:**

Most lipids contain fatty-acyl chains as part of their structure (De Carvalho and Caramujo, 2018). These biomolecules have numerous functions including structural elements of phospholipid making up the membranes of the cell, serving as a part of neutral lipids for energy storage and derivatives being implicated in cell signalling.

These parasites are unique in that they can source these FA from the host cell (FASI-fatty acid synthase I), host environment or synthesize them *de novo* by the FASII in the apicoplast. The central accepted dogma in Apicomplexan parasites is that then they can compile a patchwork assembly of lipids from these different sources (Shunmugam *et al.*, 2022).

Prior to the use of these FA in anabolic and catabolic pathways, they must be metabolically activated via the thioesterification with coenzyme A, coupled with adenosine-triphosphate (ATP) molecule. This two-step reaction is facilitated by a group of enzymes called acyl-CoA synthetases (ACSS, Figure 7) (Trisha J. Grevengoed, Klett

and Coleman, 2014). Once activated these fatty acyl-CoA molecules can participate in:

- transcriptional regulation
- phospholipid (PL) and ceramide synthesis (via Kennedy/lands pathway)
- neutral lipid synthesis (di/triacylglycerol, cholesterol, esters)
- protein acylation
- FA elongation, desaturation
- FA degradation ( $\beta/\omega$ -oxidation)

In most eukaryotic models 4-6 genes encode for ACSs, but 11 and 7 putative genes exist in *P. falciparum* and *Toxoplasma* parasite (Bethke et al., 2006; Shunmugam, Dass, et al., unpublished-see Chapter 4) is involved in neutral lipid metabolism under sub-optimal nutrient conditions). These proteins seem to localize to different parts of the parasite suggesting non-redundant functions. Once these FA are activated, they can contribute to the formation of the lipid groups described further.

### **Phosphatidylcholine (PC):**

One of the most predominant phospholipids (PLs) in all cells is phosphatidylcholine (PC) since this is a major contributor to cell membranes. This is no different in apicomplexan parasites as the relative abundance of PC is 40-70% for *T. gondii* and 40-50% for *P. falciparum* parasites (Gupta et al., 2005; Welte et al., 2007; Gulati et al., 2015). The deciding group of this class of lipids, choline, is derived from the external environment of *T. gondii* tachyzoites. A cytosolic choline-kinase facilitates the *de novo* Kennedy pathway which results in the essential synthesis of PC (Gupta et al., 2005; Sampels et al., 2012, Figure 8). A nuclear CTP: phosphocholine cytidyltransferase (*PfCCT*) in *P. falciparum* is the second rate-limiting step of the *de novo* synthesis of

PC. Phospholipase C treatment induces translocation of this protein to the cytosol along with an increase PC biosynthesis (Izrael *et al.*, 2020). Malaria parasites have also adapted a different strategy by producing PC via a pathway called decarboxylation-phosphoethanolamine methylation (SDPM) that uses host ethanolamine and serine (Pessi, Kociubinski and Ben Mamoun, 2004; Pessi *et al.*, 2005; Witola *et al.*, 2008). In phospholipase, *PfLPL20*, depleted parasites, transcript levels of enzymes in the Serine Decarboxylase-Phosphoethanolamine Methyltransferase pathway (SDPM) were altered along with up-regulation of phosphocholine and SAM levels; these results show up-regulation of alternate pathway to generate the phosphocholine required for PC synthesis through the Kennedy pathway (P. Sheokand *et al.*, 2021). In contrast *Toxoplasma* cannot use these as substrates to produce PC since parasites lack the required methyltransferase activity (Gupta *et al.*, 2005).

Phospholipases govern the balance between PC and lyso-PC (LPC). High LPC levels in malaria parasite repress RBC stage sexual conversion (Brancucci *et al.*, 2017). A *TgPL2* (phospholipase 2) was identified that is responsible for the hydrolysis of a FA from PC to form LPC. The increase in PC when this protein is disrupted is combined with a destruction of the apicoplast membrane integrity, suggesting LPC/PC levels are important in maintaining the apicoplast homeostasis (Lévêque *et al.*, 2017).



acyltransferase: AT, diacylglycerol: DAG. This figure has been adapted from Ramakrishnan et al., 2013; Hartmann et al., 2014; Flammersfeld et al., 2017; Kilian et al., 2018; Neveu et al., 2020

### **Phosphatidylethanolamine (PE):**

This phospholipid is the second most abundant in these parasites with 10-20% for *T. gondii* and 15-25% for *P. falciparum* parasite (Gupta et al., 2012; Gulati et al., 2015). PE in *Toxoplasma* is made via a mitochondrial phosphatidylserine decarboxylase (*TgPSD*, **Figure 8**) or ER fusion of CDP-ethanolamine and diacylglycerol (DAG). The loss of *TgPSD* leads to suboptimal growth of the parasite but is rescued by exogenous ethanolamine (Gupta et al., 2012; Hartmann et al., 2014). PE synthesis in *P. falciparum* is multifaceted and uses the CDP-ethanolamine/Kennedy pathway as well as a serine decarboxylase pathway (Elabbadi, Ancelin and Vial, 1997; Kilian et al., 2018). PE can also be derived from PC, the inhibition of a *PfCholine* kinase (*PfCK*) leads to a decrease in PE and ultimately parasite death (Serrán-Aguilera et al., 2016).

### **Phosphatidylserine (PS):**

This phospholipid is somewhat abundant as it comprises only 5-10% of the relative abundance of lipids in *P. falciparum* and *Toxoplasma* parasites. This lipid is exclusively localized to the inner leaflet of the lipid bilayer of cell membranes. However, during certain stresses in eukaryotes PS can flip onto the surface of cells acting as a global immunosuppressor by mimicking apoptosis to evade host immune responses (Alberts et al., 2008; Birge et al., 2016). Exposure of PS on the surface of malaria-infected red blood cells contribute to their cytoadherence/sequestration and disease pathogenesis in *P. falciparum* malaria (Eda and Sherman, 2002). This is supported by increased levels of PS in *P. falciparum* positive patients (Gulati et al., 2015).

Unlike *Plasmodium* that has a single protein (*PfPSS*), in *Toxoplasma* there are two enzymes responsible for the formation of PS (using serine as a substrate) near the ER;

*TgPSS* (phosphatidylserine synthase) and *TgPTS* (phosphatidylthreonine synthase) (Arroyo-Olarte et al., 2015, Figure 8). Malarial parasites can use host-derived PS through the degradation of host haemoglobin (Wein et al., 2018). PE can also be used as a precursor of PS synthesis via serine decarboxylation path in both these parasites (Elabbadi, Ancelin and Vial, 1997; Kilian et al., 2018).

### **Phosphatidylthreonine (PT):**

Phosphatidylthreonine (PT) is unique in *Toxoplasma* as it is a major phospholipid facilitating lipid mediated virulence in the host (Arroyo-Olarte et al., 2015). *TgPTS*, a protein which has evolved from *TgPSS* (since they are both capable of PS synthesis), is used to generate PT within the ER (**Figure 8**).

It appears that PS and PT synthesis is a fine balance in this parasite as genetic disruption of *TgPTS* resulted in increased levels of PS and correlated decrease in PT (more specifically PT species 20:4 and 20:1 which are host-derived) (Arroyo-Olarte et al., 2015). This balance influences the calcium flux and storage controlling parasite egress (Arroyo-Olarte and Gupta, 2016; Kuchipudi et al., 2016). Very little is known about the presence and role, if any, of PT in *Plasmodium* (PlasmoDB, 2022).

### **Phosphatidylinositol (PI) and related phosphoinositides**

PI, which is negatively charged, accounts for 5% of the total PL composition and has critical functions such as intracellular trafficking and cell signalling. A phosphatidyl-3-monophosphate kinase in *Toxoplasma* parasites is involved in apicoplast biogenesis and lipid trafficking to and from said-organelle (Tawk et al., 2011). Another protein, *TgPH1*, binds to the phosphoinositide PI(3,5)P<sub>2</sub> and serves as a complementary reporter of this molecule in mammalian cells (Daher et al., 2016; Chintaluri et al., 2018). A phospholipase, *TgPI-PLC*, has a high affinity for PI and localises to the plasma

membrane and cytosol of the parasite suggesting plural roles in lipid regulation. (Fang, Marchesini and Moreno, 2006; Bullen *et al.*, 2016).

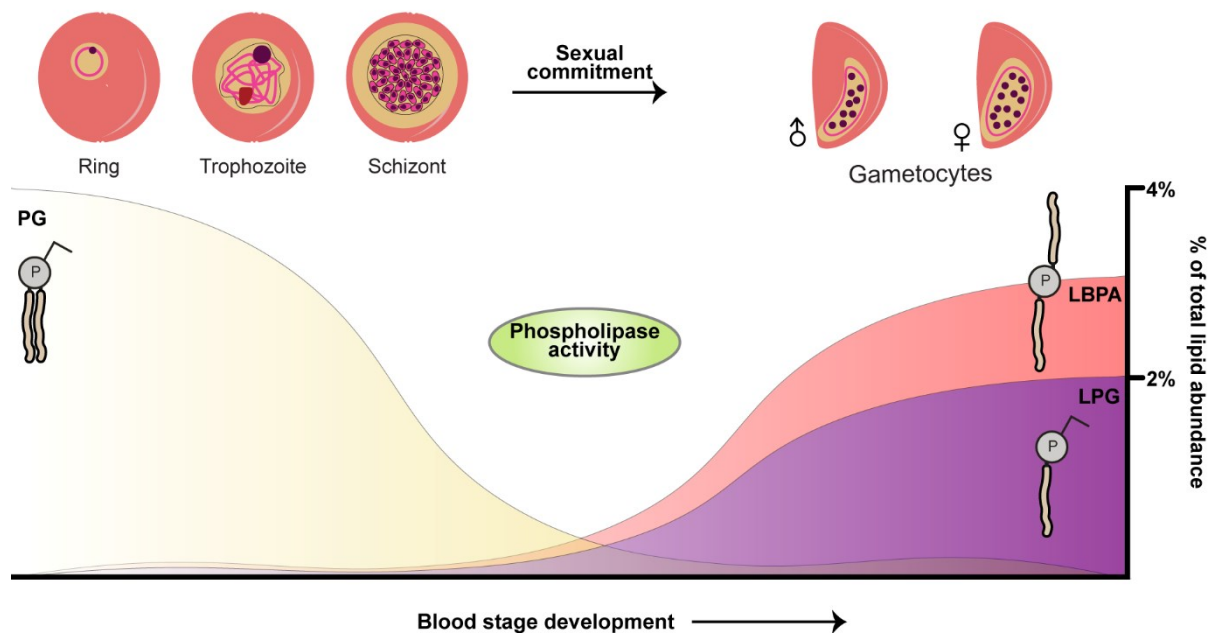
A phosphoinositide-3-kinase (PI3K) in *P. falciparum* is actively exported into the host RBC and allows for the endocytic trafficking of toxic haemoglobin from the host to the parasite food vacuole (Vaid *et al.*, 2010). A prominent drug discovery against malaria is MMV390048, an inhibitor of phosphatidylinositol 4-kinase (Paquet *et al.*, 2017; Fienberg *et al.*, 2020). The resulting product of this enzyme is a sugar-derivative of PI like glycosylphosphatidylinositol (GPI), which is known as a major pathogenicity determinant of the parasite being able to induce TNF- $\alpha$  (tumor necrosis factor alpha) production (Debierre-Grockiego *et al.*, 2006). This parasite directly channels host-scavenged myo-inositol for GPI synthesis, but uses a combination *de novo* synthesized, and host myo-inositol for other PI derivatives (Macrae *et al.*, 2014).

### **Phosphatidylglycerol (PG):**

Gulati *et al.* (2015), show that phosphatidyl-glycerol only accounts for 2-3% of the total PL content of *P. falciparum* parasites. This anionic PL increases from undetectable to 4% in infected RBC cells (Tran *et al.*, 2016), and decreases to 1% under gametocyte maturation (**Figure 9**). In other organisms, this PL is also involved in lysobisphosphatidic acids acid (LBPA, or bis (mono- acylglycerol) phosphate (BMP)) and lysophosphatidylglycerol (LPG) formation though the activity of a phospholipase A2 protein that hydrolyses a FA (Flammersfeld *et al.*, 2017). A stage-dependence dynamic of LPG abundance has been shown in malaria parasites (Tewari *et al.*, 2020). LPG is believed to be involved in calcium-cell signalling (Zhang *et al.*, 2017). Both LPG and LBPA levels increase 2/3-fold in *P. falciparum* infected RBCs (Gulati *et al.*, 2015; Tewari *et al.*, 2020). It is hypothesized that LBPA, which spike after 48 h post-infection is related to the formation of vesicles and membrane fusion for merozoite cell

remodelling. LBPA is enriched in late endosome-lysosome compartment crucial for endocytosis (Gruenberg, 2020). Human macrophages lacking Niemann-Pick C genes (displaying an autosomal disorder), lead to a disruption of endosomal lipid transport proteins and an accumulation of cholesterol, which can be rescued by exogenous PG resulting in LBPA production leading to endosomal clearing of cholesterol (Illynska *et al.*, 2021). This highlights the importance of the PG/LBPA balance.

PG synthases (from cytidine diphosphate-diacylglycerol) have not been identified in either parasite, but genes encoding homologous proteins (*PF3D7\_0820200* and *TGVEG\_005460*) do exist (Ramakrishnan *et al.*, 2013; PlasmoDB, 2022; VEuPathDB, 2022). Not much has been revealed about the role of PG in *Toxoplasma*, besides that it is essential for cardiolipin (CL) synthesis and mitochondrial homeostasis (Ramakrishnan *et al.*, 2013).



**Figure 9: The levels of phosphatidylglycerol, lysobisphosphatidic acid and lysophosphatidylglycerol in RBC stage *P. falciparum* parasites.**

Phosphatidylglycerol (PG) levels decrease as parasites transition to sexual development. The levels of lysobisphosphatidic acid (LBPA) and lysophosphatidylglycerol (LPG) seem to concomitantly increase with this conversion. It is hypothesized that a stage-dependant

phospholipase events trigger sexual commitment. Figure adapted from data in (Gulati et al., 2015; Tewari et al., 2020)

## Cardiolipin

Under plasmodial infections, cardiolipin (CL) levels and anti-CL antibodies are elevated in patients, again emphasizes that lipids are very important for the virulence of parasites (Jakobsen *et al.*, 1993; Consigny *et al.*, 2002). CL is an anionic dimeric phospholipid which usually localizes to the mitochondrial inner membrane of eukaryotic cells. It is closely associated with mitochondrial function, biogenesis, and genome stability (Mai and Altucci, 2009). CL is synthesized from phosphatidyl-CMP and phosphatidylglycerol (PG) (Schlame, 2008). Studies now show that CL induces the activation and transport of mitochondrial *P. falciparum* proteins (Nozawa *et al.*, 2020). A cardiolipin synthase (CLS) has identified but not investigated in *Plasmodium* (PF3D7\_0609400) (Ramakrishnan *et al.*, 2013).

Similarly, to *Plasmodium*, *Toxoplasma* parasites have a common mechanism in making CL from PG (TGGT\_309940) *de novo*. The fatty acid transport, acyl-CoA binding protein 2, *TgACBP2* is crucial for CL synthesis and consequently sustains mitochondrial homeostasis. A mitochondrial association factor (MAF1) mediated host mitochondrial association in host cells can restore the growth of *TgACBP2*-deficient cells, again highlighting the importance of plural-source nature of lipids like CL in this case. Additionally, cryo-EM experiments have shown that a mitochondrial ATP synthase, playing a critical role in the curvature of mitochondrial cristae in the parasite, forms a heteromer bound to CL molecules, suggesting this lipid plays a role on energy conversion (Mühleip *et al.*, 2021).

### **Cytidine diphosphate-diacylglycerol (CDP):**

Cytidine diphosphate-diacylglycerol (CDP) is a nucleotide that is at a central branchpoint of lipid metabolism. CDP synthases (CDS), the protein responsible CDP production, is often considered a gateway enzyme into the synthesis of PI and CL. CDS can regulate PA synthesis and subsequently intracellular PA signalling (Jennings and Epand, 2020). PA levels can also influence membrane curvature and hence membrane trafficking between organelles. *Toxoplasma* parasites house two distinct CDS isoforms; a eukaryotic-type *TgCDS1* found in the ER and a prokaryotic-type *TgCDS2* found in the apicoplast. Deletion of the respective proteins showed decreases in PI and PG levels, and more importantly reduced the CDP-DAG pools in the Golgi apparatus and mitochondrion. Overall, when the CDP-DAG pathway was affected, parasite virulence in mice was attenuated (Kong *et al.*, 2017). There is only a single *P. falciparum* homolog of CDS that is proteolytically cleaved. Deletion of the N-terminal end of this protein was detrimental to parasite survival (Shastri *et al.*, 2010).

### **Sphingomyelin:**

Sphingolipids *de novo* synthesis takes places within the ER-Golgi apparatus network. This class of lipids is defined by their 18-carbon amino-alcohol backbones (Adada, Luberto and Canals, 2016). Sphingomyelin synthase (SMS) is responsible for transferring the phosphorylcholine group from PC to ceramide, eventually resulting in DAG and sphingomyelin. Thereafter, this lipid is transported to other organelles for a diverse array of signal transduction pathways (Gault, Obeid and Hannun, 2010).

These parasites have retained a ceramide biosynthesis pathway via two ER-confined ceramide synthases (*TgCERS1/2*) with only one having ceramide synthase activity but both being indispensable for parasite fitness (Koutsogiannis *et al.*, 2022). *T. gondii* parasites caenge sphigomyelins but synthesis their own ceramide

phosphoethanolamines (EPC). Recent work now show that sphingolipid synthesis affects the chronic infection *in vivo* (Nyonda *et al.*, 2022).

*Toxoplasma* sphingolipids account only for 1% of the total lipid abundance, but contrastingly has an unusually high level of EPC (Welti *et al.*, 2007). A sphingolipid synthase (*TgSLS*) has been biochemically validated as an inositol phosphorylceramide (IPC) synthase (Pratt *et al.*, 2013).

Metabolic pulse-chase experiments have showed that this parasite scavenges serine and galactose for *de novo* glycosphingolipid synthesis (Azzouz *et al.*, 2002). This parasite is also highly dependent on scavenging of sphingolipids from the host. Rab-coated vesicles facilitate the recruitment of sphingolipids to the vacuolar spaces surrounding the parasite. When the sphingolipid metabolism of host cells is inhibited, by myriocin, tachyzoite replication is decreased suggesting that they still strongly rely on host-scavenging of sphingolipids (Romano *et al.*, 2013).

In *P. falciparum* parasites, the asexual stages have an abundance of 10-15% of sphingomyelin, being the second most abundant class of lipids in this stage (Gulati *et al.*, 2015). There is also the unusual presence of dihydrosphingomyelin (dhSM). An extensive tubovesicular membranes (TVM) extending from the PVM into the host cell is necessary for nutrient uptake (Lauer *et al.*, 1997). The parasite is also able to export sphingomyelin into host cells to form these TVMs (Elmendorf and Haldar, 1994; Van Wye *et al.*, 1996). Sphingomyelin synthase (SMS) activity has been seen in the Golgi-apparatus (Lauer, Ghoris and Haldar, 1995). Recent work identifies two *PfSMS* that contribute to gametocytogenesis and sex-specificity. They also show dhSM enrichment in female gametocytes and suggest that this lipid is crucial for gametocyte viability (Ridgway *et al.*, 2022).

### **Cholesterol and cholesteryl esters:**

This sterol is largely hydrophobic, localizes in eukaryotes to the plasma membrane where it is often packed with sphingolipids and GPI-anchored proteins establishing roles in the regulation of membrane trafficking, signalling and host-pathogen interactions. They are also concentrated within LD as esterified forms, i.e. cholesteryl esters (Luo, Yang and Song, 2020). Cholesterol is located within the PM and apical organelles of tachyzoites (Coppens, Sinai and Joiner, 2000).

*Toxoplasma* and *P. falciparum* parasites are auxotrophic for cholesterol and require scavenging them from the host cell and environment. Numerous studies have shown that host cholesterol levels impact *T. gondii* parasite replication (Bottova *et al.*, 2009; Hu, Binns and Reese, 2017; Nolan, Romano and Coppens, 2017a). More recently, it is now suspected that this lipid plays a role on the modulation of antigen presentation (Croce *et al.*, 2022). This parasite exploits host low-density lipoproteins (LDL) to direct exogenous cholesterol. A host membrane bound P-glycoprotein acts as an efflux pump and participates in cholesterol export towards the parasite (Bottova *et al.*, 2009).

*Toxoplasma* can make cholesteryl esters from host-derived cholesterol. A lecithin:cholesterol acyltransferase (LCAT) has been identified in *Toxoplasma*, a key enzyme that produces cholesteryl esters via transfer of acyl groups from phospholipids to the 3-OH of free cholesterol, leading to the removal of excess cholesterol from tissues (Pszenny *et al.*, 2015). Additionally, this parasite houses two acyl-CoA:cholesterol acyltransferase enzymes (ACAT) directly involved in channelling cholesterol into LD (Lige, Sampels and Coppens, 2013). Deletion of any of these ACAT enzymes leads to either a reduction in virulence/egress or parasite death due to an injurious over-production of membranes (Nishikawa *et al.*, 2005; Pszenny *et al.*, 2015; Schultz and Carruthers, 2018).

*Plasmodium* parasites do not produce any sterols and uses the human mevalonate pathway to generate IPP for sterol/sterol ester synthesis with via LDLs/HDLs as carriers (Grellier *et al.*, 1991; Coppens and Vielemeyer, 2005; Labaied *et al.*, 2011). *Pf*Niemann-Pick Type C1-Related protein (*Pf*NCR1) is suspected to be involved in this import of cholesterol, which is governed a sodium and cholesterol gradient in infected RBC found across the host membrane, PVM and PPM (Istvan *et al.*, 2019; E. H. Hayakawa *et al.*, 2020; Ahiya *et al.*, 2022). In RBC stage parasites a cholesterol transport system exists, whereby free RBC-membrane derived cholesterol is directed into the cytoplasm of the parasite via the intrusion of elongated lipid membranes that eventually fuse with the PVM (H. Hayakawa *et al.*, 2020). RBC cholesterol levels decrease upon parasite infection, but gametocyte formation leads to a relative increase in RBC-cholesterol (Tran *et al.*, 2016). Cholesterol is not stored in the form of free cholesteryl esters and is suspected to be toxic to parasites when levels are high, hence their incorporation in LD (Tran *et al.*, 2014).

### **Phosphatidic acid (PA):**

Phosphatidic acid (PA) is the simplest glycerophospholipid (negatively charged) and is considered a central precursor to all other glycerophospholipids and neutral lipids like diacylglycerol (DAG) and triacylglycerol (TAG). This lipid plays a crucial role in membrane curvature (cone shaped symmetry), thereby allowing the remodelling of the membrane for key protein interaction or events like fission and fusion (Bullen and Soldati-Favre, 2016; Tanguy *et al.*, 2019). Despite PA's importance, it only accounts of 1% in *Toxoplasma* and *P. falciparum* asexual parasites (Welti *et al.*, 2007; Gulati *et al.*, 2015). An acylglycerol-3-phosphate acyltransferase (AGPAT) can form PA through the esterification of a second FA on the sn-2 position of the LPA. PA can also be formed

via the phosphorylation of DAG via a DAG-kinase (DGK) (Bullen and Soldati-Favre, 2016; Bullen *et al.*, 2016; Shunmugam, Arnold, *et al.*, 2022; Walsh *et al.*, 2022).

A phosphatase, *Tg*LIPIN, is responsible for the conversion of PA to DAG which is essential in preventing 'lipotoxic' death by channelling host-derived FAs to LD molecules (DAG/TAG) and phospholipid membrane biogenesis (Shunmugam *et al.*, 2021). This work made up a significant part of my thesis work that was started by a previous student, Dr Sheena Dass.

PA is key for parasite biology and lipid metabolism contributing to lipid signalling (Bullen *et al.*, 2016), egress/invasion (Bisio *et al.*, 2019a), motility, cytokinesis/division (Amiar, Nicholas J Katris, *et al.*, 2020a) and ultimately what we discovered: maintaining a fine balance between lipid storage and membrane biogenesis (Shunmugam *et al.*, 2021).

### **Diacylglycerol and triacylglycerol (DAG/TAG):**

DAG and TAG are essential neutral lipids found highly concentrated in LDs. They are extremely hydrophobic and energetically dense. The FAs esterified onto these molecules can be hydrolysed and channelled toward energy metabolism via  $\beta$ -oxidation.

DAG is implicated in key signalling events (Bullen and Soldati-Favre, 2016). Bullen *et al.*, showed that *Tg*DGK1 regulates microneme secretion through a PA-mediated reaction with a microneme surface protein (Bullen *et al.*, 2016). PA acts as a lipid regulator for microneme exocytosis. DGK2 is the second kinase of this kind that is secreted into the PV and leads to PA formation from DAG, which acts as an intrinsic signal eliciting egress upstream a guanylate cyclase (GC) unique to alveolates (Bisio *et al.*, 2019a).

DAG also play a crucial role as the substrate of TAG synthesis. Two diacylglycerol acyltransferases (DGAT) exist in *Toxoplasma* (Quittnat *et al.*, 2004; ToxoDB, 2022); *TgDGAT1* and *TgDGAT2*. *TgDGAT1* localizes to the parasite ER and favourably incorporates C16:0 in TAG moieties. Oleic acid (C18:1) is the predominant FA species found in TAG of LD. Exogenous oleic acid of *Toxoplasma* parasites lead to an increase in the transcription of *TgDGAT1*, suggesting a nutrient dependant role of this protein. Additionally excessive oleic acid (more than 0.5 mM) a lipotoxic retort was provoked, which led to parasite death (Nolan, Romano and Coppens, 2017b). Parasites treated with a known DGAT inhibitor (T863), caused ER-membranous structures which can be further exaggerated with the addition of high oleic acid levels in tachyzoites. In bradyzoites this treatment reduced LD and led to poorly formed cysts (Nolan *et al.*, 2018). Our work (Shunmugam *et al.*, 2021) and this highlight how the parasite can adapt to different nutrients conditions to escape lipotoxic-induced death by channelling to TAG production for LD and preventing uncontrolled synthesis of membranes (see Chapter 3: A crucial lipid equilibrium governs the survival of asexual Apicomplexan parasites).

During my thesis I was the first author of a review entitled: 'The flexibility of Apicomplexa parasites in lipid metabolism', in which the role/pathways of lipids in Apicomplexa parasites are discussed along with a summary of recent findings on the metabolic mechanisms in host nutrient adaptation (Shunmugam *et al.*, 2022, see later in this chapter). The work discusses the following.

#### **Summary of published review (Shunmugam, Arnold, *et al.*, 2022):**

- Lipids are essential to parasites survival, especially for the stages that are rapidly dividing like blood stage *P. falciparum* parasites and *T. gondii* tachyzoites.
- These parasites conform to a widely accepted lipid metabolism dogma:

- 'MAKE' fatty acids through the apicoplast FASII pathway
- 'TAKE' fatty acids directly from the host cell or the host environment
- Combine these fatty acids into the different lipid classes contributing to membrane biogenesis, lipid signalling and lipid storage
- Scavenging of different lipids that are not made by the parasite are different in *Toxoplasma* and *Plasmodium* parasites (also depending on the life stage) and the mechanisms are described in this review.
- A fine equilibrium between lipid acquisition, membrane biogenesis and lipid storage must be maintained to promote parasite progression.
- The nutrient environment is highly variable for these parasites depending on the cells they infect and the life stages. This means that they must be able to maintain this balance while being extremely metabolically plastic.

### Résumé du travail publié (Shunmugam, Arnold, et al., 2022):

- Les lipides sont essentiels à la survie des parasites et particulièrement au cours des phases de division rapide comme la phase sanguine de *P. falciparum* et *T. gondii* au stade tachyzoite.
- Ces parasites se conforment à des mécanismes métabolique communément accepté :
  - Synthétiser des acides gras *via* la voie FASII dans l'apicoplast
  - Consommer des acides gras prélevés directement dans la cellule hôte ou son environnement
  - Combiner ces acides gras pour synthétiser les différentes classes de lipides essentielles à la biosynthèse membranaire, la signalisation lipidique et le stockage.
- Les mécanismes de capture de lipides d'origine exogène diffèrent entre les parasites *Toxoplasma* et *Plasmodium*, et en fonction de leurs stades de développement. Ces mécanismes sont décrits dans cette revue.
- Un équilibre doit être maintenu entre l'acquisition de lipide, la synthèse de membrane et le stockage de lipides pour permettre la progression du parasite.
- L'environnement nutritif est très variable pour ces parasites en fonction du type de cellules qu'ils infectent et de leur stade de développement. Ils doivent donc être capables de maintenir cet équilibre en adoptant des stratégies de plasticité métabolique.

REVIEW

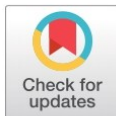
# The flexibility of Apicomplexa parasites in lipid metabolism

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## Abstract

Apicomplexa are obligate intracellular parasites responsible for major human infectious diseases such as toxoplasmosis and malaria, which pose social and economic burdens around the world. To survive and propagate, these parasites need to acquire a significant number of essential biomolecules from their hosts. Among these biomolecules, lipids are a key metabolite required for parasite membrane biogenesis, signaling events, and energy storage. Parasites can either scavenge lipids from their host or synthesize them de novo in a relict plastid, the apicoplast. During their complex life cycle (sexual/asexual/dormant), Apicomplexa infect a large variety of cells and their metabolic flexibility allows them to adapt to different host environments such as low/high fat content or low/high sugar levels. In this review, we discuss the role of lipids in Apicomplexa parasites and summarize recent findings on the metabolic mechanisms in host nutrient adaptation.

## OPEN ACCESS

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## Introduction

Lipids are one of the most predominant constituents of any living organism and are crucial for cell development and division. They serve as the building blocks of biological membranes, such as those from intracellular organelles. Lipids are also major signaling molecules and serve a central storage moiety for the generation of energy and new membranes. Lipids are therefore essential in the pathogenesis of infectious diseases [1]. Apicomplexa parasites, which are responsible for major social and economic burdens, are no different in this need for substantial amounts of lipids for survival. *Plasmodium* sp. and *Toxoplasma gondii* both belong to this phylum and are the infectious causative agents of malaria and toxoplasmosis, respectively [2,3]. These obligate intracellular parasites have 2 distinct life cycles, either alternating between asexual and sexual life stages, and/or replicative versus low replicative life stages (more particularly for *T. gondii*: highly replicative in any nucleated cells from warm-blooded animals versus chronic stages nondividing cells), with distinct organism/cellular requirements, as well as specific replicative and metabolic profiles.

All these life stages are highly dependent on large and specific amounts of lipids, as well as maintaining a specific lipid homeostasis to sustain intracellular development, membrane/organelle biogenesis, and parasite survival and propagation. Parasites also require lipids as

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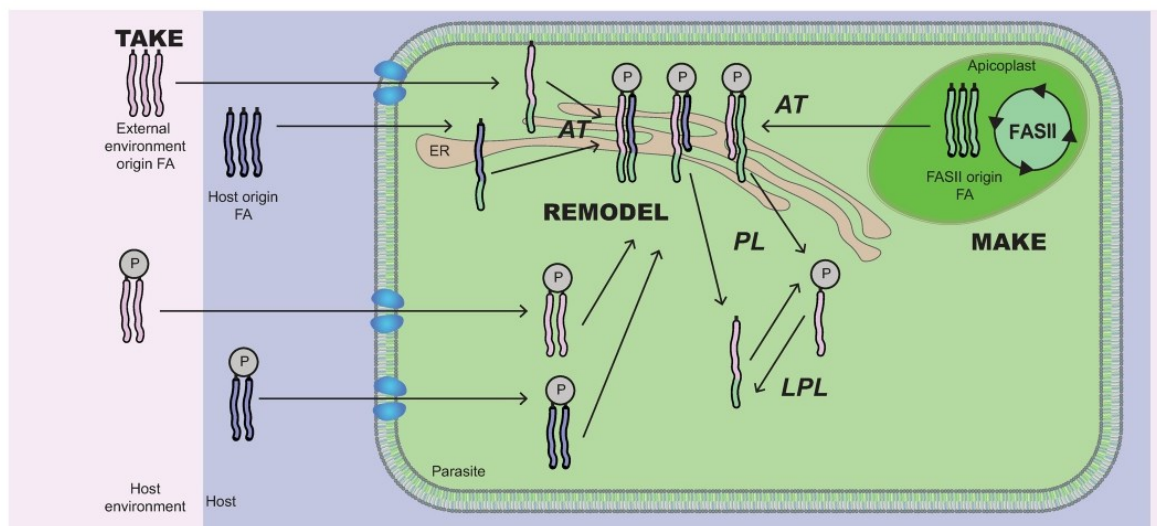
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signaling molecules, notably to regulate and control the timely release of the secretory organelles called micronemes, which trigger active invasion and egress mechanisms of the parasite in and from their host cell. Importantly, these parasites can metabolically adapt to changing host nutrient environments, setting up a unique metabolic program upon the host nutritional status and, hence, continue to optimally propagate. The unique lipid metabolism of these parasites has been thoroughly characterized by a so-called “make and take” paradigm, where lipid acquisition is met by a combination of de novo–made lipids (“make lipids”) and scavenged lipids from the host (“take lipids”) [4] (Fig 1). Furthermore, recent studies uncovered that the parasites’ survival is governed by their ability to salvage and synthesize simple fatty acyl chains and carry out an obligate “patchwork” assembly with lipids originating from both sources to generate most lipid species and thus maintain the essential parasite lipid homeostasis [5]. Importantly, recent work revealed that the parasite metabolic ability to scavenge, make de novo and reassemble lipids, are regulated, and controlled via sensing and metabolic adaptation of the parasite toward the nutritional status of the host and nutrient availability [6]. Changes in the host nutrient induces a metabolic reprogramming of the parasite where different sets of genes and proteins and not a unique one are responsible for parasite adaptation and survival.

This review will primarily cover recent developments in lipid synthesis/salvage in *Toxoplasma* with reference to other apicomplexan parasite for comparison.

### Lipids are essential in Apicomplexa parasites

Originally, these parasites were thought to solely rely on scavenging from the host for lipids, and not to being able to synthesize their own. Instead cellular investigations, genome mining, and biochemical investigations revealed the presence of a nonphotosynthetic plastid, the apicoplast, harboring a prokaryotic type II fatty acid (FA) synthesis pathway, which is essential during tachyzoite and becomes dispensable during bradyzoite development [7–11]. The pathway in sexual stages (oocyst formation in cats) remains mainly understudied, and its



**Fig 1. “Make, take, and remodel”;** the central dogma explaining how the parasite is able to scavenge lipids from the host environment, the host itself (“Take”) while also synthesize them de novo (“make”). FA elongation and a patchwork assembly mechanism in the ER contribute further to glycerolipid metabolism in the parasite (“remodel”). AT, acyltransferase; FA, fatty acid; LPL, lysophospholipase; PL, phospholipase.

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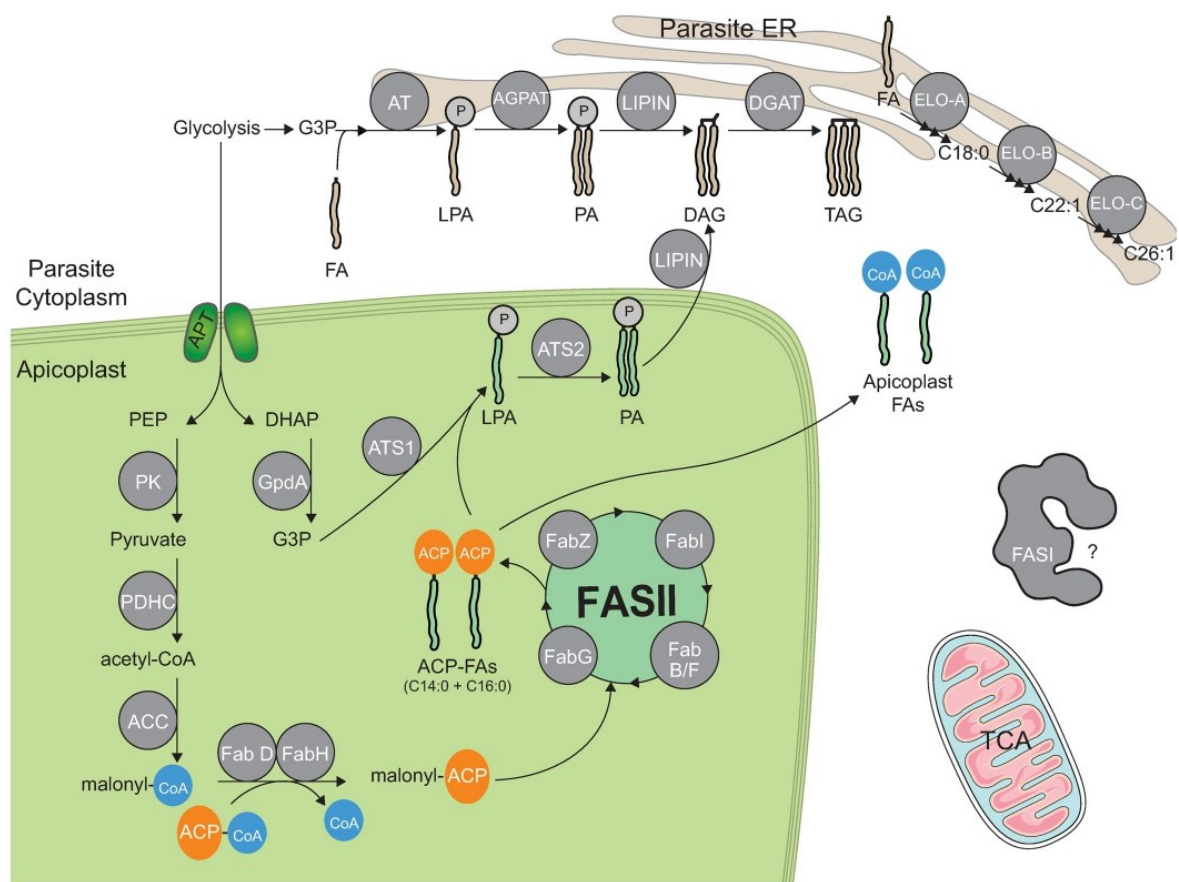
essentiality is unclear. Unlike tachyzoites, we know that *T. gondii* oocysts rely on FA breakdown to fuel the tricarboxylic acid (TCA) cycle through the production of acetyl-CoA to generate energy rather than glycolysis [12].

Further numerous analyses allowed for the “make and take” central dogma of lipid metabolism in Apicomplexa parasites to be described and accepted [4]. The FASII pathway, in *Plasmodium* parasites fast replicating blood stage, is not critical for parasite survival in nutritionally rich environments because NADH-dependent enoyl-ACP reductase (FabI) and FabB/F can be deleted. It is vital, however, during the mosquito and liver stages of the parasites in the rodent and human species, respectively [13,14].

Additionally, to the apicoplast FASII, another FA synthesis pathway can be found in *T. gondii*, namely the cytosolic eukaryotic type I FA synthase, the FASI. FASI is believed not to be essential in *T. gondii* tachyzoite life stages [15] and is completely absent in *Plasmodium* parasites. The contribution of FASI is yet to be fully determined, but it is believed to be inactive during tachyzoite life stage [15] but could play a more important role during the chronic part of the disease, for the development of bradyzoite life stages. Importantly, the parasite depends on an obligate combination of FAs to generate and maintain parasite lipid homeostasis [5]. Indeed, the parasite uses both host-scavenged FAs and de novo-synthesized FA from the relict plastid, the apicoplast, via its prokaryotic FASII pathway [4], to be assembled together and make the bulk of most parasite membrane lipids [5]. FA pools can be further elongated by the addition of 2-carbons units from acetyl-CoA via the ER-based elongases, ELO1, 2, 3 (Fig 2) [16–18]. Glycerolipids synthesis, which leads to the synthesis of phospholipid, the major membrane lipid classes present in the parasite membranes [19–21], is fueled by this essential influx of FAs from the 2 different sources (“make and take”). The synthesis of glycerolipids is composed of an FA “mix and match” process near the ER/cytosol allowing the parasites to maintain a finely tuned balance between the 2 FA sources [5]. This extraordinarily complex and seemingly redundant presence of 2 FA syntheses pathways is in fact not so complex, but instead reflects the parasite’s versatile and diverse capacities to generate and obtain lipids and precursors, crucial for its survival. Indeed, both FA/lipid scavenging and de novo syntheses pathways are essential and allow the parasite to adapt to the adverse or rich nutritional conditions met within the host. Furthermore, such complex pathway of “take, make, remodel, and adapt to the host” involves numerous proteins, which manipulate lipids as part of the general metabolic homeostasis of the parasite within host nutrient changes. Many of these proteins become essential during tachyzoite life stages, while others become involved and pivotal under certain host nutritional conditions, settings lipid acquisition metabolic programs, accordingly, to host nutrient availability.

### Lipids made by the parasite can be influenced by the nutrient environment

Apicomplexa parasites harbor a plant-like plastid acquired from the secondary endosymbiosis of a red algal ancestor [22], the apicoplast (Fig 2). This plastid has lost its photosynthesis ability but kept other essential plant-like metabolic pathways such as the isoprenoid precursor synthesis, i.e., the DOXP pathway, the iron-sulfur cluster synthesis pathway and the prokaryotic type FASII pathway [23]. Bioinformatic reconstruction of the pathway, combined to biochemical analyses, especially using stable isotope <sup>13</sup>C labeling, in both *P. falciparum* and *T. gondii*, demonstrated that the parasite imports phosphoenolpyruvate (PEP) acid generated by the parasite glycolysis pathway into the apicoplast using plant-like transporters (APT in *T. gondii* and TPTs in *P. falciparum*) [5,20,24,25]. PEP is then converted to pyruvate by the pyruvate dehydrogenase complex (PDH, which exclusively exists in the apicoplast and not in the mitochondria [26]) after which converts pyruvate to acetyl-CoA, fueling the FA synthesis pathway and



**Fig 2. An illustration of the known proteins involved in glycerolipid synthesis in Apicomplexa parasites.** PEP and DHAP generated from glycolysis occurring in the cytosol of the parasites enter the apicoplast via the apicoplast phosphate transporter (*TgAPT1*). They are then converted to pyruvate and G3P by a PK and GpdA, respectively. A PDHC (only present in the apicoplast [91]) is responsible for the formation of acetyl-CoA from pyruvate. ACC converts this to malonyl CoA. FabD/H complex leads to the attachment of an ACP and the loss of CoA resulting in ACP-malonyl. The FASII complex (comprised of FabG/B/F/I/Z) are responsible for the formation of short FA chains, mainly C14:0 and C16:0 fatty acyl (FA) ACP-chains. Esterification of FA-ACP from FASII or FA-CoAs from scavenged FAs on a G3P backbone (i.e., acylation) can occur in both the apicoplast by a plant-like acyltransferase *TgATS1* [5], and/or at the ER by a GPAT [93]. It is the first step for the de novo synthesis of all glycerolipids and results in the synthesis of LPA. LPA can be further acylated with another FA chain resulting in PA by *ATS2*-apicoplast or *AGPAT*-based ER [6]. LIPIN is responsible for the dephosphorylation of PA into DAG [39]. DAG can be further acylated into TAG by DGATs [94]. Elongation of FA chains can occur in the elongation pathway in the ER via *ELO-A/B/C* [16]. FASI is present in *T. gondii* parasite and not essential during tachyzoite stages and has not been further characterized [15]. ACC, acetyl-CoA carboxylase; ACP, acyl-carrier protein; DAG, diacylglycerol; DGAT, DAG acyltransferase; DHAP, dihydroxyacetone phosphate; FA, fatty acid; GpdA, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceral-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PDHC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TAG, triacylglycerol.

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its 4 core proteins (FabB/F, FabG, FabZ, and FabI) allowing the elongation of the growing FA chain [27]. The FASII pathway produces relatively “short” FA chains, mainly C12:0, C14:0, C16:0, and very little C18:0 [5,16]. The identification and characterization of 2 plant-like acyltransferases (so-called *ATS1*/*ApiG3PAT* and *ATS2*) located within the apicoplast revealed the capacity of Apicomplexa parasites to sequentially synthesize then esterify activated FA chains (i.e., Acyl-CoA/ACP) onto a glycerol-3-phosphate backbone resulting in the formation of lysophosphatidic acid (LPA) and, thereafter, phosphatidic acid (PA) [5,6,14,28]. Inducible disruption of *TgATS1* is lethal to tachyzoites and causes a drastic reduction of phospholipid content

(approximately 50% reduction) including bulk phospholipid synthesis, leading to the disturbance of most parasite organelles while highly affecting the apicoplast membrane integrity. Nevertheless, *TgATS1* disruption (lethal) versus *TgATS2* disruption (nonlethal) revealed that LPA C14:0 is a major apicoplast product essential for the parasite survival and addition of exogenous LPA C14:0 on *ATS1* mutant can rescue the parasite growth compare to LPA C16:0. Similar results were obtained by disrupting the *ATS1* of *P. falciparum* during liver stage, emphasizing the critical role of the apicoplast lipid synthesis during the important life stage for parasite propagation [28]. In the original chloroplast, acyltransferases are purposed to galactoglycerolipid synthesis essential for chloroplast biogenesis and photosynthetic activity [29] but the lack of such lipids in malaria and toxoplasmosis parasites [19,30] and the repurpose of *ATS1/2* [5,6] illustrate the remodeling of the organisms to be conform to an parasitic intracellular lifestyle.

The FA synthesis capacity in the apicoplast is also dependent on host nutrient availability. In *P. falciparum*, it was shown that the FASII pathway in the apicoplast is essential during liver and mosquito stage while being dispensable during blood stages [13,31,32]. Contrastingly, transcriptomics analyses in malaria patients showed that under starved conditions, the expression of the apicoplast FASII was highly up-regulated in the human host, suggesting that the pathway could be more versatile than originally thought [33]. It was shown that under minimal lipid environment, which still sustain normal parasite growth, the apicoplast FASII could be metabolically (re)activated and generate medium size FA (C14:0) de novo within the parasite [20,34].

This emphasizes the parasite's ability to sense host conditions and alter its own metabolic processes to optimize its own development and propagation according to the host environment. In *Toxoplasma*, nutrient availability directly influences the synthetic processes of the apicoplast's FASII [35–38]. For example, in harsh/low host nutritional conditions, stable isotope labeling of the FASII products showed that FASII is metabolically up-regulated and capable of producing significantly more FAs (C14:0) to compensate for the lack of lipids in the external environment. A similar mechanism was revealed in *P. falciparum* blood stages where FASII is reactivated under low host lipid environment, becoming critical for parasite survival in blood stages under these conditions over a long period. Likewise, FASII activity can also be reduced to compensate for elevated levels of FA/lipids scavenged from the host to maintain lipid homeostasis, normal parasite propagation, and avoid death by lipotoxicity [39].

Recent findings have shown that FASII disruption by antibiotics (triclosan; [11]) or gene deletion dehydrogenase complex (PDH), malonyl-CoA-[acyl carrier protein] transacylase (FabD) [40], and FabZ [37] leads to an inhibition of parasite growth, which can be rescued with the supplementation of myristic and palmitic acids, confirmation that these acyl chains are the main end products of the pathway. This was similarly observed in the *ATS1* mutant, which is rescued after LPA C14:0 supplementation. This illustrated essentiality of host lipid scavenging and the parasite's ability to compensate for FASII disruption.

Unlike *Plasmodium* and *Toxoplasma*, *Cryptosporidium* behaves atypically where in it undergoes sexual and asexual stages in a single host and lacks the apicoplast. Intracellular FA synthesis relies on the giant eukaryotic type I FA synthetase (FASI), which is also conserved in *Toxoplasma* but inactive during rapid tachyzoite life stages and completely absent in *Plasmodium*. Host scavenged "short" FA chains C16:0 are used in *CpFASI* as a substrate for synthesis of "longer" FA chain up to C22:0 that are then involved in lipid and membrane remodeling. Further, this parasite also possesses typical Apicomplexa elongases, but for elongation up to 2 carbons (not 6) from host-scavenged FAs. The absence of FASII and that the substrate of *CpFASI* is a host-derived FA, suggests that the parasite is not able to perform synthesis of de novo full-length FA chains and it solely relies on its scavenging capacities [41].

## Fatty acids are also directly scavenged from the host and extracellular environment

Parasite also obtain lipids by scavenging the host cell and its extracellular environment [42–44]. Using radioisotope-labeled lipids or precursors, and fluorescently labeled lipids, it has been shown that scavenged host lipids can be actively imported into the parasite and its organelles including the plasma membrane and the endomembrane system of the parasite [42]. These studies first showed how the parasite can import FA, PLs, and cholesterol. Sphingolipids, which are essential for parasite replication, are another important class of lipids actively scavenged by the parasite from host Golgi-derived vesicles despite the presence of a nonessential sphingomyelin synthase in *Toxoplasma* [45,46]. Furthermore, the cellular and metabolic fate of the scavenged lipids remained unknown. Recent studies showed that *Toxoplasma* can scavenge membrane/lipid material from host lipid droplets hijacked by the parasite [47,48], FA from the host mitochondria [49], and that oleic acid (C18:1) is an important scavenged FA that can kill the parasite by lipotoxicity [50]. Collectively, the existing data were suggesting that the parasite needs to scavenge lipids for survival as confirmed by disruption of the apicoplast FASII and/or complementation by external FA sources [5,6,16,37,40]. The use of metabolic labeling and lipidomic have now shed more light on the nature and the cellular destiny for such imported FA. Amiar and colleagues showed that most major phospholipid classes (PC, PE, and PI) and their molecular species were generated from an obligatory combination of FA directly scavenged from the host. Yet, this was using an indirect method labeling de novo-made FA from the apicoplast, and an actual approach to directly quantify the FA “scavengome” was missing. Dass and colleagues addressed this issue by directly fueling the host FAS pathway with  $^{13}\text{C}$  glucose to generate its own  $^{13}\text{C}$  labeled FA and phospholipids. Using such approaches allowed for monitoring and quantification of the scavenging capacity and deciphered the first parasite scavenged lipidome. This confirmed that very long chain FAs are scavenged [16]. Tachyzoites scavenge considerably more medium and long chain FAs than previously thought, notably unsaturated FAs such as palmitoleic acid (C16:1), oleic acid (C18:1), and paullinic acid (C20:1) [39]. Certain lipids and FAs are not produced by the parasite and must be obtained from the host of their environment [42,44,51]. Most importantly, this approach determined that the parasite constantly scavenges host FAs, which are then stored as triacylglycerol (TAG) in parasite lipid droplets, and then timely mobilized when the parasite most need them during parasite division. Altogether, this allows a constant access to lipid resources without the parasite being affected by potential lipotoxic death. However, using radioisotope and fluorescently labeled lipids, it has also been shown that, beyond being directed to lipid droplets, recruited host lipids can be directed also enter into the endomembrane system of the parasite [52].

Expectingly, the host nutrient environment strongly governs the scavenging capacity of *Toxoplasma* tachyzoites as well as the de novo lipid synthesis capacity. Amiar and colleagues have further shown that under host starvation, the parasites can stimulate “overscavenging,” inducing the formation of giant multivesicular bodies made from deep modifications of host organelles, further directed toward the parasite vacuole and which content is imported in the PV [6]. The identity and composition of giant multivesicular bodies (gMVBs) are still unknown at this stage. However, electron microscopy strongly suggest that they originate mainly from the host nuclear envelope/ER and may somehow allow the parasite to increase its lipid scavenging capacity in the absence of nutrient-rich serum. Further research is required to fully characterize their role in host–parasite interactions specific to the host-nutrient environment.

Mi-ichi and colleagues reported that palmitic acid (C16:0) and oleic acid (C18:1) is the sole combination of FAs for intraerythrocytic asexual proliferation of *P. falciparum* parasites

[34,53]. These FAs can be further activated to acyl-CoAs by 4 acyl-CoA synthetase (ACS) found in *Plasmodium* sp. genome. Intriguingly, *P. falciparum* harbor an additional 9 ACSs, which happen to be encoded in the subtelomeric regions where a high level of recombination occurs such as the *var* genes [54]. This emphasizes their importance for the survival of these life stages [27,54]. Of these ACSs, 2 of them have an apicoplast targeting sequence, but their localization and role in FA synthesis remain unclear. Recently, ACS11 is shown to be critical for parasites survival and been proposed as an interesting drug target, with existing MMV compounds [55].

Exogenous FAs, phospholipids, and those released by phospholipases (PLA2) can be recycled and used in phospholipid and neutral lipid assembly [56–58]. Recent work by 2 independent studies have also shown that *Pf*PLA2 is essential for gametocytogenesis during blood stages [57] and the fertilization process during mosquito stages [59]. Collectively, these studies emphasize that the synthesis of endogenous FAs is not essential for parasite growth and may in fact solely rely on exogenous FAs in blood stage parasites.

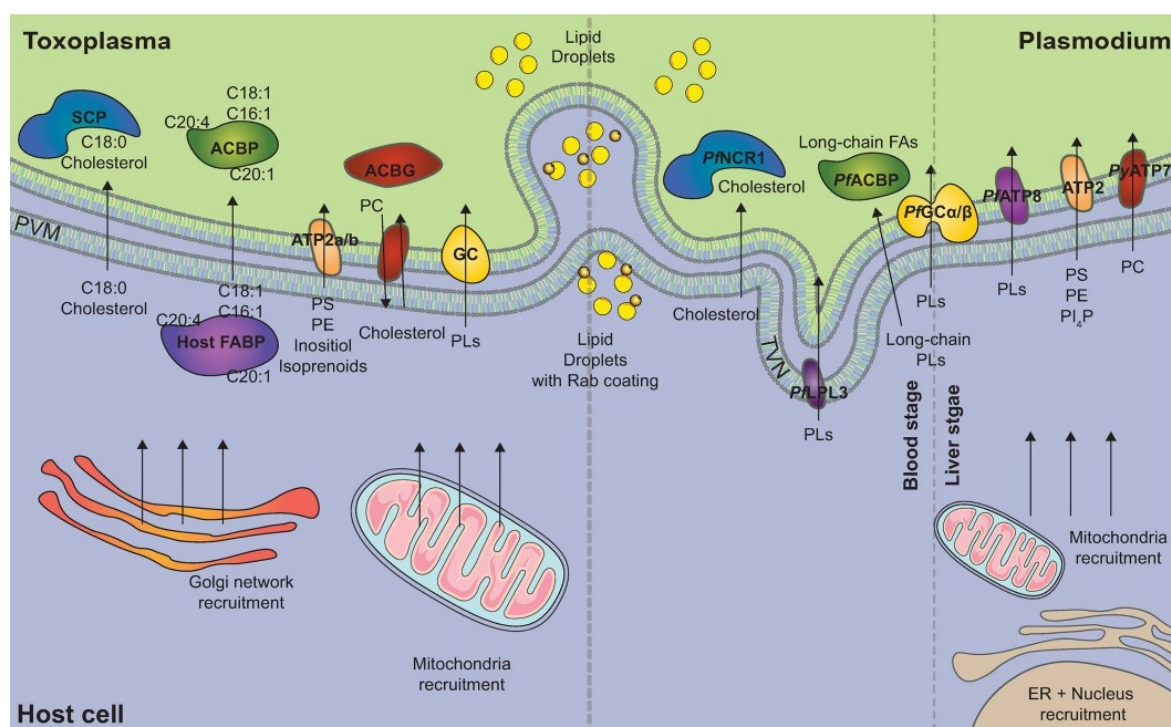
### Other classes of lipids also need to be directly scavenged from the host and host environment

*T. gondii* and *Plasmodium* parasites are auxotroph for cholesterol and must be scavenged from the host or their environment [60,61]. Nishikawa and colleagues identified an acyl-CoA: cholesterol acyltransferase localized to the *T. gondii* parasite ER that produces sterol esters and forms lipid bodies (Fig 3). Upon depletion of this protein, free FAs' levels increased. They were also able to determine that in a host lipoprotein-free environment, there was a decrease in lipid droplet numbers of the parasite. This indicates that the host lipid availability governs neutral lipid synthesis in *Toxoplasma* [62]. Recently, *T. gondii* serine hydrolases with esterase and thioesterase activities have been identified and could be acting upon lipids to release FA chains. This also highlights the potential for targeting parasite hydrolases against lipids for therapeutic applications against the parasite [63].

The process by which *Plasmodium* parasites take up cholesterol is still largely unknown. Malaria parasites have moderate need of sterols for optimal development in liver stages, but they can adapt to survive in cholesterol-restrictive conditions by exploitation of accessible sterols derived from alternative sources in hepatocytes to maintain proper infectivity [64]. Recently, a *Pf*Niemann–Pick Type C1-Related protein (*Pf*NCR1) was suggested as an essential molecule for cholesterol transfer intraerythrocytic stage parasites [65]. Tomographic imaging results suggest that *P. falciparum* initially takes up external cholesterol and/or membrane cholesterol from the inner erythrocyte membrane, after which budding lipid membranes are elongated, migrate to the cytosol, and may fuse with and/or pass through the PVM to eventually reach the parasite body [66].

### Lipid scavenging mechanisms

Identification of proteins and pathways involved in the scavenging of host lipids remain scarce due to the highly divergent and poorly conserved nature of such effectors in *Toxoplasma* and *Plasmodium* parasites. The parasite is able to manipulate the host's microtubules (Fig 3) to redirect Rab vesicles from the host Golgi network toward the PV [67]. Together with the action of GRA proteins (GRA2, GRA6 . . .), they then generate an extensive tubular network called the intravacuolar network (IVN) in the parasite parasitophorous vacuole, which contributes to host-lipid scavenging and is dependent from parasite-dense granule proteins [52]. Host lipid droplets and vesicles can also be endocytosed through this parasite-made network due to their Rab7 coating, which is redirected by the parasite. *Plasmodium* asexual blood stages are also



**Fig 3. Scavenging mechanisms known in *T. gondii* (left) and *Plasmodium* sp. (right) parasites.** *Toxoplasma* (left): *T. gondii* infection displays active recruitment of the host Golgi network and mitochondria to the parasite and is important in nutrient scavenging. Host lipid droplets can be endocytosed into the parasite through the PVM due to their Rab7 coating. Rab vesicles also facilitate the formation of the intravacuolar network found in the PVM. Stearic acid (C18:0) and cholesterol are taken up from the host via an SCP and host FABPs and parasite ACBPs modulate the importation of acyl chains into the parasite and to specific organelles. ABCGs are responsible for the influx/efflux of PC and cholesterol between the host and the parasite. Other lipid transporters are responsible for the scavenging of PS, PE (*Tg*P4-ATPase1-3), choline, sphingolipids, inositol, and isoprenoids; all involved in lipid metabolism. GC is an atypical flippase whose exact mechanism is not yet known but is proposed to be a PL transporter. *Plasmodium* (right): Lipid droplet scavenging occurs in a similar manner to that in *Toxoplasma* parasites. Malarial infection displays active recruitment of the host ER/nucleus and mitochondria to the parasite (not in blood stages). It is suspected that *Pf*NCR1 is important in cholesterol transport from the host. *Pf*LPL3 localized to the intravacuolar TVN. *Pf*LPL3 allows to hydrolyze host phospholipids to generate FA then used for DAG/TAG formation 3 *Pf*ACBPs have been identified that show an affinity to transporting phosphorylated long-chain fatty acyl chains (PLs only). A GC protein also exists in *Plasmodium* (*Pf*GCα/β) coupled to a P4 ATPase. ATP8 (in *P. falciparum*) and ATP2 (in *P. falciparum/chabaudi/berghei*) responsible for the scavenging of an assortment of PLs. *Py*ATP2 is responsible for PC transport into the parasite. ABCG, ATP-binding cassette G family transporter; ACBP, acyl-CoA binding protein; DAG, diacylglycerol; FA, fatty acid; FABP, fatty acyl binding protein; GC, guanylate cyclase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; *Pf*NCR1, *Pf*Niemann-Pick Type C1-Related protein; PL, phospholipid; PS, phosphatidylserine; PVM, parasitophorous vacuole membrane; SCP, sterol carrier protein; TAG, triacylglycerol; TVN, tubulovesicular network.

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capable for generating trafficking network within the host cell, via extensions of the PVM into the red blood cell cytosol, also known as the tubulovesicular network (TVN), which can promote nutrient exchange including lipids. Indeed, a recent study showed that the parasite expresses a lysophospholipase (LPL) (i.e., lipid-hydrolyzing enzyme) that locates at the TVN to generate FA used for timely membrane biogenesis during schizogony [68]. It has also been shown that the loss of a phospholipase can inhibit the parasites ability to scavenge lipids and further suggests that host lipids are immediately recycled at the point parasite entry at host-parasite interface [47].

The host cell mitochondria and Golgi network have shown to be actively recruited/move toward the PVM right after infection and can contribute to lipid homeostasis [49,61]. Host mitochondria are recruited in close vicinity to the parasite parasitophorous vacuole during

*Toxoplasma* infection and limit the parasites' ability to uptake FA, but it is also suggested that host mitochondria are involved in innate immunity against parasite infection [49]. In host cells lacking mitochondrial fusion, there is an increase in FA uptake [49], hence also pointing that beyond limiting FA supply, mitochondria is also an active source of FA for the parasite. The PVM's highly porous nature, which allows molecules up to 1.9 kDa into the parasite from the host cytoplasm, is accredited to the secreted parasite proteins GRA17 and GRA23 [69,70]. The parasite can mediate these processes of lipid uptake involving many different host-pathogen interactions through tubular networks and escort proteins. A sterol carrier protein (SCP) called TgHAD-2SCP2 is able to transport cholesterol and oleic acid to be incorporated into neutral lipids [71]. *Toxoplasma* also has 6 ATP-binding cassette G family transporter (ABCG), which localizes differentially within the parasite and are shown to be involved in phospholipid and cholesterol import/efflux [72]. Not only can these parasites scavenge FA, but they can also recruit whole phospholipids [42,73] and neutral lipid stores directly from the host [47]. Phospholipid synthesis is facilitated by the scavenging of precursors such as serine, ethanolamine, and choline from the host environment. These polar headgroups are used by the parasite for the production of phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) [55,74]. Chen and colleagues have shown that PE and PS are able to be taken up directly from the host environment, unlike PC that thus has to be generated directly by the parasite from host-scavenged and/or de novo-made precursors [75]. A very recent study suggests that parasite lysophospholipase could be used to generate the phosphocholine for parasite PC synthesis from existing lysolipids from the host during *P. falciparum* asexual blood stages [68].

PLs and FA are amphipathic molecules, meaning they can disrupt the membrane as they are recruited from the host to the parasite. This dilemma is overcome by carrier proteins such as acyl-CoA binding proteins (ACBPs) and FA binding proteins (FABPs) are such protein classes, which bind to fatty acyl esters and transport them across various cellular compartments [76]. The genome of *Toxoplasma* has no FABP but encodes for 2 ACBPs and are involved in lipid signaling and metabolism. In the absence of the cytosol TgACBP1, there is a decreased abundance of C18:1, which is predominantly scavenged from the host [73]. The mitochondrial TgACBP2 plays a key role in cardiolipin metabolism and is critical of the growth and virulence of type II parasites [77]. Both proteins under starvation showed no deleterious effect on tachyzoite growth, indicating it is unlikely they are involved in nutrient adaptation. ATP-dependent PL transporters for PS and PE are present in the plasma membrane of tachyzoites [75].

The *P. falciparum* genome annotation revealed the existence of 3 putative ACBPs [78]. Kumar and colleagues show that PfACBPs have a higher binding affinity to long-chain fatty acyl-CoAs than short chains. They also show that they can bind to phosphorylated lipids but not to the unphosphorylated lipid esters, such as DAG and TAG, suggesting phospholipid transport rather than that of FAs/neutral lipids. Their roles in intracellular transport or host scavenging is still not fully elucidated [79].

To cross membranes in an efficient manner, lipids can be channeled by transporters such as flippases. In *P. falciparum* parasites, there are 5 flippases whose role and putative redundancy are currently fragmented [80,81]. *Plasmodium chabaudi* ATP2 has been identified as an essential lipid flippase responsible for PS and PE transport [82]. Homologs in other *Plasmodium* sp. exist but without confirmation of function. Recently, the *Plasmodium yoelii* ATP7 ATPase has been characterized as a phospholipid transporter responsible for the uptake of PC across the plasma membrane in mosquito stage ookinetes [83]. Both *Toxoplasma* and *Plasmodium* sp. possess atypical flippases with dual domains, named GC (TgGC and PfGC $\alpha$  + PfGC $\beta$ ) consisting of a guanylate cyclase (GC) domain coupled to a P-4 ATPase (flippases). Both are involved in the processes of parasite invasion, egress, and microneme secretion

through cGMP and lipid signaling. Briefly, both enzymes can generate cGMP, which activates PKG and a signaling cascade, which includes calcium release and activation of calcium-dependent protein kinases (CDPKs). The flippase domain of GCs are known to be essential; however, their exact mechanism in the above mentioned process is unclear [55,84,85]. In a study that monitored the in vitro virulence traits of a lab-adapted *T. gondii* strain, the early fixation of a P4 flippase gene SNP supported a critical role of lipid homeostasis [35]. Other flippases have been identified in *Toxoplasma*, but their roles have not been fully elucidated [75].

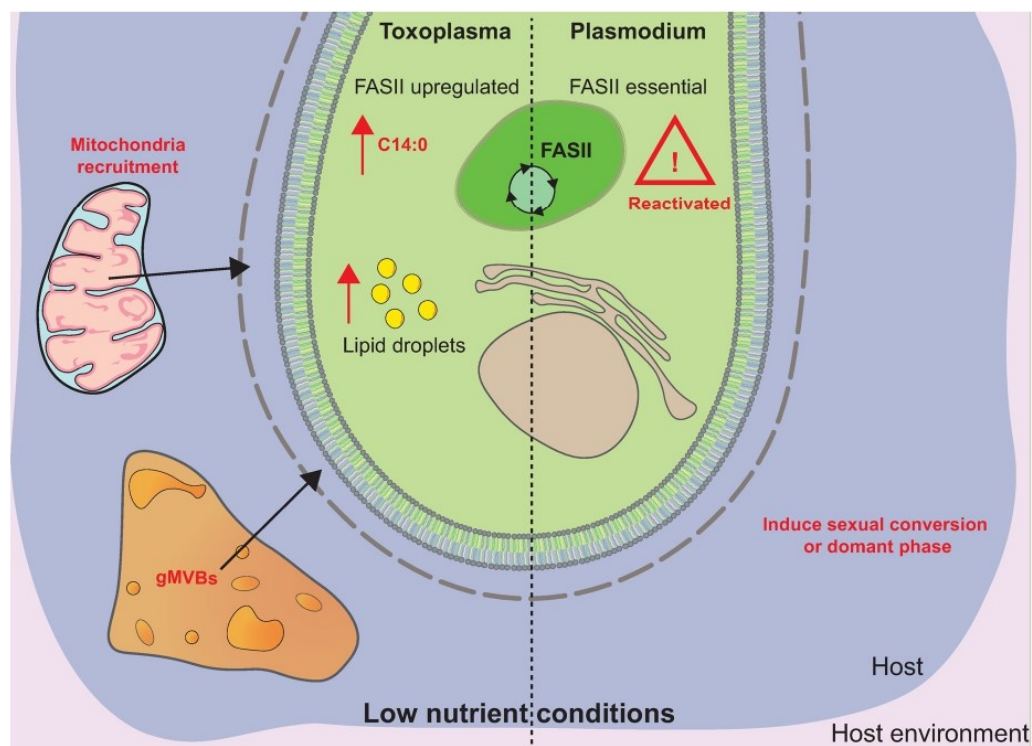
In general, *Toxoplasma* tachyzoite-infected cells are biologically flexible at many levels (genome, epigenetic, transcriptional, protein, and metabolic) to increase their ability to acquire nutrients from their environment and provide them to the parasite. As a good example at the lipid level, once within a cell, tachyzoites increase host synthesis of neutral lipids (TAG) and host lipid droplet numbers [48,49]. This lipid “hijacking” is facilitated via changes at the transcriptional levels of the host, such as the up-regulation of key host enzymes involved in TAG production like acylglycerol-3-phosphate acyltransferase (AGPAT2), acyl-CoA: diacylglycerol acyltransferase (DGAT2), and FA binding transport proteins like FABP3 and FABP5. Similarly, under starvation, parasites exhibit an increase in lipid storage abundance [6]. The low nutritional condition is sensed by the parasite, which attempts to compensate by the up-regulation of FASII lipid synthesis using glucose. The effector *TgASP5*, a Golgi-resident aspartyl protease, has been shown to play a role during FBS starvation [6].

Neutral lipids can be acquired from the host through endocytosis via the *T. gondii* parasite PVM. IVN had Rab7 coating onto their membranes. It is also proposed that probable interaction between Rab effectors and proteins present on PVM in order to promote this event of scavenging [47]. A separate study also should show that LPA accumulates in vesicles that are trafficked through the parasite's secretory pathway, suggesting that there is convergence between lipid uptake and the endocytic pathway [86]. The *ATS2* (protein involved in LPA to PA conversion) KO mutant has also shown evidence of aberrant endocytosis at the plasma membrane; however, it is presently unclear which, if any, nutrients are taken up by endocytosis during intracellular development [6].

Globally, *Plasmodium* parasites are believed not to take up lipids by typical endocytosis pathways, but characterization of *PfEHD* and *PfLPL1* could suggest that, like *T. gondii* parasites, neutral lipids are acquired via endocytosis [87,88]. During *P. berghei* liver stage development, there is host cell mitochondria recruitment at the PVM, like in *Toxoplasma*, but in this context to mediate lipoic acid scavenging, which is required for parasite growth [89].

### The balance between membrane biogenesis, lipid storage, and lipotoxicity in nutrient adaptation

Lipid storage is essential in maintaining this lipid homeostasis within the parasite and is used for the prevention of lipotoxicity or as an efficient energy source in suboptimal growth/culture conditions (Fig 4). The latter can be achieved through the process of  $\beta$ -oxidation in which lipids, in the form of FA, imported into the mitochondria or the peroxisomes, and then broken down into acetyl-CoA. These pool of acetyl-CoAs can then enter the mitochondrial TCA cycle and leads to energy production. The presence of  $\beta$ -oxidation is highly debated in the Apicomplexa field, and many questions [90,91] still exist about the fate of DAG/TAG and the dependence of this process on the host and its proteins/nutrient condition (NB: It is believed that *Toxoplasma* possess a complete beta oxidation pathway in its mitochondrion but lacks the canonical transporter allowing to initiate beta-oxidation, the Carnitine palmitoyl transferase CPT1; the situation is more complex in *P. falciparum* where not only CPT1 is missing but also some other members of the beta-oxidation pathway).



**Fig 4. Cellular changes in parasite (*Toxoplasma*: left; *Plasmodium* sp.: right) in low nutrient conditions.** Mitochondria recruitment and gMVBs are apparent and exacerbated in low nutrient conditions of *Toxoplasma* tachyzoites. The metabolic up-regulation of FASII (resulting in the increase production of C14:0), along with an increase in parasite lipid droplets, is characteristic of low nutritional conditions in the host. In similar conditions, when nutrients are limited in the host environment, *Plasmodium falciparum* asexual blood stages can up-regulate the metabolic activity of the apicoplast FASII, rendering it essential in these conditions. The lack of LPC in the host environment leads to sexual conversion of the parasite. Very little is known about the adaptation of this parasite to changing lipid statuses. gMVB, giant multivesicular body; LPC, lysophosphatidylcholine.

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A recent study by Primo and colleagues has demonstrated that laboratory adaptation of *T. gondii* GT1 strain results in augmented phenotypes driven by selection pressures in the nutrient content of the extracellular environment. They also identified medium chain FAs to be the limiting factor in the extracellular environment, which provided insight into the contribution of FAs in parasite virulence. Lab adaptation evolution experiments performed in 1% FBS, as opposed to 10% FBS showed an up-regulation of genes involved in FA metabolism, specifically those in the FASII pathway. This reiterates the importance of these genes in parasite survival in harsh conditions [35].

The physiological fluctuations of the host nutrient environment are modulated between the equilibrium of both FASII and host-scavenged lipids. Lipids taken up from the host and made de novo by the parasite are reshuffled and remodeled in many different compartments (ER/cytosol/vesicles, etc.) of the parasites by a variety of proteins. Amiar and colleagues demonstrated that disrupting LPA production through its plant-like acyltransferase in the apicoplast leads to a severe impairment of the PL bulk content and composition that leads to a drastic reduction of the abundance of both PC (major membrane component) and PI by at least 50%. To reveal the origin of the PL FA chains, stable isotope labeling of was used in combination

with LC-MS based lipidomic analyses (labeled = apicoplast; unlabeled = host). It was revealed that 70% of FA chains (mainly short chains) from both PC and PI are from the apicoplast, while the remaining 30% are from the host. The importance of this ratio of make/take lipids is important to grasp as it is severely altered depending on the nutrient availability and strongly indicates the membrane remodeling ability of Apicomplexa parasites [4,16,21,36,92].

To allow for the remodeling of these lipids, Apicomplexa parasites possess a so-called eukaryotic glycerol-3-phosphate acyltransferase (GPAT; Fig 2), which has been located in the ER (*Plasmodium*) able to assemble LPA [14,93]. This GPAT seems capable of generating LPA with a high specificity for C16:0 and C16:1, both of which originating either from de novo synthesis or being scavenged from the host. In *Toxoplasma*, a putative GPAT and 1-acylglycerol-3-phosphate acyltransferase (AGPAT) locating the parasite ER [6] have been identified but not characterized yet, suggesting that the parasite could be able to reassemble host and de novo lipids in an “eukaryotic” manner to fuel membrane biogenesis. The eukaryotic-type AGPAT was also found in the *Plasmodium* ER, and both the *Toxoplasma* and *Plasmodium* homologs seem essential for the parasite survival [6,14]. On another hand, parasites are also able to massively reroute host-scavenged lipids from membrane to storage to maintain parasite survival through active division in a timely regulation and to prevent parasite death by lipotoxicity. Indeed, recent findings showed that a PA phosphatase in *T. gondii* (TgLIPIN) can act as a “switch” and a “metabolic scale” by converting PA to diacylglycerol (DAG), ultimately sustaining bulk TAG synthesis by fueling DAG to diacylglycerol acyltransferase (DGAT) affecting formation of parasite lipid droplets [39,94]. DAG can also be converted to cytidine diphosphate-diacylglycerol (CDP-DAG, pools that are present in the apicoplast or the ER), which serves as a major precursor for phospholipid synthesis [95]. In a low lipid environment, the deletion of TgLIPIN in the parasite leads to a decrease in lipid droplets and TAG levels, ultimately leading to gross membrane malformations and parasite death by lipotoxicity. Intriguingly, this effect is exacerbated in a high host lipid environment when the TgLIPIN is depleted (i.e., the more host lipid in the absence of TgLIPIN, more membrane, less TAG, high parasite death rate) the unlike wild-type parasites, which growth and survival is typically favored under higher host lipid/nutrient content [39]. This imbalance leads to an accumulation of free FAs, which are indicative and causative of lipotoxicity, and ultimately leads to the parasite’s death. It is interesting to note that gMVBs are highly formed under suppression of this protein in both low and high nutrient environment. The formation of gMVBs was previously observed to form solely under low host nutritional conditions, as seen by Amiar and colleagues (Fig 4). The parasite thus seems tricked into a false environment of low lipid levels, thinking that no lipids are available for the conversion of PA to DAG and for the formation of storage lipid droplets/TAGs. The parasite thus seems to overcompensate by continuing to scavenge host lipids that leads to an accumulation of PA, which unnaturally fuels PLs synthesis, then diverged into the endomembrane system of the parasite, affecting the membrane/structural integrity of the parasite.

Furthermore, phospholipases are suspected to play a crucial role in the recycling and reshuffling of lipids (Fig 4). Phospholipases are lipolytic enzymes that hydrolyze phospholipid substrates at specific ester bonds, releasing lysolipids and FA. TgPL2 is one such phospholipase that localizes to the apicoplast and regulates PC and lysophosphatidylcholine (LPC) levels in the parasite [96]. This protein seems to be crucial in the lipid homeostasis of the apicoplast since its deletion leads to rapid organelle loss. Different LPLs can serve to generate FA that sources TAG and DAG synthesis, but their specific localization allows different function. PfLPL1 is a dynamic protein of *P. falciparum*, which transits from parasite periphery/PVM into cytosolic vesicles that ends in a compartment closely localized to the food vacuole and rich in neutral lipids. PfLPL1 generates FA that are used to synthesis neutral lipids, DAG, and TAG, which are used to promote hemozoin formation in the food vacuole, thus allowing

essential heme detoxification by using host lipids as substrates. Depletion of *PfLPL1* is deleterious for the *Plasmodium* blood stage but can be rescued by addition of exogenous FA in the culture media, clue of the parasite plasticity. Closer to the host, *PfLPL3* localized to the intravacuolar TVN. *PfLPL3* allows to hydrolyze host phospholipids to generate FA, then used for DAG/TAG formation. These DAG/TAGs are mobilized during blood stages schizogony and thus serve as a tightly controlled reservoir of building blocks for bulk phospholipid synthesis in the parasite. Such process is like what was uncovered through the characterization of *TgLIPIN* in *T. gondii* tachyzoites where host lipids are constantly scavenged, then stored for a timely use for membrane biogenesis specifically during asexual division.

### Physiological context

The ability of *T. gondii* to infect any nucleated cell resides in its strength/flexibility to metabolically adapt to their host (Fig 4). These intracellular parasites are in constant competition with their host for nutrient dependencies (not limited to just lipids, but includes other metabolites like glucose and glutamine), which is indirectly dependent on the environmental nutritional conditions [6,97,98]. Amiar and colleagues have also shown that *T. gondii* parasites under a low lipid environment up-regulates the FASII pathway [6]. Moreover, encountering different cell types in an organism has exposed the parasites to a wide panel of environments with different nutrient availability. Endemic areas affected by *Plasmodium*, in many cases, have a population largely affected by malnutrition. Parasites infect different cell types (hepatocytes, erythrocytes) and face certain changes in host nutrient; under amino acid starvation, parasite asexual replication within the red blood cells is reduced, and, instead, parasites enter a hibernating state [99]. Host LPC was revealed to be a critical nutrient that influences parasite growth and differentiation to the sexual stage of blood stages, with high serum levels of LPC responsible for the repression of sexual conversion [100].

Metabolic adaptation in a physiological context of these diseases caused by these parasites is becoming increasingly studied as it may holds the keys to how the parasite can sense and adapt to its host metabolic and nutritional conditions [35]. Phenotypic analyses recently indicated that only traits effected by extracellular host environment conditions evolved in *Toxoplasma* lab-adapted strains, indicating this importance in parasite propagation. Expression of genes involved in FASII pathway and ER FA elongation were up-regulated in extracellular parasites after lab adaptation.

Recent study uncovered the role of a *trans*-FA responsible for the host specificity of *Toxoplasma*. Data show that the accumulation of systemic linoleic acid (18:2) (25% to 46% in cat serum) due to the unusual absence of a  $\Delta 6$  desaturase within feline intestines allows parasite oocyst propagation [101]. Such a metabolic lipid environment therefore provides a unique platform for the development of sexual stages otherwise absent in intermediate hosts, including humans. The fact that linoleic acid is toxic for *Toxoplasma* asexual stage tachyzoites also favors sexual stage development in the cat intestine environment. The enzyme  $\Delta 6$  desaturase usually catalyzes the conversion of linoleic acid (C18:2) to arachidonic acid (C20:4), which, in turn, can participate in the formation of immune mediators like eicosanoids. Therefore, absence of this enzyme in feline intestines would also rescue the possibility of strong immune response toward parasite sexual stage.

In summary, Apicomplexa lipid metabolism is highly flexible and adapted under different host conditions. Giant strides have been taken in recent years to uncover the complex biology of this parasite and its intimate interactions with the human host. But major questions remain unanswered. Much of this is centered on the cryptic nature of host effectors, which are poorly conserved across eukaryotes and have coevolved specifically in *Toxoplasma* with the human

host. The processes by which parasites scavenge FAs and potentially whole lipids are still in question. Similarly, the proteins responsible for sensing, and adapting to, low host nutritional content are still poorly defined. Recently, a novel kinase *PfKIN1* has been found to bear homology to the *Snf1* protein found to regulate the nutritional response to starvation in yeast [102]. *PfKIN* mutant parasites struggle to adapt to low nutritional environments in *P. berghei* parasites [102]. Future efforts will likely focus on the signaling network responsible for sensing and implementing this response. Furthermore, by establishing that host nutrient content determines the extent of parasite scavenging response and host remodeling, it will be well worth experimenting with different host cell types to establish which genes are essential under which conditions and/or cell types.

Future research on Apicomplexa metabolism will follow the current trend of the parasite's adaptation in different host conditions. It will be important to elucidate this plasticity in different cell types, nutrient conditions, and how it may play a role in stage conversion. Collectively, the work described in this review and successive work is paving the road in identifying novel anti-Apicomplexa therapeutics, specifically concerning the metabolic battle between the host and parasite.

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## **CHAPTER 3: A CRUCIAL LIPID EQUILIBRIUM GOVERNS THE SURVIVAL OF ASEXUAL APICOMPLEXAN PARASITES**

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### **Summary of published article** (Shunmugam, Dass et al., 2021):

The work described here sets the precedent of the basis of my thesis work and was initiated by Dr Sheena Dass. The lipid metabolism of apicomplexan parasites is highly dependent on combining fatty acids from different sources into lipid moieties described in the previous chapter. The flux of the multi-sourced FAs is governed by the nutrient availability found in the host cell and its environment. A fine balance between lipid synthesis and storage must be maintained for efficient parasite propagation. Our work here, characterized an essential phosphatidic acid phosphatase (*Tg*LIPIN), which acts as a regulator for this equilibrium between lipid synthesis and storage. The major contributions of this study include:

- *Tg*LIPIN (the only LIPIN encoded in the *T. gondii* genome) is an essential protein for parasite survival and has active phosphatidic phosphatase activity allowing for the conversion of PA to DAG
- Down-regulation of this protein induces rapid membrane malformation, cytokinesis and replication defects which is aggravated in a high lipid environment
  - This predominantly induces excessive biogenesis of nuclear/ER membranes and the IMC
- This protein prevents lipotoxic parasite death in a high lipid host environment by channelling the flux of host fatty acids and prevents the accumulation of FFA and promotes TAG synthesis in the form of LD

### **My contribution to the work included:**

- Heterologous yeast complementation studies proving the phosphatase activity of this protein
- lipidomic and lipid flux analyses
- Illustrations and writing of the paper

### **Résumé de l'article publié** (Shunmugam, Dass et al., 2021):

Le travail présenté dans ce papier pose les fondations de ma thèse et a été initié par Dr. Sheena Dass. Le métabolisme lipidique des parasites apicomplexes demande de combiner des acides gras provenant de différentes sources pour former les fragments de lipides décrits précédemment. Les flux d'acides gras d'origines multiples sont gouvernés par la disponibilité des nutriments trouvés dans la cellule hôte et son environnement. Un équilibre précis doit être maintenu entre la synthèse de lipides et leur stockage pour permettre au parasite de se développer efficacement. Notre travail a permis la caractérisation d'une phosphatase d'acide phosphatidique (*TgLIPIN*), qui agit comme un régulateur de cet équilibre.

Les résultats principaux sont les suivants :

- *TgLIPIN*, la seule LIPIN présente dans le génome de *T.gondii*, est une protéine essentielle pour la survie du parasite et a une activité de phosphatase pour l'acide phosphatidique, permettant la conversion de l'acide phosphatidique en diacylglycérol.
- L'inhibition de cette protéine induit des malformations membranaires ainsi que des défauts de cytokinèse et de réplication qui sont aggravés dans un environnement riche en lipide.
- Cette inhibition entraîne aussi une biogenèse excessive de membrane nucléaire, endoplasmique et de complexes membranaires internes.
- *TgLIPIN* prévient la mort du parasite en le protégeant des effets toxiques des acides gras provenant d'une cellule hôte dans un environnement riche en lipide, en canalisant les flux d'acides gras issus de l'hôte et favorisant la synthèse de triglycérol pour la formation de gouttelettes lipidiques.

**Ma contribution à ce travail inclut :**






- Expérience de complémentation hétérologue prouvant l'activité phosphatase de la protéine
- Analyse des flux de lipide et lipidomique
- Illustrations et écriture de l'article.

## ARTICLE


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OPEN

# *Toxoplasma* LIPIN is essential in channeling host lipid fluxes through membrane biogenesis and lipid storage

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Apicomplexa are obligate intracellular parasites responsible for major human diseases. Their intracellular survival relies on intense lipid synthesis, which fuels membrane biogenesis. Parasite lipids are generated as an essential combination of fatty acids scavenged from the host and de novo synthesized within the parasite apicoplast. The molecular and metabolic mechanisms allowing regulation and channeling of these fatty acid fluxes for intracellular parasite survival are currently unknown. Here, we identify an essential phosphatidic acid phosphatase in *Toxoplasma gondii*, TgLIPIN, as the central metabolic nexus responsible for controlled lipid synthesis sustaining parasite development. Lipidomics reveal that TgLIPIN controls the synthesis of diacylglycerol and levels of phosphatidic acid that regulates the fine balance of lipids between storage and membrane biogenesis. Using fluxomic approaches, we uncover the first parasite host-scavenged lipidome and show that TgLIPIN prevents parasite death by 'lipotoxicity' through effective channeling of host-scavenged fatty acids to storage triacylglycerols and membrane phospholipids.

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Apicomplexa includes several pathogenic protists that are responsible for major chronic and infectious diseases with a massive social and economic burden. *Toxoplasma gondii* and *Plasmodium falciparum* parasites are responsible for toxoplasmosis and malaria, respectively. These obligate intracellular parasites have an enormous demand for lipids in maintaining their survival within their human host cells. The utilization of fatty acids (FA) to synthesize complex lipids is an essential determinant for successful host adaptation by these parasites. Apicomplexa meet their high need for lipids through de novo synthesis via type II fatty acid synthesis (FASII) within the apicoplast and via copious salvage directly from the host and extracellular environment<sup>1–6</sup>. Recent data suggest that the tight regulation of FA flux between host, parasite, and its metabolic organelles is particularly vital for intracellular development of these apicomplexa pathogens<sup>4,7</sup>. Metabolic adaptation of the parasite toward host nutritional environment is also important for parasite propagation<sup>4</sup>. For instance, in low nutrient environments, the parasite is able to form giant multivesicular bodies (gMVBs) in the host, which somehow facilitates the parasite's ability to scavenge lipids in suboptimal nutritional conditions<sup>4</sup>. However, the molecular and metabolic pathways controlling the lipid flux toward parasite membrane biogenesis and storage remain largely unknown.

Phosphatidic acid (PA), the simplest glycerophospholipid, contributes to the regulation of FA flux in apicomplexa, as it is the key intermediate balancing the biosynthesis of both glycerophospholipids and triacylglycerols (TAGs) toward membrane biogenesis and lipid storage in eukaryotes<sup>8</sup>. PA has pleiotropic roles within the parasite: (i) as a signal transducer modulating parasite invasion, motility, and egress, produced by the action of diacylglycerol kinases (DGK1 and DGK2)<sup>9–12</sup>, (ii) as a regulator of lysoPA (LPA)/PA levels for modulating membrane curvature for cytokinesis and endocytosis, formed by acyltransferase ATS2<sup>4</sup>, and (iii) as the central precursor for bulk phospholipid synthesis and membrane biogenesis, made de novo from FA and a glycerol-3-phosphate backbone by the sequential acylation catalyzed by acyltransferases, in the apicoplast/ER pathways formed by ATS1/GPAT and ATS2/AGPAT<sup>4,13</sup>. Further, PA can be catabolized to a key lipid class, diacylglycerol (DAG) by phosphatidic acid phosphatases (PAP), which play vital metabolic functions in eukaryotes<sup>14</sup>. Together, PA and DAG are central lipid precursors from which most lipid classes can be generated de novo. This includes all phospholipids made from PA and DAG via the CDP-DAG from PA and the Kennedy pathway from DAG, respectively<sup>15,16</sup>. DAG is also the precursor for the synthesis of the major storage lipids, TAG.

Since PA is pivotal for parasite lipid metabolism, the enzymes involved in PA metabolism are critical for understanding parasite pathogenesis. One such critical enzyme class is PAP, as it allows to fine-tune the balance between PA and DAG. Apicomplexa parasites possess three putative PAPs, none of which have been characterized to date.

Here, we determined that *Toxoplasma gondii* LIPIN; TgLIPIN is a PAP that localizes to the cytosolic-ER interface and is essential for the intracellular survival of tachyzoites. Its inducible disruption quickly leads to aberrant membrane anomalies, mostly at the IMC and the endomembrane system, causing division defects and parasite death. Lipidomics of the mutant reveals a time-dependent accumulation of PA and free FA (FFA), concomitant with the reduction of DAG, lipid droplets, and storage, TAG. Both lipidomic and cellular membrane phenotypes are aggravated in a high host nutrient environment. The mutant dies from a lipotoxic accumulation of FFA and major membrane phospholipids, specifically PA. Further, we conducted novel fluxomic experiments using U-<sup>13</sup>C glucose-labeled host cells to

monitor the host FA scavenging capacity of the parasite, providing the first host-scavenged lipidome of *Toxoplasma*. This approach showed that TgLIPIN channels host FA to maintain an appropriate PA/DAG synthesis ratio. TgLIPIN acts as a metabolic checkpoint, tightly regulating membrane biogenesis versus lipid storage, which is essential for proper intracellular growth division and survival.

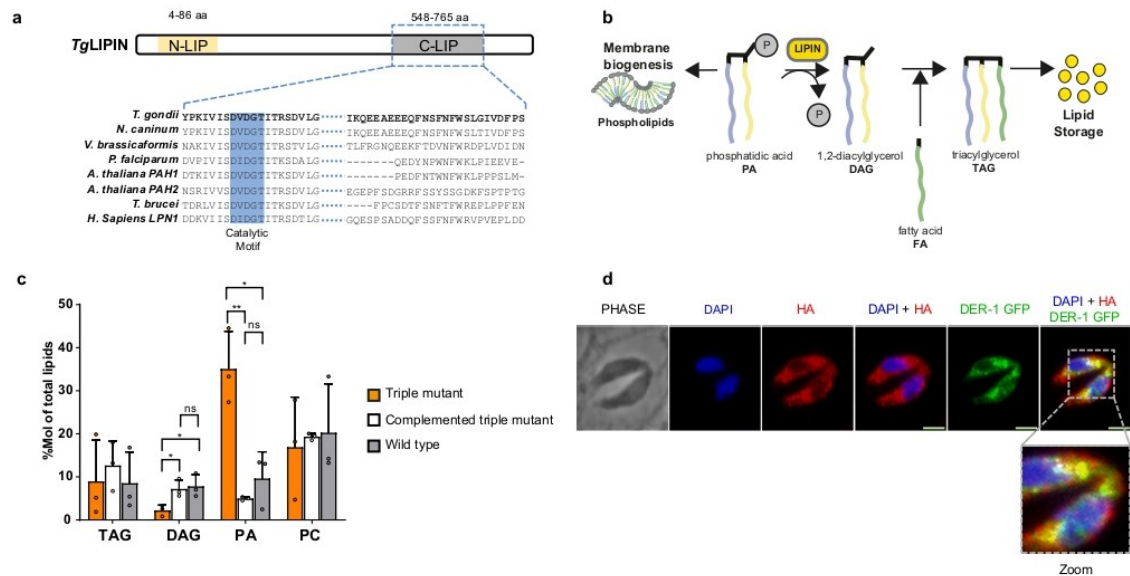
## Results

***Toxoplasma gondii* genome encodes a single lipin, TgLIPIN, which has functional phosphatidate phosphatase activity.** Bioinformatics analysis revealed that *T. gondii* parasites possess a single lipin homolog, much larger than other phosphatidate phosphatases encoded by the genome of *T. gondii*, and which we named TgLIPIN (TGGT1\_230690). TgLIPIN possesses the two typical and highly conserved domains of eukaryotic lipins, the amino-terminal N-LIP domain, and the carboxy-terminal C-LIP domain harboring its functional PA phosphatase catalytic motif DXDXT/V (HAD-like domain, Fig. 1a)<sup>17</sup>. Phylogenetic analysis confirms that the enzyme is highly conserved as a single lipin within phylum Apicomplexa, cladding specifically within a coccidian subgroup (Supplementary Fig. 1a).

To confirm the predicted phosphatidic acid phosphatase activity of TgLIPIN (Fig. 1b), we performed heterologous complementation using a *Saccharomyces cerevisiae* triple mutant, *Δdpp1Δlpp1Δpah1*, that is deficient in PAP activity with a temperature-sensitive phenotype (low growth at 37 °C)<sup>18,19</sup>. We attempted to generate the full TgLIPIN sequence and the corresponding recombinant protein but were not able to do so, due to limitations posed by the large size of the protein. Instead, we successfully generated TgLIPIN C-LIP domain, containing the putative catalytic (HAD-like) domain of the protein, and expressed it in the yeast mutant. We thus conducted lipidomic analyses on the yeast mutant, complemented or not with TgLIPIN C-LIP domain. The yeast mutant displayed a severe and significant accumulation of PA and a significant reduction of DAG compared to the wild-type yeast strain, corresponding to the loss of PA phosphatase activity (Fig. 1c). Complementation of the yeast mutant with the C-LIP domain of TgLIPIN fully restored the levels of PA and DAG to those of the wild type, thus confirming its predicted PAP activity (Fig. 1b, c).

To determine its cellular localization, TgLIPIN was endogenously tagged with 3×HA at its C-terminal end<sup>20</sup>. Immunofluorescence assay (IFA) and colocalization with known organelle markers revealed a broad cytosolic and perinuclear localization (Supplementary Fig. 1b). Interestingly TgLIPIN lacks an apparent nuclear localization signal (NLS), possessed by most eukaryotic LIPINs<sup>8,21</sup>. To further resolve its localization, we conducted IFAs of TgLIPIN-HA with a known *Toxoplasma* ER marker, DER-1-GFP<sup>22</sup> an episomally expressing plasmid, which confirmed proximity to the endomembrane system with partial ER colocalization (Fig. 1d). TgLIPIN was not further detected outside the parasite or in the host cell (Supplementary Fig. 1b).

**TgLIPIN disruption induces rapid membrane malformation, division, and replication defects leading to parasite death, all aggravated under a high host nutrient environment.** To understand the importance of TgLIPIN in parasite growth, we generated an N-terminal HA-tagged inducible knockdown parasite line based on the Tet-off system, TgLIPIN-ikD<sup>23,24</sup> (Supplementary Fig. 2a, b). Downregulation of TgLIPIN-ikD showed no detectable protein by western blot after 48 h of anhydrotetracycline (ATc) treatment (Fig. 2a). Cytoplasmic localization of the protein in the same manner as C-terminally tagged strains and its downregulation were both confirmed by IFA (Fig. 2b). This result

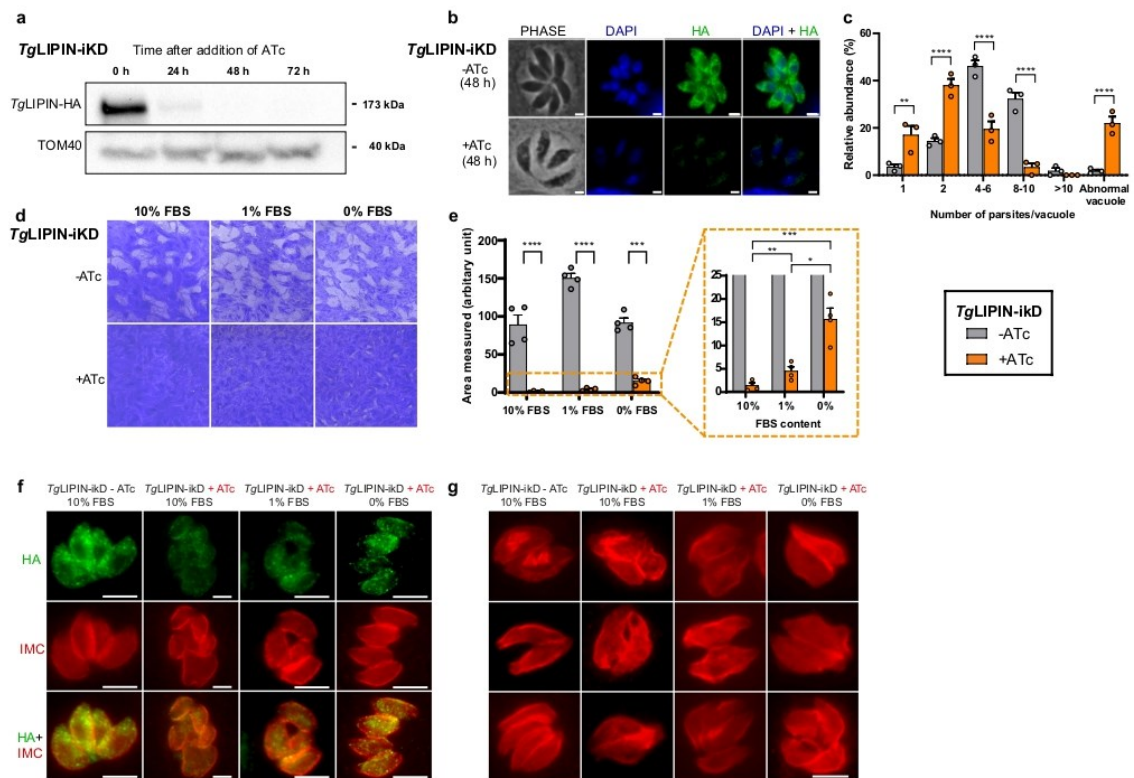


**Fig. 1** *T. gondii* LIPIN (TgLIPIN) is a phosphatidate phosphatase localized to parasite cytoplasm and endoplasmic reticulum. **a** The C-LIP domain of TgLIPIN is evolutionarily conserved among eukaryotic orthologs and harbors the catalytic motif DVDGT known to be central to PA phosphatase activity. **b** Graphical scheme of the biochemical function of LIPIN in the glycerolipid biosynthesis pathway in eukaryotes. **c** The complementation of the yeast triple KO mutant ( $\Delta dpp1\Delta pp1\Delta pah1$ ), lacking phosphatidic acid phosphatase activity, with the C-LIP domain of TgLIPIN restores PA and DAG ( $n = 3$ , unpaired  $t$  test  $P$  values where  $^*P = 0.024$  DAG triple mutant vs DAG complemented triple mutant,  $^*P = 0.032$  DAG triple mutant vs DAG wild-type,  $^{**}P = 0.0039$  PA triple mutant vs PA complemented triple mutant,  $^*P = 0.039$  PA triple mutant vs PA wild type). Data are presented as mean values  $\pm$  SEM. **d** IFA of TgLIPIN-HA (endogenous C-terminal tag) with anti-HA, DAPI, and a transiently expressed DER-1-GFP plasmid shows co-localization of TgLIPIN to the ER of the parasite. Scale bar:  $2.0\ \mu\text{m}$ .

suggests that TgLIPIN disruption dramatically impacts parasite intracellular growth and development TgLIPIN disruption caused a severe intracellular replication defect in TgLIPIN-ikD with a significant increase of small vacuoles containing 1–2 parasite vacuoles (15–20%) and vacuoles containing morphologically abnormal parasites (15–20%), alongside with a concomitant significant decrease of larger vacuoles (25–30%) containing 4–10 parasites (Fig. 2c). To further assess the effect of TgLIPIN on parasite intracellular growth, plaque assays were performed investigating the mutant's capacity to maintain proper growth with fluctuating levels of host nutrients. When cultured in regular growth conditions with 1% FBS, TgLIPIN-ikD (+ATc) exhibited a severe growth defect with few plaques (Fig. 2d, e) as expected from IFA results (Fig. 2b, f). To rescue this defect, the parasites were grown in high nutrient content with 10% FBS<sup>4</sup>. However, surprisingly, TgLIPIN-ikD (+ATc) showed a more significant decline in growth in 10% than with 1% FBS containing culture medium, as marked by the complete absence of plaques (Fig. 2d, e). Contrastingly, a decrease in the host nutritional environment with 0% FBS slightly but significantly enhanced the growth of TgLIPIN-ikD (+ATc) (Fig. 2d, e) suggesting that exogenous lipid-nutrient source of FFA or PL are somehow toxic to parasites lacking TgLIPIN. To further delineate the division defect phenotype of TgLIPIN downregulation, we performed IFAs to probe the morphology of the inner membrane complex (IMC) as a marker of parasite shape and division. IFAs were conducted prior to and after complete protein loss, at 24 h (Fig. 2f and Supplementary Fig. 2c) and 48 h (Fig. 2g) of ATc treatment, respectively, under low, normal, and high host nutrient contents. Severe membrane anomalies and division defects were observed even as early as 24-h ATc treatment. Such membrane defects were

strongly increased at 48-h ATc treatment, with membrane extrusion, loss of parasite integrity, and division arrest. Importantly, IMC and division defect were more evident when host nutrient content increased.

**Electron microscopy reveals gross membrane anomalies as an early impact of TgLIPIN downregulation.** To further resolve the cellular phenotype marked by TgLIPIN downregulation, TgLIPIN-ikD (+ATc) parasites were examined by transmission electron microscopy (TEM) at 12, 24, and 48 h post ATc treatment under low, normal, and high host nutrient content (0%, 1%, and 10% FBS, respectively). Interestingly, as early as 12 h of treatment with ATc, when TgLIPIN levels are only slightly reduced, both parasite IMC and plasma membrane displayed gross abnormalities in forming evaginations or lateral interruptions, without affecting the parasite size (Fig. 3a–c). Concordantly, IFA data of TgLIPIN-ikD (+ATc) also hinted at aberrant IMC (Fig. 2f, g). The morphology of the nuclear envelope (NE) started to adopt tubular or multilobed shapes (Fig. 3d–d<sup>1</sup> and Supplementary Fig. 3). Extensions of the NE seemed to expand in the cytoplasm in connection with the ER (Supplementary Fig. 3a) making tubular whorls in the cytoplasm (Fig. 3e–e<sup>1</sup>). At 48-h treatment, tubular or multilamellar membrane accumulation could entirely fill the cytoplasm of the parasites (Fig. 3f–f<sup>1</sup>). Local fusion of the outer membrane of different nuclear lobes could be observed (Supplementary Fig. 3b). The NE tended to adopt hairpin shapes where the inner membrane from two different regions was seen closely apposed, separated by electron-dense material (Supplementary Fig. 3c, e). Local detachment and fusion of the inner membranes could be also observed (Supplementary Fig. 3e, red arrow). Interestingly, similar expansions affecting the NE are also



**Fig. 2 TgLIPIN is indispensable for parasite replication and growth within its host.** **a** Western blot shows TgLIPIN downregulation, 48 h + ATc (0.5  $\mu$ g/mL), TgLIPIN (anti-HA), and TOM40 (control). **b** IFA of TgLIPIN-iKD, indicating loss of protein using anti-HA antibody at 48 h + ATc. Scale bar: 2.0  $\mu$ m. **c** Replication rate of TgLIPIN parasites grown with (+) or without (-) ATc measured by parasite number per parasitophorous vacuole after 24 h of growth post infection (>100 vacuoles were counted per biological replicate; n = 3, unpaired t test P values where \*\*P = 0.002 -ATc vs +ATc 1 parasite/vacuole, \*\*\*\*P < 0.000001 -ATc vs +ATc 2/4-6/8-10 and abnormal parasite/vacuole). Data are presented as mean values  $\pm$  SEM. **d** TgLIPIN-iKD plaque assays measuring parasite growth over 8-10 days (+/-ATc) in different FBS conditions. **e** shown as a bar graph (10%, 1% and 0%; n = 4, unpaired t test P values where \*\*\*\*P < 0.000001 -ATc vs +ATc 10/1/0% FBS, \*\*P = 0.026 +ATc 10% FBS vs +ATc 1% FBS, \*\*p = 0.0001 +ATc 10%FBS vs +ATc 0% FBS, \*\*P = 0.0047 +ATc 1% FBS vs +ATc 0% FBS). Data are presented as mean values  $\pm$  SEM. **f** IFA illustrating early phenotypic effects of TgLIPIN depletion (24 h + ATc) showing the presence of residual protein (HA-green). Inner membrane complex antibody (IMC- red) clearly shows aberrant IMC membrane biogenesis. **g** IFA illustrating the phenotypic effects of TgLIPIN depletion after 48 h (+ATc) using anti-IMC antibody in TgLIPIN-iKD. Scale bar: 5.0  $\mu$ m.

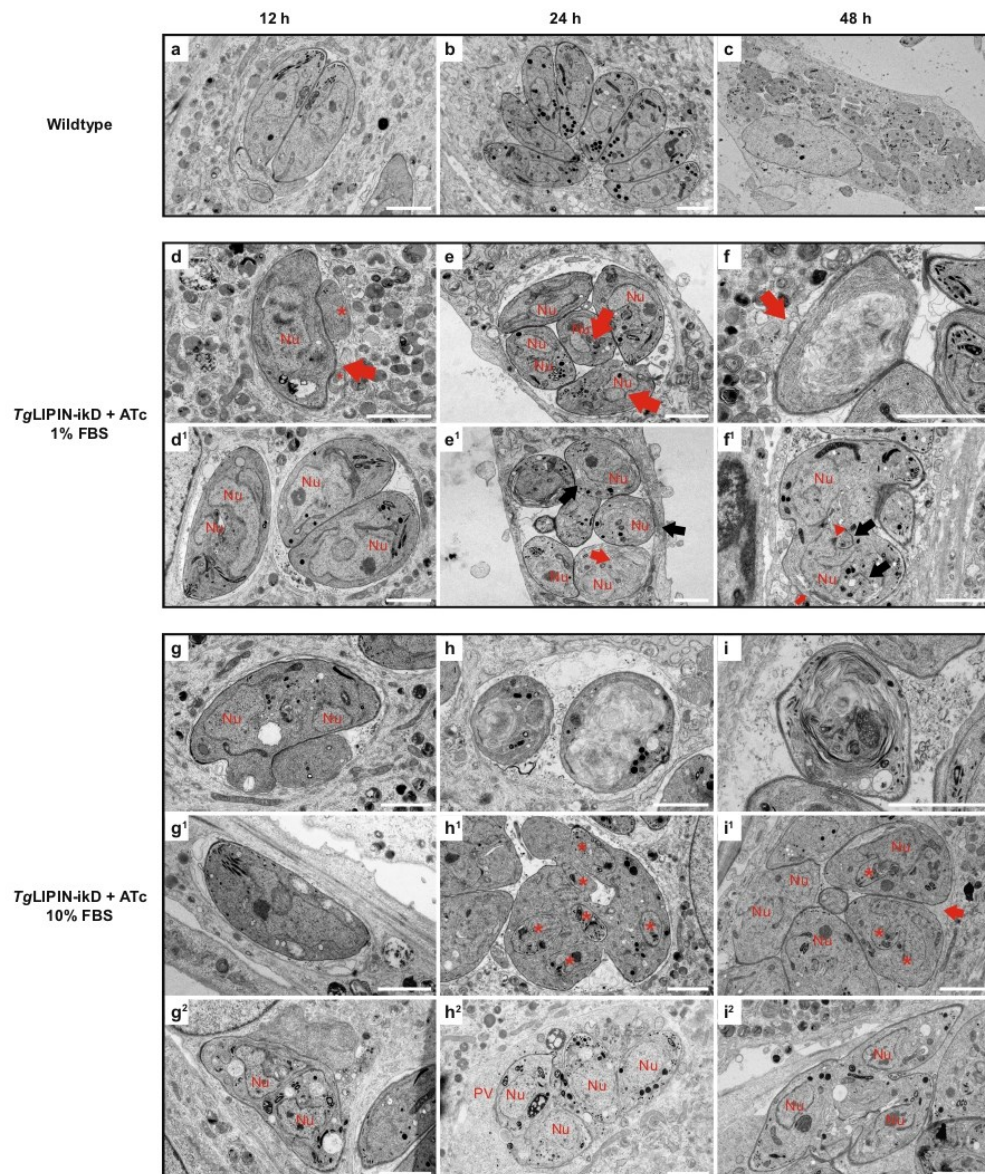
observed in the yeast LIPIN mutant coupling phospholipid biosynthesis to the nuclear membrane<sup>25,26</sup>.

Both parasite IMC and plasma membrane (PM) also displayed abnormalities in forming bumps (Fig. 3d, red arrow) or lateral evaginations (Fig. 3g-g<sup>2</sup>). Large inclusions could be observed in the vacuolar space that could correspond to such abnormal parasite evagination depending on the cutting plane. Complex structures were also observed in the cytoplasm, showing lasso-like structures formed by zippered membrane tubules (Supplementary Fig. 3f, g). It is not clear whether this type of structure originated from the ER, the nuclear membrane, or even IMC.

At 48 h, the ultrastructure of the vacuole appeared dramatically altered. Endodyogeny was strongly affected in TgLIPIN-iKD parasites, showing incomplete sister cells separation (Fig. 3e<sup>1</sup>-f<sup>1</sup>), cytokinesis failure harboring multiple daughter cells (Fig. 3h-i<sup>2</sup>, red asterisk). Local fusion of the parasitophorous membrane (PVM) with the PM of the parasite was also observed (Fig. 3f). Some vacuoles showed signs of parasite PM rupture, with cytoplasmic material reminiscent of parasite cytoplasm filling the vacuolar space (Fig. 3g<sup>2</sup>, h<sup>2</sup>, i<sup>1</sup>). Large autophagosomes with

unidentified content were also frequently observed in infected host cells suggesting that the parasites died and were eliminated by the host cell (Supplementary Fig. 3h-k). Large giant multivesicular bodies (gMVBs) and swelling of the host NE, previously described as enhanced under starvation conditions<sup>4</sup> were abundant in TgLIPIN-iKD parasites, even in the presence of 10% FBS. In some cases, the outer membrane of the NE had fused with the PVM (Supplementary Fig. 3c), occasionally leading to its rupture (Supplementary Fig. 3d).

In agreement with the replication assay data (Fig. 2c), TEM images showed that dynamics of parasite membranes was profoundly affected by the absence of appropriate levels of TgLIPIN, which seemed to start at the NE level as soon as 12 h of treatment. Further loss of TgLIPIN then induced strong aberrant membrane abnormalities mostly affecting the endomembrane system (NE, ER, IMC, and PM). This resulted in endodyogeny failure, anarchic membrane proliferation, and unexpected membrane fusion events, leading to parasite growth arrest and death. These membrane abnormalities and division phenotypes were observed in all host nutrient contents, but were always



**Fig. 3 TgLIPIN depletion results in gross membrane anomalies early in the process of ATc downregulation.** Transmission electron micrographs showing wild-type (**a–c**) and TgLIPIN-ikD parasites at 1% (**d–f**) or 10% FBS (**g–i**), after 12, 24, and 48 h of ATc treatment. After 12 h of growth, wild-type parasites showed vacuoles containing (**a**) 2–4 parasites, (**b**) 8–16 parasites at 24 h, and (**c**) after 48 h, the first round of egress/reinvasion has occurred showing cells with multiple small vacuoles showing regular nuclei and organelles. **d–f** TgLIPIN-ikD parasites show membrane anomalies as early as **d–d'** 12 h of induction. Vacuoles showed large inclusions in the parasitophorous vacuole, red asterisks. **d'** Nuclei showed a multilobed shape even in the absence of early signs of mitosis. **e–e'** At 24 h, nuclear envelope showed pronounced anomalies with elongations connected with rough ER tubules, red arrows. **e'** The ER showed exaggerated proliferation generating big membrane whorls. Incomplete cytokinesis was observed, black arrow. **f** Forty-eight hours after induction, parasites were full of ER-derived membranes and there was the fusion of the parasitophorous membrane (PVM) and parasite plasma membrane (PM), red arrow. **f'** Endodyogeny defect: a new round of mitosis started, shown by the presence of IMC buds (black arrows) and centrocone (red arrowhead), while two sister cells from the previous generation have not separated. **g–i** All membrane defects were present and enhanced in 10% FBS. **g–g'** At 12 h, nucleus elongation and cytoplasmic evaginations were observed. **g''** Cytokinesis failure was observed. **h–h'** At 24 h, ER proliferation and cytokinesis defect were observed. Vacuoles showed signs of necrosis with PM rupture and cytoplasmic leakage in the vacuolar space. **i** At 48 h, TgLIPIN-ikD did not invade, the remaining vacuoles showed profoundly abnormal parasites, **i'** PVM or **i''** PM rupture/cytokinesis failure. Nu nucleus. Scale bar: 2  $\mu$ m,  $n = 3$ .

stronger and aggravated under high host nutrient content (10% FBS). This suggests that the essential role of TgLIPIN for membrane biogenesis and parasite survival is directly linked to the nutritional status of the host.

**TgLIPIN regulates lipid homeostasis.** To determine the functional role of TgLIPIN in parasite PA synthesis and membrane biogenesis, we conducted comprehensive lipidomic analyses. To precisely assess the timely impact of TgLIPIN-ikD disruption on lipid synthesis, parasites were grown for 24 or 48 h with and without ATc. Total lipids were separated by high-performance thin-layer chromatography (HPTLC) and quantified by gas chromatography–mass spectrometry (GC–MS). Relative abundance of PA was unchanged at 24 h of +ATc (Fig. 4a). After 48 h +ATc, there was a large significant increase of PA, confirming its role for PA synthesis and our WB results for the loss of TgLipin (Fig. 4a). FA composition of PA showed an increase in the level of myristic (C14:0) and palmitic (C16:0) acids along with a decline in the levels of stearate (C18:0) (Fig. 4b). Concomitantly to PA increase, the relative abundance of DAG was decreased after 48 h of TgLIPIN downregulation by ATc (Fig. 4c) showing that TgLIPIN is an active phosphatidic acid phosphatase, controlling the levels of PA and DAG, as confirmed by our yeast heterologous complementation (Fig. 1c). Despite a strong replication defect and reduction in parasite number, the total amount of phospholipids was increased up to 1.5-fold in the TgLIPIN-ikD (+ATc) in comparison to the control (–ATc) (Supplementary Fig. 4a). Accordingly, most PL classes were further increased by TgLIPIN disruption such as the ones usually made from PA via the CDP-DAG pathway<sup>25</sup> i.e., cardiolipin (CL), PS, and PC (Supplementary Fig. 4b). This suggests that TgLIPIN controls PL homeostasis likely through PA synthesis.

In eukaryotes, TAG biosynthesis occurs at the last step of the glycerol-3-phosphate pathway through the *sn*-3 acylation of DAG catalyzed by a diacylglycerol-acyltransferase (DGAT) that uses FFA<sup>7,26,27</sup>. However, the key and limiting step of TAG synthesis is the formation of its precursor, DAG, the production of which depends on the action of a PAP/LIPIN. TAG is not a membrane but a storage lipid that accumulates within lipid droplets together with other neutral lipids such as cholesteryl esters (CE). We thus investigated the putative role of TgLIPIN in lipid-storage formation by comparing the number of parasite lipid droplets using Nile red staining<sup>28</sup> in the TgLIPIN-ikD parasites (+ATc and –ATc). This showed that the number of lipid droplets per parasite vacuole in the presence of ATc was reduced to almost 60% in comparison to the control in both normal (1% FBS) and furthermore in high host nutrient content (10% FBS) (Fig. 4d, e), suggesting that TgLIPIN is involved in the synthesis of storage lipids. According to this hypothesis, the relative abundance of TAGs was significantly reduced after 48 h of ATc treatment (Fig. 4f). Other neutral lipids making the bulk of lipid droplets, CE levels were also significantly decreased (Supplementary Fig. 4d).

TAGs and CEs are neutral storage lipids potentially implicit in the parasite's ability to cope with the excess FA<sup>7</sup>. The synthesis of TAG via enzyme DGAT requires two substrates, DAG and FFA. We, therefore, determined the FFA content of the TgLIPIN-ikD. Lipidomics revealed slight increases in total FFA levels in the TgLIPIN-ikD (+ATc/–ATc) (Fig. 4g). Interestingly, C18:1 (oleate) was the only FA found significantly increased in the parasite-free FA (FFA) pool (Supplementary Fig. 4c), suggesting C18:1 may be an important intermediate in the pathways regulated by TgLIPIN, as previously hinted<sup>7,29,30</sup>. In a high host environment (10% FBS), lipidomic analyses revealed that only TAG levels decreased under these conditions (Fig. 4h), explaining the reduction of lipid droplets. This impairment in TAG

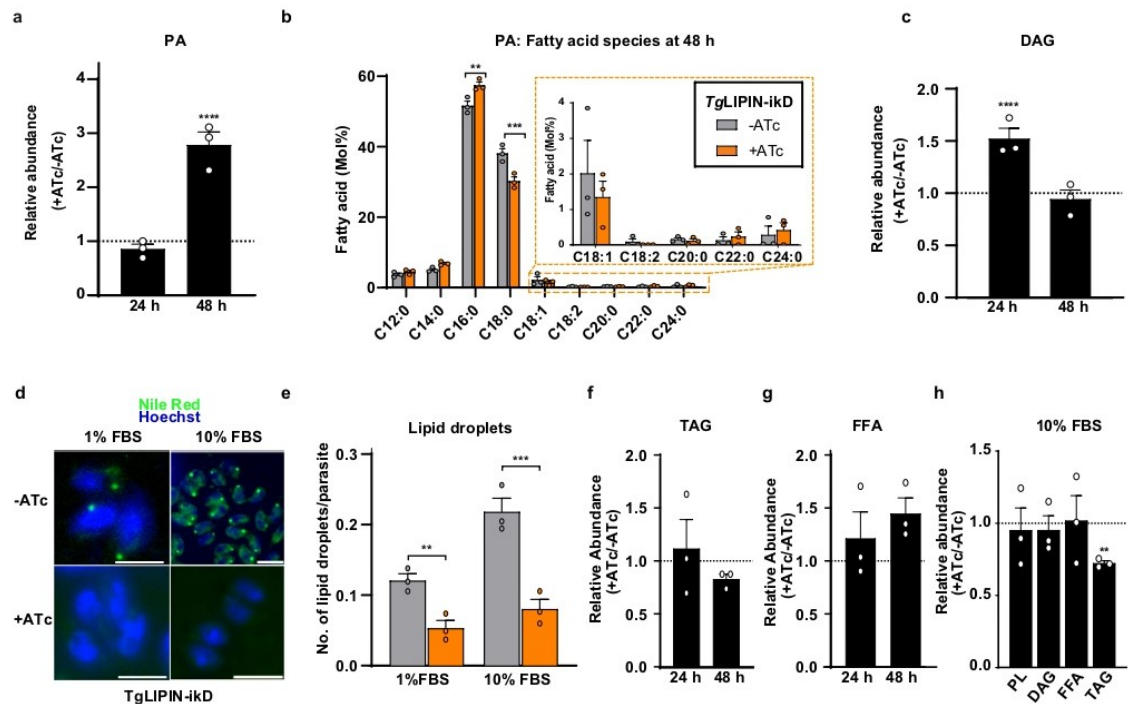
biosynthesis is consistent with the reduction in parasite viability seen under high FBS conditions (Fig. 2d, e).

**TgLIPIN channels the flux of host fatty acids to prevent the accumulation of free fatty acids levels and promotes TAG synthesis.** Our data reveal the essential role for TgLIPIN in regulating PA and DAG synthesis to control lipid homeostasis, especially the level of storage lipid molecule, TAG. TgLIPIN function seems directly connected to host nutritional status and the origin of lipid precursors used (FASII, host cell, and external environment) to maintain parasite survival.

Fatty acids required for lipid synthesis in *T. gondii* parasites are obtained from the host and/or host environment (i.e., medium)<sup>1,2,4,26,29</sup> and/or synthesized de novo via the apicoplast FASII pathway<sup>12,30,31</sup>. To identify the source of increased FA and phospholipids detected upon TgLIPIN depletion, we set up fluxomics approaches where we grew parasites and/or host cells using different stable isotope substrates-containing media (<sup>13</sup>C-U-glucose, <sup>31</sup>d-C16:0) and performed lipidomic analyses for each experimental setup (Fig. 5a–c).

To monitor apicoplast FASII activity, parasites were labeled with U-<sup>13</sup>C-glucose, which was added to a glucose-free medium, to confluent host cells together with parasites with or without ATc (Fig. 5a), as previously reported<sup>13</sup>. Total lipid content, PL, and neutral lipids were extracted to determine the ratio of <sup>13</sup>C incorporation of each FA as a measure of FASII activity. In TgLIPIN-ikD (+ATc), the amount of labeled FA made by FASII (i.e., FASII activity) decreased under the suppression of TgLIPIN (+ATc) in terms of total lipid content (Fig. 5d) as well as the pools of FFA/PL/TAG (Fig. 5e, f and Supplementary Fig. 5a, b). Interestingly, there was a significant decrease in all the signature FA from FASII (i.e., C14:0) but also in FASII-derived FA, i.e., oleic acid C18:1, reduced in total lipids, FFA, PL and TAG made from FASII (Fig. 5d, e, f and Supplementary Fig. 5a, b). There was an overall decrease in FASII-derived C18:1 in FFA (Fig. 5e) and TAG (Fig. 5f, supporting the lipid droplet analyses) and an increase in PC16:0 (Supplementary Fig. 5b). This suggests that with the loss of TgLIPIN, the increased overall PA levels with FASII-derived FAs, can be directed towards the production of excess PC. The distribution of <sup>13</sup>C incorporation to each isotopologue of FASII FA product confirmed that the pathway was nevertheless functional (Supplementary Fig. 5c–g). To monitor the FAs sourced from the external environment/culture medium, we grew parasites and confluent host cells in a medium supplemented with deuterated palmitic acid (<sup>31</sup>d-C16:0) (Fig. 5b). Parasites were harvested and total lipids were extracted to determine the incorporation of <sup>31</sup>d-C16:0 in parasite lipids. In the total lipid content, <sup>31</sup>d-C16:0 and its elongation/desaturation products <sup>31</sup>d-C18:0 and <sup>29</sup>d-C18:1 were detected in lower amounts in the TgLIPIN-ikD parasites with ATc (Fig. 5g). In addition, a drastic reduction in the incorporation of all three (<sup>31</sup>d-C16:0, <sup>31</sup>d-C18:0, <sup>29</sup>d-C18:1) to FFA species within TgLIPIN-ikD (+ATc) in comparison to the control (–ATc), further suggesting that the host external environment is not the source of increased lipids within the mutant (Fig. 5h).

To monitor the fatty acids directly scavenged from the host, we designed a novel assay using U-<sup>13</sup>C-glucose to label host lipids (Fig. 5c). Nonconfluent host cells were grown in the presence of medium containing U-<sup>13</sup>C-glucose which would theoretically fuel any of the active FA synthesis pathways within the host (FASII, elongases) via the synthesis of their substrates, acetyl-CoA, thereby generating <sup>13</sup>C-pre-labeled host metabolites including lipids and fatty acids<sup>32</sup>. These <sup>13</sup>C-pre-labeled host cells were then infected with parasites in the presence of a normal culture medium, which contains regular <sup>12</sup>C-glucose. Total lipid was



**Fig. 4** *TgLIPIN* regulates critical levels of PA and other major phospholipids while generating DAG that is directed toward neutral lipid storage. **a** Relative abundance of PA in *TgLIPIN-ikD* (nmol/parasites number, +ATc/-ATc; %;  $n = 3$ , unpaired  $t$  test  $P$  values where \*\*\*\* $P < 0.00001$  relative PA abundance ratio nmol/parasites number +ATc/-ATc at 48 h vs +ATc/-ATc = 1). **b** Fatty acid composition of PA in *TgLIPIN-ikD* (+/-ATc) in Mol%;  $n = 3$ , unpaired  $t$  test  $P$  values where \*\* $P < 0.0021$  PA C16:0 -ATc vs +ATc, \*\*\* $P < 0.0002$  PA C18:0 -ATc vs +ATc. **c** Relative abundance of DAG (+ATc/-ATc) is reduced by *TgLIPIN* depletion after 48 h + ATc ( $n = 3$ , unpaired  $t$  test  $P$  values where \*\*\*\* $P < 0.00001$  relative DAG abundance ratio nmol/parasites number +ATc/-ATc at 24 h vs +ATc/-ATc = 1). **d** Nile red staining showed the number of lipid droplet within the parasites after 24 h of +/-ATc (scale bar = 5.0  $\mu$ m) with **(e)** a representative bar graph ( $n = 3$ , unpaired  $t$  test  $P$  values where \*\* $P < 0.0093$  number of lipid droplets per parasite +ATc/-ATc at 1% FBS, \*\*\* $P < 0.0003$  at 10% FBS). **f** Relative abundance of TAGs in *TgLIPIN-ikD* (+ATc/-ATc) showing that TAG levels were relatively reduced in the *TgLIPIN-ikD* (48 h + ATc). **g** Relative abundance of free fatty acids (FFA, +ATc/-ATc in the *TgLIPIN-ikD*). **h** Relative abundance of PLs, DAG, FFA and TAG in the *TgLIPIN-ikD* +ATc to -ATc at 10% FBS ( $n = 3$ , unpaired  $t$  test  $P$  values where \*\* $P < 0.004$  relative TAG abundance ratio nmol/parasites number +ATc/-ATc at 10% FBS vs +ATc/-ATc = 1). Data are presented as mean values +/- SEM.

extracted from the parasites to determine the ratio of  $^{13}\text{C}$  incorporation to each fatty acid and determine the origin from the host or not. Using this novel approach, we were able to determine (i) the FA biosynthetic capacities of the host cells (Fig. 5i), and more importantly (ii) the first scavenged FA lipidome of the parasite (Fig. 5j-m), the scavengome. Host cells, human foreskin fibroblast (HFF), are capable of synthesizing FA ranging from C14:0 to C20:1, with the most abundant FA species being C18:1 (Fig. 5i). The wild type (*TgLIPIN-ikD* -ATc) parasites are capable of scavenging all these FA species made by the host cell with a major preference again for C18:1 (Fig. 5j). Importantly, in *TgLIPIN* mutant (+ATc),  $^{13}\text{C}$ -labeled C16:0, C18:0 and C18:1 from host cells were significantly increased in the FFAs (Fig. 5k), together with a significant increase of host-derived C18:0 in both parasite total lipids and PL (Fig. 5j, l). Therefore, since FA from both FASII and the external environment decrease in the absence of *TgLIPIN*, whilst FAs from host cell are found increasing, our data strongly suggest that the major origin of excess lipids in the *TgLIPIN* mutant is from the host cell. Correlating with the lipid droplet analyses, there was a significant decrease in the  $^{13}\text{C}$  labeled FA, specifically C16:1 and C18:1, in TAG (Fig. 5m). The distribution of  $^{13}\text{C}$  incorporation to each isotopologue of host-derived FAs was also determined

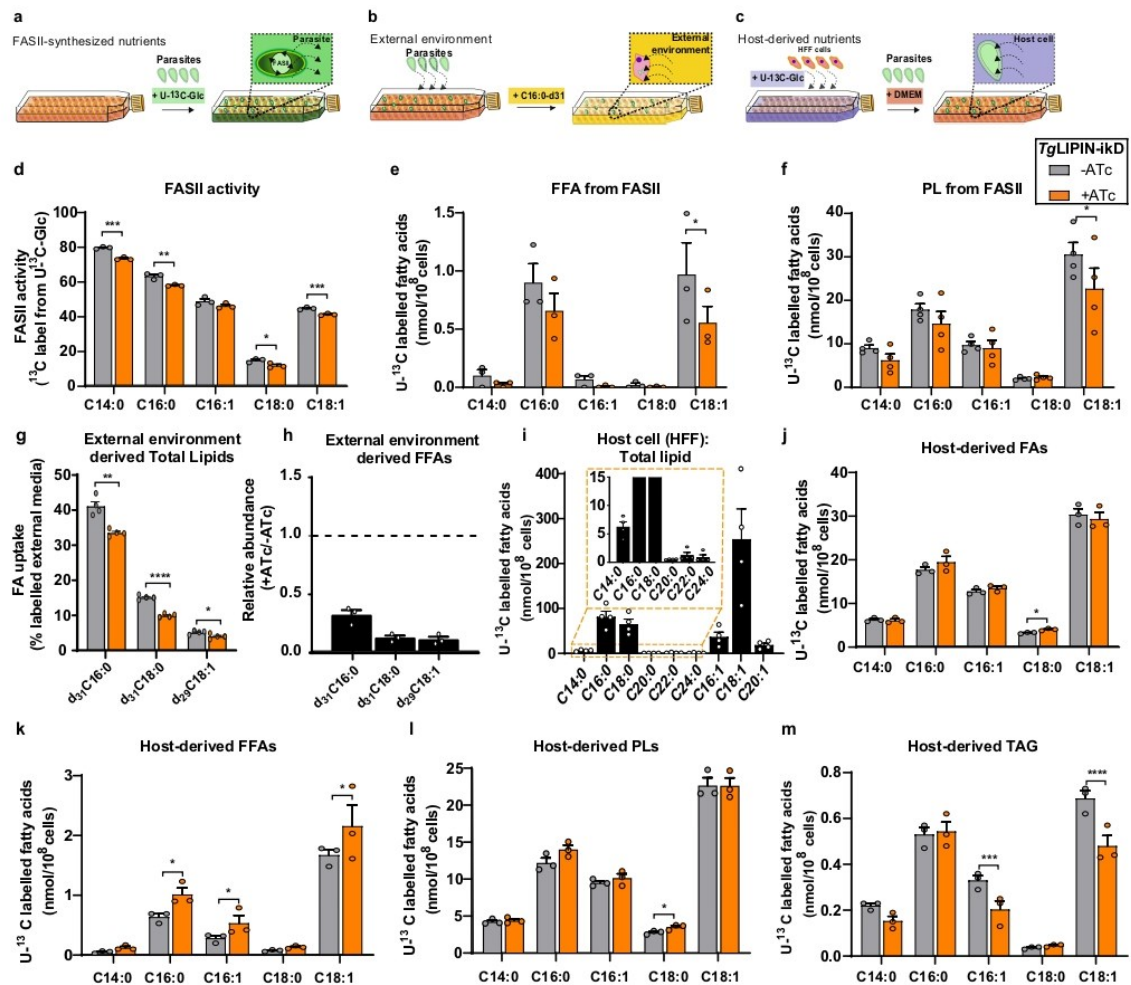
(Supplementary Fig. 5h-m). Together these data show that the disruption of *TgLIPIN* induced the massive accumulation of host cell-scavenged FA, specifically including C18:1, that cannot be used for TAG synthesis.

Hence, *TgLIPIN* regulates parasite membrane biogenesis by controlling the synthesis and content of PA and DAG, and by channeling the constant flux of host cell scavenged FA for TAG. Without *TgLIPIN*, the parasite accumulates toxic levels of PA, FFA, and PL that eventually cause massive membrane malformations, cytokinesis defect, and parasite death.

## Discussion

*TgLIPIN* plays a pivotal role in the synthesis of central lipid precursors, PA and DAG while regulating the flux of host lipids thereby controlling the equilibrium between membrane biogenesis and lipid storage. Collectively this controls intracellular development and the propagation of the parasite (Fig. 6a).

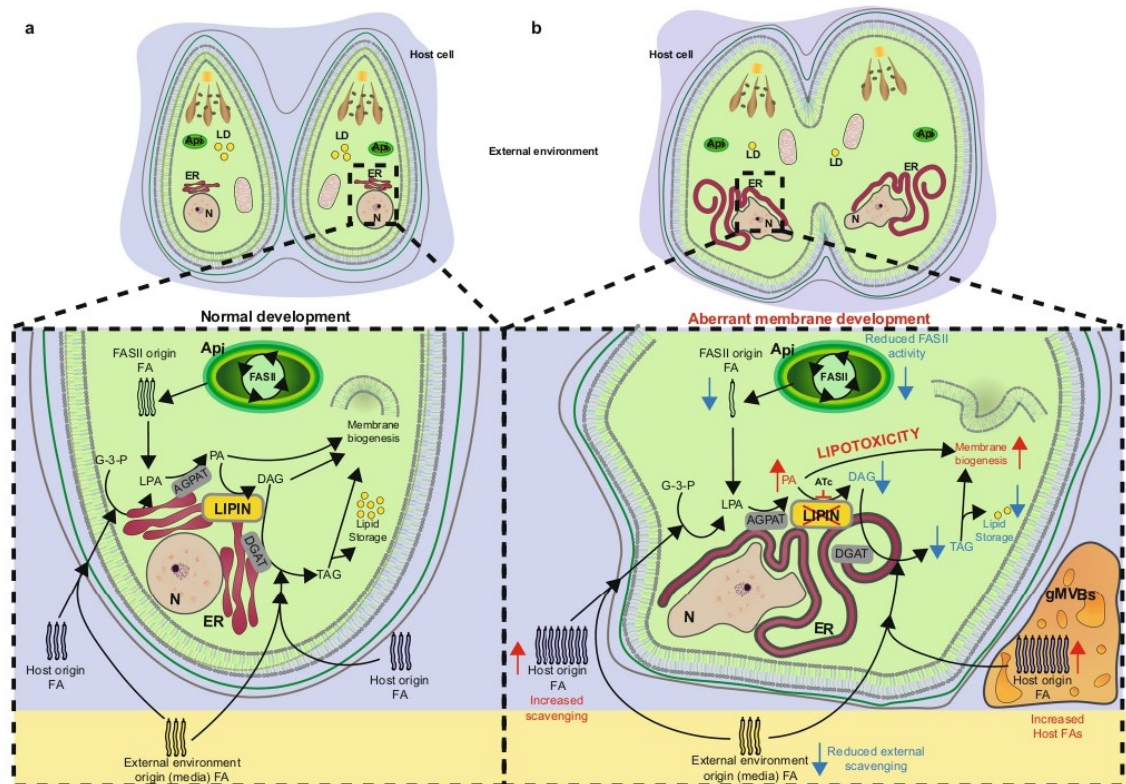
Our data here show that *TgLIPIN* is an essential PAP controlling the PA/DAG equilibrium along with the level of FFAs. Disruption of this equilibrium together with impairment of storage capacity has multiple phenotypic consequences within the parasite (Fig. 6b). In mammalian cells, LIPIN is encoded by three



**Fig. 5 Monitoring the source of fatty acids in TgLIPIN-ikD.** **a–c** Schematic for the experimental procedure of stable isotope labeling to determine the origin of fatty acids in TgLIPIN-ikD: **a** Parasite FASII derived FAs were determined by the addition of  $U-^{13}C$ -glucose (Glc) to the culture medium (1% FBS, no glucose, +/-ATc) at the time of parasite infection to the host. **b** FAs scavenged from the external environment (medium) was determined by the addition of  $^{31}d$ -palmitic acid ( $^{31}d$ -C16:0) to the parasite culture medium (high glucose, 1% FBS). **c** Host cell-derived FAs were determined by growing host cells in the presence of  $U-^{13}C$ -glucose (no glucose, 10% FBS) to confluent prior to the infection parasites with normal medium (glucose, 1% FBS, +/-ATc). **d** FASII activity in TgLIPIN-ikD as measured as %  $^{13}C$ -incorporation ( $n=3$ , unpaired  $t$  test  $P$  values; \*\*\* $P<0.0005$  FASII derived C14:0/C16:0/C18:0/-ATc vs +ATc, \*\* $P<0.007$ /\* $P<0.04$ /\* $P<0.0003$ ). **e** Abundance of apicoplast FASII derived  $^{13}C$  labeled FFAs ( $n=3$ , unpaired  $t$  test  $P$  values; \* $P<0.02$  of FASII derived FFA C18:1-ATc vs +ATc). **f** Abundance of apicoplast FASII derived  $^{13}C$ -labeled phospholipids (PLs,  $n=4$ , unpaired  $t$  test  $P$  values; \* $P<0.03$  of FASII derived PL C18:0-ATc vs +ATc). **g** FA uptake of deuterated external environment-derived FAs ( $n=4$ , unpaired  $t$  test  $P$  values; \*\* $P=0.0034$ /\* $P<0.00001$ /\* $P=0.01$  FA uptake from the external environment as  $d_3$ C16:0/ $d_3$ C18:0/ $d_3$ C18:1 -ATc vs +ATc). **h** Relative abundance of deuterated external environment-derived (+ATc/-ATc) ( $n=3$ ). **i** The abundance of  $^{13}C$ -labeled fatty acids in the host cells ( $n=4$ ). **j** Host derived FAs measured as %  $^{13}C$ -incorporation ( $n=3$ , unpaired  $t$  test  $P$  values; \* $P<0.02$  Host derived FA C18:1-ATc vs +ATc). **k** Abundance of host-derived  $^{13}C$ -labeled FFAs ( $n=3$ , unpaired  $t$  test  $P$  values; \* $P<0.05$  Host derived FA C16:0/16:1/18:1-ATc vs +ATc). **l** Abundance of host-derived  $^{13}C$  labeled PL ( $n=3$ , unpaired  $t$  test  $P$  values; \* $P<0.023$  Host derived PL C18:0-ATc vs +ATc) and **m** TAG levels ( $n=3$ , unpaired  $t$  test  $P$  values; \*\*\* $P<0.0001$  Host-derived TAG C16:0/18:1-ATc vs +ATc). Data are all presented as mean values +/- SEM.

independent genes *lipin1*, 2, 3; all of which have PAP activity modulating the levels of TAGs and phospholipids<sup>21</sup>. Genetic mutation within these LIPINs causes severe metabolic disorders, including rhabdomyolysis (*lipin1*), obesity (*lipin1*), autoimmune-inflammatory disease (*lipin2*), and impaired lipoprotein assembly in the intestines (*lipin2* and 3)<sup>33</sup>. The essentiality of LIPIN for *T. gondii* growth also suggests distinct functionality from the two

other parasite PAPs. Neither *Toxoplasma* PAPs appeared to compensate for the loss of TgLIPIN, unlike in yeast whereby deletion of genes encoding LIPIN can be partly compensated by other PAPs<sup>19</sup>. The absence of a NLS (nuclear localization signal) in the TgLIPIN sequence is consistent with a cytosolic localization, colocalization specifically with the NE membrane, ER, and other endomembrane. The localization and catalytic activity of



**Fig. 6 Proposed role of LIPIN in *Toxoplasma* lipid metabolism.** Schematic representation of the dual essential role of *TgLIPIN* in parasite lipid metabolism, including, membrane biogenesis, and lipid storage. **a** The parasite utilizes, and glycerol-3-phosphate (G-3-P) and fatty acid (FA)s derived from both apicoplast (Api) FASII and host to synthesize major lipids by forming lysophosphatidic acid (LPA) followed by phosphatidic acid (PA). PA is hydrolyzed by *TgLIPIN* to generate diacylglycerol (DAG), which is further acylated to generate triacylglycerol (TAG). The other branch of this pathway redirects PA toward the generation of major membrane phospholipids. The FA homeostasis between membrane biogenesis and storage essential for normal parasite development within its host is maintained by the PA phosphatase *TgLIPIN*. **b** Genetic ablation of *TgLIPIN* in *TgLIPIN*-iKD parasites in the presence of anhydrotetracycline (ATc) caused the PA/DAG imbalance. The increased PA is channeled towards PL synthesis resulting in excess PLS and consequent gross membrane anomalies within the parasite IMC, nucleus, and ER, while forming giant multivesicular bodies (gMVBs). Simultaneously, the reduction in DAG affected TAG biogenesis and hence the lipid storage capacity, marked by decreased lipid droplets (LD), within the parasite lacking *TgLIPIN*. The impairment of TAG biosynthesis, resulted in excess fatty acids derived from the host, within *TgLIPIN* mutant, causing lipotoxicity.

LIPIN proteins in humans and yeast are regulated by a phosphatase bearing the HAD domain as its substrate<sup>34–36</sup>.

Therefore, it is conceivable that *TgLIPIN* is quite dynamic and can be recruited on endosomal membranes to regulate the PA/DAG equilibrium, depending on metabolic demand, cell cycle, or coupled to membrane trafficking events. This hints at a probable link between metabolism and the nucleus of the parasite, however contrary to yeast and mammals, there is no evidence of a direct effect of *TgLIPIN* on gene regulation, which could explain the absence of nuclear localization.

One of the early phenotypic stresses evoked in the *TgLIPIN*-deficient mutant was the impact on major membranes including the nuclear envelope complexed with the ER and the parasite IMC. There is evidence that suggests that nuclear membrane biogenesis is linked to PA metabolism<sup>37,38</sup>. In  $\Delta$ *pah1* yeast, mutations in upstream biosynthetic steps of the glycerolipid pathway that lower PA levels also reduced the aberrant expansion of nuclear membrane<sup>37</sup>. Effects of *pah1* deletion could be phenocopied by overexpression of Dgk1p, a nuclear/ER membrane DAG kinase that generates PA<sup>37</sup>.

A recent study demonstrated that the use of DGAT inhibitor-T863, inhibiting DAG to TAG synthetic reaction in *T. gondii* parasites resulted in rapid accumulation of membranous structures accompanied by rapid parasite death due to strong replication defect<sup>6</sup>. This cytopathy was restricted mainly to the ER and the associated nuclear envelope, similarly as in the case of *TgLIPIN* depletion. The electron micrographs of the *TgLIPIN* mutant (+ATc) clearly suggest that the replication arrest was initially due to profound modifications of the nuclear membrane resulting in aberrant membrane proliferation, cytokinesis defect, and parasite death.

In the absence of *TgLIPIN*, we observed an overall increase in the major membrane phospholipids, including CL, PS, and PC which is a derivative of the CDP-DAG pathway in other eukaryotes<sup>39</sup>. In absence of DAG, PL biosynthesis was expected to be reduced. Instead, an increase in both PL and FFA is observed, which is likely due to excessive host lipid scavenging. The TAG content in *TgLIPIN* mutant decreases only 48 h post protein downregulation with ATc suggesting that due to a decrease in the substrate-specific DAG species because of *TgLIPIN* unavailability,

the parasite is unable to acylate the free fatty acids to generate its subsequent product TAG. This is supported by the drop in lipid droplet numbers in 1% and 10% FBS when TgLIPIN is suppressed and the exaggerated effect in a high host nutrient environment (Fig. 4d, e). In mammalian cells the inhibition of TAG synthesis leads to oleic acid (C18:1) induced lipotoxicity<sup>39</sup>. Thus, lipid droplets act as more than just cellular energy reserves by protecting the cells from deleterious effects of toxic excess FFAs by their incorporation into TAGs.

The prelabeling of host fibroblast cells with U-<sup>13</sup>C-Glucose, allowed us to track the source of excess fatty acid and phospholipid content in the TgLIPIN mutant. In keeping with previously reported data, using <sup>13</sup>C-fluxomics we observed that the parasite has a specific preference for scavenging oleate (C18:1) directly from host<sup>40</sup>. Existing literature provides strong evidence of the involvement of C18:1 in TAG biosynthesis and subsequent lipid droplet formation in mammals<sup>39</sup> as well as parasites<sup>7</sup>. Thus, the excess oleate in the form of FFAs within the parasite sourced directly from the host has a strong correlation to the lipid storage defect in TgLIPIN mutant (Fig. 5k). The role of TgLIPIN in the production of TAG was further strengthened by the decrease of host-derived C18:1 in TAG (Fig. 5m). In *T. gondii* parasites, excessive scavenging of C18:1 results in lipotoxicity and parasite death<sup>7</sup>. We show in the absence of TgLIPIN, C18:1 accumulates as FFA and cannot be incorporated into TAG. A significant decrease of C18:1 derived from FASII in the form of FFA is also observed. By sensing this change in nutrients, the parasite attempts to compensate by arresting FASII activity. C18:1 is likely a major FA that is typically scavenged and cannot be stored to avoid lipotoxicity in the absence of TgLIPIN. Taken together, this correlates to previous findings<sup>4</sup> showing that the parasite is capable of sensing host conditions and metabolically adapt. Similar observations on metabolic plasticity show that there is a reduction in the growth of *P. falciparum* FASII KO mutant in the presence of nutrient-deprived culture conditions<sup>4</sup>. Another study in bacteria shows that the FASII pathway is repressed in a negative feedback loop mediated by acyl-CoA<sup>41</sup>. The mechanisms of metabolic adaptation in Apicomplexa still need to be uncovered. In this study, we were able to reveal the first parasite lipid scavengome, which shows that TgLIPIN prevents parasite 'lipotoxicity' in high nutrient environments through effective channeling of host-scavenged fatty acids while maintaining parasite membrane integrity.

## Methods

**Sequence analysis.** TgLIPIN (TGGT1\_230690) was identified using EuPathDB web sources ToxoDB (<http://toxodb.org/toxo/>). A phylogenetic tree of LIPIN proteins in several eukaryotes was created using the online platform Phylogeny.fr. The organisms used for LIPIN protein sequences for generation of the phylogenetic tree include *Toxoplasma gondii* (TGGT1\_230690), *Plasmodium falciparum* (PF3D7\_0303200), *P. berghei* (PBANKA\_040180), *Homo sapiens* (NX\_Q14693), *H. sapiens* (NX\_Q92539), *H. sapiens* (NX\_Q9BQK8), *Saccharomyces cerevisiae* \_PAH1 (PAP1, SMP2, YMR165C, YMR520.14C), *Cryptosporidium parvum* (cgd3\_3210), *Cyanidioschyzon merolae* (CYME\_CMN061C), *Neospora caninum* (BN1204), *Hammondia hammondi* (HHA\_230690), *Chlamydomonas reinhardtii* (CHLRE\_12g506600v5), *Arabidopsis thaliana* \_AtPAH1 (At3g09560), *Arabidopsis thaliana* \_AtPAH2 (At5g42870), *Leishmania major* (LMJF\_06\_0830), *Trypanosoma brucei* (Tb927.7.5450), *Chromera velia* (Cve1\_24403). The first step involved the curation of these protein sequences. The protein sequences were aligned (ClustalW) and then gaps were removed from the alignment. Finally, the phylogenetic tree was constructed using the maximum likelihood method in the PhyML program (3.1/3.0 aLRT). The default substitution model (WAG) was selected. Graphical representation and edition of the phylogenetic tree were performed with Cladogram.

***T. gondii* strains and cultures.** The parasite host cells human foreskin fibroblasts (HFF) were cultured using Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco), and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>.

*T. gondii* tachyzoite parental strains RH-ΔKu80 TATi, RH-ΔKu80 as well as mutant strains TgLIPIN-ikD, TgLIPIN-3XHA were propagated by serial passage within their host HFF using DMEM supplemented with 1% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco), and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>.

**Generation of HA-tagged and inducible knockdown line for TgLIPIN.** A C-terminally tagged HA line was generated expressing from the gene's endogenous locus using the classical pLIC strategy using homologous recombination in a RH-ΔKu80 strain. For the same, a 1677-bp homology region of TgLIPIN located toward to C-terminus excluding the stop codon, was amplified from the parasite genomic DNA using the primers forward 5'-TACTTCCAATCCAATTTAATGCACGGCAGATTCTCTTACTGG-3' and reverse 5'-TCCTCCACTTCCAATTTAGCCAAATTAAGTGCATTTCGCTTTCAC-3'. The homology region was assembled into *PacI* digested pLIC-HA-DHFR plasmid using ligation-independent cloning protocol<sup>17</sup>. The assembled plasmid with linearized with single-enzyme site specific to the parasite DNA sequence within the plasmid-*NsiI* just before transfection. Parasites were selected with the drug pyrimethamine and cloned by limiting dilution.

For generation of inducible knockdown plasmid pPR2-DHFR<sup>42</sup>, two separate homology flanks were chosen. The 5' flank was amplified 1637 bp upstream of the TgLIPIN start codon using the primers F1-3' and R1. The PCR product was ligated to *PacI* and *NdeI* digested vector pPR2 using NEB assembly reaction. Next, the 3' flank was amplified as a 1739-bp fragment beginning at the start codon of TgLIPIN with the primers F2 and reverse R2. The 3' homology flank was annealed to *XmaI* and *NotI* digested pPR-HA3-DHFR vector that already contained the TgLIPIN 5' flank. The final cloned vector positions the start codon of TgLIPIN downstream of the ATc-regulatable t7s4 promoter and a 3xHA tag. The resulting vector with *NotI* and transfected this into TATiΔKu80 parasites. Parasites were selected with the drug pyrimethamine and cloned by limiting dilution.

Screening of parasite clones where the t7s4 promoter had successfully replaced the native TgLIPIN promoter, was done using the screen primers 1–6 in the combinations described in Supplementary Fig. S2. All PCRs were performed with TaKara primstar max polymerase. The knockdown of TgLIPIN was induced with 0.5 µg ml<sup>-1</sup> of anhydrotetracycline (ATc). Primer list can be found in Supplementary Table 1.

**Immunofluorescence assay.** Primary antibodies anti-HA (Rat, Roche 1:500), anti-IMC1 (mouse, 1:1000), anti-SAG1 (mouse, 1:1000), anti-MIC2 (rabbit, 1:1000), anti-CPN60 (rabbit, 1:1000), and anti-TOM40 (rabbit, 1:1000) were used at dilutions their respective dilutions. Secondary AlexaFluor 488- and 546-conjugated anti-rat, anti-mouse, and anti-rabbit antibodies (Life Technologies) were used at 1:2500. For the immunofluorescence assay (IFA), parasites were grown on confluent HFF on coverslips and fixed in PBS containing 2.5% paraformaldehyde (PFA) for 15 min at room temperature (RT). Samples were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT prior to blocking in PBS containing 3% BSA and subsequent incubation with primary antibodies then secondary antibodies diluted in the blocking solution. Labeled parasites were stained with Hoechst 33342 (1:10000, Life Technologies) for 20 min and then washed three times in PBS before final mounting of the coverslips on a glass slide using fluorogel. The IFA slides were visualized using fluorescence microscope (Axio Imager 2\_apotome; ZEISS, ×60–100 magnification).

**Western blot analysis.** Parasites were harvested for western blot analyses after complete egress from their host. To remove any host cell debris, the parasites were passed through a 3-µm filter, then counted by haemocytometer and solubilized in SDS buffer at equivalent cell densities. Equal amount of protein was separated on a 4–12% gradient SDS-polyacrylamide (Life Technologies) and transferred to a nitrocellulose membrane using the XCellIII Blot Module (Invitrogen). Primary antibodies anti-HA (rat, Roche) and anti-TOM40<sup>43</sup> (rabbit) were used at a dilution of 1:500 and 1:1000, respectively. Secondary goat anti-mouse and anti-rabbit horseradish peroxidase (HRP) conjugated antibodies (Thermo Scientific) were used at 1:2000. Protein signal was detected by chemiluminescence after membrane staining with luminata crescendo western HRP detection kit (Millipore). The signal strength of protein was quantified using a BioRad chemidoc imager (BioRad).

**Phenotypic analysis. Plaque assay:** The extracellular parasites were harvested after filtration and counted by haemocytometer. Then ~500 parasites were inoculated to a confluent HFF flask (25 cm<sup>2</sup>). TgLIPIN-ikD was grown for plaque assay in the presence or absence of Anhydrotetracycline (ATc) (0.5 µg ml<sup>-1</sup>) for 7–10 days. Plaque sizes were visualized by crystal violet staining (30–60 min) after aspiration of culture media, and cells fixation with 100% ethanol (5 min) followed by phosphate-buffered saline (PBS) wash. The plaque area was calculated ( $n = 3$ ) using Image J which calculated an arbitrary unit value assessing the viability of the parasite with and without the protein.

**Replication assay:** The parasites were grown for two days with or without ATc (0.5 µg ml<sup>-1</sup>), harvested and filtered. Equal number of parasites were allowed to invade confluent HFF grown on coverslips. Following 2 h of invasion, the coverslips were washed thrice with ED1 (1% FBS containing DMEM), to remove

extracellular parasites and promote synchronized replication. ATc (0.5  $\mu\text{g ml}^{-1}$ ) was added at the start of the experiment, allowing the treatment for 24 h, alongside control parasites without ATc. These coverslips were then fixed and processed for IFA using anti-HA, anti-SAG1 antibodies wherein the parasite number per parasitophorous vacuole was analyzed.

**Electron microscopy.** The TgLIPIN-ikD parasites were grown for 12, 24 and 48 h in the presence and absence of ATc, in labteks (Nunk, Thermofisher). The parental  $\Delta\text{Ku80}$  strain treated with ATc was used as the wild-type control. The labteks containing parasite-infected HFF were fixed in 0.1 M cacodylate buffer with 2.5% glutaraldehyde for 2 h and kept at 4 °C until further processing. During processing, the sample was fixed again for 1 h with 1% osmium tetroxide in cacodylate buffer followed by overnight treatment in 2% uranyl acetate in distilled water. After dehydration in graded series of acetonitrile, samples were progressively impregnated in Epon812, the wells were then filled with fresh resin and allowed to polymerize 48 h at 60 °C. Ultrathin 70-nm sections were obtained with a Leica UC7 ultramicrotome and collected on copper grids. Grids were post-stained with uranyl acetate and lead citrate before their observation on a Jeol1200 EXII Transmission Electron Microscope on the Electron microscopy facility of the University of Montpellier (MEA). Chemicals and consumables were from Electron Microscopy Sciences.

**Nile red staining of lipid droplets.** The TgLIPIN-ikD parasites were allowed to infect and grow in confluent monolayer HFF grown on coverslips in 1% and 10% FBS, in the +/− ATc conditions for 24 h and 48 h. Similar to IFA, these coverslips were fixed using 2.5% PFA, permeabilized with 0.25% Triton X-100 and then stained with primary rat anti-HA antibody followed by detection with secondary AlexaFluor 488-conjugated goat anti-rat antibody. Thereafter, the sample coverslips were incubated for 1 h with Nile red (1:2000) in 1× PBS before proceeding to DNA staining with Hoechst. The coverslips were mounted onto a glass slide in fluorogel prior to imaging using a fluorescence microscope (Axio Imager 2, apotome; ZEISS). For visualizing Nile red-stained droplets yellow-gold fluorescence (excitation, 450–500 nm; emission, greater than 528 nm<sup>30</sup>) was used on the axio-imager. Quantification in +/− ATc condition was done by counting the number of lipid droplets per parasite vacuole.

**Heterologous complementation.** The codon-optimized carboxy terminal sequence of TgLIPIN (548–765 a.a., C-LIP) harboring the catalytic HAD domain (DVDGT), obtained from GenScript, was ligated to *NotI/MluI* digested pD0170 yeast expression vector (obtained from Carman's lab) to yield pD0170-CLIP. This plasmid was transformed into yeast strain  $\Delta\text{dpp1}\Delta\text{pp1}\Delta\text{pah1}$  (*pah1::URA3 dpp1::TRP1/Kanr lpp1::HIS3/Kanr, triple mutant*) or  $\Delta\text{dpp1}\Delta\text{pp1}\Delta\text{pah1}\Delta\text{app1}$  (*app1::natMX4 pah1::URA3 dpp1::TRP1/Kanr lpp1::HIS3, quadruple mutant*) (gifts from Dr. George Carman's lab, Rutgers Center for Lipid Research, New Jersey), and transformants were selected on solid SD medium (YNB, 2% glucose, agar) lacking leucine<sup>19</sup>.

#### Lipidomic analysis

*Saccharomyces cerevisiae.* The triple mutant strain was transformed with vector pD0170-CLIP or pD01050 (lacking the HAD domain) and the cells were grown in YNB glucose medium at 30 °C until OD<sub>600</sub> reached 1–3. Cultures were centrifuged for 5 min at 1000 × g, washed with PBS thrice, and normalized by cell weight.

**Total lipid analysis:** Total lipids were extracted in chloroform/methanol/water (1:3:1, v/v/v) containing FFA (free fatty acids C13:0, 10 nmol) and PC (21:0/21:0, 10 nmol) as internal standards for extraction. Next, the polar and apolar metabolites were separated by phase partitioning by adding chloroform and water to give the ratio of chloroform/methanol/water as 2:1:0.8 (v/v/v). For lipid analysis, 50  $\mu\text{l}$  of the extract was directly dried and dissolved in 2:1 chloroform:methanol and trimethylsulfonium hydroxide (TMSH, Macherey-Nagel) for total fatty acid content. Resultant FAMES were then analyzed by GC-MS as previously described<sup>44</sup>. All FAMES were identified by comparison of retention time and mass spectra from GC-MS with authentic chemical standards. The concentration of FAMES was quantified after initial normalization to different internal standards.

**Phospholipid, DAG/TAG analyses:** The extracted total lipid extracted (as above) was separated with 5 nmol DAG/PA(C16:0/C18:1) (Avanti Polar lipids) by one-dimensional silica gel high-performance thin-layer chromatography (HPTLC, Merck). The 1st and 2nd solvent system used were chloroform/methanol/water/acetic acid, 25:15:2:4 (v/v/v/v) and hexane/MTBE/acetic acid, 35:15:0.5 (v/v/v), respectively. For DAG, TAG, FFA, and CE analysis, total lipid fraction was separated by 1D-HPTLC using hexane/diethyl ether/formic acid, 80:20:2 (v/v/v) as solvent system. The spots correlating to TAG, DAG, PA, and PC on the HPTLC plate were scraped off, and lipids were methanolized with 200  $\mu\text{l}$  0.5 M methanolic HCl in the presence of 1 nmol pentadecanoic acid (C15:0) as internal standard at 85 °C for 4 h. The resulting FAMES were extracted with hexane and analyzed by GC-MS (Agilent).

*Toxoplasma gondii.* The parasites were grown for 24 h and 48 h in +/− ATc conditions within a confluent monolayer of HFF in flasks (175 cm<sup>2</sup>). At each time point, parasites were harvested as intracellular tachyzoites (1 × 10<sup>7</sup> cell equivalents

per replicate) after syringe filtration with 3- $\mu\text{m}$  pore size membrane. These parasites were metabolically quenched by rapid chilling in a dry ice-ethanol slurry bath and then centrifuged down at 4 °C. The parasite pellet was washed with ice-cold PBS thrice, before transferring the final pellet to a microcentrifuge tube. Then total lipids were extracted in chloroform/methanol/water (1:3:1, v/v/v) containing PC (C13:0/C13:0), 10 nmol and C21:0 (10 nmol) as internal standards for extraction. Polar and apolar metabolites were separated by phase partitioning by adding chloroform and water to give the ratio of chloroform/methanol/water as 2:1:0.8 (v/v/v). For lipid analysis, the organic phase was dried under N<sub>2</sub> gas and dissolved in 1-butanol to obtain 1  $\mu\text{l}$  butanol/10<sup>7</sup> parasites.

**Total lipid analysis:** The extracted total lipid sample was then added with 1 nmol pentadecanoic acid (C15:0) as an internal standard as stated before using TMSH for total fatty acid content. Resultant FAMES were analyzed by GC-MS as previously described<sup>44</sup>. All FAMES were identified by comparison of retention time and mass spectra from GC-MS with authentic chemical standards. The concentration of FAMES was quantified after initial normalization to different internal standards and finally to parasite number.

**Free fatty acid and cholesterol analysis:** Total lipid samples were dried and derivatized with BSTFA + TMCS, 99:1 (Sigma) to generate trimethylsilyl (TMS-) fatty acids and TMS-cholesterol. These TMS derivatives were analyzed by GCMS as described above.

**Phospholipid and neutral lipid analysis:** For phospholipid analysis, the extracted total lipid extracted (as above) was separated with 1 nmol PA(C17:0/C17:0) (Avanti Polar lipids) by two-dimensional silica gel high-performance thin-layer chromatography (HPTLC, Merck). The solvent system used for the 1st and 2nd dimension was chloroform/methanol/28% ammonium hydroxide, 12:7:1.6 (v/v/v) and chloroform/acetone/methanol/acetic acid/water, 10:4:22.6:1 (v/v/v/v/v), respectively. For DAG, TAG, Free fatty acids (FFA) and cholesteryl ester (CE) analysis, total lipid fraction was separated by 1D-HPTLC using hexane/diethyl ether/formic acid, 80:20:2 (v/v/v) as solvent system. Then each lipid spot on the HPTLC plate was scraped off, and lipids were methanolized with 200  $\mu\text{l}$  0.5 M methanolic HCl in the presence of 1 nmol pentadecanoic acid (C15:0) as internal standard at 85 °C for 3 h. The resulting FAMES were extracted with hexane and analyzed by GC-MS (Agilent).

#### Stable isotope metabolic labeling experiment

**Tracking FASII origin fatty acids (monitoring de novo FA synthesis by the parasite apicoplast FASII).** The TgLIPIN parasites were infected to a confluent monolayer of HFF in glucose-free-DMEM (1% FBS) supplemented with U-<sup>13</sup>C-glucose or U-<sup>12</sup>C-glucose at a final concentration of 800  $\mu\text{M}$ , with or without ATc (0.5  $\mu\text{g ml}^{-1}$ ). The parasites were harvested up to 48 h post depletion of TgLIPIN and metabolically quenched in a dry ice and ethanol slurry in a tube until the sample reached 4 °C. Lipids were extracted, derivatized using TMSH (Macherey-Nagel) and analyzed by GC-MS as described above. <sup>13</sup>C incorporation to each fatty acid was calculated as the percent of the metabolite pool containing one or more <sup>13</sup>C atoms after correction for natural abundance and the amount of <sup>13</sup>C-carbon source in the culture medium. The degree of the incorporation of <sup>13</sup>C into fatty acids (% carbon incorporation) was determined by the mass isotopomer distribution (MID) of each FAMES. MID was obtained from the shift in isotopic mass dependent on the amount of <sup>12</sup>C carbons compared to the integration of <sup>13</sup>C carbon atoms. The total abundance of <sup>13</sup>C-labeled fatty acids was obtained by calculating the concentration of all isotopomers of <sup>13</sup>C-labeled FAMES and finally normalizing to authentic internal standards and parasite number.

**Tracking host-derived fatty acids (monitoring parasite scavenging capacities).** The HFF cells were grown (1 × 10<sup>8</sup> cell equivalents per replicate) to confluency in the presence of stable isotope U-<sup>13</sup>C-glucose at a final concentration of 800  $\mu\text{M}$  added to a glucose-free DMEM. These <sup>13</sup>C-pre-labeled HFF were then infected with TgLIPIN-ikD parasites in the presence of normal-glucose containing DMEM under +/− ATc (0.5  $\mu\text{g/ml}$ ). The host HFF and parasites were metabolically quenched separately, and their lipid content was quantified by GC-MS as described above. As described previously, the degree of the incorporation of <sup>13</sup>C into fatty acids (%carbon incorporation) is determined by the mass isotopomer distribution (MID) of each FAMES. The total abundance of <sup>13</sup>C-labeled fatty acids was analyzed initially for HFF to check labeling of the metabolites (described previously). Later, the same was calculated for parasites to confirm direct uptake of <sup>13</sup>C-labeled fatty acids from the host.

**Tracking uptake of deuterated fatty acid from medium (monitoring extracellular host environment).** Deuterated palmitic acid (<sup>31</sup>d-C16:0) was dissolved in 10 mM in fatty acid-free bovine serum albumin/PBS solution by sonication in a water bath for 30 min followed by incubation at 55 °C for 30 min. Freshly egressed TgLIPIN parasites were allowed to invade a confluent monolayer of HFF for at least 2 h under conditions of +/− ATc. Following the invasion, the uninvaded parasites were washed off with DMEM and further allowed to grow in the normal culture medium-DMEM (1% FBS) containing <sup>31</sup>d-C16:0 at a final concentration of 0.1 mM in +/− ATc until 24 and 48 h of growth. The parasites were harvested by metabolic quenching as described previously. Lipids were extracted, derivatized using TMSH as well as TMS, and further analyzed by GC-MS. All raw data were quantitatively and qualitatively analyzed using Agilent MassHunter Software: MS B.07.00.

**Statistics and reproducibility.** The graphical data for this study was generated using GraphPad Prism software. Three biological replicates were used per experiment ( $n = 3$ , unless stated otherwise). The error bars are representative of the standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by unpaired  $t$  tests using GraphPad Prism. The range of statistical significance was signified as per the  $P$  value, where  $0.01-0.05 = *$ ,  $0.01-0.001 = **$ ,  $<0.001 = ***$ , and  $<0.0001 = ****$ .

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability

Authors can confirm that all relevant data are included in the paper and/or its Supplementary/Source data files. Protein and gene sequences were acquired from EuPathDB web sources ToxoDB (<http://toxodb.org/toxo/>). Source data are provided with this paper.

### Code availability

No codes were used to analyze the data in this study.

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### Author contributions

C.Y.B., Y.Y.B., and S.D. contributed to the design of the work. C.Y.B., Y.Y.B., L.B., S.S., F.B., M.F.C., and S.Da. contributed to the interpretation of data. S.Da., S.S., S.Du., F.B., C.S.A., N.J.K., L.B., Y.Y.B., and C.Y.B. contributed the acquisition and analysis of data. M.F.C. and C.Y.B. financed the project.

### Competing interests

The authors declare no competing interests.

### Additional information

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# SCIENTIFIC QUESTION OF THE THESIS

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## ***Hypothesis:***

Apicomplexa parasites cause major human diseases such as malaria and toxoplasmosis, that still impose major economic and health burdens of large parts of the world. Large amounts of lipids are essential for parasite progeny and persistence within their hosts. FAs are central to lipid production and the parasites are unique in that they can scavenge FAs directly from the host cell and their environment and newly-synthesize FAs themselves via an exclusive prokaryotic type II fatty acid synthase or FASII within a non-photosynthetic essential relict plastid, named the apicoplast. The complex life cycles of these parasites require them to invade a multitude of different host under varied conditions, meaning they must be able to adapt to the host conditions to survive. Their prevalence and drug resistance emphasizes the need to understand the parasites biology and identify novel drug strategies. Recent work is only starting to uncover that these parasites can significantly adapt to the nutrient availability of the extracellular host environment (Zuzarte-Luís and Mota, 2018; Primo *et al.*, 2021; Shunmugam *et al.*, 2021; Shunmugam, Arnold, *et al.*, 2022; Walsh *et al.*, 2022). Many questions remain unanswered about the lipid metabolism of these parasites:

- How are FAs activated for their use in the lipid-processing pathways of the parasite?
- How does different nutrient environments affect the lipid synthesis within the parasite?
- How does the parasite recycle lipids and contribute to the dynamic remodelling of their lipid metabolism strategies?

My PhD project focused on answering the above questions by identifying three protein families, which we hypothesized play major roles in these mechanisms:

- **Acyl-CoA synthetases** (ACSSs), which can activate FA for their metabolic use by the parasite
- **Phospholipases** that can release FA from existing or recycled lipids
- **Acyltransferases** (ATs) that (re)incorporating FA to generate new lipid species

### ***Aim and Objectives:***

Their putative roles and importance in the lipid synthesis pathway are not fully understood as is critical to the propagation of the parasite.

I aimed to elucidate and characterize the roles of prioritized protein candidates in Apicomplexan parasites, specifically *Toxoplasma gondii* and *Plasmodium falciparum*.

The completion of my PhD followed the objectives presented below:

1. Generate molecular tools (CRISPR-Cas9) to characterize the lipid processing pathway in the two Apicomplexa models *P. falciparum* and *T. gondii*
2. Assess the essentiality, localization, and cellular context of each target protein in both models
3. Determine the function of the target protein at the parasite lipid synthesis level using core lipidomics by GC-MS, and lipid flux analyses by stable isotope labelling combined to lipidomics
4. To understand the global contribution of each protein class to the parasite's nutrient-adaptability in lipid synthesis, storage, and utilization processes

## **CHAPTER 4: PUTATIVE *TOXOPLASMA GONDII* ACYL-COA SYNTHETASES ARE INVOLVED IN NEUTRAL LIPID METABOLISM UNDER VARIED NUTRIENT CONDITIONS**

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### **Summary of manuscript (Submitted to Cell Reports):**

#### **A putative peroxisomal *Toxoplasma gondii* acyl-CoA synthetase (*TgACS1*) is involved in neutral lipid metabolism under suboptimal nutrient conditions**

Lipid biosynthesis in parasites, as with all other organisms is reliant on the metabolic activation of fatty acids (FAs) by the parasite. Acyl CoA synthases (ACS) catalyse the thioesterification of FA, allowing their metabolic use by any enzymes handling FAs in the parasite. Beside the crucial functions of FA for parasite survival, the presence and roles of ACS is yet to be determined. In *P. falciparum* and *C. parvum* 13 and 3 ACS proteins have been defined, respectively. We identified 7 putative ACSs encoded by the genome of *T. gondii* (*TgACS*). The differential localization of these proteins suggests that ACS in this parasite have varied functions. We prioritized a protein which seemed to have an important role in parasite survival when lipid levels are scarce in the host, hence suggesting a function in parasite metabolic adaptation; *TgACS1*.

The major findings of this are listed below:

- *TgACS1* is important in FFA formation and TAG utilization in intracellular parasites in low-nutrient conditions for the host.
- This is also true for extracellular parasites, where the phenotype of *TgACS1* disruption is even more marked than in intracellular parasites
- *TgACS1* is important for the utilization of LD in extracellular parasites and its disruption affects parasite motility/energy production
- *TgACS* may be important for directing FAs toward the  $\beta$ -oxidation pathways under low nutrient levels
- Heterologous complementation in yeasts conclude that *TgACS1* has peroxisomal acyl-CoA synthétase function



## **Résumé du manuscrit :**

**Une acyl-CoA synthétase (*TgACS1*) péroxisomale putative chez *Toxoplasma gondii* est impliquée dans le métabolisme des lipides neutres en conditions nutritives défavorables.**

La biosynthèse de lipides chez les parasites, comme pour les autres organismes, requiert l'activation préalable des acides gras. Les acyl-CoA synthétase (ACS) catalysent la thioestérification des acides gras libres, permettant leur utilisation dans le métabolisme par toutes les enzymes dont ils sont le substrat. Outre le rôle crucial des acides gras pour la survie des parasites, la présence et fonctions des ACS restent à déterminer. Treize ACS ont été identifiées chez *P. falciparum* et 3 ACS chez *C. parvum*. Nous avons identifié 7 ACSs putatives dans le génome de *T. gondii* (*TgACS*). Les différences de localisation de ces protéines suggèrent que les ACS de ce dernier occupent des fonctions variées. Nous avons priorisé la protéine *TgACS1* dont le score d'essentialité évalué était élevé et pour laquelle la déplétion avait le plus fort impact sur la viabilité du parasite en conditions de stress nutritif, suggérant ainsi une fonction dans l'adaptation métabolique du parasite

Les résultats principaux sont les suivants :

- *TgACS1* est importante pour la formation des acides gras libres et pour la consommation des triglycérols chez le parasite intracellulaire en conditions de stress nutritif.
- Cette observation se vérifie également chez les parasites extracellulaires pour lesquels la disruption de *TgACS1* induit un phénotype encore plus sévère que pour les parasites intracellulaires.

- *TgACS1* permet la consommation des gouttelettes lipidiques chez les parasites extracellulaires et sa disruption diminue la mobilité, et donc production d'énergie, du parasite
- *TgACS* pourrait contribuer à la consommation d'acides gras via les voies de  $\beta$ -oxydation en conditions de stress nutritif.
- Des expériences de complémentation hétérologue conduites chez les levures confirment que *TgACS1* possède une fonction d'acyl-CoA synthétase peroxysomale.

***A putative peroxisomal Toxoplasma gondii acyl-CoA synthetase (TgACS1) is involved in neutral lipid metabolism under sub-optimal nutrient conditions***

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**ABSTRACT**

Apicomplexa parasites cause immense health and economic burdens such as toxoplasmosis and malaria. They mostly comprise obligate intracellular protists that heavily depend on lipid metabolism for the survival within their hosts. The lipid synthesis within these parasites relies on an essential combination of fatty acids (FAs) obtained from both *de novo* synthesis and scavenging from the host. The constant flux of scavenged FA needs to be channelled towards parasite lipid storage and are timely mobilized during parasite division. In eukaryotes, the utilization of FA relies on an obligate metabolic activation mediated acyl-CoA synthases (ACS), which catalyse the thioesterification of FA to a CoA. Beside the essential functions of FA for parasite survival, the presence and roles of ACS is yet to be determined. Here, we identified that TgACS1 is a vesicular cytosolic protein (peroxisomal) protein which is involved in lipid metabolism of the parasite during low host nutrient conditions. The protein was present in cytosolic punctuates and revealed a host-nutrient-dependent role during

both intracellular and extracellular tachyzoite life stages. We show initial evidence that this protein is involved in FFA metabolism for peroxisomal processes such as  $\beta$ -oxidation.

## **IMPORTANCE**

*Toxoplasma gondii* is an apicomplexa parasite and model within this phylum that hosts major infectious agents, such as *Plasmodium* spp., responsible for malaria. The diseases caused by apicomplexans are responsible for major social and economic burdens on more than 1/3 of the world's population. Lack of efficient vaccines, rapid emergence of resistance to existing treatments, and toxic side effects of current treatments, all argue for the urgent need to develop new therapeutical tools to combat these diseases. Understanding the key metabolic pathways sustaining host-intracellular parasite interactions is pivotal to develop new efficient ways to kill these parasites. Current data support parasite lipid synthesis and trafficking as pertinent target for novel treatments. Many processes of this essential lipid metabolism in the parasite are not fully understood. The capacity for the parasites to sense and metabolically adapt to the host physiological conditions has only recently been unravelled. Our results clearly indicate the role of acyl-CoA synthetases for the essential metabolic activation of FA used to maintain parasite propagation and survival. The significance of our research is 1) the identification and cellular localization of 7 of these enzymes in *T. gondii* parasites, 2) using lipidomic techniques we also identified a nutrient dependent role of *TgACS1*, 3) yeast complementation assay showed that ACS1 has acyl-activating function and possible implication in the  $\beta$ -oxidation 4) and has a important role for extracellular parasite motility and energy production.

## INTRODUCTION

Apicomplexa parasites are a group of unicellular eukaryotes that include obligate intracellular parasites responsible for major human and cattle diseases. These include *Toxoplasma gondii*, *Plasmodium sp.* and *Cryptosporidium parvum*, which are the causing agents of the infectious diseases toxoplasmosis, malaria and cryptosporidiosis, respectively. Eradicating these diseases is a worldwide priority, and the renewal of our therapeutic arsenal relies on understanding host-parasite interactions sustaining parasite survival. Specific metabolic pathways allowing nutrient acquisition, intracellular development, and parasite propagation represent ideal targets for drug development. Lipid synthesis, acquisition and homeostasis have been shown as one of the key metabolic pathways for parasite survival and pathogenicity (1, 2).

*Toxoplasma gondii* is capable of infecting virtually any nucleated cell from warm-blooded animals, thereby reflecting its metabolic capacities and plasticity in adapting to different nutritional environments. During the intracellular development of *T. gondii*, lipids play an essential role by providing structural building blocks for membrane biogenesis(3, 4), as signalling molecules participating in key events like active invasion and egress through timely microneme secretion (5–7) and as storage fuels, which plays critical role to maintain proper intracellular development (8, 9). To cope with a continuous need for lipids within the different host cells environments, *T. gondii* has evolved so it harbours three *de novo* synthetic pathways to generate fatty acids (FA), central lipid building blocks: (i) the apicoplast resident prokaryotic type-II fatty acid biosynthesis pathway (FASII) (3, 10), (ii) the ER-based FA elongation pathway (4) and (iii) the eukaryotic type-I fatty acid synthase present in the parasite cytosol(10). Additionally, the parasite is also heavily reliant on acquiring FA directly from their hosts (4, 8, 9, 11–13). Current consensus on parasite lipid metabolism suggests that both

FA flux derived from apicoplast FASII (3, 10), and FA obtained from direct scavenging from the host (4, 8, 9, 11–13), are critical for intracellular parasite viability. Interestingly the metabolic input of both FA sources can be regulated upon the host nutrient availability. Under high host lipid content, FASII can be downregulated and maintained at a basal level for the parasite to rely more on host scavenging. On the other hand, under low host lipid content, the FASII metabolic activity is significantly upregulated concomitantly with an upregulation of the scavenging of host membrane material, both to meet and maintain the parasite demand for lipids (13).

Interestingly, major phospholipid classes making the bulk of *T. gondii* lipid composition are obligate “patchwork molecules” composed of FA from the host as well as made *de novo* by the parasite(3). Most importantly, to optimise the use of host nutrient and avoid death by lipotoxic accumulation of lipids, the parasite channels the constant flux of scavenged FA towards its own lipid storages, i.e. lipid droplets (LD) in the form of neutral lipids, mainly triacylglycerols (TAG) (9). LD and their TAG content are then timely mobilized to provide an essential boost of FA for membrane biogenesis specifically during parasite division (9). Taken together, FA, and most importantly their acquisition, mobilization from sources/storages, and utilization are pivotal pathways for sustaining parasite survival.

One of the key biochemical steps required for general FA utilization and trafficking is their metabolic activation by thioesterification to a Co-Enzyme A (CoA). This metabolic activation step is catalysed by the essential acyl-CoA synthetase (ACS or acyl-CoA ligase) family (14). Biochemically, ACS catalyses a two-step reaction to form fatty acyl-CoA (Fig. 1a). The initial step involves the formation of an adenylated intermediate through the hydrolysis of an ATP molecule, thus releasing pyrophosphate (15). The ATP-activated enzyme then binds to the carboxyl group of an incoming free FA (FFA)

moiety through an acyl bond to the phosphoryl group of AMP. Final fatty acyl-CoA product is formed after the transfer of the fatty acyl group to the sulfhydryl group of coenzyme A, thereby releasing AMP (15).

ACSs exist as different isoforms in eukaryotes depending upon on FA substrate specificity, (based on FA chain length and degree of unsaturation), metabolic or cellular function (FA storage,  $\beta$ -oxidation etc.). Humans have 26 enzymes that comprise the large ACS family activating short-chain, medium chain, long chain and very long chain fatty acid substrates (14). There are 9 long chain ACSs (LACS) that have been well characterized in *Arabidopsis thaliana* (16). This family of AMP-binding domain containing enzymes to a larger extend are indispensable for fatty acid metabolism in various eukaryotes. ACSs have been shown to be predominantly involved in glycerolipid biosynthesis as well as catabolic  $\beta$ -oxidation pathway and transport of fatty acids across organellar membranes (14, 17).

The phylum apicomplexa is actively engaged in FA metabolism and hence the role of the ACSs in parasite survival within its host cannot be undermined individually/apart from the host. ACSs in apicomplexa potentially activate fatty acids derived from both *de novo* synthetic machinery and from the host. This can be supported with the existence of ACS encoding genes in the genome of *Cryptosporidium* and *Theileria* which lack the *de novo* fatty acid biosynthetic machinery (10). *C. parvum* has been reported to have three AMP-binding domains containing long-chain fatty acid CoA ligases, of which *CpACS1* and *CpACS2* have been biochemically characterized as functional ACSs (18). *Plasmodium falciparum* on the other hand has a large family of ACSs encoded by 13 genes (*PfACS1a-PfACS12*), which functions remain to be determined (19). Interestingly, *P. falciparum* ACSs are also the only family of metabolic enzymes that are encoded by genes expanded into the sub-telomeric region, usually

harbouring parasite virulence genes (19). Drugs specific to ACSs in *C. parvum* and *P. falciparum* have shown potent parasite killing activity, thereby hinting that this enzymatic reaction is a potential Achilles heel for apicomplexans (18, 20). Although the presence of an ACS was reported in *T. gondii* by bioinformatics analysis (21), the number of ACS or their functional roles in *T. gondii* are yet unknown.

Here, we identified that *T. gondii* encodes and harbour 7 putative ACSs by phylogenetic analysis. *TgACS1* was shown to have a specific role for FA utilization when parasites face low nutrient environment, especially during extracellular stages. Moreover, we found that *TgACS1* belongs to the ACS bubble gum family and can only compensate a yeast homologue specific for beta oxidation. Therefore, this strongly suggests that *TgACS1* is involved in providing energy through FA mobilization, potentially  $\beta$ -oxidation in a peroxisomal-like compartment in low nutrient content.

## **RESULTS**

### **Identification seven genes encoding putative acyl-CoA synthetase (ACS) enzymes within the *Toxoplasma* genome**

To identify putative members of ACS gene family in *Toxoplasma gondii*, we searched for genes bearing the typical AMP-binding domain found in eukaryotic ACS by mining the ToxoDB database (<http://toxodb.org/toxo/>). We were able to identify several candidate genes. Within these candidates bearing the AMP-binding domain, seven proteins were identified bearing highly conserved signature ACS motifs, named Motif I-V (15). We assigned the 7 putative *T. gondii* ACS (*TgACS*) candidates as *TgACS1* (TG GT1\_297220), *TgACS2* (TG GT1\_310150), *TgACS3* (TG GT1\_310080), *TgACS4* (TG GT1\_243800), *TgACS5* (TG GT1\_247760), *TgACS6* (TG GT1\_232580), *TgACS7* (TG GT1\_276155) (Fig.1b). Phylogenetic analysis allowed the cladding of the different *TgACS*s to specific clusters based on their homology to other eukaryotic counterparts

(Fig. 1b). *TgACS1* cladded together with the human ACSs belonging to the bubble-gum gene family (ACSBG), also known to be involved in  $\beta$ -oxidation, *i.e.* FA catabolic pathway allowing the generation of reducing power and energy to the cell (22). *TgACS1* has already been predicted to be involved in the possible  $\beta$ -oxidation by its peroxisome transit sequence PTS (21). Based on their CRISPR-based phenotype scores (23) and predicted functions based on sequence homology and phylogenetic analyses, we decided to focus on a specific candidate *TgACS1* with its putative role as bubble-gum ACS, thus potentially involved in FA mobilization for a putative and long-debated  $\beta$ -oxidation in *T. gondii*.

### **The PTS sequence in *TgACS* is crucial for correct protein localization**

In order to identify putative functional domains in *TgACS1* protein sequence, Smart Protein program ([www.smart.embl-heidelberg.de](http://www.smart.embl-heidelberg.de)) and Phyre 2 were used to compare to known domains. The presence of bubble-gum domain was identified in *TgACS1* and this supports the phylogenetic analyses described earlier (Fig. 2a) suggesting similar functionality of this protein. We also identified a highly conserved peroxisomal targeting sequence (PTS) present in the C-terminal end of the protein (tripeptide, AKL). Other alveolates such as *Hammondia* and *Neospora*, and a known peroxisomal ACS in *Saccharomyces cerevisiae* also contain this specific PTS (Fig. 2b). Using Phyre 2 and a 3D crystal structure of a bubble-gum-domain-containing ACS found in *Mycobacteria tuberculosis* (24), we generated a 3D model prediction, which had a sequence identity of 51% with *TgACS1* (Fig. 2c). These protein structures seem to have similarly placed hydrophobic regions suggested to be the AMP-binding cavity and similar placement of arginine residues which suggest common functionality (Fig. 2c ; (24)).

To characterize and identify the localization of *TgACS1*, we generated an AID-based (25) inducible knock down mutant of *TgACS1* with HA-tag. Immunofluorescence

analyses (IFA) revealed that this protein had a punctuated/vesicular cytosolic localization, which we originally thought to be overlapping with lipid droplets (LD) of the parasite (Fig. 2d). LTox staining, which stains LD, showed close association of *TgACS1* with LD but no direct co-localization. A similar mutant line was developed lacking the predicted PTS1 (C-terminal AKL, consensus of the tripeptide is [(S/A/C)(K/R/H)(L/M)]). Immunofluorescence microscopy of both lines revealed that when the PTS signal is removed the protein loses its cytosolic/punctuated localization and instead localizes to the apical end of the parasite, close to the Golgi apparatus (Fig. 2e). Further detailed localisation analysis, however, shows no direct localization with the Golgi apparatus (GRASP45-RFP) or micronemes (anti-AMA1) of the parasite since it was thought to start aggregating in an anterograde manner towards the apical pole randomly (Fig. 2e).

### ***TgACS1* is important for intracellular development in low nutrient environment**

We also confirmed the protein size of HA-tagged *TgACS1*-iKD by Western blot analysis with its expected size of 100 kDa (Sup. 1b). The downregulation of *TgACS1* by the addition of 100  $\mu$ M auxin was also confirmed by Western blot and IFA analyses both after 30 min of treatment (1% FBS, Sup. 1b and Fig. 3a), with complete degradation of the protein within 1 h of treatment. To determine if the depletion of this protein influenced parasite growth, a replication assay was performed and showed a slight but not significant increase in small vacuoles (1-4 parasites per vacuoles) and decrease in larger vacuoles (10 parasites or more per vacuole) upon *TgACS1* degradation (Sup. 1c).

Since the host nutrient environment affects the development and metabolism of the parasite as shown in our previous work (7, 12), the parasites were grown in different concentration of FBS. Seeing that the phenotype of *TgACS1* at normal FBS conditions

(1%) was not indicative of essentiality, as proposed by the phenotypic score (33), we further investigated how the loss of this protein would affect the parasite in differential host nutrient environments. We thus performed plaque assays in the presence of different host nutritional conditions, i.e. at 0, 1, or 10% FBS in DMEM as previously described. A significant difference was seen in the growth of plaques under starvation lipid restrictive conditions (0% FBS) and in normal conditions (1% FBS, Fig. 3b). Parasites grown in the presence of auxin in these two conditions showed to have less plaque area on average and hence a delayed growth in the absence of *TgACS1*. In 10% FBS, parasites were able to survive without the protein (Fig. 3b) suggesting that *TgACS1* does not have an essential role in high nutrient conditions. Our results on parasite growth, confirmed that *TgACS1* is involved in parasites development in a nutrient-dependent manner, this was further confirmed by relative quantification of mRNA levels of this protein (Sup. 1d), which showed an increase in expression of this protein in nutrient poor conditions and specifically in extracellular parasites.

Since *TgACS1* grows differently in response to the nutrient availability, it is possible that its flux of storage may be altered. In addition, LDs are not only the main source for lipid storage, but they also can serve to peroxisomes mediated lipid-based energy conversion via  $\beta$ -oxidation (26). Hence, we decided to analyse how these lipid energy stores were affected under the suppression of this protein in different nutrient conditions. Here, instead of LTox, we used Nile red to stain the LDs in various nutrient content, (0-1% FBS). The results showed an increasing accumulation of lipid droplet number as FBS concentration increased but no difference in the number of lipid droplets per parasite with or without the *TgACS1* protein (Fig. 3c). Closer analyses revealed that only in a high host nutrient environment was there no changes in lipid

droplet volume (Fig. 3d), suggesting *TgACS1* is not important in intracellular parasites in a high lipid content.

***TgACS1* is important in FFA formation and TAG utilization in intracellular parasites depending on the availability of host nutrients.**

Although there was an increase in the lipid droplet number by the change in the nutrient condition, there was no difference between the parasite with or without the *TgACS1* protein. Here, to determine if there is any change in the major lipid content and so as the lipid changes were reflected in the lipid metabolism of the parasite, we conducted lipidomic analyses by gas chromatography-coupled to mass spectrometry (GC-MS) and separation of neutral lipid groups via high-performance thin layer chromatography (HPTLC) on *TgACS1*-iKD intracellular parasites with and without auxin.

Firstly, we examined the lipidomic profiles of intracellular parasites at 0-10% FBS (Fig. 3e) and noticed no changes in total lipid abundance when the protein was depleted, which was suspected since we saw little to no changes in LD formation in the Nile red analyses (Fig. 3c-d). To further investigate the nutrient dependent role of *TgACS1* we quantified the phospholipid and neutral lipid content of intracellular parasites in different nutritional conditions (namely 0-10% FBS, Fig. 3e-i, Sup. 2a-c, g/h).

The fatty acid profile of intracellular parasites in 0% FBS media revealed that under suppression of *TgACS1* there was a significant increase in the mol% of C18:1 and decrease in the mol% of C18:0 (Fig. 3f). This suggests a role with- or higher substrate-binding affinity to C18:0/C18:1. To further determine the precise function of *TgACS1*, the total lipid fractions were subjected to separation by HPTLC and the composition and content of total phospholipids (PLs), triacylglycerol (TAG, LD constituent), diacylglycerol (DAG) and free fatty acids (FFA) were quantified by GC-MS. This detailed lipidomic analyses revealed a slight but not significant increase in TAG levels

and a significant decrease in FFA levels when the protein is degraded in 0% FBS (Fig. 3g). This suggests that FFAs are the final product of this enzyme, with a slight hint to TAG moieties being the substrate. At 1% FBS, intracellular parasites without *TgACS1* displayed no changes in their total lipid fatty acyl profile. Additionally, there were no noticeable changes in neutral lipid content when the protein was knocked down under these same conditions (Fig. 3h-i). There were no changes in the fatty acid profile of the individual lipid groups in intracellular parasites when the protein was depleted in 1% FBS (Sup. 2b).

### ***TgACS1* is important for extracellular parasite lipid droplet utilization**

Over the general surveillance of *TgACS1*-ikD we noted that under normal 1% FBS conditions, IFAs indicated a punctuated cytosolic localization in extracellular parasites (Fig. 4a). mRNA quantification of this gene under different conditions indicated expression was highest in intracellular parasites but, equally as high in extracellular parasites at 0% FBS, or under low nutrient availability (Sup. 1d). This persuaded us to determine if we see the same trend in extracellular parasites and whether *TgACS1* plays a role in lipid droplet utilization in these sub-optimal conditions. Interestingly, lipid droplet number/volume is higher in extracellular parasites as compared to intracellular parasites (2-3-fold), suggesting that the parasite increases lipid droplet formation in the absence of a host (Fig 3c, and Fig 4b). The number of lipid droplets per parasite at 0% FBS and the lipid droplet volume at 1% FBS nutrient conditions were respectively increased in extracellular parasites under protein loss (Fig. 4b-c).

To determine if this observation is also present under complete loss of nutrients, extracellular parasites were incubated with PBS alone for less than 2 h (to avoid parasite death) and stained them with Nile red (Sup. 1e). In PBS conditions, lipid droplet numbers were significantly less than in 1% FBS conditions, and further diminished

when *TgACS1* was degraded under auxin treatment in extracellular parasites. LD analyses on intracellular and extracellular parasites suggests a role of the protein in the utilization of lipid droplet constituents such as TAGs. It also suggests that this role is highly dependent on nutrient availability.

### ***TgACS1* is important for extracellular parasite motility and energy production**

Fatty acid stored in a form of TAG can be hydrolysed and used for energy production through the process of  $\beta$ -oxidation. We determined if *TgACS1* could be involved in energy-production in the parasite. Therefore, we analysed the viability energy-producing state of the *TgACS1*-ikD parasites under 'stressful' conditions that may potentially induce  $\beta$ -oxidation i.e. without nutrients and outside the host. We performed parasite gliding (motility) assays of extracellular parasites in different nutrient conditions (0-1% FBS with and without glucose), wherein we measure the slime trail length formed by the release of SAG1. It revealed that under *TgACS1* depletion, in all conditions, parasite motility in terms of average trail length ( $\mu$ M) was significantly decreased (Fig. 4d-e). When measuring the motility rate (nominal data) we notice that only at low nutrient conditions (0% FBS and PBS) is motility diminished for extracellular parasites (Sup. 1f). Interestingly it was noted that in the absence of glucose (which is the primary source for ATP-production, i.e., glycolysis) loss of the protein did not change the total lipid abundance of the parasites (Sup. 2j). This suggest that the role of *TgACS1* is rather based on the level of FBS, hence nutrients and most likely its main constituent lipids, in the extracellular media.

This confirms the role of *TgACS1* in energy-requiring processes like motility and emphasizes that the role is most important in low lipid conditions. The relationship between the parasite and the putative  $\beta$ -oxidation process occurring is uncharacterized and there is still much debate on whether the process even occurs in the parasite with

or without a host. These data now suggests that the parasite is still able to produce energy to facilitate gliding in sub-optimal conditions, hinting that even without the host the parasite can produce its own energy from its own reserves.

Secondly, due the cellular phenotypic changes in terms of lipid droplet formation, we investigated the lipidomic profile of *TgACS1* depleted extracellular parasites. By doing so we have also been able to first identify the lipidome of the intermediate extracellular phase of the *T. gondii* tachyzoite development. Surprisingly we noticed no changes in the total lipid abundance of extracellular parasites at 0-10% FBS (Fig. 4f) when *TgACS1* was depleted.

We noticed that the lipid content was higher (2-3-fold) in extracellular parasites than intracellular parasites (Fig. 2e and Fig. 3f), which reinforces the same observations seen in LD number (Fig. 3c-d and Fig. 4b-c). This suggest that more lipids are stored within extracellular parasites than when they exist within a host. Detailed investigation into the fatty acyl profiles of extracellular parasites in 0% and 1% FBS revealed changes in mol% of specific length FA. In no FBS conditions the loss of the protein results in a decrease of C14:0 (most derived from the apicoplast) and an increase in FA species normally scavenged from the extracellular environment/host (C18:1 and C20:1). A largely significant increase in C18:1 in 0% FBS again suggests a strong substrate specificity of the protein (Fig. 3g) as was seen for intracellular parasites (Fig. 3f). As in intracellular parasites, we again see a decrease in FFA levels in extracellular parasites in 0% FBS (Fig. 4h), affirming that FFA moieties may be the final product of the enzymatic reaction facilitated by *TgACS1*.

We also quantified the fatty acid abundance of extracellular parasites in different nutrient condition. In extracellular parasites at 1% FBS, under depletion of the *TgACS1*, there is a similar trend where there is an increase in C18:0 and C16:0 and a decrease

in C18:1, suggesting *TgACS1* has a substrate specificity to these chains (Fig. 4i). Most interestingly, the detailed analyses of the neutral lipids again show a slight but not significant increase in TAG levels with a decrease in FFA moieties (Fig. 4j). These data collectively suggest *TgACS1* plays an important role in low nutrient conditions, namely 0-1% FBS, specifically in the mobilization of acyl chains from TAG/lipid stores and furthermore the formation of FFAs.

Overall, these results suggest that *TgACS1* plays a more important role in the activation of FFA in extracellular parasites under low nutrient conditions. Moreover, this protein seems to have a substrate specificity to longer, unsaturated FA species that are normally externally derived.

### ***TgACS1* complements a yeast strain defective of peroxisomal acyl-CoA synthase function**

In order to determine the acyl-CoA synthase activity of *TgACS1*, we performed heterologous complementation in yeast *Saccharomyces cerevisiae* strain defective of the acyl-CoA synthases. In yeast, it is known that acyl-CoAs can be made by the endogenous FAS pathway or by the activation of exogenous fatty acyl chains by ACSs. Johnson *et al.*, (1994) have shown that in yeast two major enzymes (Faa1 and Faa4) are involved in the activation of long chain acyl-CoA chains. Only when FAS is inactivated (with the addition of cerulenin) the double mutant strain can no longer grow even with exogenous FAs.

We transfected the mutant yeast strain (*faa1Δfaa4Δ*) with a pRS high copy (*ura3*) plasmid expressing *TgACS1* with a His tag. The WT and mutant strain transfected with the empty vector (no *TgACS1*) were used as controls. All experiments were performed in synthetic YNB media without uracil, supplemented with glucose (2%) and Tween20 (0.2%, to induce  $\beta$ -oxidation). Growth curves were determined at 30°C (Fig. 4). The

WT strain displayed a phenotype as expected, where the inhibition of FA synthesis with the addition of cerulenin lead to a decreased growth rate of this strain. There was no rescued phenotype under cerulenin treatment and addition any FA species (Sup. 3), suggesting *TgACS1* is not an ACS of this nature dependent on exogenously acquired FFAs (37).

This prompted us to investigate other types of ACS proteins found in yeast. ScPCS60 is an ACS involved in peroxisomal fatty acid metabolism of budding yeast. This protein is integrated in the peroxisomal  $\beta$ -oxidation system of this organism (38). Addition of doxorubicin (anti-cancer drug targeting peroxisomal  $\beta$ -oxidation) has shown to inhibit growth of yeast lacking this protein (39) on the  $\Delta$ PCS60 yeast strain (Fig. 5). In the absence of doxorubicin, the  $\Delta$ PCS60 strain grows optimally and similarly as expected with the empty vector plasmid and pRS-*TgACS1*-His. When treated with doxorubicin, the strain with the empty vector still grows put at a slower rate, but not to the same OD<sub>600</sub> reading as the untreated cells. Growth is almost completely restored in the doxorubicin-treated  $\Delta$ PCS60 strain complemented with pRS-*TgACS1*-His, suggesting that *TgACS1* can rescue the effects caused by this drug. This strongly indicates that this protein has an acyl activating capacity and plays a pivotal role in peroxisomal  $\beta$ -oxidation.

## **DISCUSSION**

Acyl CoA synthetases catalyse a fundamental and limiting reaction in FA metabolism (Fig. 5a): the activation of free FA via thioesterification to coenzyme A (CoA). This key reaction allows the fatty acyl CoA intermediates to participate further in trafficking, assembly into complex lipids, post-translational modification of membrane proteins, and/or the catabolism of FAs via  $\beta$ - oxidation (14).

Here we identified 7 acyl-CoA synthetase (ACS) enzymes found in *T. gondii* parasites, all of which possess an AMP-binding domain (27, 28). Multiple sequence alignment (Fig. 1b) of these proteins with known ACS proteins in other eukaryotic models suggest an overall identity of up to 30-40% specifically within the putative AMP-binding domains. Phylogenetic analysis of the identified *Tg*ACSs with known ACS from other eukaryotes clustered them to various clades, potentially based on their enzymatic substrate- specificity and function (Fig. 1b). The interesting separate sub-grouping of *Tg*ACS7 alongside its homologs from coccidia and chromerida may indicate a coccidian-photosynthetic origin of *Tg*ACS7 potentially lost during the evolution of haemosporidians (*P. falciparum*).

The existence of several ACSs in the *Plasmodium* and *Toxoplasma* genome are indicative of their unique and central functions for maintaining parasite survival. These parasites harbour extensively diverse range of FA varying in chain lengths and degree of saturation/unsaturation. Thus, it is not surprising that the parasites have a long list of ACS encoding genes. Similarly, there are 26 ACSs in humans, 11 of which are long-chain (ACSL) and very long chain acyl-CoA synthetases (ACSVL) (15). These ACSL and ACSVL have the specificity to activate fatty acid up to 16-22 carbons in length (14). The ACSs, however typically activate the highly abundant fatty acids in nature including palmitate (C16:0) and oleate (C18:1). Others include short-chain ACSs (ACSSs) that typically activate acetate, propionate, or butyrate and medium-chain ACSs (ACSMs) that activate fatty acids ranging in chain lengths from C6 to C10 (14). Lastly, the bubble gum-ACSs called ACSBG gene family (also known as lipidotin) that can activate FAs of chain length 16-24 (29).

The diverse sub compartmental localization of putative *Toxoplasma* ACSs indicates non-redundancy and their highly specific roles in fatty acid activation. The correlation

between protein localization and its function has been established with extended family of ACSs in humans (30–32) and prostate ACSL1 localized at mitochondria, has a significant contribution to FA  $\beta$ -oxidation (28, 33). ACSL3, which has been found on lipid droplets and ER, participates in FA uptake and glycerolipid biosynthesis (34). The localization of *Cryptosporidium parvum* CpACSL1 at the apical end of cell-free sporozoites has been linked to FA biosynthesis required during process of invasion, and/or early-stage development (18).

Additionally, the localization of the ACS could also direct the fate of the acyl-CoAs by allowing the ACSs to interact with different proteins involved in the direct downstream processing. For example, human ACSL1 coimmunoprecipitates with mitochondrial outer membrane proteins carnitine palmitoyl transferase 1a (CPT1a) and voltage-dependent anionic channel (VDAC) (33, 35). No CPTa homologue exists in apicomplexa parasites (8), however recent data now provides evidence for the presence of an active acyl-CoA binding protein-2 (ACBP) that localizes to the mitochondria of the parasite (11, 13) and *TgACBP2* are potentially coherently involved in FA activation and transport within the parasite mitochondria. However, whether they participate in mitochondrial membrane biogenesis and/or rather  $\beta$ -oxidation is yet to be determined. It is also possible that this parasite favours a peroxisomal  $\beta$ -oxidation system instead which can explain the loss of CPT1a but the existence of other  $\beta$ -oxidation proteins .

Amongst several functions of the ACSs includes channelling of FA towards energy yielding process of  $\beta$ -oxidation (14, 16, 36). The mouse ortholog of human *HhsACSBG1*, called mBG1 localizing in the form of vesicles in close proximity to mitochondria of neuronal cells was shown to be involved in mitochondrial  $\beta$ -oxidation of the long chain fatty acid palmitate (22). Interestingly, based on phylogenetic analysis

*TgACS1* clusters together with the *hsACSBG*-gene family known to be involved in  $\beta$ -oxidation (14). The presence of FA  $\beta$ -oxidation in apicomplexa has been a relatively unexplored. In eukaryotes, this energy yielding process compartmentalizes to mitochondria and vesicular organelles called peroxisomes. There is genomic and bioinformatic evidence for the presence of peroxisomes in *T. gondii* (37, 38). Proteins imported into the peroxisomal lumen bear two canonical targeting sequences- peroxisomal targeting sequence type 1 (PTS1), a C-terminal tripeptide with the consensus sequence [SAC]-[KRH]-[LM] and peroxisomal targeting sequence type 2 (PTS2), an N-terminal peptide comprised of the amino acids [RK]-[LVIQ]-X-X-[LVIHQ]-[LSGAK]-X- [HQ]-[LAF] (21). In *A. thaliana* LACS6 is targeted by a type 2 (PTS2) peroxisomal targeting sequence whereas LACS7 inhabits both functional PTS1 as well as a PTS2 (39). This peroxisomal LACS activity has been shown to be essential for seedling growth (39). Moog *et al.* provides bioinformatic evidence for the presence of classical PTSs in the protein factors potentially involved in  $\beta$ -oxidation in apicomplexan parasites. These proteins are subjected to stage specific expression supporting the existence of this process during the stage of sporulation or encystment within the parasites (21). *TgACS1* was one of the proteins defined within a “high confidence” PTS1-targeted peroxisomal proteome for *T. gondii*. Vesicular localization and predicted PTS1 at the C-terminus of *TgACS1* further strengthen its putative role in the elusive  $\beta$ -oxidation pathway in *T. gondii*. Co-localization studies with peroxisomal markers like catalase can provide further confirmation. Electron microscopy of extracellular parasites under nutrient-stress conditions would be able to confirm the existence of peroxisomal-like vesicles, previously not known.

Our complementation experiments of an acyl-CoA synthase deficient yeast strain (Fig. 5), illustrates acyl-CoA synthase activity of *TgACS1*, but that that this protein has a

preference in the activation of FAs (namely C18:1 which induces expression of ScPCS60) destined for  $\beta$ -oxidation, as all experiments were performed using Tween20 (a polysorbate with a C12:0, lauric acid attached), which induces  $\beta$ -oxidation. These experiments strongly indicate that this parasite may have a very 'minimalistic' version of  $\beta$ -oxidation process in the parasite peroxisome, but it would be critical to investigate these metabolites under conditions that induce the process such as nutrient deprivation. Co-localization studies with peroxisomal markers like catalase and lipidomics can provide further confirmation.

In this study we show the first lipidome of extracellular parasites and parasites with differing host nutrient conditions. These data now show that extracellular parasites seem to have 2-3-fold more lipids internalized when extracellular as opposed to being within a host (Fig. 3e and Fig. 4f) and are supported by the increase in lipid droplet numbers of extracellular parasites (Fig. 3c and Fig. 4b). The consistent increase in TAG and decrease in FFA levels under the depletion of *TgACS1* in intracellular and extracellular parasites, specifically in low nutrient conditions (0-1% FBS), highlights the nutrient dependent role of this protein in the mobilization of TAG moieties into activated FFAs. FFAs can be hydrolysed from TAG molecules via phospholipases. Thereafter we suspect ACSs are responsible for the activation of these recycled FFAs for other metabolic process. If these FFA are not incorporated, they will become toxic to the parasite cell. Hence why we see an accumulation of LDs as an attempt by the parasite to escape lipotoxic death.

The substrate-specificity of this protein to long chain fatty acids such as C18:1 seen in the lipidomic analyses of mutant parasites was reinforced by the yeast complementation experiment (we supplemented with C18:1 FFA), which showed growth of the  $\Delta TgACS1$  complemented mutant only with this FFA. The decrease in

motility under low nutritious conditions additionally supports *TgACS1* being involved in the utilization/mobilization of TAGs for energy production in non-ideal conditions (Fig. 5b). Recent findings on a putative ACSBG protein like *TgACS1* in the microalgae *Microchloropsis gaditana*, shows a similar increase in TAG levels when the protein is disrupted and hypothesizes a role in TAG utilization and  $\beta$ -oxidation (37). It has been reported that one of the sources of energy for the *T. gondii* motility is gamma amino-butylic acid (GABA, (40)). In our study, there was always some glutamine included which could be used to serve for the TCA cycle directly or via GABA synthesis. In comparison to the ultimate nutrient deficient condition, i.e. PBS, the parasite probably still had very low motility DMEM medium without glucose or FBS. Here, the presence of *TgACS1* with FBS supplying to the possible beta oxidation showed the high increase of motility suggesting that the *TgACS1* dependent activation for the beta oxidation is the major supply for the energy to the parasite motility.

Further experiments in the role of *TgACS1* in peroxisomal  $\beta$ -oxidation are necessary in understanding its role in lipid metabolism. We already know from previous work that the nutritional availability (9, 13) and governs the parasites survival. This work brings insight to the parasites ability to adapt to changing host environments and in depth understanding of the diverse roles of *TgACS*s in this process to allow for parasite propagation.

## **MATERIALS AND METHODS**

### **Protein sequence analysis and Phylogeny: Identification of *Tg*ACSs**

Members of the Acyl CoA synthetase (ACS) gene family within *T. gondii* were identified using various online bioinformatic tools, Clustal omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), (<https://www.uniprot.org>), EuPathDB (<https://eupathdb.org/eupathdb>). A phylogenetic tree of ACS proteins in several eukaryotes including apicomplexa, humans and plants was created using the online platform Phylogeny.fr. The organisms used for ACS protein sequences for generation of the phylogenetic tree include: *T. gondii\_TgACS1* (TGGT1\_297220), *T. gondii\_TgACS2* (TGGT1\_310150), *T. gondii\_TgACS3* (TGGT1\_310080), *T. gondii\_TgACS4* (TGGT1\_243800),

*T. gondii\_TgACS5* (TGGT1\_247760), *T. gondii\_TgACS6* (TGGT1\_232580), *T. gondii\_TgACS7* (TGGT1\_276155), *P. falciparum\_PfACS1a* (PF3D7\_1479000), *P. falciparum\_PfACS2* (PF3D7\_0301000), *P. falciparum\_PfACS3* (PF3D7\_1253400), *P. falciparum\_PfACS4* (PF3D7\_1372400), *P. falciparum\_PfACS5* (PF3D7\_0731600), *P. falciparum\_PfACS6* (PF3D7\_0401900), *P. falciparum\_PfACS7* (PF3D7\_1200700), *P. falciparum\_PfACS8* (PF3D7\_0215300), *P. falciparum\_PfACS9* (PF3D7\_0215000), *P. falciparum\_PfACS10* (PF3D7\_0525100), *P. falciparum\_PfACS11* (PF3D7\_1238800), *P. falciparum\_PfACS12* (PF3D7\_0619500), *A. thaliana\_LACS1* (At2g47240), *A. thaliana\_LACS2* (At1g49430), *A. thaliana\_LACS3* (At1g57920), *A. thaliana\_LACS4* (At4g23850), *A. thaliana\_LACS5* (At4g11030), *A. thaliana\_LACS6* (At3g05970), *A. thaliana\_LACS7* (At5g27600), *A. thaliana\_LACS8* (At2g04350), *A. thaliana\_LACS9* (At1g77590); *Homo sapiens\_ACSS1* (NX\_Q9NUB1), *H. sapiens\_ACSS2* (NX\_Q9NR19), *H. sapiens\_ACSS3* (NX\_Q9H6R3), *H. sapiens\_ACSVL1* (NX\_O14975), *H. sapiens\_ACSVL2* (NX\_Q9Y2P4), *H. sapiens\_ACSVL3*

(NX\_Q5K4L6), *H. sapiens*\_ACSVL4 (NX\_Q6P1M0), *H. sapiens*\_ACSVL5 (NX\_Q6PCB7), *H. sapiens*\_ACSVL6 (NX\_Q9Y2P5), *H. sapiens*\_ACSL1 (NX\_P33121), *H. sapiens*\_ACSL3 (NX\_O95573), *H. sapiens*\_ACSL4 (NX\_O60488), *H. sapiens*\_ACSL5 (NX\_Q9ULC5), *H. sapiens*\_ACSL6 (NX\_Q9UKU0), *H. sapiens*\_ACSS3 (NX\_Q9H6R3), *H. sapiens*\_ACSM3 (NX\_Q53FZ2), *H. sapiens*\_ACSM2B (NX\_Q68CK6), *H. sapiens*\_ACSM1 (NX\_Q08AH1), *H. sapiens*\_ACSM4 (NX\_P0C7M7), *H. sapiens*\_ACSM5 (NX\_Q6NUN0), *H. sapiens*\_ACSM6 (NX\_Q6P461), *H. sapiens*\_ACSBG1 (NX\_Q96GR2), *H. sapiens*\_ACSBG2 (NX\_Q5FVE4); *Chromera velia* (Cvel\_26948), *Chromera velia* (Cvel\_29920), *C. velia* (Cve1\_20092), *C. velia* (Cvel\_8845)), *C. velia* (Cvel\_24841), *C. velia* (Cvel\_29002), *C. velia* (Cvel\_9659), *C. velia* (Cvel\_3410), *C. velia* (Cvel\_2306), *C. velia* (Cvel\_26948) ; *Cryptosporidium parvum* (cdg5\_3200), *C. parvum* (cdg3\_2870), *C. parvum* (cdg3\_2870) *C. parvum* (cdg3\_640) ; *Hammondia hammondi* (HHA\_310150), *H. hammondi* (HHA\_310080), *H. hammondi* (HHA\_276155), *H. hammondi* (HHA\_232580), *H. hammondi* (HHA\_297220), *H. hammondi* (HHA\_243800), *H. hammondi* (HHA\_247760); *Neospora caninum* (NCLIV\_063970), *N. caninum* (NCLIV\_006300), *N. caninum* (NCLIV\_006990), *N. caninum* (NCLIV\_054250), *N. caninum* (NCLIV\_018500). First step involved the curation of these protein sequences. The protein sequences were aligned (Clustalomega) and then gaps were removed from the alignment. Finally, the phylogenetic tree was constructed using the maximum likelihood method in the PhyML program. The default substitution model (WAG) was selected. Graphical representation and edition of the phylogenetic tree were performed with cladogram.

### ***T. gondii* strains and cultures**

The parasite host cells human foreskin fibroblasts (HFF) were cultured using Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco) and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>. *T. gondii* tachyzoite parental strains RH-ΔKu80 Tir as well as the mutant strain were cultured by serial passage within their host HFF using DMEM supplemented with 1% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco) and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>.

### **Generation of inducible knockdown line for *TgACS1***

A C-terminally tagged HA line with inducible post-translational protein degradation was generated using the Auxin-inducible Degron system (AID). A 2685 bp homology region of the C-terminal end of *TgACS1* excluding the stop codon was amplified using the primers in Table 1. A guide sequence (Table 1) was selected near the C-terminal end and was less than 100 bp away from at least one homology region. The guide sequence was inserted into Cas9 U6 universal plasmid Q5 mutagenesis.

The PCR product and the Cas-9 guide plasmid were used for transfection of a RH TIR1-3FLAG parental line (34). After 24 hours the newly transfected line was treated with 25 µg/ml mycophenolic acid and 50 µg/ml xanthine. Parasites were selected with the drug and cloned by limiting dilution. Screening of parasite clones for correct insertion of the mAID sequence, HX drug cassette and overall insertion/orientation were done with the primers in Table 1. All PCRs were performed with TaKara primestart Max polymerase. The knockdown of *TgACS-1* was induced with a final concentration of 100 µM of indole-3-acetic acid (auxin) made up in 100% ethanol.

Table 1:

TgACS Gene ID	Primers (gene specific sequence underlined)	Molecular system used
TgACS1 (TGGT1_297220)	Fw: GCAGCATGAGAAAACCGTTGCTGCGCTGCAG GCAAAGCTGGCTAGCAAGGGCTCGGGC  Rw: CCTTGACCGCCGCAGAGATGTTCTTCTCCCTT TTGCCAGGATAGGGCGAATGGAGCTCC  Guide sequence: CGAATTCAGAAGCTGCCGA  SS55: CGCTCTCCCCTGCCCTTCTTG  SS48: ATACATTCGAAATCAATCTTTCGCAGGTACG GC  SS49: TTGAAGTCGTAGCAGCAACCAACGA  SS56: CGCACACAAGGAAATGTGATGAAGG	mAID
TgACS1 (TGGT1_297220)	Fw: TACTTCCAATCCAATTTAATGCAGCCTGGGGA <u>TGCCGATCAACT</u>  Rw: TCCTCCACTTCCAATTTTAGCCAGCTTTGCCCT <u>GCAGCGC</u>	pLIC HA tagging
TgACS1 (TGGT1_297220)	Fw: TCCCTTTCGTCA TGAAACTCA  Rw: GCCGTAAATGGAGTTGATCG	mRNA relative expression

All PCRs were performed with TaKara Primestar Max polymerase.

### Immunofluorescence assay

Primary antibodies anti-HA (Rat, Roche), anti-IMC1, anti-SAG1 were used at dilutions 1:500, 1:1000 and 1:1000 respectively. Secondary Alexa Fluor 488- and 546-

conjugated anti-mouse, anti- rat and anti-rabbit antibodies (Life Technologies) were used at 1/2500. For the immunofluorescence assay (IFA) parasites were grown on confluent HFF on coverslips and fixed in PBS containing 2.5% paraformaldehyde (*PFA*) for 15 min at room temperature (RT). Samples were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT prior to blocking in PBS containing 3% BSA and subsequent incubation with primary antibodies then secondary antibodies diluted in the blocking solution. Labelled parasites were stained with Hoechst (1/10000, Life technologies) for 20 min and then washed three times in PBS before final mounting of the coverslips on a glass slide using fluorogel. The fluorescence was visualized using fluorescence microscope (Axio Imager 2\_apotome; ZEISS).

### **Nile red staining of lipid droplets**

Parasites were allowed to infect and grow in confluent monolayer HFF grown on coverslips, in the +/- ATc or Auxin conditions for 24 h in 0%/1%/10% FBS ED1 medium. As with IFA, these coverslips were fixed using 2.5% *PFA*, permeabilized with 0.25% triton X-100. Thereafter, coverslips were incubated for at least 1 h with Nile red (or LipidTOX™ HCS, 1 : 200) in 1X PBS before proceeding to DNA staining with Hoechst. The coverslips were mounted onto a glass slide in fluorogel prior to imaging using fluorescence microscope (Axio Imager 2\_apotome; ZEISS). For visualizing Nile red stained droplets yellow-gold fluorescence (excitation, 450-500 nm; emission, greater than 528 nm) was used on the Axio Imager. Quantification of lipid droplet number and volume was performed based on iterative image processing as described by Exner *et al.*, 2019 (41).

### **Western blot analysis**

Parasites were harvested for western blot after complete egress from their host. In order to remove any host cell debris, the parasites were passed through a 3 µm filter, then

counted by hemocytometer and solubilized in SDS buffer at equivalent cell densities. Equal amount of protein was separated on a 4-12% gradient SDS-polyacrylamide (Life Technologies) and transferred to Nitrocellulose membrane (check this) using the XCellIII Blot Module (Invitrogen). Primary antibodies anti-HA (Rat, Roche) and anti-TOM40 (Rabbit) (Van Dooren *et al.* 2008) were used at a dilution of 1:500 and 1:1000, respectively. Secondary goat anti-mouse and anti-rabbit horse radish peroxidase (HRP) conjugated antibodies (Thermo Scientific) were used at 1:2000. Protein signal was detected by chemiluminescence after membrane staining with Luminata Crescendo Western HRP detection kit (Millipore). The signal strength of protein was quantified using a BioRad Chemidoc imager (BioRad).

### **Phenotypic analysis**

*Plaque assay*- The extracellular parasites were harvested after filtration and counted by hemocytometer. Then approx. 500 parasites were inoculated to confluent HFF flask (25 cm<sup>2</sup>). The mutant parasites *TgACS1-iKD* and *TgAC2-iKD* were grown for plaque assay in the presence or absence of ATc or auxin (0.5 µg ml<sup>-1</sup> or 100 µM) for 7-10 days. Plaque sizes were visualized by crystal violet staining (30-60 min) after aspiration of culture media, and cells fixation with 100% ethanol (5 min) followed by phosphate-buffered saline (PBS) wash.

*Replication assay*- The parasites were grown for two days with or without ATc or auxin, harvested and filtered. Equal number of parasites were allowed to invade confluent HFF grown on coverslips. Following 2 h of invasion, the coverslips were washed thrice with ED1 (1% FBS containing DMEM), in order to remove any uninvaded parasites and promote synchronized replication. ATc or auxin (same concentration as above) was added at the outset of the experiment, allowing the treatment for 24 h, alongside control

parasites without auxin. These coverslips were then fixed and processed for IFA wherein the parasite number per parasitophorous vacuole was analysed.

*Motility assay-* Slides were prepared before start of the experiment whereby, 1.5 mm glass slides were flamed and smeared with 0.1% polyethyleamine. Slide were placed in 6 well plates and incubated with a layer of FBS for 2-3 hours at 37°C. Freshly egressed *TgACS-1* iKD parasites were passes through a 3 µM filter and spun down for 10 mins at 1800 rpm. Cells were washed with PBS three times and resuspended in 1 ml of ED1 media (100 mM HEPES) or PBS (100 mM HEPES), in conditions with or without 100 mM auxin. Before placing the cell suspension, slides were washed three times with PBS to remove excess FBS on the glass slides. Cells where incubated added to the wells for 10 mins at room temperature to allow cells to settle and for another 1 hour at 37°C. Samples were fixed with 5% paraformaldehyde/PBS for 15 min and processed for an IFA using anti-SAG1 antibody. The experiment was performed three independent times.

*Relative mRNA expression quantification-* RH TIR1-3FLAG parasites were grown on confluent monolayer of HFF in flasks (175 cm<sup>2</sup>) and allowed to grow for 48 hours (mainly intracellular) or until completely egressed (extracellular parasite) in 0, 1, or 10% FBS normal DMEM conditions. Parasites were then harvested by scaping cells of the bottom of the flask and moved to 50 ml tubes; intracellular tachyzoites were pushed through a 0.2 mm needle and syringe filtration with 3-µm pore size membrane to remove host cell debris. A NucleoSpin RNA kit (Macherey-Nagel) was used for mRNA extraction and whereafter cDNA was synthesized using a SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit. Primers specific (Table 1) to each gene of interest, including a house keeping gene GAPDH1 (*TGGT1\_289690*) which was used to calculate the relative mRNA expression in the different conditions used in this study.

## Lipidomic analysis

The parasites were grown for 24 h or until completely extracellular in conditions of +/- auxin on the confluent monolayer of HFF in flasks (175 cm<sup>2</sup>). For extracellular parasite lipid analyses completely, egressed parasites were collected, and host cells were filtered out with a with 3-µm pore size membrane, after which parasites were incubated for 6 hours in the respective media conditions 0/1/10% FBS). At each time point, parasites were also harvested as the intracellular tachyzoites ( $1 \times 10^7$  cell equivalents per replicate) after syringe filtration with 3-µm pore size membrane. These parasites were metabolically quenched by rapid chilling in a dry ice-ethanol slurry bath and then centrifuged down at 4°C. The parasite pellet thus obtained was washed with ice-cold PBS thrice, before transferring the final pellet to a microcentrifuge tube. Then total lipids were extracted in chloroform/methanol/water (1:3:1, v/v/v) containing PC (C13:0/C13:0), 10 nmol and C21:0 (10 nmol) as internal standards for extraction) for 1 h at 4°C, with periodic sonication. Then polar and apolar metabolites were separated by phase partitioning by adding chloroform and water to give the ratio of Chloroform/Methanol/Water, 2:1:0.8 (v/v/v). For lipid analysis, the organic phase was dried under N<sub>2</sub> gas and dissolved in 1-butanol to obtain 1 µl butanol/10<sup>7</sup> parasites.

*Total lipid analysis* – Total lipid was then added with 1 nmol pentadecanoic acid (C15:0) as internal standard and derivatized to give fatty acid methyl ester (FAME) using trimethylsulfonium hydroxide (TMSH, Machenery Nagel) for total glycerolipid content. Resultant FAMES were then analysed by GC-MS as previously described (Dubois *et al.* 2018). All FAME were identified by comparison of retention time and mass spectra from GC-MS with authentic chemical standards. The concentration of FAMES was quantified after initial normalization to different internal standards and finally to parasite number.

*Phospholipid and neutral lipid analysis*- For phospholipid analysis, total lipid extracted (as mentioned above) was separated with 1 nmol PA(C17:0/C17:0) (Avanti Polar lipids) by one-dimensional silica gel high-performance thin layer chromatography (HPTLC, Merck). The solvent system used for the 1<sup>st</sup> and 2<sup>nd</sup> dimension was chloroform/methanol/28% ammonium hydroxide, 12:7:1.6 (v/v) and chloroform/acetone/methanol/acetic acid/water, 10:4:2:2.6:1 (v/v/v/v/v), respectively. Total PL, DAG, TAG, Free fatty acids (FFA) and cholesteryl ester (CE) analysis, total lipid fraction was separated by 1D-HPTLC using hexane/diethyl ether/formic acid, 80:20:2 (v/v/v) as solvent system. Thereafter, each lipid spot on the HPTLC plate was scrapped off and lipids were methanolized with 200 µl 0.5 M methanolic HCl in the presence of 1 nmol pentadecanoic acid (C15:0) as internal standard at 85°C for 3 h. The resulting FAMES were extracted with hexane and finally analysed by GC-MS (Agilent).

### **Yeast complementation assay and growth curves**

A pRS426-MET25 vector was used as the backbone for a plasmid containing the entire cDNA sequence of *TgACS1*. The plasmid was generated by GeneCust by cloning the sequence of interest using *EcoRI* and *KpnI* restriction sites. All strains were transfected either with this plasmid of the pRS426-MET25 vector lacking *TgACS1* as a control.

The isogenic *S. cerevisiae* strains WT (YB332: MATa ura3 leu2 his3A200 ade2 lys2-801) and *faa1Δfaa4Δ* (YB525: MATa ura3 leu2 his3D200 ade2 lys2-801 *faa1Δ::HIS3*; *faa4Δ::LYS2*) used in this study were kindly provided by Dr. Juliette Jouhet (CEA, Grenoble, France) and have been described earlier (52, 53). After transfection, all the strains described in the present work were grown on synthetic media containing 6.7 g/l of Yeast Nitrogen Base (YNB, MP biomedical), 0.77 g/l of CSM-URA (MP Biomedicals) and g/l of glucose, as described. In complementation experiments, the

media were supplemented with 22.5 mM cerulenin (Sigma–Aldrich), 100 mM FFA, and 0.2% (v/v) final concentration TWEEN 20 (Sigma–Aldrich). The different FFAs were dissolved in 100% TWEEN 20 at a concentration of 50 mM. Cerulenin was dissolved in acetone at 45 mM. As controls, media without FFAs and/or cerulenin were also supplemented with 0.2% (v/v) TWEEN 20 and/or acetone.

Three clones per transformation were used for complementation tests. For each experiment, one colony was inoculated in 6 mL of CSM-URA + glucose (2%) and grown overnight at 30°C at 180 rpm. Growth assays were performed in 96-well microplates (Thermo Scientific Nunc MicroWell 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate) filled with 200  $\mu$ L of culture in glucose medium supplemented or not by FA or cerulenin and set at an OD<sub>600</sub> = 0.1. The microplate was incubated at 30°C in an Infinite M2000 PRO microplate reader (Tecan) set for plate orbital shake for 10 s and absorbance measurements (OD<sub>600</sub>) every 20 min, with four measurements per well. OD<sub>600</sub> readings from experimental wells minus the original OD<sub>600</sub> values resulted in the  $\Delta$ OD<sub>600</sub> values shown in Sup. Fig. 5.

Since no complementation was seen in the aforementioned strains, we began to investigate another acyl-CoA synthetase activities. We transfected the pRS426-MET25-*TgACS1* plasmid into a yeast strain lacking a peroxisomal-CoA synthetase,  $\Delta$ PCS60 (38, 39) These experiments were also performed in TWEEN 20, with and without 6  $\mu$ g/ml doxorubicin; a cancer-drug inducing lipid peroxidation and peroxisome proliferation in yeast cells (23, 54). OD<sub>600</sub> were calculated as described above.

### **Statistical analysis for all experiments**

Entire graphical data for this study was generated using GraphPad prism software. Three biological replicates were used per experiment (n=3). The error bars are

representative of standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by t-test using GraphPad Prism (version 8). Range of statistical significance was signified as per the *p* value, wherein 0.01-0.05=\*, 0.01-0.001=\*\* and <0.001=\*\*\*.

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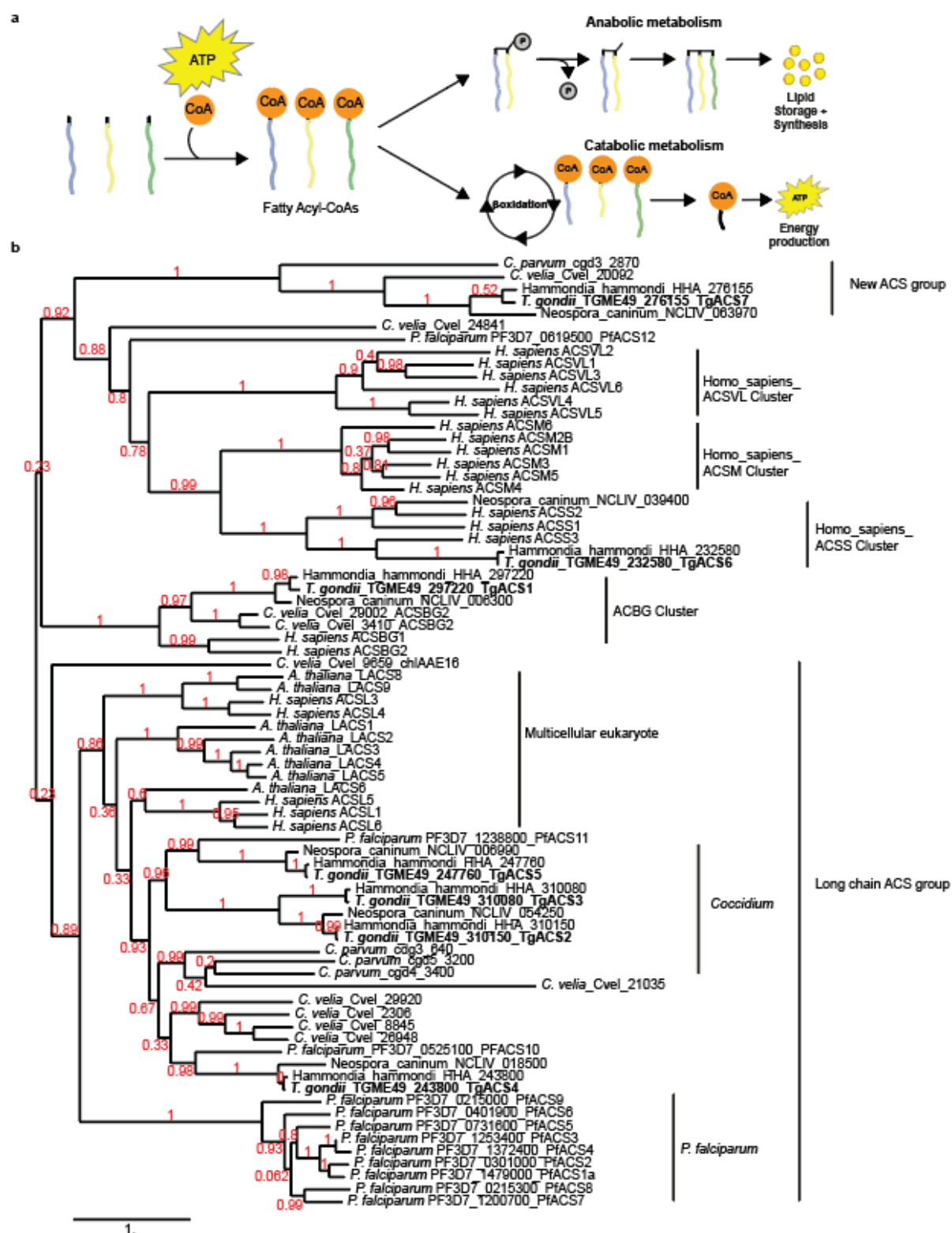
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**TABLE 1**

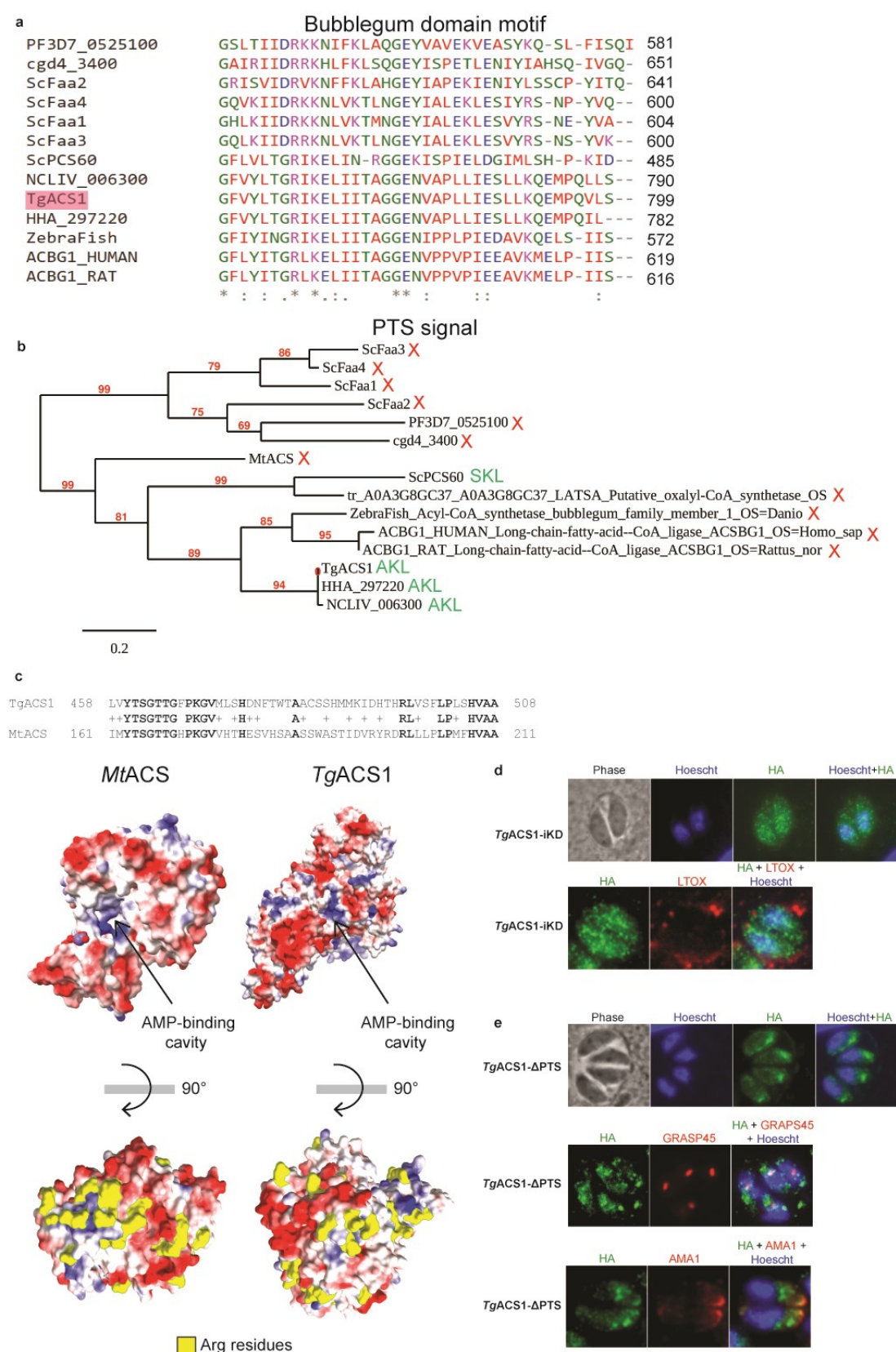
<i>Tg</i> ACS Gene ID	<b>Primers</b> (gene specific sequence underlined)	Molecular system used
<i>Tg</i> ACS1 (TGGT1_297220)	Fw: GCAGCATGAGAAAACCGTTGCTGCGCTGCAG GCAAAGCTGGCTAGCAAGGGCTCGGGC  Rw: CCTTGACCGCCGCAGAGATGTTCTTCTCCCTT TTGCCAGGATAGGGCGAATGGAGCTCC  Guide sequence: CGAATTCAGAAGCTGCCGA  SS55: CGCTCTCCCCTGCCCTTCTTG  SS48: ATACATTCGCAAATCAATCTTTCGCAGGTACG GC  SS49: TTGAAGTCGTAGCAGCAACCAACGA  SS56: CGCACAACAAGGAAATGTGATGAAGG	mAID
<i>Tg</i> ACS1 (TGGT1_297220)	Fw: TCCCTTTCGTCATGAAACTCA  Rw: GCCGTAAATGGAGTTGATCG	mRNA relative expression

## FIGURES



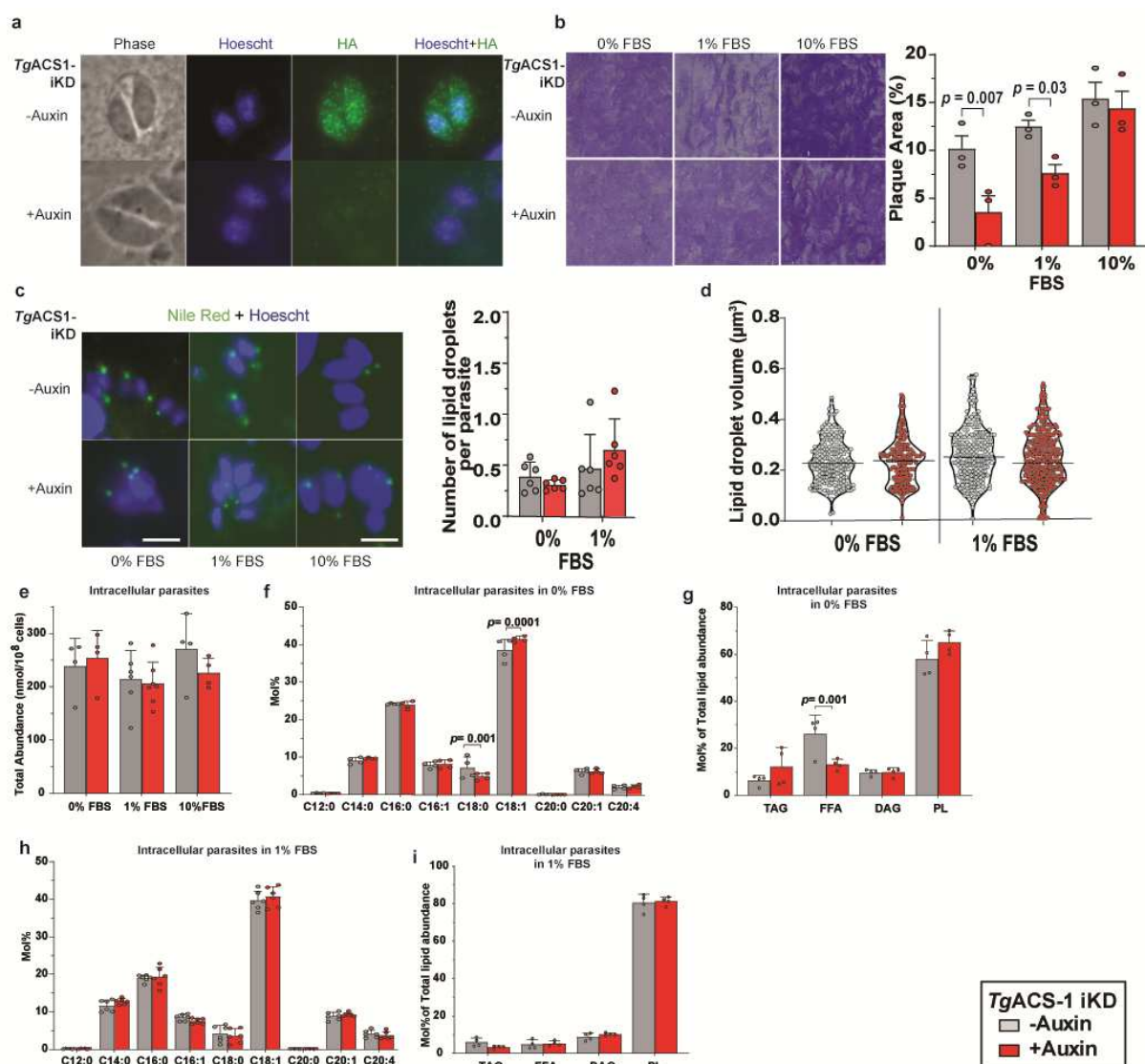
**Figure 1: Identification of seven acyl CoA synthetase in *T. gondii* and their cellular localizations** a) Anabolic and catabolic processes resulting from the

thioesterification of a fatty acid by an acyl-CoA synthetase (ACS) using ATP. **b)** Phylogenetic analyses of all seven ACS found in the genome of *Toxoplasma gondii* parasites. All 7 *Tg*ACSs appear in specified clusters based on their homology with their eukaryotic counterparts: *Tg*ACS1 in ACBG cluster; *Tg*ACS2, *Tg*ACS3, *Tg*ACS4, *Tg*ACS5 in the long chain ACS group; *Tg*ACS6 in the short chain ACS cluster; *Tg*ACS7 in a separate cluster specific to coccidia and chromerids. This phylogenetic tree was generated online using Phylogeny.fr.



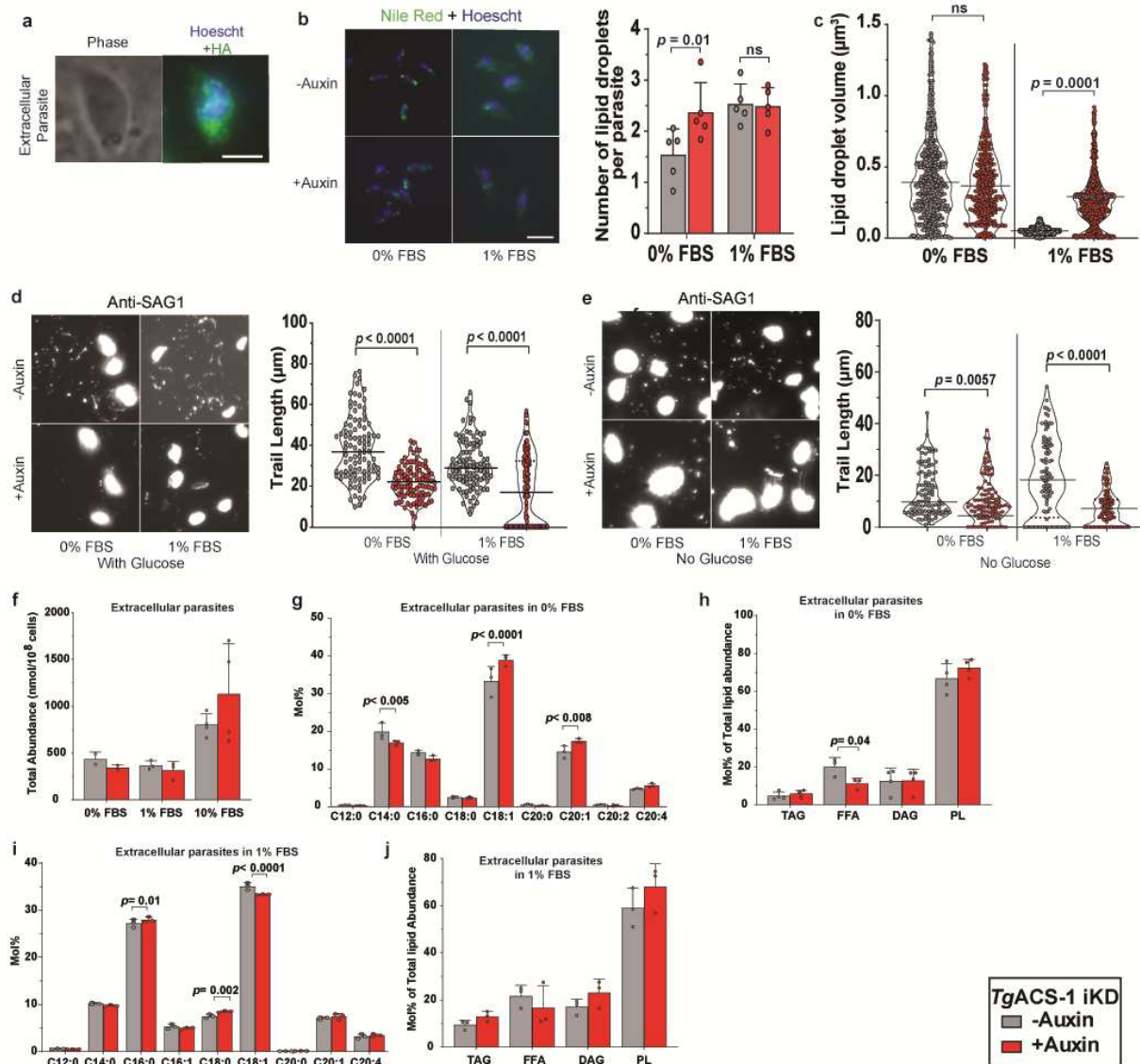
**Figure 2: *TgACS1* is a protein containing a bubble-gum domain and PTS1 signal sequence which is important for correct localization of this protein. a)**

Phylogenetic analyses and Blastp analyses revealed a bubble-gum domain present in this protein with high sequence similarity to other acyl-CoA synthetases from other organisms. b) A cladogram of homolog ACS proteins from other proteins shows to contain a peroxisomal targeting sequence (PTS1) tripeptide sequence (in green) is present at the C-terminal end of *TgACS1* and *ScPCS60*, a known peroxisomal ACS (X: does not contain a A/SKL PTS1). c) 3D comparison of model *TgACS1* (Phyre2) and 3D crystal structure of *MtACS* (sequence identity of 51%), shows a hydrophobic area (hydrophobic) of a putative AMP-binding pocket in *TgACS1* and similar arrangement of Arg residues (yellow). d) Immunofluorescence microscopy revealed in intracellular *TgACS1*-iKD parasites that the protein localizes to the cytosol in a punctuated/vesicular formation. LTox staining (lipid droplet) showed close association but no overlapping of *TgACS1* to the parasite lipid droplets (bottom row). e) Deletion of the PTS1 (AKL), resulted in random apical pole mis-localization of *TgACS1*, which did not show overlapping to the Golgi-apparatus (anti-GRASP45: middle row) or microneme (anti-AMA1: lower row)



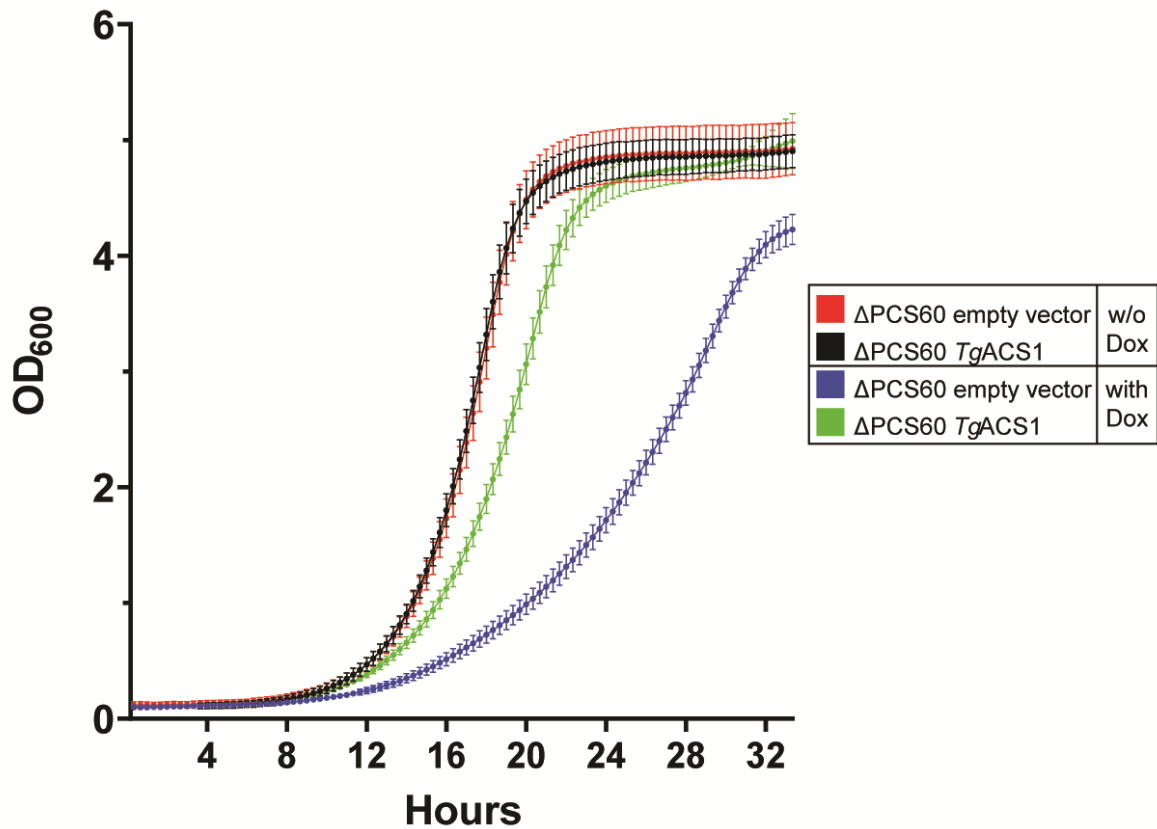
**Figure 3: *TgACS1* is important for intracellular propagation in poor lipid environments.** a) Immunofluorescence microscopy revealed downregulation of *TgACS1*-iKD (100 kDa) in the presence of auxin (100  $\mu\text{M}$ ) after 24 h, and that the protein localizes to the cytosol of intracellular parasites. b) Plaque assay with an accompanying (right) bar graph showing inhibition of parasite growth when *TgACS1* is degraded (red) at 0% and 1 % FBS. c) Immunofluorescence microscopy using Nile Red indicates that the number of lipid droplets (LD-green) per parasite were not affected under auxin treatment. A gradual increase in lipid droplet number is seen from 0-10% FBS. d) Lipid droplet volume analyses revealed no changes in intracellular parasites

when *TgACS1* was depleted. e) Total lipid abundance (nmol/cell number) of intracellular in low, normal and high FBS (0/1/10%) conditions with (grey) and without (red) *TgACS1* protein. f) Fatty acid (FA) profile (mol%) of intracellular parasite in 0% FBS with (grey) and without (red) *TgACS1*. g) Neutral lipid mol% of total lipid abundance in intracellular parasites in 0% FBS without (grey) and with (grey) auxin treatment, namely triacylglycerol (TAG), free fatty acid (FFA), diacylglycerol (DAG) and phospholipid (PL). Error bars indicate standard deviation (SD), Experiments were conducted in triplicates, unpaired t-test *p* are indicated only when statistically significant.

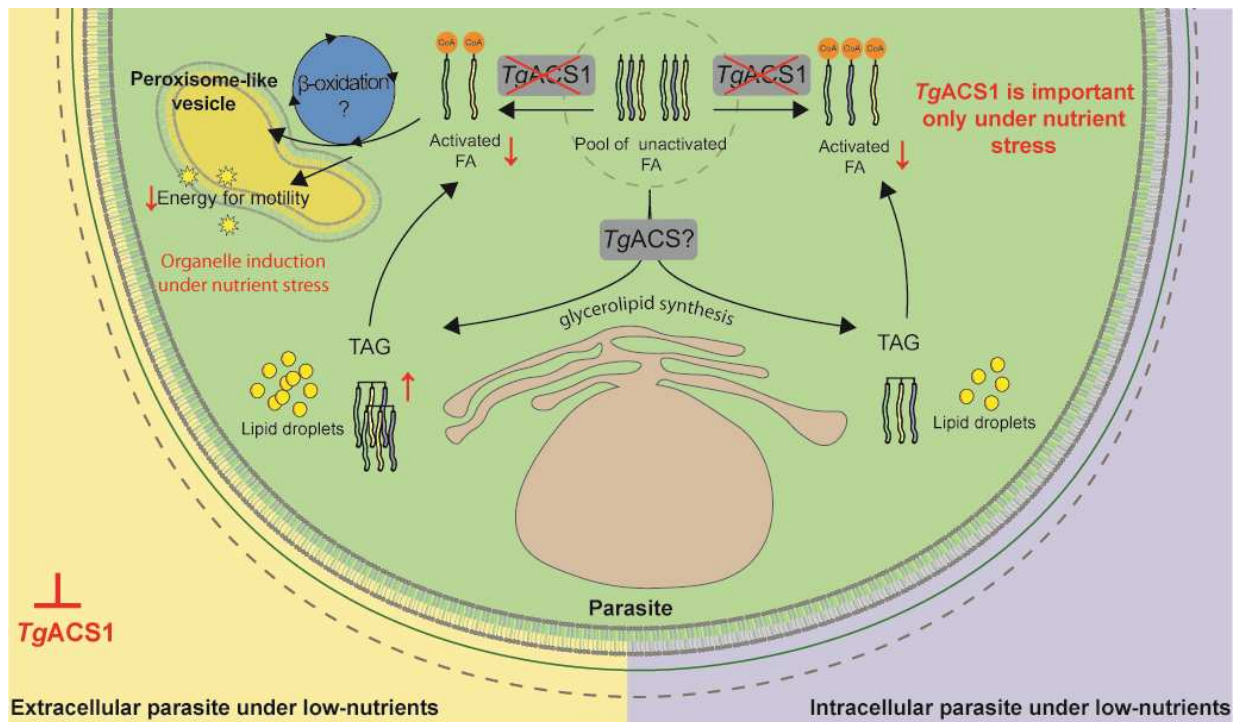


**Figure 4: *TgACS1*-iKD is important for extracellular parasite motility and lipid droplet mobilization for energy production.** a) Immunofluorescence microscopy also revealed *TgACS1* expression and localization in the cytosol of extracellular parasite (1% FBS). b) Nile red staining and immunofluorescence microscopy also revealed that (right) lipid droplet number per extracellular parasite is increased at 0% FBS and not 1% FBS when the protein is degraded (auxin treatment, red). c) Further Nile Red analyses with (grey) and without (red) the protein shows that LD volume is increased at 1% FBS conditions for extracellular parasites. d) Motility assay using anti-SAG-1 revealed that (right) that at 0-1% FBS with glucose and e) without glucose

conditions the length of slime trails per extracellular parasite is significantly diminished under suppression of *TgACS1*. f) Total lipid abundance of extracellular parasite in 0/1/10% FBS. g) Fatty acid (FA) profile (mol%) of extracellular parasite in 0% FBS with (grey) and without (red) *TgACS1*. h) Neutral lipid mol% of total lipid abundance in extracellular parasites in 0% FBS without (grey) and with (grey) auxin treatment, namely triacylglycerol (TAG), free fatty acid (FFA), diacylglycerol (DAG) and phospholipid (PL). i) Fatty acid (FA) profile (mol%) of extracellular parasite in 1% FBS with (grey) and without (red) *TgACS1*. j) Relative abundance ratios calculated for TAG, FFA, DAG and PL levels without/with the protein in 1% FBS in extracellular parasite. Error bars indicate standard deviation (SD), Experiments were conducted in triplicates, unpaired t-test p values are only indicated when statistically significant.

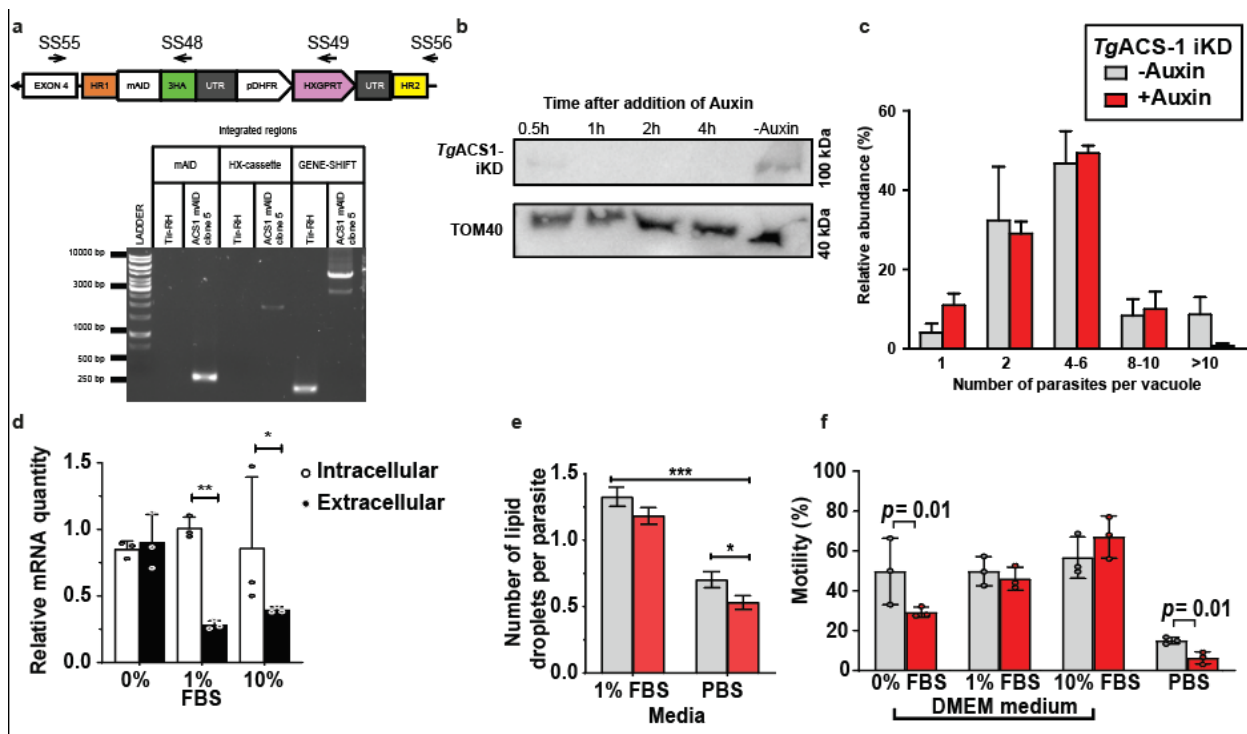


**Figure 5: Growth complementation of a yeast mutant deficient of peroxisomal acyl-CoA activity by *TgACS1*.** In the absence of doxorubicin, the  $\Delta$ PCS60 strain grows (30°C for 32 h) as expected with the empty vector (red) and pRS-*TgACS1*-His (black). When treated with doxorubicin, the strain with the empty vector (blue) grows put at a slower rate. Growth is almost completely restored in the doxorubicin-treated  $\Delta$ PCS60 strain complemented with pRS-*TgACS1*-His (green), suggesting that *TgACS1* can rescue the effects of doxorubicin.

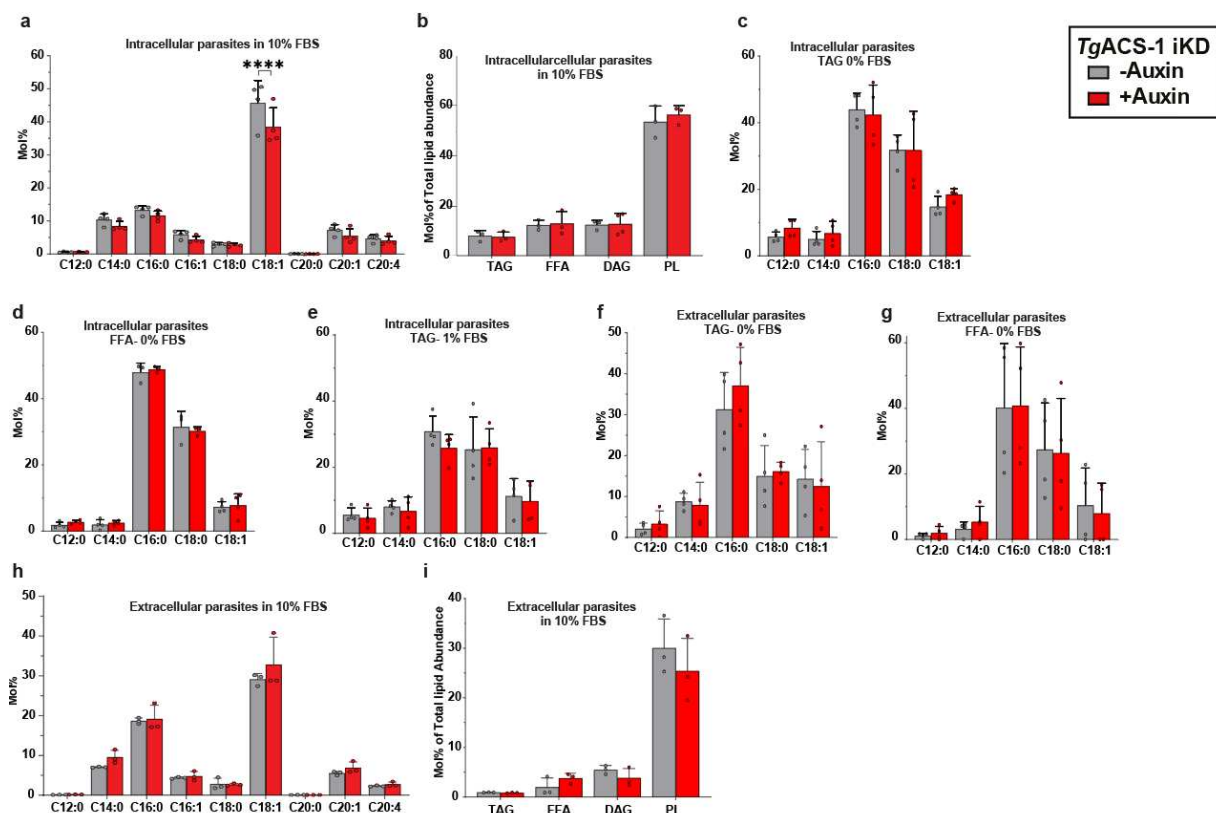


**Figure 6: Model explaining the proposed role of *TgACS1* in parasite host-adaptation and lipid metabolism.** Fatty acids (FA) from the host, extracellular environment and directly from the parasite are incorporated into the glycerolipid pathway of the parasite. Under the depletion of *TgACS1* in extracellular parasites there is an increase in TAG levels and decrease in FFA levels in a low nutrient environment. Concomitantly there is an evident increase in lipid droplet formation in a low nutrient environment. In this study *TgACS1* has been showed to have acyl-CoA synthase activity and we speculate specifically in the activation of long chain FFA destined for their use in  $\beta$ -oxidation. We propose that these acyl chains are derived by the mobilization of TAG moieties by *TgACS1* and is involved in energy processes in sub-optimal conditions like  $\beta$ -oxidation most probably in putative peroxisomal bodies.

## SUPPLEMENTARY FIGURES:

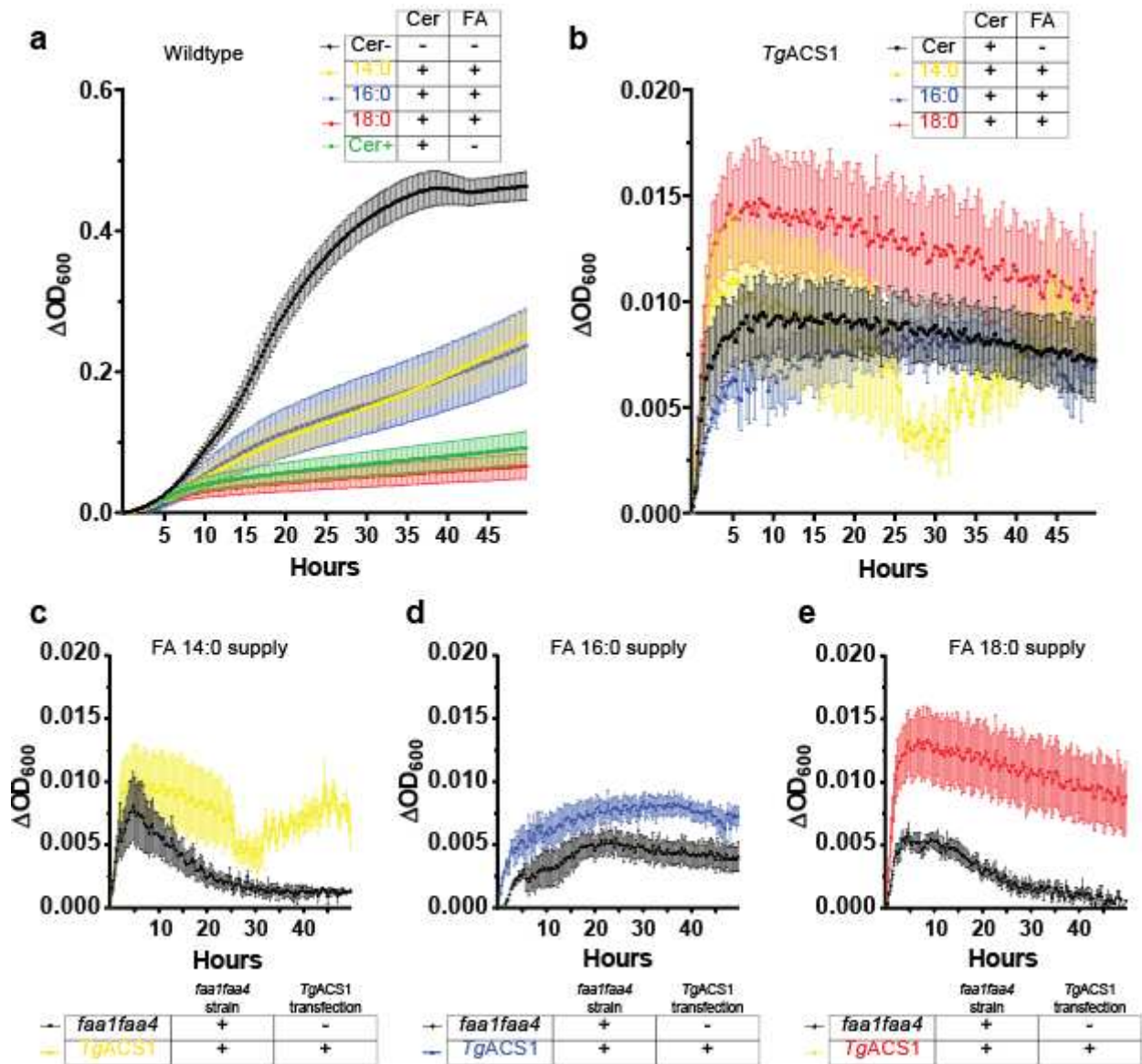


**Supplementary Figure 1:** a) PCR confirmation of *TgACS1*-iKD clone 5 using the mAID system, showing correct integration of the mAID sequence, HX-drug cassette, and the gene-shift compared to the parental line. b) Western blot analyses showing down regulation of *TgACS1* (100 kDa) after 30 mins of auxin treatment (100 μM), TOM40 (40 kDa) antibody used as loading controls. c) A replication assay of this line without (grey) and with (red) auxin revealed no effect on intracellular parasite replication. d) Relative mRNA expression of *TgACS1* (compared to *TgGAPDH*) in intracellular (white) and extracellular (black) parasites in different FBS conditions. e) The number of lipid droplets per intracellular parasite were not affected at 1% FBS but significantly reduced under complete nutrient loss (PBS), with (grey) and further decreased without (red) the protein of interest. f) A gliding motility assay showed that under only under complete nutrient loss (PBS, 2 h) and low nutrient conditions like 0% FBS, is the motility rate reduced without *TgACS1* (red), in extracellular proteins.



**Supplementary Figure 2:** a) Lipidomic profiles showing the mol% of each fatty acid comprising the total lipid fraction of intracellular 10% FBS without (grey) and with (red) auxin treatment in the *TgACS1-iKD* mutant line. b) Neutral lipid mol% of total lipid abundance in intracellular parasites in 10% FBS without and with auxin treatment, namely triacylglycerol (TAG), free fatty acid (FFA), diacylglycerol (DAG) and phospholipid (PL). Mol% of c) TAG and d) FFA levels in intracellular parasites at 0% FBS and e) TAG levels in intracellular parasites at 1% FBS. Mol% of f) TAG and g) FFA levels in extracellular parasites at 0% FBS. h) Lipidomic profiles showing the mol% of each fatty acid comprising the total lipid fraction of extracellular 10% FBS without (grey) and with (red) auxin treatment in the *TgACS1-iKD* mutant line. Neutral lipid mol% of total lipid abundance in extracellular parasites in 10% FBS without and with auxin treatment





**Supplementary Figure 3: *TgACS1* does not rescue the growth of a yeast strain deficient in acyl-CoA activity of exogenous fatty acids.** a) Wildtype yeast strain growth curves in a glucose (2%) and tween20 (0.2%, to induce  $\beta$ -oxidation) medium. (Cer-, black, positive control to allow for FA synthesis), with the addition of 22.5  $\mu$ M cerulenin only (Cer+, green, negative control), or additionally in the presence of 100  $\mu$ M of FFAs C14:0 (yellow), C16:0 (blue) or C18:0 (red), respectively. b) Growth curves of a *TgACS1* complemented *faa1* $\Delta*faa4* $\Delta$  double yeast mutant in a glucose (2%) and Tween20 (0.2%) media supplemented with 22.5 $\mu$ M cerulenin and 100 $\mu$ M of FA as before. Medium with 22.5 $\mu$ M cerulenin but FFA was used as a negative control (black).$

c-e) Growth curves of the complemented strain under the supply of different FFAs; c) C14:0 (yellow), d) C16:0 (blue) or e) C18:0 (red), with their negative controls of *faa1* $\Delta$ *faa4* $\Delta$  double mutant without complementation in the presence of the respective FFAs. Error bars indicate standard deviation (SD), experiments were conducted in independent triplicates.

### ***Summary of manuscript:***

#### ***Characterization of *Toxoplasma gondii* acyl-CoA synthetases reveal the critical role of *TgACS3* in adapting to the host nutrient environment***

FA biosynthesis is crucial to lipid metabolism which is required for growth and division of apicomplexa parasites. This work is closely related to the work of *TgACS1* found earlier in this chapter and will be submitted as ‘back-to-back’ articles. We looked to further characterize *TgACS3*, an essential protein for parasite growth that seemed to be most important at 10% FBS, or high host nutrient content.

The major findings of this study were:

- *TgACS3* is critical for tachyzoite intracellular development, especially when host nutrients are in excess
- *TgACS3* is involved in TAG formation in 10% FBS, which we hypothesize is through the activation of FFA destined to LD biogenesis
- Disruption of *TgACS3* leads to an increase in host derived FFA levels in a high host nutrient environment and this decrease TAG and PL levels
- This protein has a preference to exogenous FAs destined to TAG/PL formation

**This work was initially started by a previous student, Dr Sheena Dass, and I finished most of the functional experimentation and writing of the article during my own PhD thesis. My contribution to this paper includes:**

- Lipid droplet analyses and quantification
- Fluxomic (Host-labelled experiments) sample preparation, analyses, and interpretation
- Writing and final illustrations of the paper

## **Résumé du manuscrit :**

### ***La caractérisation d'une acyl-CoA synthétases chez Toxoplasma gondii révèle le rôle critique de TgACS3 dans l'adaptation du parasite aux condition nutritive de son hôte.***

La synthèse d'acides gras libre est essentielle au métabolisme lipidique requis pour la croissance et propagation des parasites apicomplexes. Ce travail est intimement lié aux études retrouvées dans ce même chapitre sur *TgACS1* et sera soumis successivement au premier article. Nous avons caractérisé *TgACS3*, une protéine essentielle à la croissance des parasites, qui semble jouer un rôle majeur dans des conditions nutritives favorables (10% FBS).

Les résultats principaux sont les suivants :

- *TgACS3* est primordiale pour le développement du tachyzoite intracellulaire mais particulièrement en conditions d'excès nutritif (10% FBS).
- *TgACS3* est impliquée dans la formation de triglycérols en conditions d'excès nutritif. Nous supposons que cette observation découle de l'activation des acides gras libres destinés à la biogenèse de gouttelettes lipidiques.
- La disruption de *TgACS3* entraîne une augmentation des niveaux d'acides gras libres issus de l'hôte, qui s'accompagne également d'une diminution des niveaux de triglycérols et de phospholipides en conditions d'excès nutritif
- Cette protéine montre une préférence pour les acides gras exogènes destinés à la formation triglycérols et phospholipides

J'ai continué le travail initié par Dr Sheena Dass en complétant la majorité des expérimentations fonctionnelles et en rédigeant l'article au cours de ma thèse. Ma contribution inclut :

- Analyse et quantification des gouttelettes lipidiques
- Préparation de marquage isotopiques, réalisation des analyses et interprétation des résultats d'études fluxomiques.
- Ecriture de l'article et création des illustrations.

***TITLE: Characterization of Toxoplasma gondii acyl-CoA synthetases reveal the critical role of TgACS3 in adapting to the host nutrient environment***

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***ABSTRACT***

Apicomplexa comprise several pathogenic protists that heavily depend on lipid metabolism for the survival within their hosts. The lipid synthesis within these parasites relies on an essential combination of fatty acids (FAs) obtained from *de novo* synthesis and scavenging from the host. The metabolic utilization of these fatty acids is mediated by acyl-activating enzymes. These include acyl CoA synthases (ACS) that catalyze the activation of fatty acids, primarily through the formation of a thioester bond. The role of these enzymes in FA utilization in *T. gondii* has been unforeseen. Previously we identified 7 putative ACSs encoded by the genome of *T. gondii* (*TgACS*). Interestingly, the *TgACS*s localize to different sub-cellular compartments of the parasite, suggesting their exclusive functions. Now we show that the perinuclear/cytoplasmic *TgACS3*

regulates replication and growth of *Toxoplasma* tachyzoites. Lipidomic analysis of parasites lacking *TgACS3*, suggests its role in activation of host-derived FAs directed towards parasite phospholipid and triacylglycerol synthesis in high lipid conditions. Altogether, the results presented here confirm the importance of ACS enzymes in *T. gondii* parasite survival and lipid activation and metabolism.

## **INTRODUCTION**

Apicomplexa parasites are a group of unicellular eukaryotes that include obligate intracellular parasites responsible for major human and cattle diseases. These include *Toxoplasma gondii*, *Plasmodium* sp. and *Cryptosporidium parvum*, which are causing agents of the infectious diseases toxoplasmosis, malaria and cryptosporidiosis, respectively. There is currently no fully efficient vaccines, and, depending on the parasite and the related diseases, current treatments can be toxic, losing efficiency and or emergence and fast spreading of resistance amongst parasite populations. There is thus an urgent need to renew our therapeutic arsenal to further push for the eradication of these important diseases. One important step for this is to understand and identify the key metabolic pathways sustaining host-parasite interactions and parasite survival. Metabolic pathways allowing nutrient acquisition, intracellular development, and parasite propagation represent ideal targets for drug development. Lipid synthesis, acquisition and homeostasis have been shown as one of the key metabolic pathways for parasite survival and pathogenicity (1,2).

*T. gondii* is capable of infecting virtually any nucleated cell in mammals and birds, thereby reflecting its metabolic capacities and plasticity in adapting to different nutritional environments. During the intracellular development of *T. gondii*, lipids play an essential role by providing structural building blocks for membrane biogenesis (3,4), as signaling molecules participating in key events like active invasion and egress

through microneme secretion (5,6) and as storage fuels, which plays critical role to maintain proper intracellular development (7,8). To cope with a continuous need for lipids within the different host cells environments, *T. gondii* has evolved so it harbors three *de novo* synthetic pathways to generate fatty acids (FA), central lipid building blocks: (i) the apicoplast resident prokaryotic type-II fatty acid biosynthesis pathway (FASII) (4,9), (ii) the ER-based FA elongation pathway (10) and (iii) the eukaryotic type-I fatty acid synthase present in the cytosol (9). Additionally, the parasite is also heavily reliant on acquiring FA directly from their hosts (7,8,11–14). Ongoing research on parasite lipid metabolism suggests that the FA flux derived from both apicoplast FASII (4,9) as well as from direct scavenging from the host (10,13–15) are both critical for intracellular parasite viability. Furthermore, in regular growth conditions, most major phospholipid classes making the bulk of *T. gondii* lipid composition are obligate “patchwork molecules” composed of one FA scavenged from the host and one FA made *de novo* (4). The metabolic activities of both pathways to acquire and generate FAs are regulated by host nutrient/lipid availability (16–18) (Amiar and Katris, Krishnan *et al.* Cell Host Soldati, +JBC Gupta). Nutrient/lipid deprivation in the host induces significant upregulations of the apicoplast FASII activity in both *T. gondii* and *P. falciparum* (16). On the other hand, lipid scavenging is a constant phenomenon that renders FASII dispensable when the nutrient/lipid content is in excess in the host.

Furthermore, this constant flux of FA scavenged from the host, which can be lipotoxic for the parasite, needs to be used timely for phospholipid synthesis and membrane biogenesis, specifically during parasite division (8,19,20)The parasite accomplishes this by directing excessive scavenged FA towards triacylglycerol (TAG) formation which facilitates the biogenesis of lipid droplets (LD). These LD can be present

throughout the asexual intracellular developmental stages and can be mobilized specifically during parasite division.

FAs need to be metabolically activated to be used by the cell for trafficking, metabolic utilization, or any other cellular function. This key biochemical step is performed by thioesterification of the FA to a Co-Enzyme A (CoA) that is catalyzed by the essential acyl-CoA synthetase (ACS or acyl-CoA ligase) family (21). Biochemically, ACSs catalyze a two- step reaction to form fatty acyl-CoA (Fig. 1a). The initial step involves the formation of an adenylated intermediate through the hydrolysis of an ATP molecule, thus releasing pyrophosphate (22). The ATP-activated enzyme then binds to the carboxyl group of an incoming free FA (FFA) moiety through an acyl bond to the phosphoryl group of AMP. Final fatty acyl-CoA product is formed after the transfer of the fatty acyl group to the sulfhydryl group of coenzyme A, thereby releasing AMP (22).

The phylum apicomplexa is actively engaged in FA metabolism, and hence a putatively critical role of ACSs for parasite survival is highly expected. Any putative ACSs candidate encoded in the Apicomplexa genome can theoretically activate FAs derived from both *de novo* synthetic machinery and/or from the host. Such importance and expected functions are supported with the existence of ACS encoding genes in the genome of *Cryptosporidium* and *Theileria*, which lack the *de novo* fatty acid biosynthetic machinery (9). *Cryptosporidium parvum* has been reported to have three AMP-binding domain containing long-chain fatty acid CoA ligases out of which two *CpACS1* and *CpACS2* have been biochemically characterized as functional ACSs (23). *Plasmodium falciparum* on the other hand has a large family of ACSs encoded by 13 genes (*PfACS1a-PfACS12*) (24). They also describes ACSs as the only family of metabolic enzymes that are encoded by genes expanded into the sub telomeric region which usually harbors parasite virulence genes (24). Drugs specific to ACSs in

*C. parvum* have shown potent parasite killing activity, thereby hinting that this enzymatic reaction is a potential Achilles heel for apicomplexans (23). Nothing is currently known on the function of ACSs in *T. gondii*.

We have identified 7 enzymes in *Toxoplasma gondii*, which we classify as putative acyl-CoA synthases that have non-overlapping localizations, suggesting non redundant roles (*TgACS*(25)). In this study, we identified one of them, *TgACS1*, whose phenotypic score initially suggested it as not important for parasite growth. However, we further showed that the protein's function becomes critical under specific physiological conditions in the host. We reveal that it actually activates FA for their mobilization and use by a peroxisomal-like pathway to provide energy to the parasite under poor/deleterious host nutritional environment, notably during the tachyzoites extracellular stages. This indicates that the role of such enzymes might be directly linked to the capacity of the parasite to have a strong metabolic plasticity upon host nutritional conditions that strongly governs the metabolic pathways chosen by the parasite (1,2,8,17,25–28). The function and potential nutrient-dependent roles of the other ACSs in *T. gondii* are still unknown. Here, we reveal the essential function of another ACS candidate, *TgACS3*(29).

Downregulation of *TgACS3*, via a Tet-off-based inducible KD parasite line, revealed that the protein is critical for tachyzoite intracellular development. Cellular characterization shows that *TgACS3* is required to maintain intracellular propagation of *T. gondii* tachyzoites, especially under high host nutrient environments. Lipidomic and fluxomics analyses further determined that the disruption of *TgACS3* significantly reduces the parasite lipid content, phospholipid content, and concomitantly accumulates host FA within the parasite. Together, our data reveal that *TgACS3* is responsible for channeling the constant flux of host FA to generate the bulk of

phospholipid for parasite membrane biogenesis, hence maintaining tachyzoite division and survival.

## **RESULTS**

### **Cellular localization of seven genes encoding putative acyl-CoA synthetase (ACS) enzymes within the *Toxoplasma* genome**

To determine the physiological localization of the 7 putative *TgACS*, we generated *T. gondii* tachyzoite parasite lines expressing each protein fused to a C-terminal triple haemagglutinin (3×HA) epitope-tag under control of their endogenous promoter (30). Immunofluorescence assays (IFA) using anti-HA antibody revealed that the 7 *TgACS*s localize to distinct sub-cellular compartments of the parasite (Fig. 1a). *TgACS1*, *TgACS6* and *TgACS7* were localized widely within the parasite cytoplasm, mostly as vesicular/punctate patterns. *TgACS2* localized in a compartment reminiscent of the parasite mitochondria. *TgACS3* was broadly localized at the cytosol, accumulating around, and avoiding the nuclear area of the parasite. *TgACS4* showed a distinct and interesting peri-nuclear/ER-like localization. Finally, *TgACS5* displayed a cytoplasmic localization at, particularly enriched at the basal end of the parasite. Such specific and diverse localizations suggest non-redundant functions of ACSs within the different endomembrane compartments and cytoplasm of *T. gondii* similarly to the ACSs in other eukaryotes, which have different fatty acid substrate specificities and functions depending on their differential localizations (21,31,32). The endogenously tagged parasite lines were further confirmed by western blot analysis using anti- HA antibody (Sup. 1b). The migration of all the *TgACS*-HA was in accordance with their annotated protein sizes on ToxoDB, except for *TgACS2*, which migrated approximately 10 kDa less than predicted.

## ***TgACS3* is critical for tachyzoite intracellular development especially in high host nutrient environments**

To determine the function and importance of *TgACS3* (*TGGT1\_310080*), which is predicted to be important for intracellular growth with a phenotypic score of -2.53 (29,33), we generated an inducible knockdown line of *TgACS3* (Fig. 2) by promoter replacement using the Tet-off system in *TATi\_ΔKu80* background (34,35) with an additional N-terminal HA-tagging. Western blot and IFA analyses both confirmed the downregulation of *TgACS3*-iKD-HA (Fig. 2a, b) parasite line by anhydrotetracycline (ATc) treatment. Complete protein depletion was observed at 48 h after ATc treatment for *TgACS3* (Fig. 2a).

IFA confirmed *TgACS3* is located at the cytosol/perinuclear compartment (Fig. 2b). The disruption of *TgACS3* had a direct deleterious effect on tachyzoite intracellular morphology, especially on large vacuoles (>10 parasites) where parasites appeared round and unhealthy (Fig. 2b, lower panel: phase). The growth phenotype was confirmed by a replication assay where *TgACS3* depleted parasites had significantly fewer large vacuoles (7-10 parasites) and a significantly higher number of smaller vacuoles (3-6 parasites) (Fig. 2c), indicating that intracellular propagation was significantly impaired in the mutant. We further performed plaque assays in the presence of different host nutritional environments (0,1 and 10% FBS containing growth media/DMEM) to probe the mutant's capacity to maintain normal growth under these conditions. We previously showed that wild type parasites could sense host nutritional status and grow better with higher levels of host nutrient (10% FBS) (14). The disruption of *TgACS3* strongly disrupted parasite growth, which escalated with increasing levels of FBS in the growth culture medium. When grown in regular culture conditions with 1% FBS, parasites lacking *TgACS3* had significantly smaller and lesser

plaques, although the growth phenotype was significantly lesser than the control (Fig. 2d, e). Importantly, when grown in high nutrient environment, at 10% FBS, parasites lacking *TgACS3* displayed a much stronger growth phenotype than at 0 and 1% FBS, with significantly lesser and smaller plaques than in the control (Fig. 2d, e). Furthermore, *TgACS3*-iKD parasites also displayed a significant, yet mild egress defect in the presence of ATc, which may or may not be an underlying effect of replication defect (Sup. Fig. 1b).

To further determine the cellular phenotype induced by the absence of *TgACS3*, we monitored the content of parasite lipid storage, i.e. lipid droplets (LD), which play an important role for channeling the massive flux of host scavenged FA. Nile red staining of *TgACS3* intracellular parasites at 0%, 1% and 10% FBS indicated that the disruption of *TgACS3* induced a significant reduction in the number of lipid droplets per parasite (Fig. 2f, g). This suggests that *TgACS3* plays a role in the formation of neutral lipids and storage either directly or indirectly.

### **Disruption of *TgACS3* leads to decrease of TAG levels in a high host nutrient environment**

Our data suggest that *TgACS3* has an important role for the division, overall intracellular tachyzoite development and potential metabolic adaptation of the parasite to different host nutritional environments. To determine its precise function, we performed lipidomics analysis on *TgACS3*-iKD +/-ATc (48 h time point) using GCMS approaches. We first determined the overall FA abundance and composition of total lipid fraction (including FAs from all glycerolipids, neutral lipids and free FA). Overall, we did not observe any significant difference in the total FA abundance of *TgACS3*-iKD (+ATc and - ATc) (Fig. 3a). However, the detailed profile of the total FA composition showed a significant increase in palmitic acid (C16:0) with a decrease in

C20:1 content in the mutant (*TgACS3*-iKD +ATc) (Fig. 3b). To assess function of *TgACS3*, the total lipid content was separated by HPTLC, and the composition and content of phospholipids (PL), free fatty acid (FFA), and storage lipid triacylglycerol (TAG) were quantified by GCMS. Interestingly, this detailed lipidomic analysis FFA were significantly increased in the mutant (*TgACS3*-iKD +ATc, 1% FBS) (Fig. 3c). The FFA excess in *TgACS3*-iKD mutant (+ATc) correlated with the growth defect phenotype in the presence of 1% FBS (Fig. 2e). However, disruption of *TgACS3* resulted in no changes in TAG levels (Fig. 3c). Quantification of the FA composition of the FFA fraction revealed significant decrease in short chain FAs like C14:0 and C16:0 and an increase in long chain FAs like stearic acid (C18:0) and C22:1 under the depletion of *TgACS3* (Fig. 3d). Analysis of the FA composition from TAG and DAG levels in the *TgACS3*-iKD mutant (+ATc), showed no significant changes in individual FA molecular species (Sup. Fig. 3a, b). Overall, this suggested *TgACS3* depletion within the parasite, leads to an increase in inactive FFA, likely due to the loss of ACS activity to incorporate FA, potentially leading to a lipotoxic effect causing parasite death.

The exaggerated growth defect seen at 10% FBS under the suppression of *TgACS3* (Fig. 2d, e) lead us to further investigate the lipidomic profile of parasites lacking this protein under a high lipid content. The total lipid FA composition analyses revealed that there was an increase in FA chains that are normally scavenged directly from the host (C18:1, C20:1, C20:4, Fig. 3e) (8,14). Investigation of neutral lipids and PL levels by 1D-HPTLC, revealed a significant decrease in the relative abundance of TAG levels without and with *TgACS3* (Fig. 3f). There was an increase in C12:0 and decrease in C16:0 FA chains in the TAG levels when the protein was suppressed (Fig. 3g), suggesting that most of the TAGs are coming directly from the parasite and not

scavenged as lipid droplets from the host (8). An increase in the relative abundance of C18:0 and decrease of C18:1 in DAG levels was observed (Fig. 3h). Despite no changes in DAG and PL relative abundance in the absence of *TgACS3*, there was a general trend of a decrease in unsaturated FAs chains (C18:1, C20:1) and a compensating increase of saturated FAs (C18:0, C16:0) in both lipid fractions (Fig. 3h, i).

### ***TgACS3* is responsible for channeling the bulk of host scavenged FA**

Based on the lipid data showing major effect of *TgACS3* depletion on long chain fatty acids (>16:0/18:0), previously shown to be mostly scavenged from the host (8,14) These results prompted us to further examine the source of FA dealt with/activated by *TgACS3* within the parasites. We performed novel flux analysis approach, using U-<sup>13</sup>C-glucose (Glc) combined to GCMS-lipidomics to monitor and quantify the FA scavenged from the host, i.e. the parasite “FA scavengeome” (8). <sup>13</sup>C-Glc-pre-labelled host cells were infected with *TgACS3*-ikD parasites (+/ATc) in the presence of normal culture medium, which contains regular <sup>12</sup>C-Glc (8). The total lipid content was extracted from the parasites to determine the amount of <sup>13</sup>C incorporation to each FA originating from the host. The wild type *TgACS3*-ikD parasites were able to scavenge fatty acids from the host, with a significant decrease in host-derived C18:1 in the absence of the protein (Fig. 4a). There was a decrease in scavenging of C16:0 and increase in C16:0 and C18:1 of TAG and FFA pools, respectively (Fig. 4b, c). There was an overall decrease in FAs scavenged from the host for DAG (C16:0) and PL (C16:0, C18:1) lipid pools (Fig. 4d, e) in 10% FBS. This data suggests that the *TgACS3* majorly acts on FA scavenged directly from the host especially in a high host lipid environment balancing between PL synthesis and lipid storage. The increase in host

derived FFA suggests without this protein they cannot be incorporated to form PL and other storage lipids like DAG/TAG.

## ***DISCUSSION***

Acyl CoA synthetases catalyze a fundamental and limiting reaction in FA metabolism: the activation of free FA via thioesterification to coenzyme A (CoA). This key reaction allows the fatty acyl CoA intermediates to participate further in trafficking, assembly into complex lipids, post-translational modification of membrane proteins, and/or the catabolism of FAs via  $\beta$ -oxidation (21). We prioritized *TgACS3*, due to their largely distinct roles in lipid metabolism and nutrient adaptation in other models (nutrient scarcity and excess) and its high predicted phenotypic score (25,29,36–39).

Eukaryotic TAG biosynthesis occurs as the last step of the glycerol-3-phosphate pathway by the sn-3 acylation of DAG by a diacylglycerol-acyltransferase (DGAT, Fig. 5a) (40,41). Here we tested the roles of *TgACS3* this process by comparing the levels of neutral lipids within the reservoir organelles called lipid droplets by Nile red staining (42). This suggests that *TgACS3* plays a role in the formation of neutral lipids and storage either directly or indirectly.

In metabolically active hepatocyte cells Huh7, ACSL3 has been shown to specifically channel activated FA (esp. C18:1, oleic acid) into phosphatidylcholine. A siRNA mediated knockdown of ACSL3 decreases oleate incorporation to PC with concomitant rise in the levels of cellular-free oleic acid (40). Like this study, the measured decrease of the total levels of FA content derived from phospholipids in the *TgACS3*-iKD alongside concomitant increase of free FA both corroborate with the functional role of the enzyme (Fig. 3c). We see a drop in TAG levels under depletion of the protein in 10% FBS which is supported by the droplet in lipid droplet formation by Nile red staining (Fig. 2g

and 3g). Lipidomic analysis of *TgACS3*-iKD +ATc illustrated an upregulation of the normally FASII-derived fatty acids to compensate for the decrease in host-derived fatty acids like C20:1. In a high host nutrient environment we see increases in the total lipid abundance of even more long chain unsaturated FAs that are normally host-derived like C18:1, C20:1 and C20:4. This suggests that *TgACS3* probably is a long chain fatty acyl-CoA ligase which thioesterifies long chain FA scavenged from the host rather than the apicoplast-FASII specific short chain fatty acids (C14:0 and C16:0). This was supported by our <sup>13</sup>C-Glc based flux analyses, where we were able to identify whether the increase of FFAs is host-derived. There was an overall decrease in host-derived FAs for total lipids, which accounted for the decrease in host-derived FAs of TAG, DAG and PLs pools (Fig. 4).

The growth defect for the *TgACS3*-iKD was significantly more pronounced in 10% serum conditions. This observation can be explained by linking it to lipidomic data showing increase in the free FA content within *TgACS3*-iKD (+ATc) and more specifically an increase in host-derived FFAs (Fig. 5). As per its expected function, due to loss of *TgACS3* within the parasite there is a reduction in the metabolic utilization of fatty acids via thioesterification, and instead of an equilibrium in lipid storage and synthesis we see a decrease in PL synthesis and lipid storage. In the presence of lipid enriched 10% serum DMEM, the parasites normally uptake more FAs, however, are unable to balance its utilization due to absence of an important acyl-CoA synthetase *TgACS3*. This led to 'over scavenging' of host FFAs, which seemed to accumulate instead of being used to form PL/DAG or TAGs in the absence of this protein (Fig. 5).

Despite having a negative phenotype score on *Toxoplasma* genome wide CRISPR-Cas9 screen(29), the measured growth defect of *TgACS3*-iKD in normal nutrient conditions at 1% FBS was not as pronounced as predicted. However, in the presence

of high host nutritional environment, the phenotype of *TgACS3*-iKD (+ATc) was more pronounced, correlating reported phenotype scores. These differences might be explained by a certain level of redundancy by the other *TgACS*s when *TgACS3* is depleted. However, the replication assay and egress defect demonstrated the importance of *TgACS3* for the parasite intracellular development.

Inflammatory cytokine (TNF- $\alpha$ ) elevation in humans, seems to induce the expression of ACSL1, ACSL3, and ACSL5 (44). Increase ACSL3 levels were also accompanied with increase oleoyl-CoA (activated FA C18:0) levels, mirroring our results of increased oleic acid (FFA) when *TgACS3* is absent. The downstream effect of TNF- $\alpha$  increasing ACSL3 levels promoted lipid droplet formation in human endothelial cells which may also be the case in *TgACS3* favouring activation of FFA to be used for TAG formation (44). In a recent study, ACSS3 (the short chain paralogue of ACSL3) was identified as a gene regulating the stability of lipid droplet-associated protein, PLIN3, and causing the reduction of LD deposits in the prostate (45). These studies suggest that ACS3 proteins are critical of LD metabolism and interact with partner proteins to regulate FFA activation and TAG formation.

This work brings insight into the nutrient-dependent role of *TgACS3*, as it is different from *TgACS1* and most probably the other ACSs that exist in *T. gondii* parasites (25). This protein is important in a high nutrient environment, again illustrating the parasite adapts different metabolic pathways in different conditions, rendering them highly plastic (1,2,8,26–28).

## **MATERIALS AND METHODS**

### ***T. gondii* strains and cultures**

The parasite host cells human foreskin fibroblasts (HFF) were cultured using Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco) and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>. *T. gondii* tachyzoite parental strains RH-ΔKu80 TATi, RH-ΔKu80 as well as the mutant strains were cultured by serial passage within their host HFF using DMEM supplemented with 1% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco) and 25 µg/mL gentamicin (Gibco) at 37°C and 5% CO<sub>2</sub>.

### **Generation of an inducible knockdown line for *TgACS3***

For generation of an inducible knockdown parasite line for the gene *TgACS3*, plasmid pPR2-DHFR (46) was used. For homologous recombination in RH-ΔKu80 TATi strain two separate homology flanks/regions were chosen. The 5' flank was amplified 1298 bp upstream of the *TgACS3* start codon using the primers in Table 1. The PCR product was ligated to PacI and NdeI digested vector pPR2 using NEB assembly reaction. Next, the 3' flank was amplified as a 1263 bp fragment beginning at the start codon of *TgACS3* with the primers in Table 1. The 3' homology flank was annealed to XmaI and NotI digested pPR-HA3-DHFR vector that already contained the *TgACS3* 5' flank. The final cloned vector positions the start codon of *TgACS3* downstream of the ATc-regulatable t7s4 promoter and a 3xHA tag.

The resulting vector were linearized with NotI and transfected this into TATiΔku80 parasites. Parasites were selected with the drug pyrimethamine and cloned out by limiting dilution. Screening of parasite clones *TgACS3*-iKD where the t7s4 promoter had successfully replaced the native *TgACS3* promoter, was done using the primers in Table 1 in the combinations described in supplementary figures (Sup. 4f). All PCRs

were performed with TaKara Primestar Max polymerase. The knockdown of *TgACS3* was induced with 0.5  $\mu\text{g ml}^{-1}$  of anhydrotetracycline (ATc).

### **Immunofluorescence assay**

Primary antibodies anti-HA (Rat, Roche), anti-IMC1, anti-SAG1 were used at dilutions 1:500, 1:1000 and 1:1000 respectively. Secondary Alexa Fluor 488- and 546-conjugated anti-mouse, anti-rat and anti-rabbit antibodies (Life Technologies) were used at 1/2500. For the immunofluorescence assay (IFA) parasites were grown on confluent HFF on coverslips and fixed in PBS containing 2.5% paraformaldehyde (PFA) for 15 min at room temperature (RT). Samples were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT prior to blocking in PBS containing 3% BSA and subsequent incubation with primary antibodies then secondary antibodies diluted in the blocking solution. Labelled parasites were stained with Hoechst (1/10000, Life technologies) for 20 min and then washed three times in PBS before final mounting of the coverslips on a glass slide using fluorogel (reference). The fluorescence was visualized using fluorescence microscope (Axio Imager 2\_apotome; ZEISS).

### **Nile red staining of lipid droplets**

Parasites were allowed to infect and grow in confluent monolayer HFF grown on coverslips, in the +/- ATc or Auxin conditions for 24 h in 0%/1%/10% FBS ED1 medium. As with IFA, these coverslips were fixed using 2.5% PFA, permeabilized with 0.25% triton X-100. Thereafter, coverslips were incubated for 1 h with Nile red in 1X PBS before proceeding to DNA staining with Hoechst. The coverslips were mounted onto a glass slide in fluorogel prior to imaging using fluorescence microscope (Axio Imager 2\_apotome; ZEISS). For visualizing Nile red stained droplets yellow-gold fluorescence (excitation, 450-500 nm; emission, greater than 528 nm) was used on the Axio Imager.

Quantification in +/-ATc or Auxin conditions were done by counting the no. of lipid droplets per parasite.

### **Western blot analysis**

Parasites were harvested for western blot after complete egress from their host. To remove any host cell debris, the parasites were passed through a 3  $\mu\text{m}$  filter, then counted by hemocytometer and solubilized in SDS buffer at equivalent cell densities. Equal amount of protein was separated on a 4-12% gradient SDS-polyacrylamide (Life Technologies) and transferred to Nitrocellulose membrane (check this) using the XCellIII Blot Module (Invitrogen). Primary antibodies anti-HA (Rat, Roche) and anti-TOM40 (Rabbit) (47) were used at a dilution of 1:500 and 1:1000, respectively. Secondary goat anti-mouse and anti-rabbit horse radish peroxidase (HRP) conjugated antibodies (Thermo Scientific) were used at 1:2000. Protein signal was detected by chemiluminescence after membrane staining with Luminata Crescendo Western HRP detection kit (Millipore). The signal strength of protein was quantified using a BioRad Chemidoc imager (BioRad).

### **Phenotypic analysis**

*Plaque assay*- The extracellular parasites were harvested after filtration and counted by hemocytometer. Then approx. 500 parasites were inoculated to confluent HFF flask (25  $\text{cm}^2$ ). The mutant parasites *TgACS3-ikD* was grown for plaque assay in the presence or absence of ATc (0.5  $\mu\text{g ml}^{-1}$ ) for 7-10 days. Plaque sizes were visualized by crystal violet staining (30-60 min) after aspiration of culture media, and cells fixation with 100% ethanol (5 min) followed by phosphate-buffered saline (PBS) wash.

*Replication assay*- The parasites were grown for two days with or without ATc (0.5  $\mu\text{g ml}^{-1}$ ), harvested and filtered. Equal number of parasites were allowed to invade

confluent HFF grown on coverslips. Following 2 h of invasion, the coverslips were washed thrice with ED1 (1% FBS containing DMEM), in order to remove any uninvaded parasites and promote synchronized replication. Anhydrotetracycline (ATc) ( $0.5 \mu\text{g ml}^{-1}$ ) was added at the outset of the experiment, allowing the treatment for 24 h, alongside control parasites without ATc. These coverslips were then fixed and processed for IFA wherein the parasite number per parasitophorous vacuole was analyzed.

*Egress assay*- Similar to replication assay, the parasites were treated with ATc prior to the experiment. Equal number of parasites were allowed to invade confluent HFF grown on coverslips and allowed to grow for 24-30 h for having big vacuoles with 16-32 parasites. For the egress assay, the culture media (ED1) was removed and replaced with either  $5 \mu\text{M}$  of calcium ionophore A23187 or DMSO control. This was followed by a 4 min incubation at  $37^{\circ}\text{C}$ . The parasites were immediately fixed with 2.5% paraformaldehyde+0.02% glutaraldehyde and processed for an IFA using anti-SAG1 antibody.

### **Lipidomic analysis**

The parasites were grown for 48 h or until completely extracellular in conditions of +/- Auxin/ATc on the confluent monolayer of HFF in flasks ( $175 \text{ cm}^2$ ). For extracellular parasite lipid analyses completely, egressed parasites were collected, and host cells were filtered out with a with  $3\text{-}\mu\text{m}$  pore size membrane, after which parasites were incubated for 6 hours in the respective media conditions 0/1/10% FBS). Parasites were also harvested as the intracellular tachyzoites ( $1 \times 10^7$  cell equivalents per replicate) after syringe filtration with  $3\text{-}\mu\text{m}$  pore size membrane. These parasites were metabolically quenched by rapid chilling in a dry ice-ethanol slurry bath and then centrifuged down at  $4^{\circ}\text{C}$ . The parasite pellet thus obtained was washed with ice-cold

PBS thrice, before transferring the final pellet to a microcentrifuge tube. Then total lipids were extracted in chloroform/methanol/water (1:3:1, v/v/v) containing PC (C13:0/C13:0), 10 nmol and C21:0 (10 nmol) as internal standards for extraction) for 1 h at 4°C, with periodic sonication. Then polar and apolar metabolites were separated by phase partitioning by adding chloroform and water to give the ratio of Chloroform/Methanol/Water, 2:1:0.8 (v/v/v). For lipid analysis, the organic phase was dried under N<sub>2</sub> gas and dissolved in 1-butanol to obtain 1 µl butanol/10<sup>7</sup> parasites.

*Total lipid analysis* – Total lipid was then added with 1 nmol pentadecanoic acid (C15:0) as internal standard and derivatized to give fatty acid methyl ester (FAME) using trimethylsulfonium hydroxide (TMSH, Machenery Nagel) for total glycerolipid content. Resultant FAMES were then analyzed by GC-MS as previously described (Dubois et al. 2018). All FAME were identified by comparison of retention time and mass spectra from GC-MS with authentic chemical standards. The concentration of FAMES was quantified after initial normalization to different internal standards and finally to parasite number.

*Phospholipid and neutral lipid analysis*- For phospholipid analysis, total lipid extracted (as mentioned above) was separated with 1 nmol PA(C17:0/C17:0) (Avanti Polar lipids) by one- dimensional silica gel high-performance thin layer chromatography (HPTLC, Merck). Total PL, DAG, TAG, Free fatty acids (FFA) and cholesteryl ester (CE) analysis, total lipid fraction was separated by 1D-HPTLC using hexane/diethyl ether/formic acid, 80:20:2 (v/v/v) as solvent system. Thereafter, each lipid spot on the HPTLC plate was scrapped off and lipids were methanolized with 200 µl 0.5 M methanolic HCl in the presence of 1 nmol pentadecanoic acid (C15:0) as internal standard at 85°C for 3 h. The resulting FAMES were extracted with hexane and finally analyzed by GC-MS (Agilent).

### *Tracking host-derived fatty acids – (monitoring parasite scavenging capacities)*

The HFF cells were grown ( $1 \times 10^8$  cell equivalents per replicate) to confluency in the presence of stable isotope U- $^{13}\text{C}$ -glucose at a final concentration of 800  $\mu\text{M}$  added to a glucose-free DMEM (10% FBS). These  $^{13}\text{C}$ -pre labelled HFF were then infected with *TgACS3-ikD* parasites in the presence of normal-glucose containing DMEM under +/- ATc (0.5  $\mu\text{g/ml}$ ). The host HFF and parasites were metabolically quenched separately, and their lipid content was quantified by GC-MS as described above. As described previously, the degree of the incorporation of  $^{13}\text{C}$  into fatty acids (%carbon incorporation) is determined by the mass isotopomer distribution (MID) of each FAMES. The total abundance of  $^{13}\text{C}$ -labelled fatty acids was analysed initially for HFF to check labelling of the metabolites (8). Later, the same was calculated for parasites to confirm direct uptake of  $^{13}\text{C}$ -labelled fatty acids from the host.

### **Statistical analysis for all experiments**

Entire graphical data for this study was generated using GraphPad prism software (version 8). Three biological replicates were used per experiment ( $n=3$ ). The error bars are representative of standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by t-test using GraphPad Prism. Range of statistical significance was signified as per the p value, wherein 0.01-0.05=\*, 0.01-0.001=\*\* and  $<0.001$ =\*\*\*.

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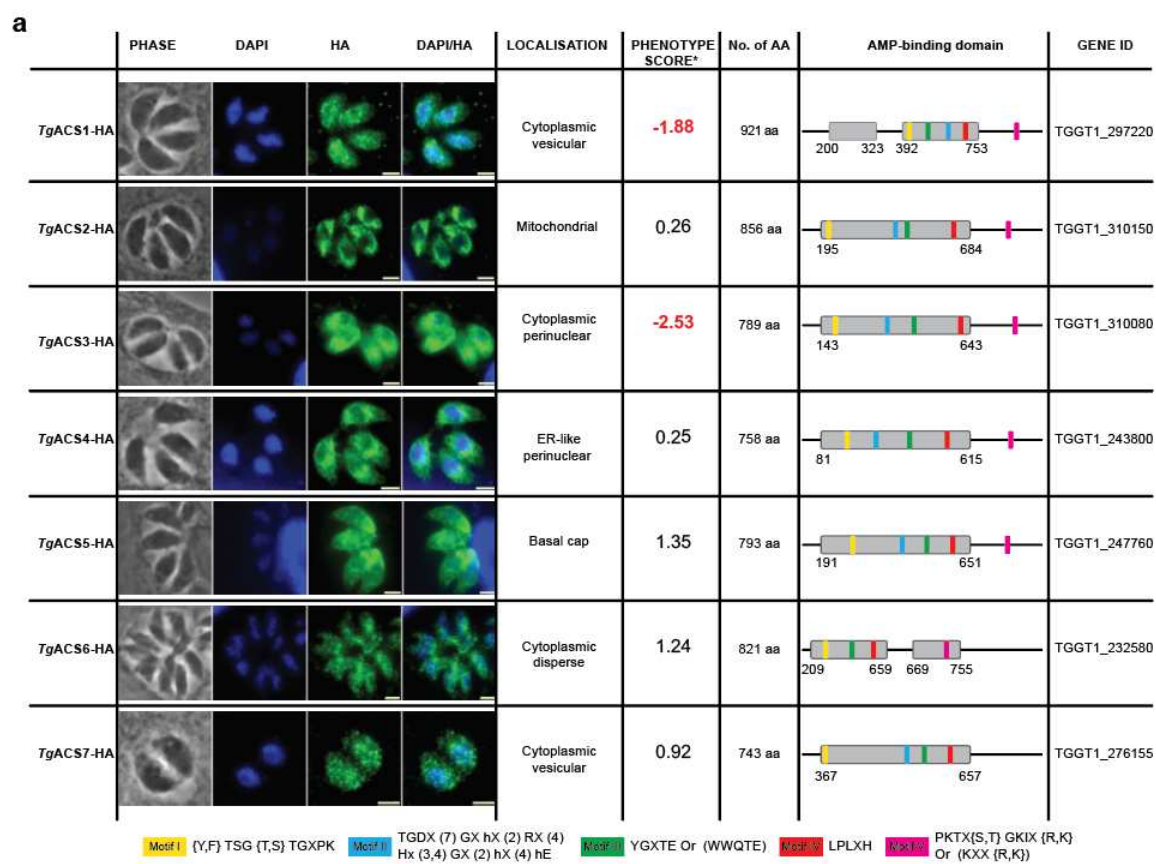
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**Table 1**

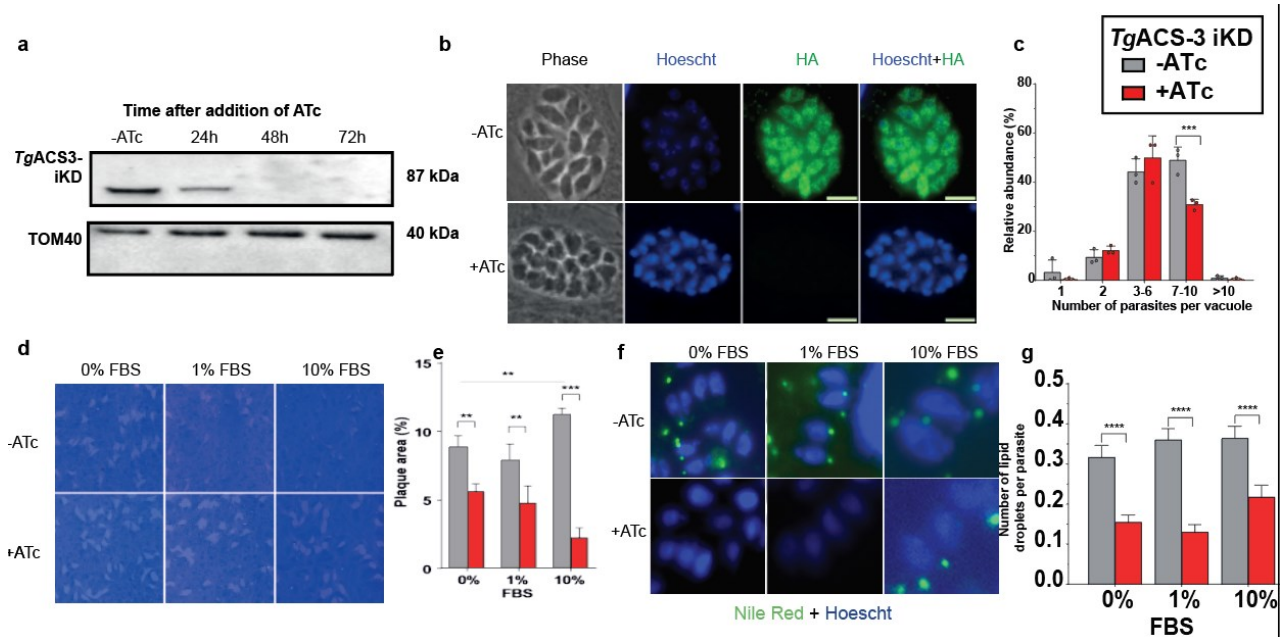
<i>Tg</i> ACS Gene ID	<b>Primers</b> (gene specific sequence <u>underlined</u> )	Molecular system used
<i>Tg</i> ACS3  ( <i>TGGT1_310080</i> )	5' flank Fw:  GGGCGCGCCGGATCCTTAATTAATC  GCTGCGATGTCGATCGTTTTC	TATI-Tet off
	5' flank Rw:  CGCCATGCATGGCCGGCCCATATGG  CGACTACGAAAGACAAACGCC	
	3' flank Fw:  TGTTCCAGATTATGCCTTACCCGGGA  TGGCACTCCAGTACGCCTACC	
	3' flank Rw:  TGGAGCTCCACCGCGGTGGCGGCC  GCCTGCTTCGAGGAGAAATGGCTTTC	
	SD01:  CGATGACCTGTGTCTGACCTGT	
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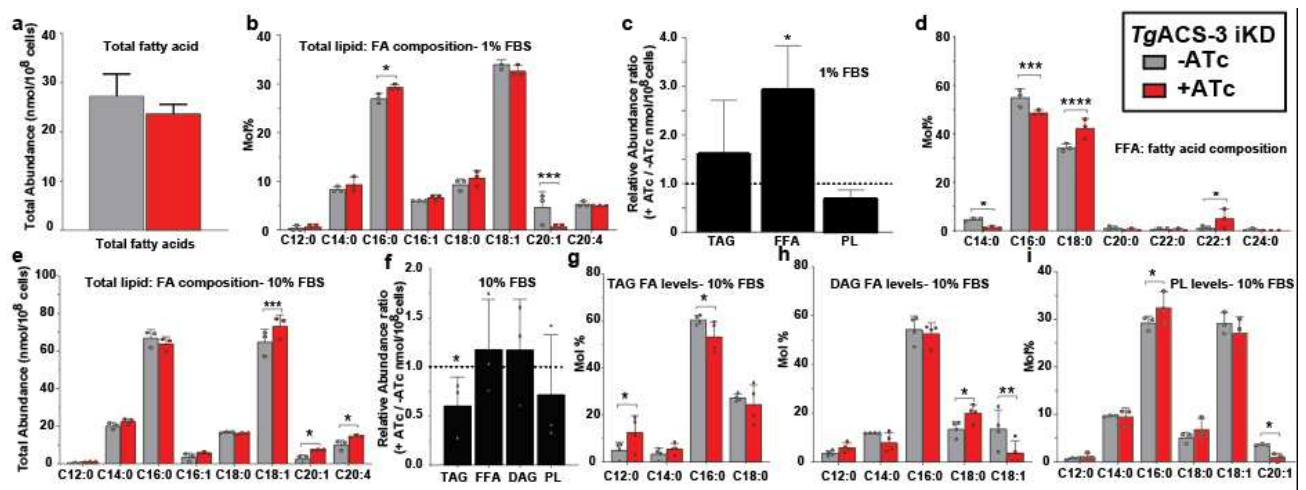
## Figures



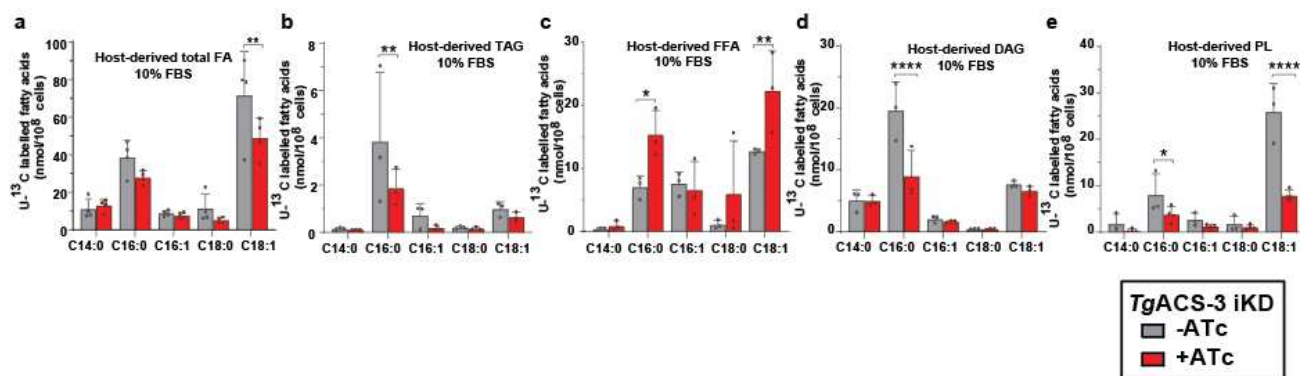
**Figure 1: Identification of seven acyl CoA synthetase in *T. gondii* and their cellular localizations** a) Immunofluorescence of TgACS1-7 with their respective parasite cellular localization, phenotype score (with the most essential in red) (55), amino acid size, AMP-binding domain location and gene ID. The motif in the AMP-binding domain with their respective amino acid sequences.



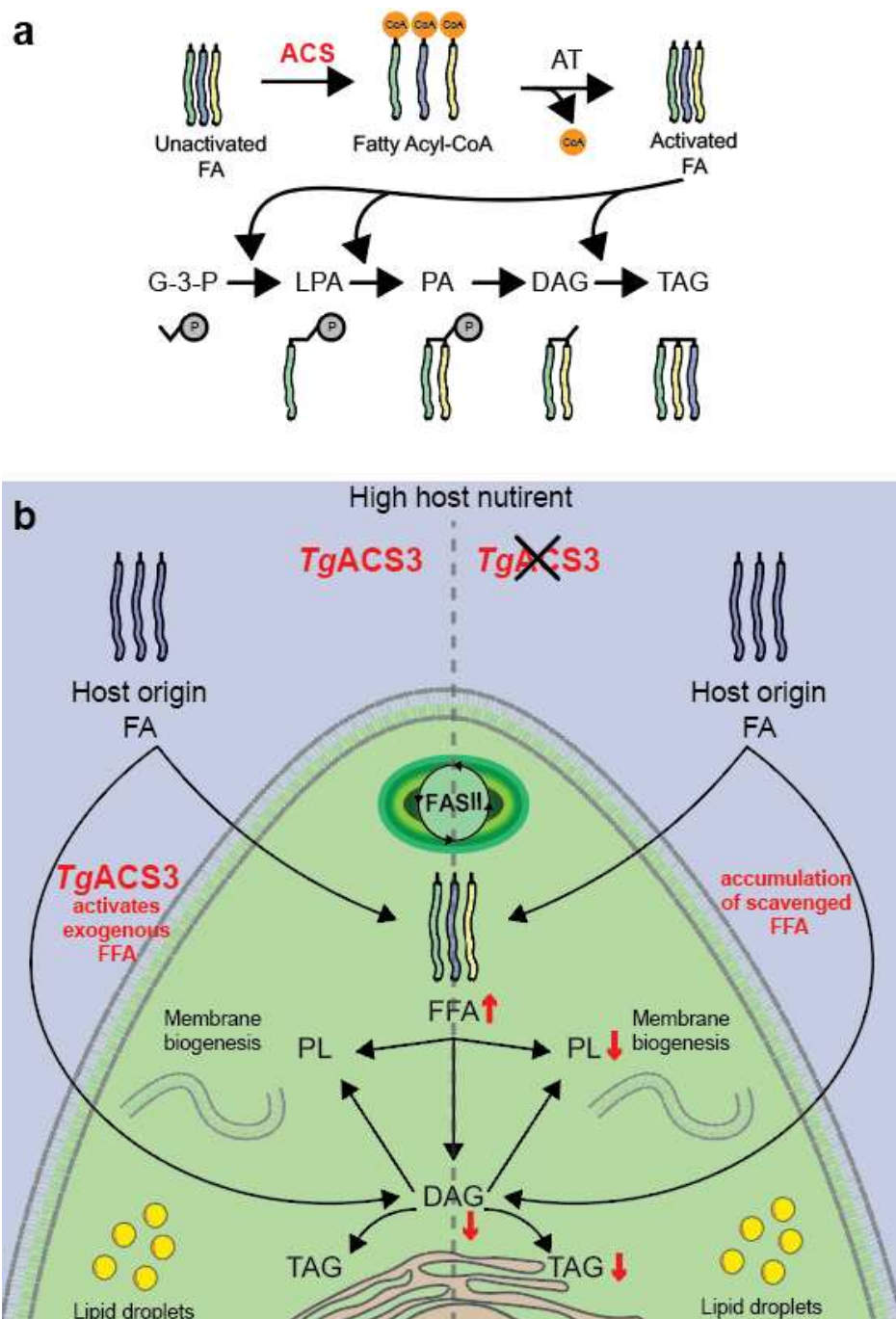
**Figure 2: Cellular characterization of *TgACS3-iKD* reveal its critical role for intracellular growth and LD formation.** a) Immunoblot analyses revealed downregulation of *TgACS3-iKD* (87 kDa) in the presence of anhydrotetracycline (ATc) after 48 hr with a TOM40 (40 kDa) loading control. b) *TgACS3* localizes to the cytosol of intracellular parasites with complete loss of signal under ATc-treatment. c) A replication assay revealed an increase in vacuoles of 2-4 parasites and a decrease in larger vacuoles (>8 parasites). d) Plaque assay with an accompanying e) bar graph showing inhibition of parasite growth when *TgACS3* is degraded at 0%, 1% and 10 % FBS. f) Lipid droplet analyses using Nile red staining (green), showed under *TgACS3* suppression there was a decrease in lipid droplet number per parasite g) in the accompanying graph at 0%, 1% and 10% FBS. Error bars indicate standard deviation (SD), Experiments were conducted in triplicates, unpaired t-test p values where 0.01-0.05=\*, 0.01-0.001=\*\*, <0.001=\*\*\* and <0.0001=\*\*\*\*.



**Figure 3: Lipidomic analyses of *TgACS3*-iKD mutant under high and low host nutrient content shows its pivotal role for FA activation, and for TAG and PL synthesis.** a) Under anhydrotetracycline (ATc) treatment (red), the loss of *TgACS3* does not affect the total fatty acid (FA) content of intracellular tachyzoite parasites. b) The FA molecular composition in 1% FBS shows an increase in C16:0 and decrease C20:1 under suppression of the protein. c) Relative abundance ratios calculated for TAG, FFA and PL levels without/with the protein in low level (1% FBS), with d) the molecular compositions of the FFA fraction showing a short chain FA (C14:0, C16:0) and increase in long chain FA (C18:0, C22:1) under *TgACS3* depletion. e) Under 10% FBS conditions the analyses of the total FA abundance revealed an increase in long chain FA (C18:1, C20:1, C20:4). f) Relative abundance ratios calculated for TAG, FFA, DAG and PL levels without/with the protein in high level (10% FBS), with the mol% of the FA composition of h) DAG and i) PL levels. Error bars indicate standard deviation (SD), Experiments were conducted in triplicates, unpaired t-test *p* values where 0.01-0.05=\*, 0.01-0.001=\*\*, <0.001=\*\*\* and <0.0001=\*\*\*\*.



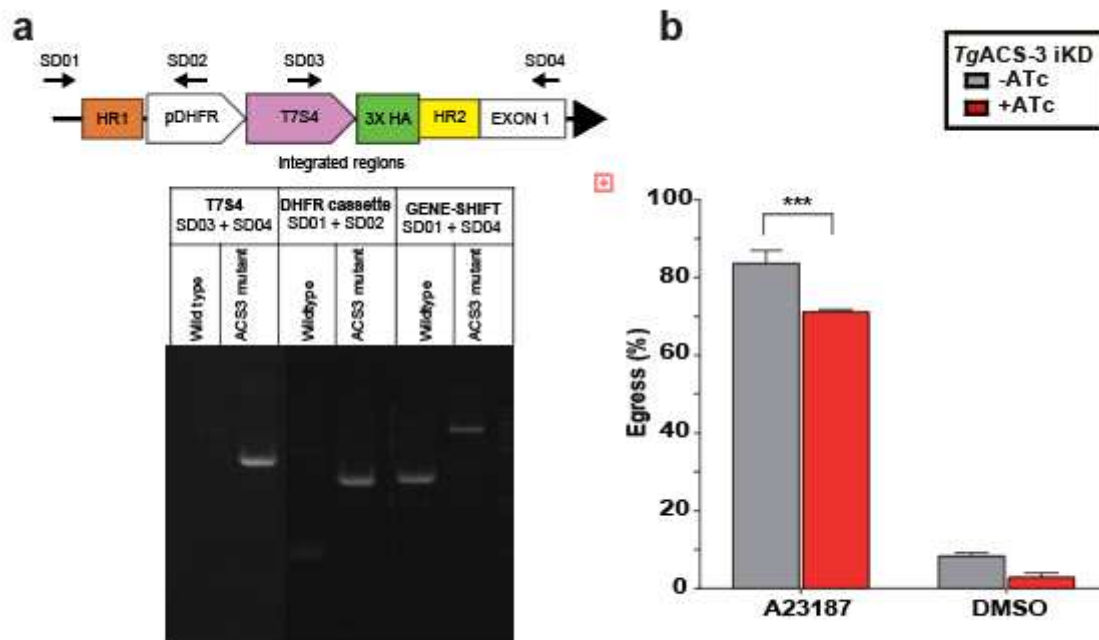
**Figure 4: Lipid flux analyses of host-derived lipids in the *TgACS3-iKD* mutant under high host nutrient content.** Using  $^{13}C$  stable isotope-labelled host cells, lipid abundance derived from the host and channelled into the parasite membranes were quantified by GCMS approach for a) total FA, b) TAG, c) FFA, d) DAG and e) PL fractions in 10% FBS. Error bars indicate standard deviation (SD), Experiments were conducted in triplicates, unpaired t-test p values where 0.01-0.05=\*, 0.01-0.001=\*\*, <0.001=\*\*\* and <0.0001=\*\*\*\*.



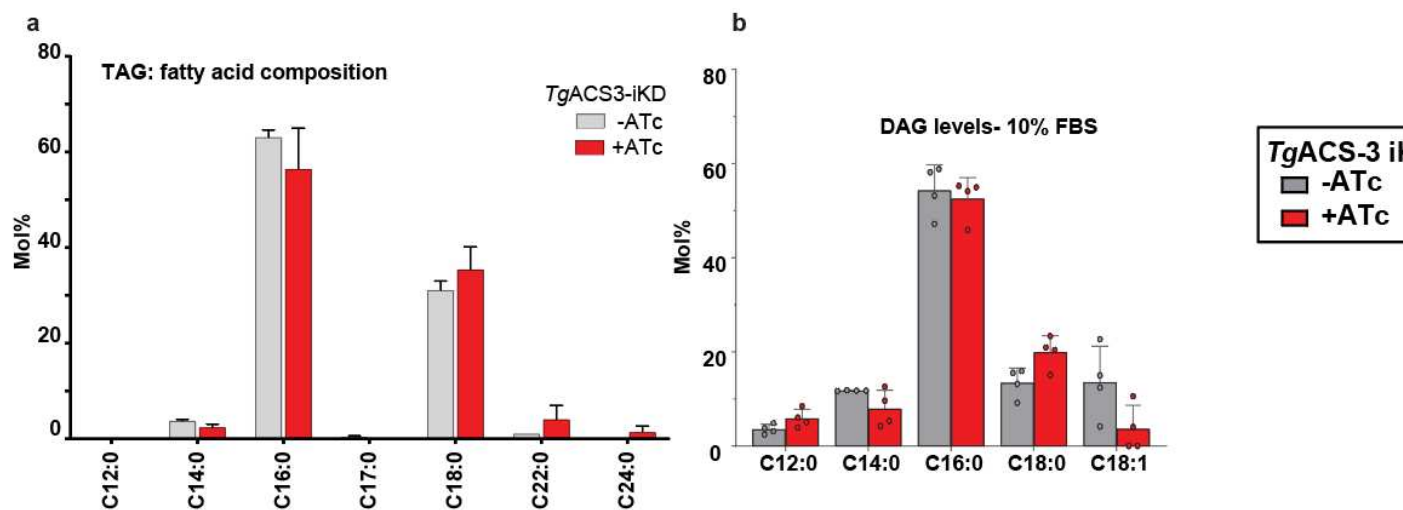
**Figure 5: Model explaining the proposed roles of *TgACSs* in parasite host-adaptation and lipid metabolism.** a) Fatty acids (FA) from the host, extracellular environment and directly from the parasite are incorporated into the glycerolipid pathway of the parasite. They are esterified onto a glycerol-3-phosphate (G-3-P) by acyltransferase (ATS) like ATS1, ATS2 and AGPAT to form lysophosphatidic acid (LPA) with backbone and phosphatidic acid (PA), respectively. LIPIN is a phosphatase

responsible for dephosphorylation of PA to diacylglycerol (DAG) onto which another FA is attached to form triacylglycerol (TAG) by DGAT. FA must be activated to allow for their utilization by ATSS, this is facilitated by acyl-CoA synthetases (ACSS). b) The downregulation of *TgACS3* leads to affects in intracellular parasites in a high host nutrient environment. There is a 'over scavenging' effect that leads to an increase in host derived and global FFA levels, but this is coupled with a decrease in host-derived TAG, DAG, and phospholipid (PL) levels.

## Supplementary Figures



**Supplementary Figure 1:** a) PCR confirmation of *TgACS3*-iKD that uses the TATI-Tet-Off system, showing correct integration of the T7S4 sequence, DHFR-drug cassette, and the gene-shift compared to the parental line.



**Supplementary Figure 2: Lipidomic analyses of *TgACS3-iKD* mutant 10% FBS.**

Under anhydrotetracycline (ATc) treatment (red), the loss of *TgACS3* does not affect the total fatty acid (FA) content of a) TAG or b) DAG levels.

**CHAPTER 5: THE PATATIN-LIKE PHOSPHOLIPASE  
*PFPNPLA2* IS INVOLVED IN THE MITOCHONDRIAL  
DEGRADATION OF PHOSPHATIDYLGLYCEROL DURING  
*PLASMODIUM FALCIPARUM* BLOOD STAGE  
DEVELOPMENT**

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***Summary of submitted article:***

**The patatin-like phospholipase *PfPNPLA2* is involved in the mitochondrial degradation of phosphatidylglycerol during *Plasmodium falciparum* blood stage development**

This article was submitted to Frontiers in Cellular and Infection Microbiology (IF: 6.073) for an invited special edition on “Lipids in Host and Protozoan Parasite Interaction”. It is currently under minor revision.

The malaria parasite causes half a million deaths every year, specifically in impoverished and developing countries. Novel therapeutic strategies are needed considering the emergence of drug-resistance and neglected treatment services. New evidence points towards exploiting the unique lipid metabolism of this parasite.

During the asexual cycle, *P. falciparum* parasites can sense nutrient changes and adapt to varying metabolic states whereby it can manage a fine balance between lipid storage (neutral lipids) and lyso/PL synthesis required for parasite proliferation or sexual commitment to gametocyte stages facilitating parasite transmission to the mosquito vector. A reduction of LPC levels induces sexual conversion and the start of gametocytogenesis. Not only is lipid acquisition required, but to maintain lipid homeostasis, PL and other lipid groups must be degraded to generate FA and polar heads, a reaction that is usually catalysed by phospholipase enzymes. Here we look at a patatin-like phospholipase type A2 that is involved in phosphatidylglycerol metabolism.

The major findings of this study include:

- *Pf*PNPLA2 localizes to the mitochondrion **and** the apicoplast of the parasite
- This protein is important in asexual stage parasites, mostly under limiting lipid availability
- Lipidomic analysis by GC-MS revealed that under reduction of this protein, the lipidomic profiles were only altered in ring and schizont stage parasites
- Separation of neutral lipids by thin-layered chromatography (TLC), showed that when the protein is disrupted:
  - TAG levels were always higher
  - FFA levels increase in rings staged parasites but decrease in schizont stages
- Despite no overall changes in PL levels, when we looked at the individual lipid species a major finding was:
  - Increase in PG
  - Decrease in LBPA and not LPG levels

Our data here suggests that *Pf*PNPLA2 is important for the balance between PG and LBPA (not LPG) in asexual parasites. It also strongly suggests that the enzyme is involved in parasite lipid recycling, to generate FA from mitochondrial PG and LBPA as by-products. These recycled FA can be further used for phospholipid synthesis under low host lipid environment. This further supports the hypothesis that PL/lyso-PL balances and nutrient availability are crucial to maintaining asexual development and/or sexual conversion of RBC parasites.

### **Résumé de l'article soumis :**

**La phospholipase « *patatin-like* » PfPNPLA2 est impliquée dans la dégradation mitochondriale du phosphatidylglycérol au cours du développement sanguin de *Plasmodium falciparum***

Cet article a été soumis au journal « *Frontiers in Cellular and Infection Microbiology* » (IF: 6.073) pour une édition spéciale sur le thème des lipides dans un contexte d'interaction entre les parasites protozoaires et leur hôte. Cet article est actuellement en cours de révision.

Le paludisme cause plus d'un demi-million de morts chaque année et spécifiquement dans les pays émergents ou pauvres. De nouvelles stratégies thérapeutiques doivent être développées pour faire face à l'émergence de résistance aux thérapies traditionnelles. De nouvelles études orientent ces stratégies vers l'exploitation des mécanismes lipidiques qui sont uniques à ce parasite.

Au cours de son cycle de reproduction asexué, *P. falciparum* perçoit les variations de l'environnement nutritif et adapte son métabolisme pour maintenir l'équilibre entre la synthèse de lipides de stockages (neutres) et de (lyso)-phospholipides qui sont nécessaire à la prolifération du parasite ou sa transition au stade gamétocyte qui facilite la transmission au moustique, son vecteur principal.

La réduction des niveaux de lyso-Phosphatidyl choline induit le passage en cycle sexué et initie la gametocytogenèse. L'acquisition de ce lipide est non seulement requise, mais afin de maintenir l'homéostasie lipidique, des phospholipides ainsi que d'autres classes de lipides doivent être dégradés pour

généraliser des acides gras et des têtes polaires, réactions habituellement catalysées par des phospholipases. Dans cette étude, nous avons analysé une phospholipase « patatin-like » de type A2 qui est impliqué dans le métabolisme des phosphatidylglycérols.

Les résultats principaux sont les suivants :

- *PPNPLA2* est localisée dans la mitochondrie ainsi que dans l'apicoplaste du parasite.
- Cette protéine est importante lors de la phase de reproduction asexuée et spécifiquement dans des conditions où les lipides sont limitants.
- Des analyses lipidomiques par GC-MS indiquent que la déplétion de cette protéine affecte seulement les profils lipidomiques des parasites au stade annulaire ou schizonte.
- La séparation de lipides neutres par chromatographie sur couche mince montre que lorsque la protéine est déplétée :
  - Les niveaux de triglycérols sont systématiquement plus élevés
  - Les niveaux d'acides gras libres augmentent au stade annulaire mais décroissent au stade schizonte.
- Malgré l'absence de changement des niveaux globaux de phospholipides, lorsque nous analysons les lipides individuels, nous observons :
  - Une augmentation des niveaux de phosphatidylglycérol
  - Une décroissance des niveaux d'acide lysobiphosphatidique sans modification des niveaux de lysophosphatidylglycérol.

Nos données suggèrent que *Pf*PNPLA2 contribue au maintien de la balance entre les phosphatidylglycérols et les acides lysobiphosphatidiques (mais pas les lysophosphatidylglycérol) au cours des phases du cycle asexué du parasite. Nos données suggèrent également que cette enzyme est impliquée dans le recyclage des lipides du parasite, permettant la génération d'acides gras à partir de phosphatidylglycérol provenant des mitochondries ainsi que des acides lysobiphosphatidiques. Ces acides gras recyclés peuvent ainsi être utilisés pour la synthèse de phospholipides en conditions de stress nutritif. Ces observations supportent l'hypothèse que la balance phospholipides/lysophospholipides et la disponibilité des nutriments sont essentielles au maintien du parasite en cycle asexué et/ou à leur passage au cycle sexué.

**TITLE: The patatin-like phospholipase *Pf*PNPLA2 is involved in the degradation of phosphatidylglycerol during *Plasmodium falciparum* blood stage development**

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**SHORT TITLE:** Mitochondrial PLA2 degrades PG in malaria

***FUNDING SOURCES***

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## **ABBREVIATIONS**

CTCF	corrected total cell fluorescence
DAG	Diacylglycerol
GC-MS	Gas chromatography mass spectrometry
(F)FA	(Free) fatty acid
FASII	Fatty acid synthase II
HPTLC	High performance thin layer chromatography
glmS	glucosamine-induced ribozyme
GlcN	Glucosamine
LBPA	Lysobisphosphatidic acid
LPG	Lysophosphatidyl glycerol
PC	Phosphatidyl choline
PG	Phosphatidyl glycerol
PM	Plasma membrane
PL	Phospholipid
PNPLA	Patatin-like Phospholipase
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
TAG	Triacylglycerol

## **KEYWORDS**

Apicomplexa, malaria, lipid, phospholipase, metabolism, lipidomic

### ***Contribution to the field statement***

Malarial infections cause more than half a million deaths a year, mostly in children in inter-tropical regions. Emergence of drug-resistant strains in combination with other pandemics are draining resources (COVID19), all urging for novel malarial drug therapies. Understanding the biology of the unicellular obligate parasite *Plasmodium falciparum* is the key in combating the disease. This parasite undergoes a complex lifestyle in human red blood cells, hepatocytes, and mosquitoes. This renders this organism metabolically plastic,

allowing the parasite to survive in very different host environments. One specific nutrient that is consistently needed, especially when the parasite divides to form the new progeny (hence allowing for disease propagation), is lipids. Parasite lipid metabolism relies on scavenging host resources and parasite lipid synthesis/recycling upon physiological variation of available nutrients. Therefore, enzymes manipulating lipids constitute pertinent drug targets. Here, we show that one such lipase allows the parasite to recycle existing lipids for survival when host resources are lacking. This brings novel insight into how the parasite can sense its host environment to metabolically adapt, hence revealing potential hidden drug target. This will ultimately contribute to the fight against malaria.

## **ABSTRACT**

*Plasmodium falciparum* is an Apicomplexa parasite causing human malaria, a major disease causing more than ½ million deaths every year, against which there is no fully efficient vaccine. The current rapid emergence of drug resistances emphasizes the need to identify novel drug targets. Current evidence shows that lipid synthesis and trafficking are essential for parasite survival and pathogenesis, and these pathways represent potential points of attack. Large amounts of phospholipids are needed for the generation of membrane compartments for newly formed parasites. Parasite membrane homeostasis is achieved by an essential combination of *de novo* parasite lipid synthesis/recycling and host lipid scavenging. Recent data suggest that the mobilization and channeling of lipid resources is key for asexual parasite survival within the host red blood cell. Enzymes remodeling lipids such as phospholipases are likely involved in these mechanisms. *P. falciparum* possess

an unusually large set of phospholipases, whose functions are largely unknown. Here we focused on the putative patatin-like phospholipase *PfPNPLA2*, for which we generated an GlmS-inducible knockdown strain and investigated its role during blood stages. Interestingly, the mitochondrial *PfPNPLA2* had a significant parasite replication and survival defect under low host lipid content. Lipidomic analyses revealed that *PfPNPLA2* specifically degrades parasite phosphatidylglycerol to generate lysobisphosphatidic acid. *PfPNPLA2* knockdown resulted in an increased host lipid scavenging accumulating in the form of storage lipids and free fatty acids. These results suggest that *PfPNPLA2* is involved in the recycling of parasite PG to sustain optimal asexual development when the host resources are scarce. This work strengthens our understanding of the complex lipid homeostasis pathways to acquire lipids and allow asexual parasite survival.

## **INTRODUCTION**

There are 241 million cases of malaria per year worldwide, of which 627 000 (World malaria report, 2021) result in death, mostly children in Sub-Saharan Africa and South-East Asia (1). Malaria control has struggled to improve due to the rapid emergence of drug-resistant strains, and even more considering the current COVID19 pandemic, which dramatically hampered the current situation. The global and rapid emergence of drug resistant parasite strains has made the search for novel drug targets even more urgent and pertinent in recent years (2). Malaria is caused by an obligate intracellular parasite called *Plasmodium* spp, which belong to the phylum Apicomplexa. The clinical symptoms and lethal consequences of the disease are due to the asexual intracellular development of the parasite within the host red blood cell. These parasites must develop and

divide within erythrocytes and understanding the metabolic interactions by which the parasite can acquire essential host nutrients, especially during blood stages, is crucial to identify key pathways that could be targeted as potential therapies.

Transmission of malaria parasites is achieved by a blood feed of an infected female *Anopheles* mosquito on a human host, releasing *Plasmodium* spp. sporozoites that travel towards the liver where they invade hepatocytes. Parasites then undergo an intense asymptomatic asexual schizogonic division cycle, generating tens of thousands of parasites within hours. Newly formed merozoites then egress hepatocytes to enter the blood flow where they can invade host red blood cells to embark on the asexual intraerythrocytic stages to proliferate. During the intraerythrocytic replication phase of the parasite, they develop from ring stages to trophozoite and schizont stages in which new merozoites are formed. Interestingly, this intense asexual schizogonic division relies on the levels of host nutrient and more particularly, lipids. Indeed, lysophosphatidylcholine (LPC) present in the host serum is the key limiting nutrient allowing parasite division through provision of important lipid precursor for parasite membrane biogenesis (3,4). Reduction of LPC in cultures *in vitro* induces parasite conversion from highly dividing asexual merozoites to nondividing sexual gametocytes. During a new blood feed, mosquitos can then ingest stage V gametocytes.

The intraerythrocytic cycle demands the parasite to be able to extend and modify the plasma membrane (PM) and the parasitophorous vacuole membrane (PVM) and to replicate its organelles like the mitochondria and apicoplast, which are both sites of lipid synthesis for the formation of daughter

cells. This is a phase of intense membrane biogenesis and current evidence shows that large amounts (5-fold more) of lipids are needed for the survival and replication of the parasite during this asexual cycle (5–8).

The building blocks of all lipids, the fatty acids (FAs), are predominantly scavenged from the host but can also be fully made *de novo* via an active prokaryotic type II FA synthase, called FASII, that is present in the relict non-photosynthetic plastid of the parasite (i.e. the apicoplast). The FASII pathway provides specific FA and is particularly essential during intense schizogonic division of the parasite occurring during the liver-stage and for sporozoite development during mosquito stages (9–11). However, the activity of the apicoplast FASII is also directly influenced by the level of host nutrient during asexual blood stages, as it can be upregulated and become essential if host nutrient and scavenging are scarce (12,13). Membrane biogenesis is therefore dependent on a combination of host lipid scavenging and *de novo* parasite lipid synthesis relying on the acquisition of FA building blocks from FASII *de novo* synthesis and host scavenging (10,11,14,15). Phospholipid (PL) synthesis through assembly of these FA, and trafficking are needed for the generation of membrane compartments for newly formed parasites.

The bulk of PLs, their precursors like the polar heads choline and ethanolamine as well as FA, especially the sole FA species required from the host, C16:0 and C18:1, need to be scavenged from the host serum (10,11,16–18). This supports the latest postulate that the parasite is highly dependent on the nutritional status of the host cell and its environment, which has been extensively studied in *Toxoplasma gondii*, another apicomplexan parasite (19–21). Currently, this lipid flux in *Plasmodium* is poorly understood and requires further investigation.

During the lytic cycle, the parasite must be able to sense nutrient changes and adapt to varying metabolic states whereby it can manage a fine balance between lipid storage (neutral lipids) and lyso/PL synthesis required for parasite proliferation or sexual commitment to gametocyte stages facilitating parasite transmission to the mosquito vector (22). Not only is lipid acquisition required, but to maintain lipid homeostasis, PL and other lipid groups must be degraded to generate FA and polar heads, a reaction that is usually catalyzed by phospholipase enzymes (17).

Phospholipases are enzymes which hydrolyze specific ester bonds in PLs and are classified into four groups, A, B, C, and D, based on their hydrolytic activity (17). Over 20 putative phospholipase enzymes are present in *P. falciparum* and only two have been shown to play a key role in intracellular parasite development, namely phosphoinositide specific-phospholipase C and *Pf*PNPLA2 (23). The latter enzyme belongs to the group of patatin-like phospholipase proteins that are poorly understood thus far. Their putative roles suggested by bioinformatic analyses and databases suggest pivotal and putatively redundant functions in lipid homeostasis at all stages of the development of the malaria parasite (17,24,25). One key question is to determine the function, substrate, and products for each of these phospholipases to unravel the complex lipid metabolism by which the parasite depends for its survival. Here we investigated the phospholipase *Pf*PNPLA2 and compared its expression with two other *Pf*PNPLA proteins, *Pf*PNPLA3 and *Pf*PNPLA4. We reveal their non-overlapping localizations and further elucidate the role of *Pf*PNPLA2 at the parasite mitochondria using lipidomics approaches. We further demonstrate that *Pf*PNPLA2 has a critical role during low host

nutrient content, when the protein becomes essential for asexual blood stage survival, especially at the schizont-to-ring transition. In addition, we show that *PfPNPLA2* degrades phosphatidylglycerol (PG) to allow the synthesis of lysobisphosphatidic acid (LBPA). This likely allows the generation of FA for the boost of synthesis for membrane PLs, potentially facilitating parasite division and progeny formation. Our work further emphasizes the importance of mobilization and channeling of lipid resources, here PG degradation/recycling, for asexual blood stage parasite development within the human host RBC.

## **MATERIALS AND METHODS**

### **Primary antibodies**

In this study, the following primary antibodies and antisera were used: Rabbit anti-HA antibody (Sigma-Aldrich); rat anti-HA antibody (Roche); mouse anti-GFP antibody (Sigma-Aldrich); rabbit polyclonal antisera against *Pfs230* (Biogenes), *Pfs25* (ATCC), *Pfs16*, AMA1(26); mouse polyclonal antisera against falcilysin (27), *Pf39* (28) and actin I (29). The generation of polyclonal mouse antisera against *PfPNPLA2*, 3 and 4 is described below (see Sup. Fig. 2C).

### **Generation of mouse antisera**

Recombinant proteins of *PfPNPLA2* (spanning aa 571-998), *PfPNPLA3* (spanning aa 589-763) and *PfPNLPA4* (spanning aa 2-187) were expressed for the generation of antisera. *PfPNPLA2* was expressed with an N-terminal maltose-binding protein (MBP)-tag using the expression vector pIH902. Cloning was mediated by the addition of the restriction sites EcoRI and PstI (for primer sequences see Table 1). *PfPNPLA3* was expressed with an C-terminal His-tag using pET28c (+) and cloning was mediated by the addition of NcoI/XhoI

restriction sites. *PfPNPLA4* was expressed as a fusion protein with an N-terminal MBP-tag using pMalc5x. Cloning was mediated by XmnI/PstI restriction sites. Recombinant fusion proteins were expressed using *E. coli* BL21 (DE3) RIL (Stratagene) and purified via affinity chromatography using amylose resin (New England Biolabs) for the MBP-tagged proteins and cOmplete™ His-tag purification resin (Sigma-Aldrich) for the His-tagged proteins according to the manufacturers' protocols. After PBS buffer exchange via filter centrifugation using Amicon Ultra 15 (Sigma-Aldrich), protein concentrations were determined by Bradford assay. 100 µg recombinant proteins emulsified in Freund's incomplete adjuvant (Sigma-Aldrich) were injected into 6 weeks-old NMRI mice (Charles River Laboratories) via subcutaneous injection. After 4 weeks, a boost with 50 µg recombinant proteins was injected. Ten days after the boost, mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (Sigma-Aldrich) according to the manufacturer's protocol and immune sera were collected via heart puncture. Sera were pooled from three mice immunized with the same antigen. Experiments for the generation of antisera in mice were approved by the animal welfare committees of the District Council of Cologne, Germany (ref. no. 84-02.05.30.12.097 TVA).

### **Generation of the *PfPNPLA2*- and *PfPNPLA4*-KD parasite lines**

*PfPNPLA2*- and *PfPNPLA4*-KD parasite lines were generated via single cross-over homologous recombination using the pARL-HA-glmS vector (30). A 1015-bp gene fragment homologous to the 3' end of the gene coding for *PfPNPLA2* and a 840-bp gene fragment of the gene coding for *PfPNPLA4* (excluding the stop codons) were amplified using corresponding primers (for primer

sequences see Table 1). Ligation of the inserts with the vector backbone was mediated by NotI/AvrII restriction sites. A synchronized wildtype (WT) NF54 culture containing 5 % ring stages was loaded with 100 µg vector in transfection buffer via electroporation as described. Parasites carrying the vector were selected by the addition of WR99210 (Jacobus Pharmaceuticals) in a final concentration of 4 nM and successful integration of the vector was confirmed by diagnostic PCR using (1) 5'-integration primer, (2) 3'-integration primer, (3) pARL-HA-glmS forward primer and (4) pARL-HA-glmS reverse primer (for primer location see Sup. Fig. 2a; for primer sequences see Table 1).

### **Diagnostic RT-PCR**

Total RNA was isolated from synchronous ring, trophozoite and schizont cultures, as well as from Percoll-gradient enriched immature, mature and activated gametocytes as described before (30). RNA preparation and subsequent cDNA synthesis were performed as described (30). Transcripts for *PfPNPLA2* (250 bp), *PfPNPLA3* (250 bp) and *PfPNPLA4* (250 bp) were amplified in 25 cycles using the corresponding primers (for primer sequences see Table 1). To confirm purity of the asexual blood stage and gametocyte samples, transcript amplifications of *ama1* (407 bp) and of *ccp2* (286 bp) were performed using the corresponding primers. Amplification of the housekeeping gene *aldolase* (378 bp) was used as loading control.

### **Indirect immunofluorescence assay and live cell imaging**

Synchronized asexual cultures of a wildtype (3D7) strain and the PNPLA2-iKD line were maintained as previously described (31,32). Parasite cultures (200 µl of compact infected RBS) were collected by centrifugation (5 min at 1 500 rpm at room temperature). Parasites were fixed with 4% paraformaldehyde and

0.0075% glutaraldehyde for 30 min at room temperature. Cells were centrifuged at 3000 rpm for 2 mins and the supernatant was discarded. The sample was then washed twice with PBS and the supernatant removed. The pellet was resuspended gently in 1 ml of 1% FBS (fetal bovine serum) in PBS and spun again as previously. Cells were permeabilized using 0.1% Triton X-100 in PBS for 10 mins. Cells were pelleted and the sample was washed twice with PBS. The pellet was saturated with 3% FBS in PBS and incubated at room temperature for 1 h. Thereafter the primary antibodies of HA-tag or anti-PNPLA2/PNPLA3/PNPLA4 were added at dilutions of 1:500 or 1:50, respectively. The antibodies were then washed away twice with PBS. The secondary antibodies (AlexaFluor488/546 anti-rat/mouse) were used at 1:1000 dilutions in PBS and incubated for 1 h. Hoechst stain was used at 1:25000 dilution for 20 mins and finally samples were washed with PBS thrice. Pellets were resuspended in 150  $\mu$ l of PBS and then gently mixed with the same amount of Fluorogel. 15  $\mu$ l of each sample was pipetted on a microscope slide and overlayed with a square coverslip. Samples were allowed to solidify in the dark at 37°C for 1 h. Specimens were examined with an Axio Imager; ZEISS,  $\times$ 100 magnification. Images were treated using ImageJ software. Indirect immunofluorescence images were obtained for gametocytes as previously described (30).

For IFAs using anti-LBPA antibodies (Echelon Biosciences), a dilution of 1:500 was used, and parasites were incubated for at least 1 hour. To determine the corrected fluorescence intensity experiments were performed in triplicate and Image J was used to identify the cell area, mean fluorescence intensity, minimum/maximum fluorescence intensity and integrated fluorescence

intensity (33). The same data was then determined concerning an area outside the cell (used as the background). The corrected total cell fluorescence (CTCF) was calculated with the following equation:

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background readings})$$

### **Western blotting**

Western blotting was performed as previously described (30). Briefly, total sorbitol synchronized schizont stage parasite samples underwent saponin lysis to remove red blood cell membrane (34). The sample was then resuspended in a lysis buffer containing an anti-protease cocktail (0.5% Triton X- 100, 4% w/v SDS, 0.5× PBS). SDS-PAGE buffer was added, and samples were denatured at 95°C for 10 mins. Equal sample volumes were then separated by SDS-PAGE and proteins were transferred onto a Hybond ECL nitrocellulose membrane (Amersham Biosciences) according to the manufacturer's protocol. The blot was blocked using Tris-buffered saline containing 5% w/v skim milk, pH 7.5, followed by overnight incubation at 4°C with polyclonal mouse immune sera specific for *Pf39* (dilution 1:5,000) or using polyclonal rabbit immune sera specific to anti-HA (1:1,000). Subsequent washing was followed by incubation with alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) for 1 hr at RT to reveal the blot. Blots were scanned and processed using the Adobe Photoshop CS software. Band intensities were measured using the ImageJ programme version 1.51f.

### **Asexual blood stage replication assay**

To analyze asexual blood stage replication and length of the intraerythrocytic replication cycle in the PNPLA2-KD parasite line, experiments were set-up as follows: Expression of PNPLA2 was knocked-down in transgenic PNPLA2-KD parasite line by treatment of the culture with GlcN (D-(+)-glucosamine hydrochloride; Sigma Aldrich) at a final concentration of 2.5 mM. GlcN treatment started one cycle before the experimental set-up. The GlcN-treated culture was compared to the untreated culture cultivated in normal cell culture medium. This assay was also done in minimal media only including RPMI, glucose, hypoxanthine (with 1M NaOH), FA-free bovine serum albumin (60 µM) and FFA C16:0 (30 µM) and C18:1 (30 µM) . To exclude an unspecific effect of GlcN, the parental WT line was used as control. For each assay, three experiments were performed, each in triplicate.

### **Gametocyte development assay**

To induce knockdown of *Pf*PNPLA2, a synchronized parasite culture of the *Pf*PNPLA2-KD line was treated with 2.5 mM GlcN for 72 h. Gametocytogenesis was subsequently induced with lysed blood cells while the GlcN treatment was continued. The gametocytemia was evaluated by Giemsa smears over a period of six days. Untreated *Pf*PNPLA2-KD cultures were used as controls.

### **Lipidomics analysis**

Lipidomics analysis was performed on 3-4 independent cell harvests of the PNPLA2-iKD line. Highly synchronous parasite cultures grown for at least 48 hours with or without glucosamine (ring/trophozoite or schizont stage) were harvested by transferring the cultures to pre-warmed 50-ml tubes and the total amount of parasites was determined by Giemsa staining and via counting on a

hemocytometer. The cultures were metabolically quenched via rapid cooling to 0°C by suspending the tube over a dry ice/100% ethanol slurry mix, while continually stirring the solution. Following quenching, parasites were constantly kept at 4°C and liberated from the enveloping RBCs by mild saponin treatment with 0.15% w/v saponin/0.1% w/v BSA PBS for 5 min. After three washing steps with 1 ml ice-cold PBS followed by centrifugation each time, the parasite pellet was subjected to lipid extraction using chloroform and methanol (28) in the presence of butylhydroxytoluene, PC (C21:0/C21:0), C13:0 or C15:0 FA standards (Avanti). Lipid extraction, separation and analyses were all performed as previously described in Flammersfeld *et al.*, 2020 (30). FA methyl esters were detected by GC-MS (Agilent 5977A- 7890B) according to the method described in Ramakrishnan *et al.* (2012) (6) and quantified by Mass Hunter software (Agilent). Each FA was quantified according to a calibration curve generated with authentic fatty methyl ester standards.

### **Statistical analysis**

Data are expressed as mean  $\pm$  SD. Statistical differences were determined using unpaired two-tailed Student's t test, as indicated. Values of  $p < 0.05$  were considered statistically significant. Significances were calculated using GraphPad Prism 5 and are represented in the figures as follows: ns, not significant  $p > 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ .

## RESULTS

***Pf*PNPLA2 is a non-redundant parasite phospholipase that localizes to both apicoplast and mitochondrion and is important for asexual parasite development under nutrient poor environment**

Gene *PF3D7\_1358000* encodes a putative patatin-like phospholipase (17), termed *Pf*PNPLA2. The protein is predicted to be a phospholipase A2 (PLA<sub>2</sub>) with a patatin-like phospholipase domain like that of *Pf*PNPLA1 (30) and an estimated molecular weight of 238.2 kDa (Fig. 1a). *Pf*PNPLA2 comprises a transmembrane domain at the N-terminal region of the protein. Further, the characteristic GX SXG motif is present in this protein between 1166 aa- 1171 aa, as well as a conserved signature catalytic aspartate found in most eukaryotic PLA<sub>2</sub>, which is found at residue 1201 (Fig. 1A) (35). As previously shown, all plasmodial patatin-like phospholipase homologs share the above-mentioned motif and catalytic aspartate, supporting the classification of PNPLA2 (17,30). In addition to *Pf*PNPLA1 and *Pf*PNPLA2, two more members of the *Pf*PNPLA family were previously identified, i.e. *Pf*PNPLA3 (151 kDa, encoded by gene *PF3D7\_0924000*) and *Pf*PNPLA4 (283 kDa, encoded by gene *PF3D7\_0218600*). *Pf*PNPLA3 comprises two N-terminal transmembrane domains in addition to the central patatin-like phospholipase domain. (Sup. Fig. 1A).

Diagnostic reverse transcriptase (RT)-PCR was performed to investigate expression of *pnpla2*, *pnpla3* and *Pfpna4* in the *P. falciparum* blood stages and the synthesis of transcripts in the ring and trophozoite stages and schizonts were demonstrated for all three genes.. Similar transcript levels were detected in immature (stage II-IV) and mature (stage V) gametocytes as well as in

gametocytes at 15' post-activation (p.a.) (Sup. Fig. 1B). Transcript analysis of the housekeeping gene *aldolase* was used as a loading control, and purity of the asexual blood stage and gametocyte samples was demonstrated by amplification of transcripts for the asexual blood stage-specific gene *Pfama1* (apical membrane antigen 1) (36) and for the gametocyte-specific gene *Pfccp2* (LCCL-domain protein 2) (37). The cDNA preparations lacking the reverse transcriptase were used to verify that samples were devoid of gDNA (Sup. Fig. 1B).

For functional characterization of *PfPNPLA2*, we generated a conditional *PfPNPLA2*-HA-KD line (using the pARL-HA-glmS vector) by fusing the sequences coding for a hemagglutinin A (HA)-tag to the 3'-coding region of *Pfppnpla2* (Sup. Fig. 2A). In the *PfPNPLA2*-HA-KD, the *Pfppnpla2* mRNA is expressed under the control of the glmS ribozyme, which catalyzes its own cleavage in the presence of GlcN and thus triggers transcript degradation. Vector integration was confirmed by diagnostic PCR (Sup. Fig. 2B). Immunoblotting of asexual blood stage lysate with an anti-HA antibody confirmed the synthesis of a HA-tagged *PfPNPLA2* fusion protein in the untreated *PfPNPLA2*-HA-KD line, running at a molecular weight of 238 kDa (Fig. 1B). When the conditional knockdown of *PfPNPLA2*-HA synthesis was induced by treating asexual blood stage parasites with 2.5 mM GlcN for 72 h, *PfPNPLA2*-HA levels were reduced compared to the untreated control (Fig. 1B).

The untreated *PfPNPLA2*-HA-KD line was used for in-depth protein expression analysis. Furthermore, a HA-tagged *PfPNPLA4* parasite line was generated, using the above mentioned pARL-HA-glmS vector. In addition, mouse antisera

were generated against recombinant *Pf*PNPLA2, *Pf*PNPLA3 and *Pf*PNPLA4 peptides to be used for protein expression analysis. Immunofluorescence assays (IFA) revealed that *Pf*PNPLA2 is expressed throughout all asexual and sexual blood stages (Fig. 1C). PNPLA2 is predominantly expressed in the schizont stages and localizes to vesicular structures of individual merozoites formed within the parasite vacuole. *Pf*PNPLA2 could further be detected at distinct loci within the cytoplasm of gametocytes (Fig. 1C). Closer co-localization studies revealed that *Pf*PNPLA2 does not colocalize with *Pf*PNPLA3 or *Pf*PNPLA4 (Fig. 1D-E). We performed further co-localization IFA, using an apicoplast-tagged parasite line, the *Plasmodium falciparum* apicoplast triose phosphate transporter TPT-HA line (12,38), with antibodies against native *Pf*PNPLA2. This revealed that *Pf*PNPLA2 colocalizes with the apicoplast marker, especially during ring and trophozoite stage (Fig. 1F). However, IFA using Mitotracker™ also revealed that, in some rings to schizont-stage parasites, this protein largely localizes to the mitochondria. This supports previous data showing mitochondrial association (23). Our data points at a dual localization and suggests a potential dual/complementary function around/within the apicoplast and the mitochondrion (Fig. 1G).

To determine the importance of *Pf*PNPLA2, we performed growth assays upon presence or absence (+GlcN) of the protein. The reduction of *Pf*PNPLA2 in the mutant line by treatment with 2.5 mM GlcN revealed no significant effect on intraerythrocytic replication during a period of 72 h in regular *in vitro* culture conditions, when compared to untreated *Pf*PNPLA2-HA-KD parasites and GlcN-treated or untreated WT NF54 parasites (Fig. 2A). In addition, quantification of ring, trophozoite and schizont stages at different time points

did not reveal a major effect of GlcN-treatment on parasite stage development of *Pf*PNPLA2-HA-KD and WT NF54 parasites (Sup. Fig. 3), suggesting that the reduction of *Pf*PNPLA2 does not delay the blood stage cycle. (Fig 2A). According to likely predicted essentiality scores for the protein in a piggyBac study of the *P. falciparum* genome, these data were surprising (39). It could be that the incomplete reduction of protein expression was sufficient to allow for parasite survival.

We previously showed that the host nutritional and lipid content can influence the essentiality of a single protein, such as one protein can be non-essential in normal/rich lipid conditions, while becoming essential under scarce nutritional conditions (13). The mild phenotype due to incomplete protein expression reduction prompted us to set up an experiment to analyze this. Parasites were cultivated in minimal media (11) containing the minimal FA requirement for *in vitro* culture, i.e. C16:0 and C18:1 (in combination to FA-free BSA). Interestingly, in these lipid-limiting conditions, the *Pf*PNPLA2-HA-KD line displayed a significant growth delay after 96 hours of intracellular development. This led to a decrease in ring stage parasites (Fig. 2B). In sexual stage parasites, the depletion of this protein did not affect the gametocyte population (Fig. 2C). These data may suggest that PNPLA2 is more important for asexual blood stage development than for gametocytogenesis, and the essentiality of this protein cannot be ruled out, as we were only able to achieve 50% protein reduction. More importantly, this protein seems to be important under nutrient poor conditions and may strongly be dependent on the extracellular environment of the host.

## ***Pf*PNPLA2 depletion differentially affects neutral lipid synthesis in ring and schizont stage parasites**

To investigate the effect the loss of PNPLA2 has on overall parasite lipid metabolism, we conducted global lipidomic analyses using gas-chromatography mass spectrometry on all asexual parasite stages (synchronized ring, trophozoite and schizont stages). It should be noted that despite PNPLA2 depicting essentiality in a lipid poor environment, all lipidomic experiments were conducted in normal culture conditions (with Albumax) as cultures did not survive long enough or yield a high enough parasitaemia when the protein was knockdown under limiting conditions.

We first sought to assess any potential defects at the total lipid content and then quantified major lipid classes of sustaining parasite survival, free FAs (building blocks of most lipids; FFA), diacylglycerols (major lipid precursors, DAG), PLs (major membrane lipids), and triacylglycerols (the main component of lipid storage bodies; TAG).

During ring stage development, the knockdown of *Pf*PNPLA2 in the *Pf*PNPLA2-HA-KD line had no significant effect on the global FA profile from the total parasite lipid content (Fig. 3A). However, further analyses, through separation of neutral and total PLs groups via 1D thin-layer chromatography (TLC), revealed significant increases in all lipid classes constituting neutral lipid content: TAG, FFA and DAG, whereas there was no impact on the total parasite PL content upon *Pf*PNPLA2 knockdown (Fig. 3B). Further analyses to determine the FA composition of each lipid class showed that both FFA and DAG displayed significant increases in their C16:0 and C18:1 content (Fig. 3C-D), which are predominantly scavenged from the extracellular media (10,11).

This also correlates to our findings that growth defects following *Pf*PNPLA2 knockdown occur under low lipid content only containing C16:0 and C18:1. However, no changes could be detected in the FA profiles of TAG or total PL levels (Sup. Fig. 4A-B) under *Pf*PNPLA2 deficiency.

Knockdown of *Pf*PNPLA2 in trophozoite stage parasites exhibited limited changes in the overall FA profile of the total lipid content of the parasite (Fig. 3E), namely a significant increase in C18:0 and a significant decrease in C18:1. Further analyses of parasite lipid classes showed no changes in total neutral lipid content (either FFA, DAG or TAG) and PL content (Fig. 3F). This lack of phenotype might be directly linked to a lower activity of the enzyme during trophozoite stages as its RNA expression is the lowest during trophozoite stages (25).

The most severe effects were observed during schizont stages when the protein is most expressed. First, in the total lipid FA profile, the loss of PNPLA2 in schizont stage resulted in a significant decrease of the relative abundance of palmitic acid (C16:0) (Fig. 3G). Similarly, as in ring stages, knockdown of *Pf*PNPLA2 in schizonts resulted in a significant increase of the storage lipid, TAG. However, unlike in ring stages, the levels of both FFA and DAG were significantly reduced in schizonts (Fig. 3H), but there were no changes in their FA species profile (Sup. Fig. 4C-D). The reduction of *Pf*PNPLA2 levels did not lead to any changes in the relative amount of total PL levels (Fig. 3H). Analysis of the FA content of TAG accumulating in schizont stage in the absence of *Pf*PNPLA2 revealed a significant increase of TAG molecular species containing C16:0 and C18:1 (Fig. 3I), as previously observed in rings, suggesting increased host scavenging. It should also be noted that there were no changes

in the total lipid abundance at any stage, despite seeing clear changes in individual lipid groups (Sup. Fig. 4E).

These data collectively suggest that PNPLA2 is most important for FFA generation/acquisition as well as the synthesis of neutral lipids likely for storage and timely mobilization during the transition from the schizont to the ring stage parasites.

### ***Pf*PNPLA2-deficiency results in an overall increase of PG and decrease of LBPA**

To further investigate the putative phospholipase activity of PNPLA2, notably determine its potential lipid substrates and products, we sought to analyze each PL class and their molecular content in schizonts by 1D or 2D HPTLC coupled to GCMS lipidomic analyses.

The knockdown of *Pf*PNLA2 significantly impacted parasite PL homeostasis (Fig. 4A). There were slight, yet significant reductions in the content of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) that seemed more marginal. PLA2 usually catalyze the hydrolysis of one of the FA moieties from PL, hence generating lysoPL and FA. The levels of major lysolipids, lysophosphatidylcholine (LPC), and lysophosphatidic acid (LPA) were not significantly impacted in the absence of *Pf*PNPLA2, suggesting they might not be the direct products of the enzyme. The most striking change, however, was a significant increase of phosphatidylglycerol (PG). PG is usually a low represented class of membrane PL that is also the substrate for the essential *de novo* synthesis of the mitochondrial-signature lipid class, cardiolipin. However, no change was

observed in the levels of cardiolipin (CL) in the absence of *Pf*PNPLA2, again suggesting CL not being the product of the phospholipase. Interestingly, PG can also be the substrate of PLA2s to generate lysophosphoglycerol (LPG), or LBPA/bismonoacylglycerolphosphate (LBPA/BMP), both of which are important lipid precursors/intermediates also present in apicomplexa in low amounts (13,40–42). LBPA/BMP can also be made from cardiolipin but the usual substrate in mammalian cells is usually PG (40). We thus sought to quantify the levels of LPG and LBPA and determine their potential change in content upon the disruption of *Pf*PNPLA2 (Fig. 4B). Our approach thus reveals that upon the loss of the enzyme, there was a significant accumulation of PG together with a very significant decrease of LBPA, whilst LPG levels were not significantly impacted (though slightly reduced). This significant decrease in LBPA was further confirmed by immunofluorescence (using an anti-LBPA antibody), which showed a significant decrease in fluorescence within the parasite when the protein expression was inhibited by GlcN treatment (Fig. 4C-D). Taken together, the data strongly suggest that *Pf*PNPLA2 is responsible for the degradation of PG to form LBPA, which further induces PL homeostasis disruption (see hypothesis model in Fig. 5).

## **DISCUSSION**

Phospholipases contribute to the virulence of many pathogens, and more specifically to *Plasmodium* parasites (30). This family of proteins is pivotal for PL recycling, which direct a diversity of physiological roles via the regulation of lipid homeostasis for membrane synthesis but also through the control of key signaling pathways that govern host egress, secretion and developmental switching (3,17,30,43–45). Knowing the importance of lipids, membrane

biogenesis and homeostasis for the development and survival of *P. falciparum*, the enzymes manipulating lipids must play central essential parts. Phospholipases are such enzymes as they can hydrolyze membrane PL, either existing from the host or not, to enable the release of FA and LPL that can be used for parasite membrane homeostasis. Interestingly, phospholipases rare metabolic genes located in the sub-telomeric region of *P. falciparum* genome, potentially favoring gene duplication as for *var* and *rifin* surface antigens, reflecting their putative importance for the parasite (46). Their unusually high numbers (at least 22 putative phospholipase genes) compared to typical eukaryotes also reflect their importance for *P. falciparum* (17,45). Their exact roles for the parasite remain poorly understood but recent studies have started revealing differential localization and non-redundant and major functions such as hemozoin formation, parasite division or gametocyte formation (30,44,47,48). The few that have been investigated also show potential as drug targets (49,50). To date however, the exact functions and mechanisms of most phospholipases remain to be unraveled.

As with PC and LPC, PL and lysoPL balances in the parasite can govern their cell development and are at the mercy of phospholipase (3,17). This study focused on a putative phospholipase enzyme containing a patatin-like phospholipase domain (Fig. 1A). We generated an inducible knock-down line for *PfPNPLA2* (*PF3D7\_1358000*) using the glmS-ribozyme system (51), but were unable to achieve complete protein degradation. This could suggest why we did not see a decrease in parasite viability as previously described in Burda *et al.*, (2021). We revealed that *PfPNPLA2* also seems to be important for parasite replication in asexual parasites under nutrient stress, specifically when

exogenous lipids are limited (Fig. 2A). This seems to be in accordance to the essentiality data from a genome-wide saturation mutagenesis screen in *P. falciparum* (52).

PNPLA1 (PF3D7\_0209100), has been previously identified as critical for gametocyte induction (30) and the process of gametocytogenesis itself (48,49). *Pf*PNPLA2-HA-tagged parasites in this study have been able to prove dual/proximal localization of this protein to both the mitochondria and the apicoplast of the parasite. This is supported by the predicted mitochondrial targeting sequence found in N-terminal end of the protein (48). These data correlate to the decreased transcription of this protein in gametocytes and could suggest this protein is not critical during sexual blood stages (25).

Localization of the enzyme to the apicoplast might be explained by the close vicinity and the shared metabolic pathways between the this organelle and the mitochondrion (53,54). Currently are there is no known shared lipid metabolism pathways between the two organelles, even if this could be occurring, notably for potential lipid exchange between them. The *T. gondii* homologue of *Pf*PNPLA2 (*Tg*PL2) does solely localize to the apicoplast and is involved in apicoplast biogenesis and parasite lipid homeostasis, more precisely by affecting levels of PC and LPC (55). The proximity to the mitochondria suggests that *Pf*PNPLA2 could be involved in mitochondrial membrane biogenesis through the generation of FA and lysoPL precursors. We hypothesized that this could be linked to cardiolipin synthesis as it only occurs at the mitochondrial level (56). The full cardiolipin synthetic pathway is yet to be characterized in *P. falciparum*, but in other eukaryotes, it requires PG as a substrate (57). Lipidomics revealed that PG was the most strikingly affected PL class in the

absence of *Pf*PNPLA2, yet without significantly affecting cardiolipin levels. The disruption of the protein resulted in the accumulation of PG instead, unlike in the knockout of the *T. gondii* homologue of *Pf*PNPLA2, which displayed increased levels of PC (55). However, PG, and or LBPA can be degraded through the action of PLA2s to form LBPA (i.e. BMP-bis(monoacylglycerol)phosphate). In eukaryotes, LBPA is a lipid class normally involved in slow catabolism, such as degradation of lipid stores for energy production in nutrient poor conditions (58). LBPA is negatively charged at the acidic pH of lysosomes, and these charges are central to its role in the degradation of lipids and membranes in the lysosome. In effect, these negative charges facilitate the adhesion of the soluble positively charged hydrolases and activator proteins (such as PLAs), thus allowing them to degrade the lipids at the interface of the inner membranes of the lysosome (57). Here, our results indicate that *Pf*PNPLA2 acts on the mitochondrial degradation of PG to form LBPA. Whether the synthesis of LBPA is important for blood stage parasite survival is an open question.

Another important point is that the most significant growth phenotype occurred in low host lipid environment. We and others showed that host nutrient levels directly influence parasite metabolic activity, especially lipid metabolism, which can be rewired/adapted by the parasite upon host environment (3,19,24,59,60). Indeed, *de novo* lipid synthesis and host scavenging activities can be used alternatively to compensate one another (13,19). Host lipid scavenging constitutes the major source of parasite lipids and can be upregulated if *de novo* synthesis is insufficient. Scavenged lipids, specifically their FA moieties, are usually channeled towards lipid storages, usually lipid droplets, in the form of

TAG, which can be timely mobilized for membrane biogenesis during parasite division (19,50). Here, we see that disrupting *PfPNPLA2* affects parasite growth only under low host lipid environment, indicating that the enzyme likely plays a role to boost lipid synthesis when scavenging is scarce (Fig. 5). This is further correlated by the increase in C16:0 and C18:1 that are accumulating in TAG during schizont, and ring stages in the absence of *PfPNPLA2* in high host lipid content. This suggests that the parasite increases scavenging and lipid storage to compensate for the loss of *PfPNPLA2*, as previously observed for the loss of another *P. falciparum* lysophospholipase, *PfLPL3* (50). This is further correlated by the additional accumulation of all neutral lipids, TAG, DAG and FFA during ring stages, reflecting the accumulation of scavenged material normally channeled through the storage lipid synthetic pathway, as previously observed (19,50).

One possible hypothesis (Fig. 5) for the reduction of parasite population in the absence of *PfPNPLA2* under low host lipid content is that, similarly as in the *PfLPL3* mutant, the parasite does not have enough supply of FA by the degradation of its lipid substrate, in this case, PG. This, in return, could prevent the parasite lipid synthesis machinery to form PLs for parasite membrane biogenesis during division, hence reducing progenies and overall parasitaemia. This is further supported by the significant decrease of major PL classes PC, PE, PI and PG when *PfPNPLA2* is absent (Fig. 5). A potential explanation is that the degradation of PG can provide FA moieties for the synthesis of major lipid classes when host lipid resources are scarce, hence enabling optimal progeny formation even under sub-optimal physiological conditions.

Taken together, our results point to the importance of *Pf*PNPLA2 to maintain proper parasite lipid homeostasis during blood stage replication, especially under low host nutrient content. It also points to the diversity and importance of the phospholipase activities that the parasite has developed and uses to maintain optimal asexual division and likely for other life stages. The number and diversity of them advocate for their importance and probable redundancy as well. Antibodies raised in this study against native putative phospholipases revealed differential localization of phospholipase, suggesting a redundancy/varied functionality (Fig. 1C-G). Phospholipase functional redundancy is well known and illustrated in *Listeria monocytogenes*, where mutation of a single phospholipase C resulted in moderate effects on mice infectivity and was severely enhanced when both were disrupted (61). This interplay of phospholipases could be extrapolated to malaria parasites, but further gene-knock out studies are needed to understand the essentiality of this family of proteins.

We investigated 3 different putative patatin-like phospholipase enzymes, all which had different cellular localization and did not co-localize. This may suggest that each phospholipase has a non-redundant function. Further characterization of the other phospholipases must be done to prove this hypothesis.

### **DATA AVAILABILITY STATEMENT**

All data generated or analyzed during this study are included in this published article.

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## TABLES

Table 1: Primers used in this study

### Primer sequences (5'-3')

#### ***PF3D7\_1358000***

Cloning for pARL-HA-gImS	Fw primer	TAGCGCGGCCGCGGAAGAAGGCGATAAATTGATCT	Restriction site: NotI	Amplified fragment size:	1015 bp
	Rev primer	TACGCCCTAGG7GTTTCTTTTTAAAAAGAAACATCTTAATAAGT	Restriction site: AvrII		

Integration-PCR of the KD					
5' Integration	Fw primer	TAAGAAGAAGAAGAAATTAATTCCTCC		Amplified fragment size:	1173 bp
	Rev primer	CCTTAGAGCTCGGCATAATCTGG			
3' Integration	Fw primer	GCTTTACACTTTA7GCTTCCGGCTCG		Amplified fragment size:	1578 bp
	Rev primer	TTACTTTTTTTTAATATCATTTTTACGTCC			

Episome	Fw primer	GCTTTACACTTTA7GCTTCCGGCTCG		Amplified fragment size:	1250 bp
	Rev primer	CCTTAGAGCTCGGCATAATCTGG			
WT locus	Fw primer	TAAGAAGAAGAAGAAATTAATTCCTCC		Amplified fragment size:	1501 bp
	Rev primer	TTACTTTTTTTTAATATCATTTTTACGTCC			

Rec. Protein for antibody	Fw primer	GCGTAGAATTCTCGATTAACATGGGAT7GATG	Restriction site: EcoRI	Amplified fragment size:	1284 bp	AA 571 - 998	vector: pIH902 (MBP-Tag)
(#359)	Rev primer	TACGCCTGCAGTTAGACACTTAAAGGAGGTTCTTC	Restriction site: PstI				

RT-PCR	Fw primer	CGAATTTTATGTCTAGATGG		Amplified fragment size:	250 bp
	Rev primer	ATCTGGTATTTCTTACAGCA			

**PF3D7\_09240****00**

Rec. Protein for antibody	Fw primer	GCGTACCATGG <b>ATG</b> CCACTTACCAAAAATAATGAAAAC	Restriction site: NcoI	Amplified fragment size:	525 bp	AA 589 - 763	vector: pET28c(+) (His-Tag)
(#378)	Rev primer	TACGCCTCGAGCATAAATCTATACACATTTTCTTT	Restriction site: XhoI				

RT-PCR	Fw primer	AATACTATGGATTTTCAAGATAGCTT		Amplified fragment size:	250 bp
	Rev primer	CATCAATTCATTTCTAATACTCGA			

**PF3D7\_02186****00**

Cloning for pARL-HA-glmS	Fw primer	TAGCGCGGCCG <b>CCT</b> CGCATCGATTGAATATGCTG	Restriction site: NotI	Amplified fragment size:	840 bp
	Rev primer	TACGCCCTAGGAAAACCAAAATATCCAGATAA	Restriction site: AvrII		

Integration-PCR of the KD					
5' Integration	Fw primer	ATGATTTAGGTAGGAGCGATGCTC		Amplified fragment size:	1120 bp
	Rev primer	CCTTAGAGCTCGGCATAATCTGG			
3' Integration	Fw primer	GCTTTACACTTTATGCTTCCGGCTCG		Amplified fragment size:	1160 bp
	Rev primer	TGATAACTTCAAAAATAACATTATG			
Episome	Fw primer	GCTTTACACTTTATGCTTCCGGCTCG		Amplified fragment size:	1075 bp
	Rev primer	CCTTAGAGCTCGGCATAATCTGG			
WT locus	Fw primer	ATGATTTAGGTAGGAGCGATGCTC		Amplified fragment size:	1205 bp
	Rev primer	TGATAACTTCAAAAATAACATTATG			

Rec. Protein for antibody	Fw primer	TAGCGAAGGATTTCA <b>TG</b> TTTGAGCGTAGGATTAAGT	Restriction site: XmnI	Amplified fragment size:	424 bp	AA 704 - 804	vector: pMalc5x (MBP-Tag)
(#451)	Rev primer	TAGCCTGCAGATAATCAGAATCAAACGGAGATAC	Restriction site: PstI				

Rec. Protein for antibody	Fw primer	TAGCGAAGGATTTCTGGTAATACGAACAGAAAAGATATA	Restriction site: XmnI	Amplified fragment size:	561 bp	AA 2 - 187	vector: pMalc5x (MBP-Tag)
(#460, #461)	Rev primer	TAGCCTGCAGTTAAATTTCTATTTTACATTTGTAGTATC	Restriction site: PstI				

RT-PCR	Fw primer	CATAATACACATAATATACATAATACACA		Amplified fragment size:	250 bp
	Rev primer	AACAACATGCACAGATACTAC			

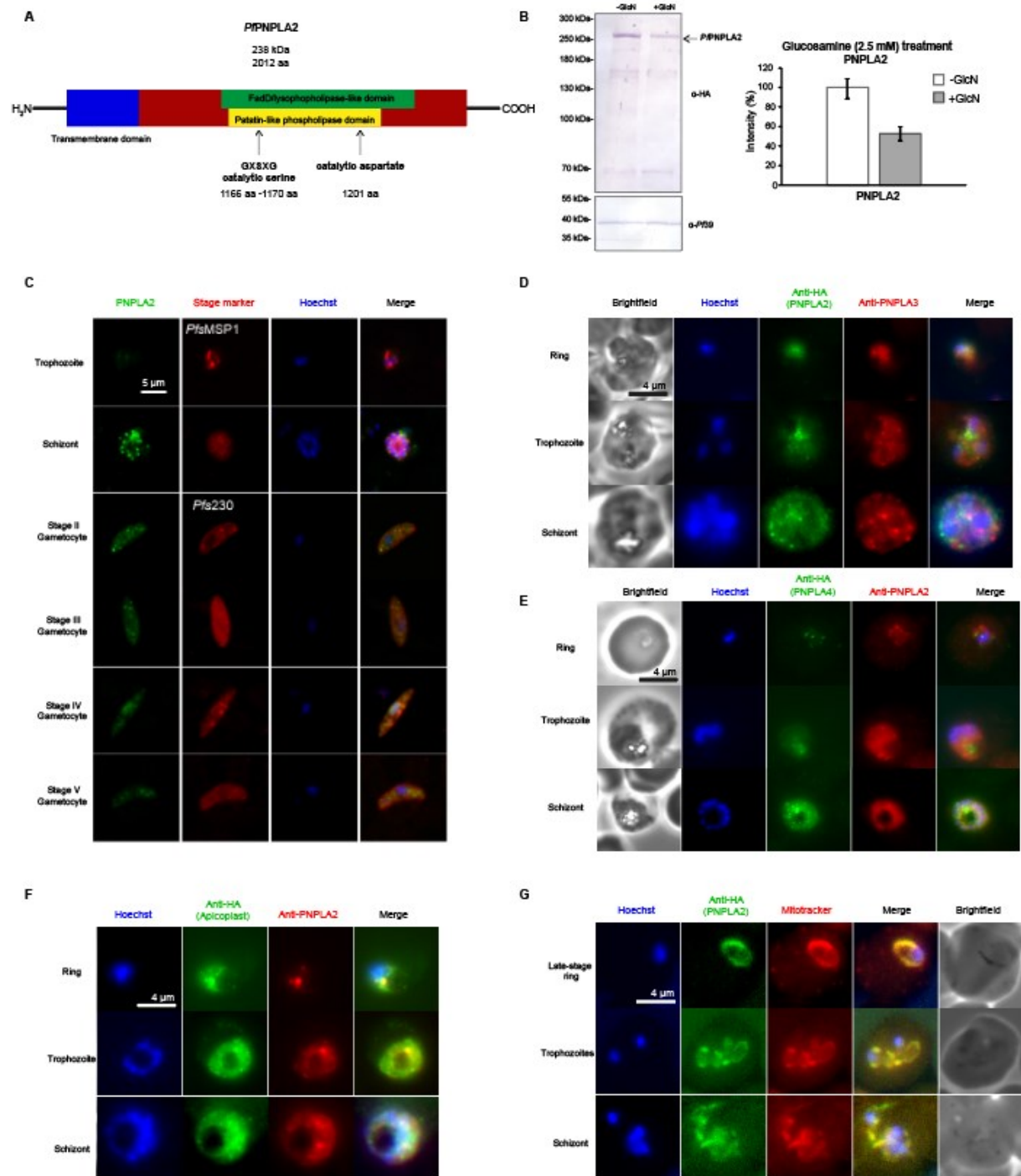
### RT-PCR controls

Ama1	Fw primer	CGGTAGCTACGGGAAATCAA		Amplified fragment size:	407 bp
	Rev primer	AGGGCAAACTTTTCCAGT			

CCp2	Fw primer	AGTTGTTGATGGGCTTTTGG		Amplified fragment size:	286 bp
	Rev primer	ATTCGGTGCCATTAGGGTTA			

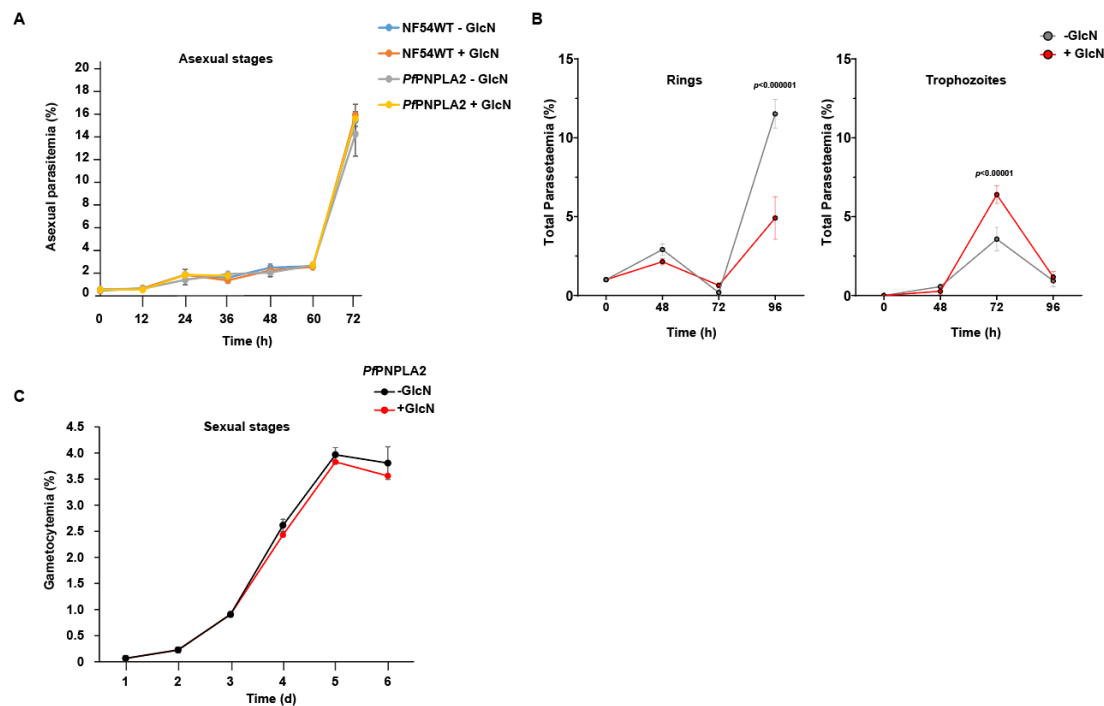
Aldolase	Fw primer	TAGATGGATTAGCAGAAAGATGC		Amplified fragment size:	378 bp
	Rev primer	AGAAACCAACCATCTTGAGTAGTGG			

## FIGURES AND FIGURE LEGENDS



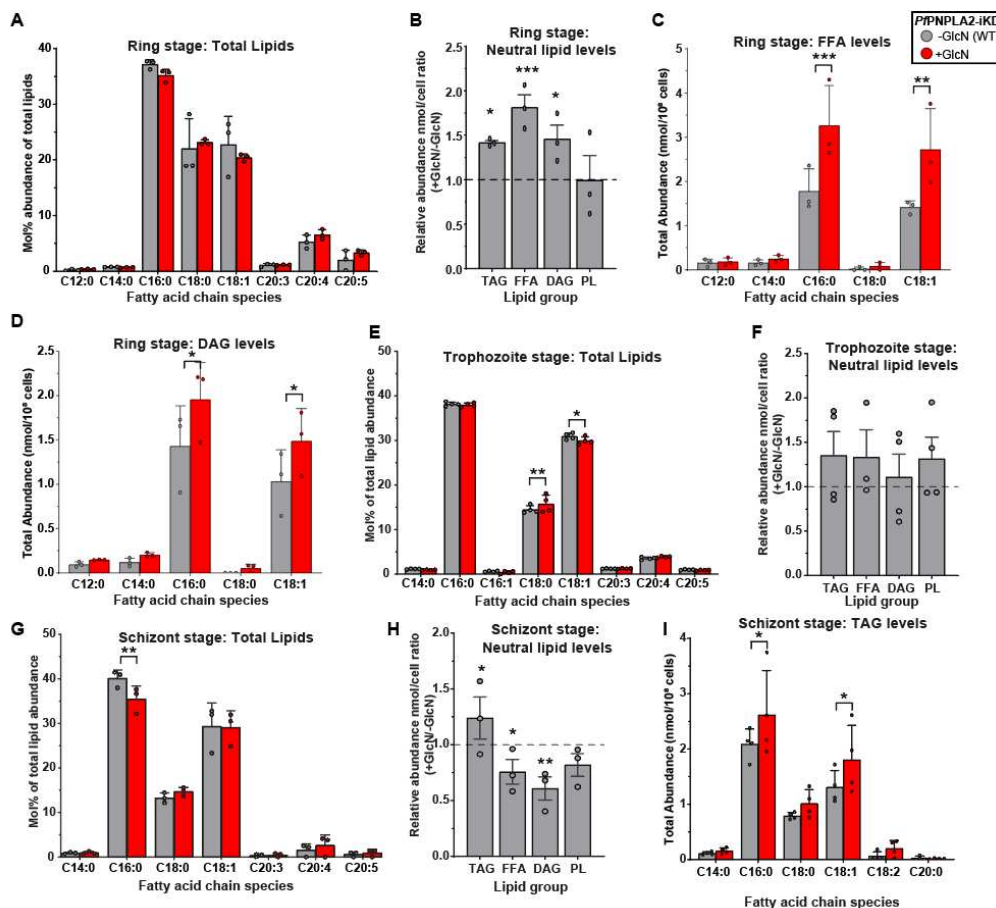
**Figure 1: *Pf*PNPLA2 localizes to the apicoplast and mitochondria during different parasites stages.** (A) A putative patatin-like phospholipase, *Pf*PNPLA2

(PF3D7\_1358000) contains a FabD-like domain (green) and a patatin-like phospholipase domain (yellow) along with the GX SXG-motif and catalytic aspartate characteristic of this family of proteins. (B) Downregulation of *Pf*PNPLA2-HA levels. Schizont cultures of the *Pf*PNPLA2-HA-KD parasite line were treated with 2.5 mM GlcN for 72 h to determine knockdown of *Pf*PNPLA2. Lysates were immunoblotted with rat anti-HA antibody to detect *Pf*PNPLA2-HA (~240 kDa); immunoblotting with rabbit anti-*Pf*39 antisera (39 kDa) was used as loading control (left panel). Quantification of *Pf*PNPLA2-HA levels following knockdown. *Pf*PNPLA2-HA levels were evaluated between the GlcN-treated and untreated *Pf*PNPLA2-HA-KD parasites in three independent Western blots based on the band intensity as estimated with ImageJ software (right panel). *Pf*39 levels were used for normalization (set to 100%). (C) Using a mouse anti-*Pf*PNPLA2 antibody (green) antibody for immunolabelling, *Pf*PNPLA2 is detectable in perinuclear regions in schizonts and gametocytes of stages II-V. Counter labeling of the asexual blood stages was done using rabbit anti-*Pf*MSP1 antisera and gametocytes with rabbit anti-*Pf*s230 antisera (red). The nuclei were labeled with Hoechst 33342 nuclear stain (blue). Bar; 5  $\mu$ m. (D) *Pf*PNPLA2-HA, as detected by rat anti-HA antibody (green) does not colocalize to *Pf*PNPLA3 (red), which was immunolabelled using a mouse anti-*Pf*PNPLA3 antibody, and (E) *Pf*PNPLA4-HA (green), detected by rat anti-HA antibody, does not localize with *Pf*PNPLA2, immunolabelled with a mouse anti-*Pf*PNPLA2 antibody (red). (F) *Pf*PNPLA2 has an apicoplast (green) co-localization in ring stage parasites using the TPT-HA line using an anti-PNPLA2 antibody (red). (G) Co-localization microscopy using Mitotracker (red), revealed that PNPLA2 (green) also shows to be clearly present in the mitochondria during asexual life stage parasites.



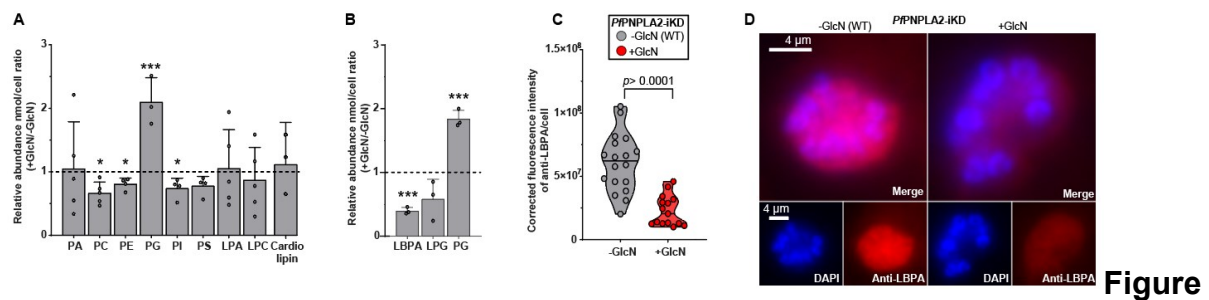
**Figure 2: *PfPNPLA2* is important for blood stage replication under limiting lipid conditions.** (A) Asexual blood-stage replication following *PfPNPLA2* knockdown. A highly synchronized *PfPNPLA2*-HA-KD parasites ring stage culture was set up with a parasitemia of 0.25% and treated with 2.5 mM GlcN. The parasitemia was determined by Giemsa smears at 0 h, 24 h, 48 h, and 72 h. Untreated *PfPNPLA2*-HA-KD cultures as well as GlcN-treated and untreated WT NF54 cultures were used as controls. (B) In a lipid scarce environment (only C18:1 and C16:0), the depletion of *PfPNPLA2* displays a reduction in ring stage parasites after 96 hours (left) and in trophozoite stage parasites after 72 hours (right) of glucosamine (GlcN) treatment. (C) Gametocyte development following *PfPNPLA2* knockdown. A synchronized parasite culture of the *PfPNPLA2*-HA-KD line was treated with 2.5 mM GlcN for 72 h, and gametocytogenesis was subsequently induced with lysed blood cells while the GlcN treatment was continued. The gametocytemia was evaluated by Giemsa smears over a period of six days. Untreated *PfPNPLA2*-HA-KD cultures were used as controls.





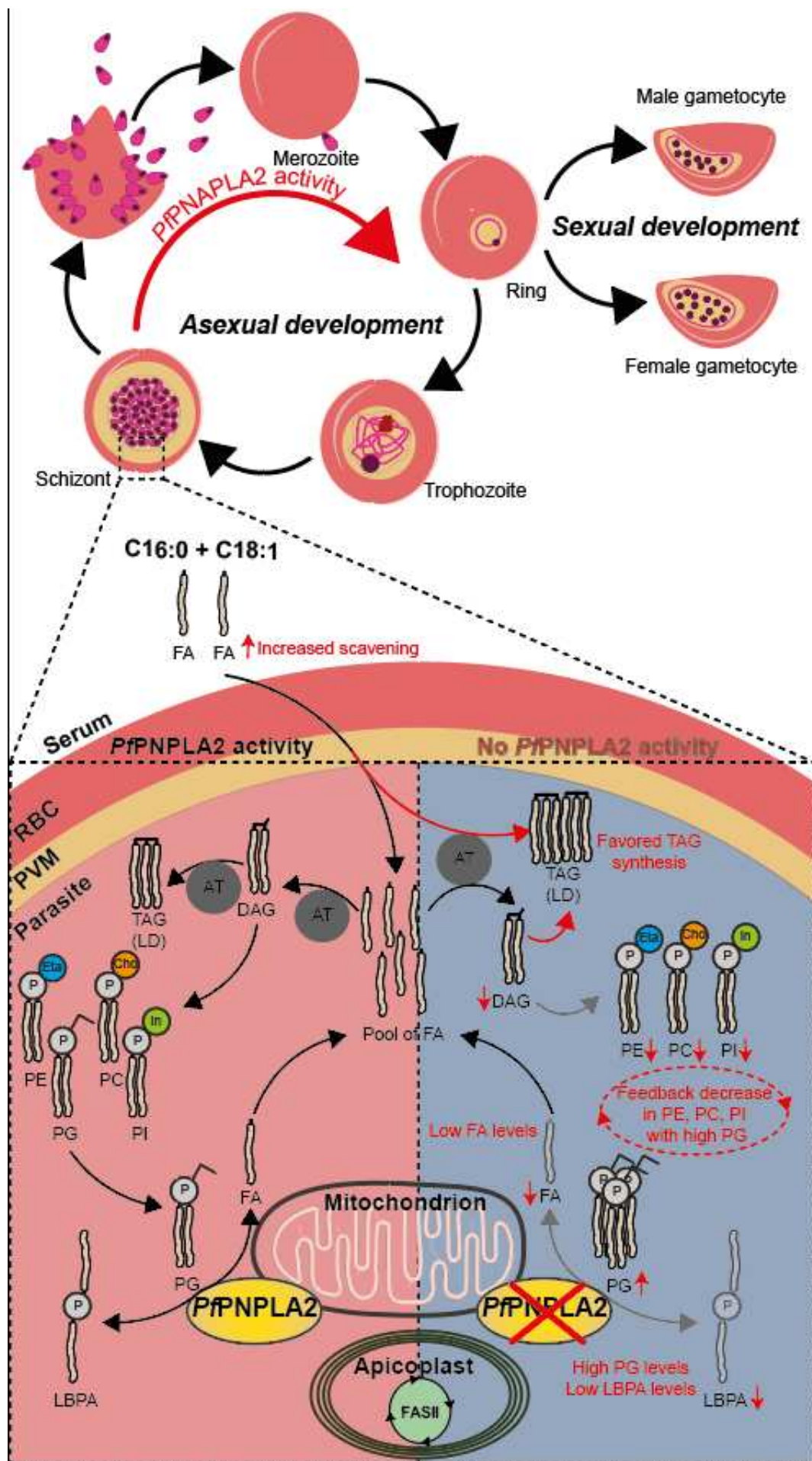
**Figure 3: *Pp*PNPLA2 depletion influences the lipidomic profile of asexual stage parasites.** (A) Under the depletion of *Pp*PNPLA2 (+GlcN, red) the lipid profile of ring stage parasites is not altered. (B) The relative neutral lipid content (TAG-triacylglycerol, FFA-free FAs, DAG-diacylglycerol) was increase when PNPLA2 was inhibited, but the PL levels remained unaltered. (C) FFA and (D) DAG total abundance levels increased in ring stage parasites specifically for C16:0 and C18:1 fatty acyl chain species. (E) In trophozoite stage parasites the total lipid profile shows a significant mol% increase in C18:0 and decrease in C18:1, with no changes in (F) total neutral lipid abundance under the depletion of PNPLA2. (G) In schizont stage parasites the depletion of this proteins results in a decrease in the mol% of C16:0. (H) There was an increase in overall TAG levels and a decrease in FFA and DAG levels

under *Pf*PNPLA2 knockdown. (I) TAG FA profiles were altered in schizonts under depletion of PNPLA2 as displayed by increases in C16:0 and C18:1.



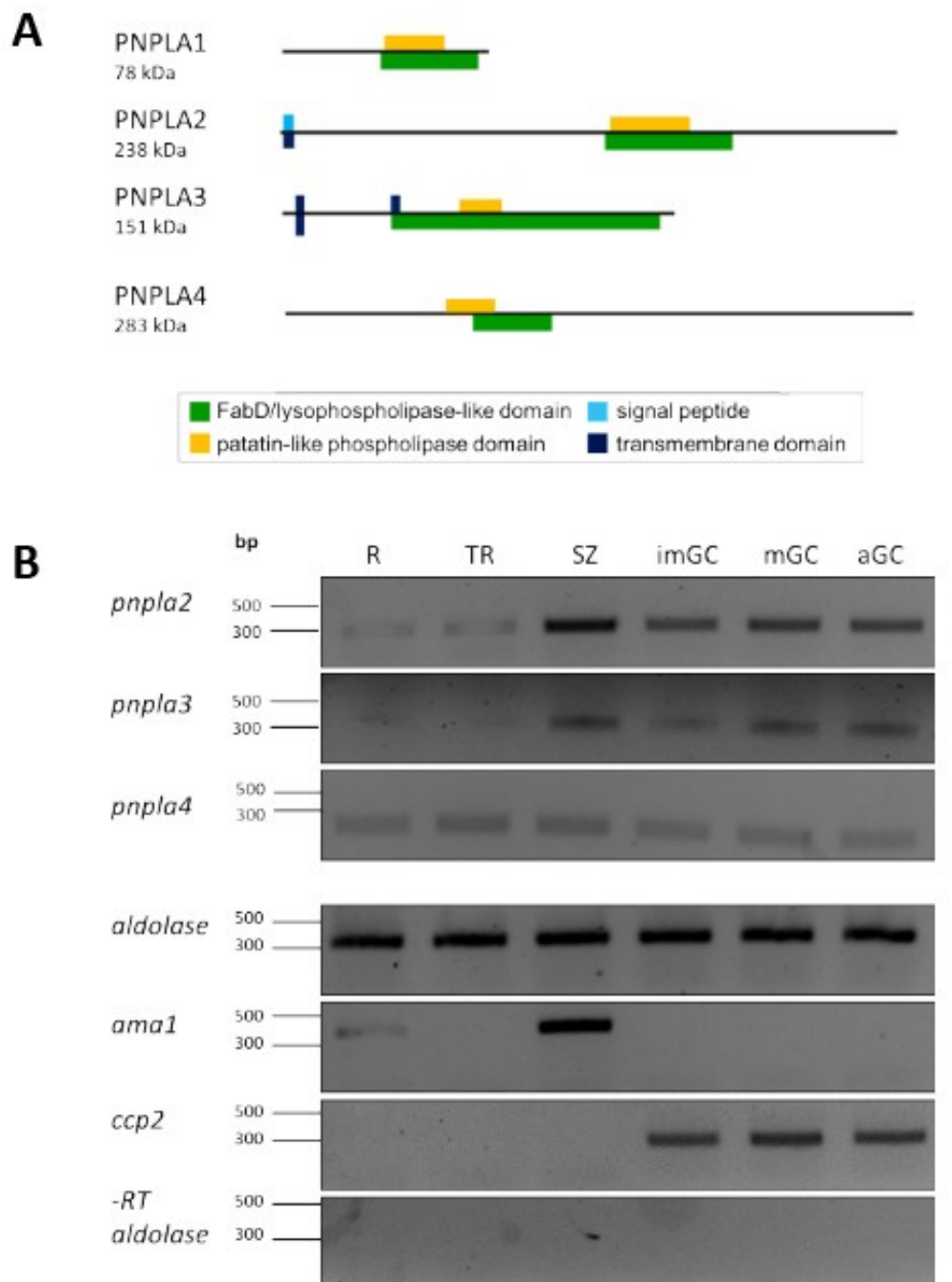
**Figure**

**4: *PPNPLA2* is involved in the hydrolysis of phosphatidylglycerol to lysobiphosphatidic acid (n=3).** (A) Individual phospholipid species (separated by 2D-thin layer chromatography) were analyzed by GCMS and revealed that *PPNPLA2* reduction resulted in an increase in the relative abundance of (+Glucosamine (GlcN)/-GlcN) phosphatidylglycerol (PG) and a decrease in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI). (B) Closer analyses shows a decrease in relative abundance of lysobiphosphatidic acid (LBPA) and not lysophosphatidylglycerol (LPG). (C) The decrease in LBPA was additionally confirmed by an (D) anti-LBPA antibody immunofluorescence assay which showed a decrease in corrected total cell fluorescence under glucosamine (GlcN) treatment (red, protein knock-down). These data were determined using fluorescence intensity values from ImageJ.



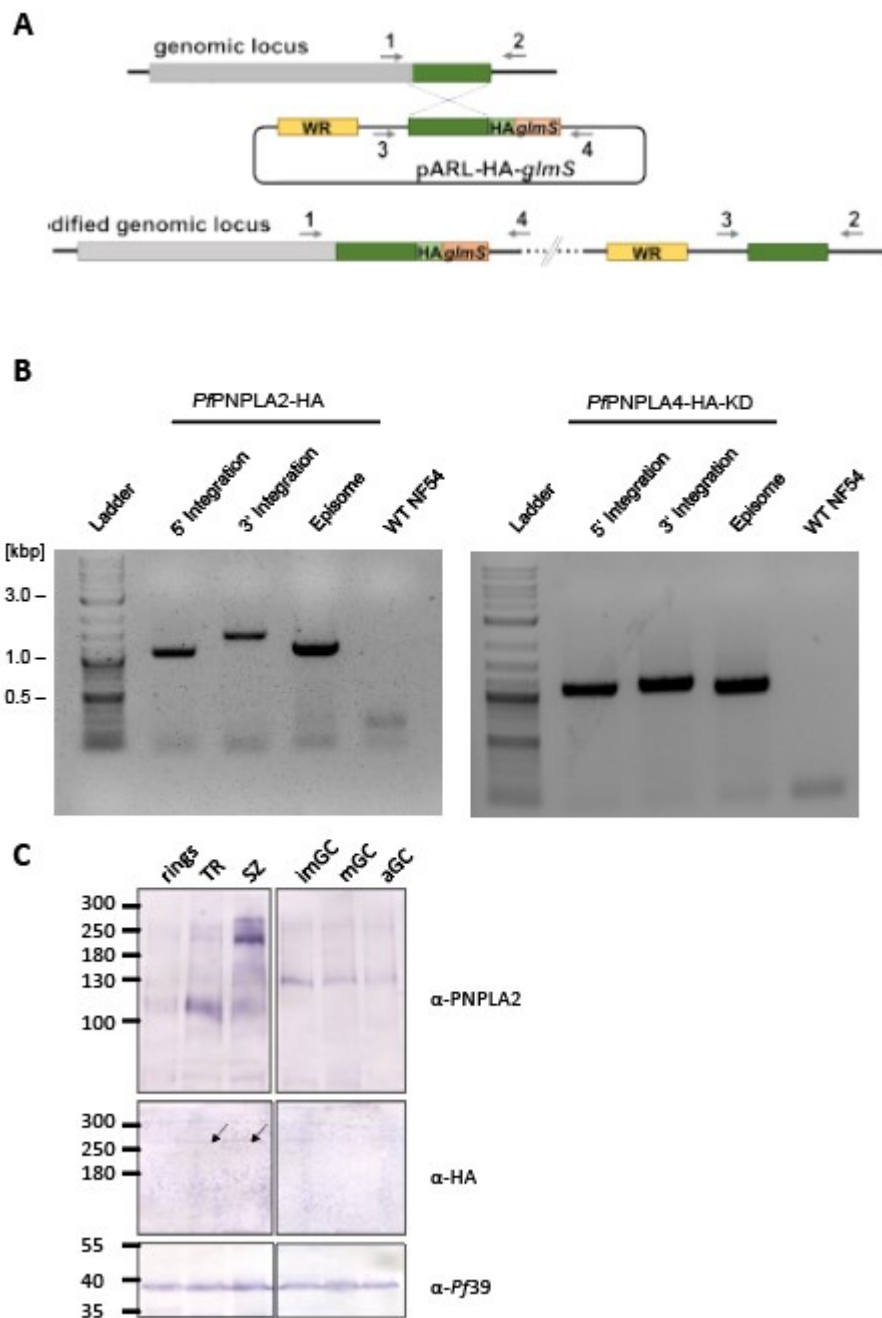
**Figure 5: *Pf*PNPLA2 is involved parasite development via the conversion of PG to LBPA.** Normal *Pf*PNPLA2 activity (during schizont and ring stages near the mitochondria) facilitates the utilization of phosphatidylglycerol (PG) into free FA and LBPA/BMP. Pool of FAs generated by FASII, host scavenging and this process can then contribute to lipid storage generation (triacylglycerol, TAG) and PL synthesis via the CDP-DAG pathway (PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol). In the absence of *Pf*PNPLA2 PG levels are increased with lowered levels in LBPA and FFA. We hypothesize that the lack of sufficient FFA prompts the parasite to increase host FFA scavenging of C16:0 and C18:1. This does not restabilize FFA to normal and instead TAG formation (LD: lipid droplet) is favored to escape lipotoxic death. The increase in PG has a negative feedback effect and PL synthesis (PC, PI, PE) is inhibited.

## SUPPLEMENTARY INFORMATION



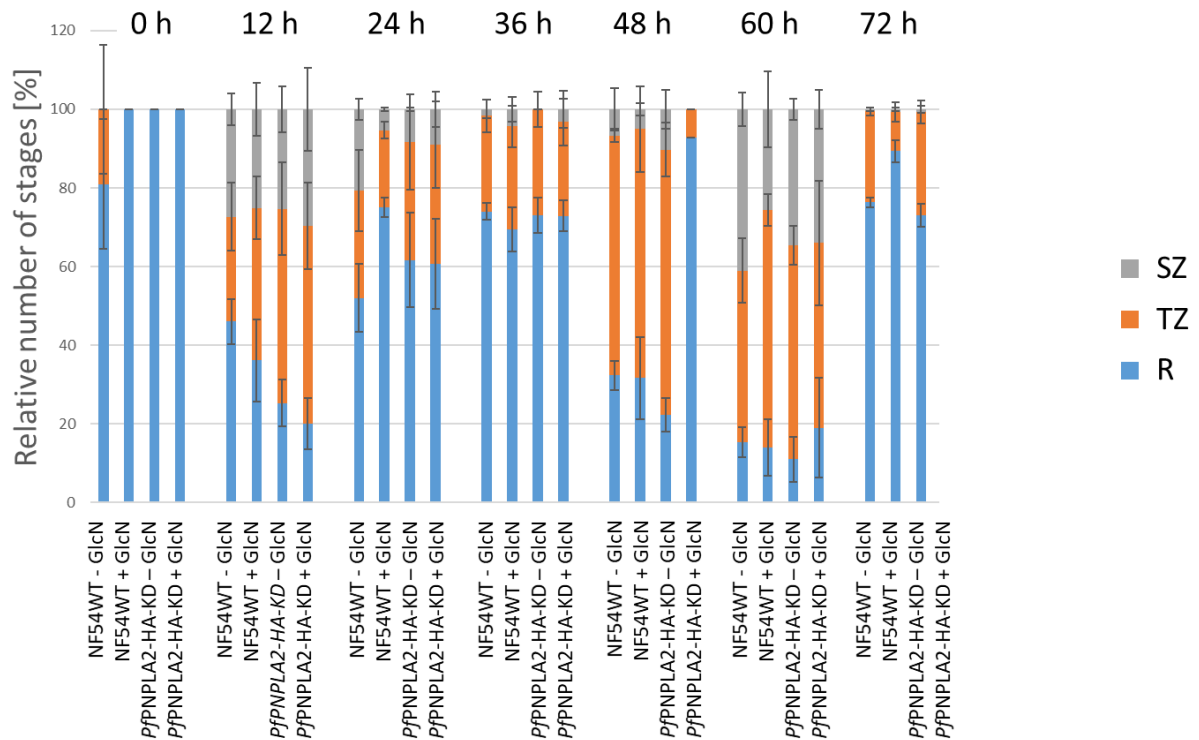
**Supplementary Figure 1: The PNPLA family of *P. falciparum*.** (A) Schematic depicting the four members of the *P. falciparum* PNPLA family. (B) Transcript expression of *Pf*PNPLA2-4 in the asexual and sexual blood stages. Diagnostic RT-PCR was used to amplify transcript of *Pf**pnpla2* (250 bp), *Pf**pnpla3* (250 bp), and *Pf**pnpla4* (250 bp) from cDNA generated from total RNA of rings (R), trophozoites (TR),

schizonts (SZ), as well as immature gametocytes (imGC; stages II-IV), mature gametocytes (mGC; stage V) and activated gametocytes (aGC; 15' p.a.). Transcript analysis of *ama1* (407 bp) and *ccp2* (286 bp) were used to demonstrate purity of the asexual and sexual blood stage samples. Transcript analysis of *aldolase* (378 bp) was used as loading control, RNA samples lacking reverse transcriptase (-RT) were used to prove the absence of gDNA. For transcript expression of *Pfprnpla1*, see Flammersfeld *et al.*, 2020.

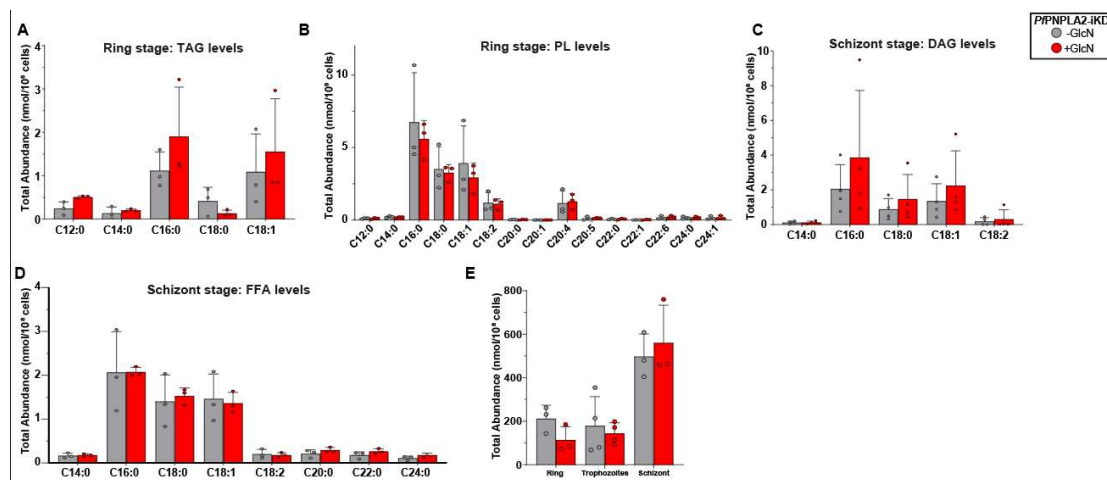


**Supplementary Figure 2: Generation of the *Pf*PNPLA2-HA-KD and *Pf*PNPLA4-HA-KD lines.** (A) Schematic depicting the single-crossover homologous recombination strategy for the generation of the *Pf*PNPLA2-KD and *Pf*PNPLA4-KD lines. The coding region of *Pf**pnp*la2 or *Pf**pnp*la4 was fused at the 3'-region to a HA-encoding sequence followed by the *glmS*-ribozyme sequence. The numbered arrows indicate positions of primers used to confirm integration of the pARL-HA-*glmS* vector. WR, *hDHFR* gene conferring resistance to WR99210. (B) Confirmation of gene locus

integration of the pARL-HA-*gImS* vector. Diagnostic PCR using gDNA of the *Pf*PNPLA2-HA-KD and the *Pf*PNPLA4-HA-KD lines demonstrates successful 5' (primers 1 and 4; 1,182 bp for *Pf*PNPLA2-HA-KD; 1,129 pb for the *Pf*PNPLA4-HA-KD) and 3' (primers 2 und 3; 1,575 fpr *Pf*PNPLA2-HA-KD; 1,166 bp for the *Pf*PNPLA4-HA-KD) integration. As a control, amplification of WT NF54 gDNA was aimed (primers 1 and 2; 1,501 bp for the *Pf*PNPLA2-HA-KD; 1,205 bp for the *Pf*PNPLA4-HA-KD). Episomal DNA was further detected (primers 3 and 4; 1,250 bp for the *Pf*PNPLA2-HA-KD; 1,075 for the *Pf*PNPLA4-HA-KD). (C) Western blot analyses of rings, trophozoites (TR), schizonts (SZ), immature gametocytes (imGC, stage I-IV), mature gametocytes (mGC) and activated gametocytes (aGC, 30 min p. a.) of the (untreated) PNPLA2-KD using mouse anti-PNPLA2, anti-HA and anti-*Pf*39.



**Supplementary Figure 3: The effect of *PfPNPLA2*-HA deficiency on asexual blood stage development.** Synchronized ring stage cultures of WT NF54 and the *PfPNPLA2*-HA-KD parasite line were treated with 2.5 mM GlcN at a starting parasitemia of 0.25% and the cultures were maintained in cell culture medium with GlcN over a time-period of 72 h. Rings (R), trophozoites (TZ), and schizonts (SZ) were determined in a total number of 100 iRBCs in triplicate every 24 h via Giemsa-stained blood smears.



**Supplementary Figure 4: *Pf*PNPLA2 depletion influences the lipidomic profile of asexual stage parasites.** Under the depletion of *Pf*PNPLA2 (+GlcN, red) the lipid profile (in nmol/cell) of (A) ring stage TAG and (B) PL levels remain unaltered. The same is true for (C) schizont stage DAG and (D) FFA levels under depletion of the protein. (E) No changes in Total lipid abundance were seen in any asexual stages of the parasite.

**CHAPTER 6: *TOXOPLASMA GONDII*  
ACYLTRANSFERASES, *TGGPAT* AND *TGDGAT2*, ARE  
INVOLVED IN THE BALANCE BETWEEN BULK  
MEMBRANE BIOGENESIS AND LIPID STORAGE  
DEPENDENT ON HOST FATTY ACID FLUXES**

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### ***Summary of manuscript:***

This work described my final thesis project which aims to characterize the cellular biochemical role of putative acyltransferases (AT), specifically glycerol-3-phosphate acyltransferase (*TgGPAT*) and diacylglycerol acyltransferase 2 (*TgDGAT2*). This project reiterates the metabolic flexibility of these parasites as the global theme of my thesis work. We developed two inducible lines of the above-mentioned proteins and there after conducted a series of experiments to determine their importance under different nutrient conditions.

The major findings of this study are:

- *TgGPAT* is the eukaryotic homolog of the apicoplast and plant-like *TgATS1*
- *TgDGAT2* is previously uncharacterized and is not like *TgDGAT1*
- *TgGPAT* and *TgDGAT2* are in close association with the endomembrane network of the parasites.
- *TgGPAT* is a critical protein needed for parasite development, which is responsible for lysophosphatidic acid (LPA) formation, which is pivotal to membrane biogenesis and neutral lipid synthesis.
- *TgGPAT* is essential under starved **and** excessive FBS conditions, unlike other ATs like *TgAGPAT* or *TgATS2* (which are responsible for the second esterification of a FA to the glycerol-3-phosphate backbone forming phosphatidic acid)
- This suggests that GPATs' (*TgGPAT* and *TgATS1*) catalytic roles govern an indispensable reaction for parasite survival
- *TgDGAT2* on the other hand, only seems important in low nutrient conditions and is crucial for lipid droplet (LD) biogenesis.

- In this study we also show that the deletion of this protein and a paralogue protein, *TgDGAT1*, leads to an increase in parasite development suggesting that these proteins play a collective role in controlling parasite progression
- The loss of these proteins both result in an accumulation of free FAs that cannot be 'detoxified' by incorporating them into LDs, leading to parasite death.
- The essentiality of these two proteins was nutrient dependent showing that the parasite can reprogram its metabolic activity and adapt to a changing host environment

In summary, we identified that the first esterification step of the ER glycerolipid pathway forming LPA is crucial for parasite propagation. We also show that LD biogenesis/metabolism is crucial under low nutrients as these storage moieties serves as indispensable deposits of fuel for parasites. Finally, the nutrient-dependency of these proteins provides insight into the parasite's nutrient flexibility, in that they can adapt to different environmental conditions rendering proteins that were once non-essential, essential, or vice-versa.

## ***Résumé du manuscript:***

Ce manuscrit décrit mon dernier projet de thèse, qui vise à caractériser le rôle d'acyltransférases putative, la glycérol-3-phosphate acyltransférase (*TgGPAT*) et la diacylglycérol acyltransférase 2 (*TgDGAT2*). Ce projet renforce la thématique de flexibilité métabolique retrouvée chez *Toxoplasma gondii* abordé au cours de ma thèse. Nous avons développé des lignées parasitaires permettant l'expression inductible des protéines mentionnées pour étudier leur activité dans des conditions nutritives variées.

Les résultats principaux sont les suivants :

- *TgGPAT* est la protéine eucaryote homologue à la protéine *TgATS1* retrouvé dans l'apicoplaste.
- *TgDGAT2* n'est pas caractérisée mais diffère de *TgDGAT1*
- Ces deux protéines sont associées au réseau endomembranaire du parasite
- *TgGPAT* est essentielle au développement du parasite et permet la formation d'acide lysophosphatidique qui joue un rôle clé dans la biogenèse des membranes et des lipides neutres.
- *TgGPAT* est essentielle en conditions de stress nutritive et en situation d'excès nutritif, à l'inverse des autres acyltransférases comme *TgAGPAT* or *TgATS2* (qui sont responsables de la seconde étape d'estérification des acides gras au squelette de glycérol-3-phosphate pour former des acides phosphatidiques).
- Ces observations suggèrent que le rôle catalytique des GPATs (*TgGPAT* and *TgATS1*) gouverne une réaction indispensable à la survie du parasite.
- *TgDGAT2* en revanche semble seulement importante en conditions de stress nutritif est permet la formation de gouttelette lipidiques.

- Dans cette étude, nous montrons également que la délétion de cette protéine et de son paralogue *TgDGAT1* favorise le développement du parasite, suggérant un rôle conjoint dans le contrôle de la croissance parasitaire.
- La délétion de chacune de ces protéines mène à l'accumulation d'acides gras libres qui ne peuvent pas être incorporés dans les gouttelettes lipidiques et deviennent toxiques pour le parasite.
- Ces deux protéines sont essentielles seulement dans des conditions nutritives spécifiques, indiquant que le parasite peut moduler son activité métabolique et s'adapter aux changements environnementaux.

En résumé, nous avons montré que le déroulement de la première étape d'estérification de la voie métabolique formant les acides lysophosphatidiques est indispensable à la propagation du parasite. Nous montrons également que le métabolisme et la biogenèse des gouttelettes lipidique sont essentiels en conditions de stress nutritif puisqu'elles servent de réserve énergétique pour le parasite. Enfin, ces observations nous informent sur la capacité du parasite à moduler son métabolisme pour s'adapter à son environnement en utilisant des protéines qui ne sont essentielles que dans des conditions nutritives spécifiques.

***Toxoplasma gondii* acyltransferases, TgGPAT and TgDGAT2, are involved in the balance between bulk membrane biogenesis and lipid storage dependent on host nutrient availability**

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**Abstract**

Current evidence points at Apicomplexa parasites being highly flexible in adapting their metabolic program in response to nutrient availability from their hosts. Lipids are crucial nutrients for parasite division and hence parasite survival and pathogenesis. These eukaryotic parasites can readily scavenge fatty acids (FAs) directly from their host but can also synthesize them *de novo*. Both pathways are crucial for the parasite, and their respective activities are up/downregulated depending on host nutrient availability. Such metabolic plasticity controls parasite proliferation but the molecular pathways allowing this are poorly understood. The FAs that are either made or scavenged are used by acyltransferases to generate key lipid classes used as lipid storages and central precursors, sustaining parasite development and survival in a timely regulated as lipid storages and central precursors used for such as phosphatidic acid (PA), Diacylglycerol (DAG), and triacylglycerol (TAG). In this study we investigated the function of two acyltransferases; glycerol-3-phosphate

acyltransferase (*TgGPAT*) and diacylglycerol acyltransferase 2 (*TgDGAT2*) that localize to the endomembrane system and cytosol of the parasite. We emphasize the nutrient role of these proteins as in different lipid environments the parasite can render a protein that was once non-essential, essential, and vice versa. *TgGPAT* is a critical protein needed for parasite development. Lipidomic analyses showed this protein is responsible for lysophosphatidic acid formation, which is pivotal to membrane biogenesis and neutral lipid synthesis. Lipid droplet (LDs) IFA also indicated that this protein has a role in TAG formation, the main constituent of LDs. *TgDGAT2* is a non-essential cytosolic protein that is involved in LD biogenesis as confirmed by IFA. Lipidomic approaches revealed that its disruption impacted the lipidomic profile of the parasite, specifically DAG levels. This study revealed that these proteins are essential for glycerolipid metabolism and act in a regulatory manner in different nutrient conditions. The loss of these proteins both result in an accumulation of free FAs that cannot be 'detoxified' by incorporating them into LDs, leading to parasite death.

## ***Introduction***

Apicomplexa parasites, such as *Plasmodium falciparum* and *Toxoplasma gondii*, are unicellular eukaryotes responsible for major human diseases affecting about a third of the global population. Traditionally toxoplasmosis is not life threatening but can be even lethal to those suffering from immunological impairment, cancer, AIDS, and organ transplant recipients (1,2) No vaccine or efficient drug exist to treat this disease. Current evidence shows that lipid synthesis and trafficking is pivotal for parasite-host interactions and critical for parasite survival, hence representing a pertinent drug target (3,4).

Parasites need a large amount of lipids for the generation of membrane compartments for parasite propagation and pathogenesis within the human host. Fatty acids (FA) are

the building blocks of lipids and are acquired by the parasite either through the FASII pathway (*de novo*) or by directly scavenging them from the host and host environment (5,6). These FAs are then channelled into phospholipid (PL) synthesis for membrane biogenesis or storage as lipid droplets (LD) for timely controlled usage and parasite survival (7,8). *Toxoplasma* tachyzoite parasites exemplify the need for new membranes to maintain parasite propagation. This is achieved by the continuous FA synthesis and scavenging (8). We recently identified a nexus protein, TgLIPIN, that is responsible for channelling FAs to either membrane biogenesis or lipid storage.

The main metabolites of these pathways lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG) and triacylglycerol (TAG). LPA and PA are the simplest PLs/PLs precursors and are crucial for a) cell signalling to induce parasite invasion, motility, and egress and production of DAG (via DGK1/2) (9,10), b) membrane curvaton for cytokinesis and endocytosis (11), and c) are considered the central precursors for all PL like phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositols (PIs) (7). LPA/PA can be further catabolized to for storage lipids like diacylglycerol (DAG) and triacylglycerol (TAG). DAG can be directed through the Kennedy or CDP-DAG pathway for PL synthesis, or it can continue through the glycerolipid pathway and be esterified to form TAG, the main constituent of LDs. These pathways are governed by nutrient availability in terms of the physiological environment of the host cell (12–15). The fate and incorporation of these FAs into PLs or LDs are reliant on the activity of specific acyltransferase (ATs). There are many different ATs identified and studied (16–25), the ATs responsible in determining the fate of fatty acids to PLs or LDs upon the response to the nutrient availability are yet to be determined.

One of the first acyltransferase involved in glycerolipid precursor synthesis is named glycerol-3-phosphate acyltransferase (GPAT(16)). It facilitates the rate limiting reaction involving the conversion of glycerol-3-phosphate (G3P) to LPA by esterifying a fatty acyl-CoA (FA) to the sn-1 position of the G3P backbone. Then to add another fatty acid to the LPA, there is another AT, Acylglycerol-3-phosphate acyltransferase (AGPAT). This enzyme is responsible for adding the second activated acyl-CoA to LPA on the sn-2 position of the G3P backbone, forming PA. In eukaryotic organisms, many isoforms of GPAT/AGPATs exist with different cellular localization and function. GPAT1/2 in humans are mitochondrial and seem to modulate fatty acid oxidation (16). GPAT3/4 in most models are found near the ER (16). GPAT3 in humans has a controversial identity as it seems to function as both a GPAT and AGPAT(26).

Thereafter another group of ATs exist, which transfer an activated FA to sn-3 position of G3P. Since sn-3 position is usually allocated to the head group of phospholipids, these ATs are specific to DAG to generate TAG, i.e. diacylglycerol acyltransferase (DGAT). Both TAG and DAG can be then used as energy storage and/or of FA to fuel the phospholipid synthesis machinery (27). DAG to be acylated can be *de novo* synthesized from PA by the removal of its phosphate group by Phosphatidic acid phosphatase or LIPIN (7).

In most eukaryotic models, at least two DGATs coexist and contribute to synthesis and metabolism of TAG (18,28). DGAT1, which is part of the membrane-bound O-acyltransferases or MBOAT super family) is typically more involved in the recycling of hydrolysed TAGs by re-esterifying FAs (29). This enzyme may also be important for preventing lipotoxicity and protecting the cell from highly levels of FAs, having a preference to exogenous FAs. In contrast to DGAT1s, the model of DGAT2 suggest this protein is instead more active when FA levels are low. This enzyme is frequently

associated to endogenous/*de novo* FA synthesis and esterification (17). In terms of sequence motifs, DGAT1s are more conserved than DGAT2s(30).

In *T. gondii* parasites several ATs have been identified and characterised. Previously, we identified an apicoplast-derived GPAT (*TgATS1*) and *TgAGPAT* (*TgATS2*) as well as an ER-localised *TgAGPAT* (24,25). *TgATS1* is an essential protein and is lethal when knocked out, unlike *TgATS2* (31). Both *TgATS1* and *TgATS2* are responsible for the bulk lipid synthesis using FAs from the FASII for rapid membrane biogenesis supporting parasite survival (31,32). These enzymes are also identified and partially characterised in *Plasmodium spp.* (32,33). In *P. yoelli* ATS1 has shown to be essential for liver stage parasites (34). A GPAT in *P. falciparum* has also been identified but not characterized lipidomically (35). In *T. gondii* a DGAT, *TgDGAT1*, was found not to be essential in this parasite. Its disruption exhibited a decrease in LDs and an accumulation of palmitic acid (C16:0). *TgDGAT1* may additionally have a role in the ancient eukaryotic type of diacylglycerol esterification in Apicomplexa parasites (36). The roles the above-mentioned ATs in the transfer of host-scavenged FAs is not known. Moreover, we do not understand how Apicomplexan parasites can orchestrate continuous bulk PL synthesis and lipid storage under constant FA scavenging which is dependent on the nutrient environment.

To investigate how the parasite uses or stores FA, through the synthesis of LPA/PA and TAG, respectively, we then searched for putative GPATs and DGATs. In *T. gondii*, there are still uncharacterised ATs such as GPAT (*TGGT1\_256980*, phenotype score = -2.83, (37,38), and DGAT2 (*TGGT1\_275600*, phenotype score = 0.35, (37,38). It is possible that these acyltransferases maybe involved in the transfer of FAs in the response to the nutrient condition. Here, we identified *TgGPAT* and *TgDGAT2* to study their role in the synthesis of TAG under different lipid environments. IFA analyses

show these proteins either in proximity with the endomembrane system or cytosol of the parasite. Viability surveillance showed that the *TgGPAT* is critical for parasite survival, whereas *TgDGAT2* seems to be less crucial but still affects the lipid metabolism according to our lipidomic analyses. Our metabolic labelling and mass-spectrometry-based experiments revealed these proteins play important roles in PL and TAG synthesis that are highly governed by the host nutrient environment.

## **Material and Methods**

### **Sequence analyses of *TgGPAT* and *TgDGAT2***

Sequence alignments were generated using ClustalW using predicted protein sequences of GPAT3 and DGAT2 subfamily proteins from different species namely: *Homo sapiens* GPAT3 (Q53EU6), *Saccharomyces cerevisiae* GPT2 (P36148), *Saccharomyces cerevisiae* SCT1 (P32784), *Trypanosoma brucei* GPAT (Tbg972.10.3920), *Eimeria falciformis* GPAT (ASN64451.1) *Plasmodium falciparum* GAT (PF3D7\_1212500), *Neospora caninum* GPAT (NCLIV\_029980), *Hammondia hammondi* GPAT (HHA\_256980, *Homo sapiens* DGAT2 (Q96PD7), *Arabidopsis thaliana* DGAT2 (Q9ASU1), *Saccharomyces cerevisiae* DGAT2 (Q08650), *Plasmodium falciparum* DGAT (PF3D7\_0322300), *Neospora caninum* DGAT2 (NCLIV\_029090) and *Hammondia hammondi* DGAT2 (HHA\_275600). Secondary structure and putative residues are indicated on ToxoDB (37). Putative transmembrane domains were predicted using Deep TMHMM (39). Consensus sequences were found using work by Cao *et al.*, (2011) and Yamashita *et al.*, (2014) (26,30).

3D-structure modelling was done by threading the sequence of *TgGPAT* in the online software Swiss-Model (<https://swissmodel.expasy.org/>), which lead to the identification of the crystal structure of *CmATS1* (40). The sequence surrounding the

motif III of this group of proteins was then aligned to *TgGPAT* and visualized using ChimeraX 1.4.

### ***Toxoplasma gondii* strains and cultures**

*T. gondii* tachyzoites (RH TIR1-3FLAG and inducible knock-down strains) were maintained in human foreskin fibroblasts (HFF) using Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 1% foetal bovine serum (FBS) (Life Technologies), 2 mM glutamine and 25 µg/mL gentamicin at 37°C and 5% CO<sub>2</sub>.

### **Generation of inducible knockdown GPAT and DGAT2 lines in *T. gondii***

A C-terminally tagged HA line with inducible post-translational protein degradation was generated using the Auxin-inducible Degron system (AID). Homology regions of the C-terminal end of *TgGPAT/TgDGAT2* excluding the stop codon were amplified using the primers found in **Tables**

**Table 1.** Guide sequence for each protein (*Table 1*) were selected near the C-terminal end and was less than 100 bp away from at least one homology region. The guide sequence was inserted into Cas9 U6 universal plasmid Q5 mutagenesis or by enzyme digestion with BsaI and ligation.

The PCR product and the Cas-9 guide plasmid were used for transfection of a RH TIR1-3FLAG parental line (Brown, Long and Sibley, 2017). Parasites were electroporated in a 2-mm cuvette using BTX ECM 630 (Harvard Apparatus, at 1.100 V, 25Ω, and 25 µF).

After 24 h the newly transfected line was treated with 25 µg/ml mycophenolic acid and 50 µg/ml xanthine. Parasites were selected with the drug and cloned by limiting dilution. Screening of parasite clones for correct insertion of the mAID sequence, HX drug cassette and overall insertion/orientation were done with the Forward primer

SSTg35 Reverse primers SSTg48, SSTg49 and SSTg38 for *TgGPAT* and SSTg39 for *TgDGAT2* (Table 1). All PCRs were performed with TaKara primestar Max polymerase (Sup. Figure 2/3). The knockdown of these lines was induced with a final concentration of 100  $\mu$ M of indole-3-acetic acid (auxin) made up in 100% ethanol.

### **Generation of a double mutant $\Delta$ DGAT1-DGAT2-iKD line in *T. gondii***

For the double mutant, we attempted to knock out ( $\Delta$ ) *TgDGAT1* in the *TgDGAT2* mAID strain (above).

A guide sequence (Table 1) was selected near the within the gene sequence and was less than 100 bp away from at least one homology region (with the other homology region up stream of the start of the gene). The guide sequence was inserted into Cas9 U6 universal plasmid enzyme digestion with BsaI and ligation.

This  $\Delta$  strain was generated by amplification of a GFP signal sequence and a DHFR-cassette (1  $\mu$ g/ml pyrimethamine) from pDTG plasmid. This sequence was flanked by two homologous regions of *TgDGAT1* on the 5' and 3' ends of this sequence. The primers used for this amplification are Table 1.

Transfection the *TgDGAT2* mAID strain with the amplicon and U6 plasmid described for  $\Delta$ *TgDGAT1* was performed by electroporation as described above. After 24h, parasites were put under drug selection using 1  $\mu$ M pyrimethamine.

### **Immunofluorescence**

Primary antibodies anti-HA (rat, Roche), anti-CPN601 (rabbit), Nile red were used at dilutions 1:250, 1:1000 and 1:1000 respectively. Secondary AlexaFluor 488- and 546-conjugated anti-mouse/rat or anti-rabbit antibodies (Life Technologies) were used at 1:2000. For the immunofluorescence assay (IFA) parasites were grown on confluent HFF on coverslips and fixed in PBS containing 2.5% paraformaldehyde (PFA) for 15

min at room temperature (RT). Samples were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT prior to blocking in PBS containing 3% BSA and subsequent incubation with primary antibodies then secondary antibodies diluted in the blocking solution. Labelled parasites were stained with Hoechst (1:2000, Life technologies) for 15 min and then washed three times in PBS before final mounting of the coverslips on a glass slide using fluorogel. The IFA slides were visualized using fluorescence microscope (Axioimager, Zeiss).

### **Nile Red staining of lipid droplets**

The parasites were allowed to infect and grow in confluent monolayer HFF grown on coverslips, with and without auxin for 24 h. These coverslips were prepared as above and then incubated for 1 h with Nile red in 1X PBS before proceeding with DNA staining with Hoechst. For visualizing Nile red stained droplets yellow-gold fluorescence (excitation, 450-500 nm; emission, greater than 528 nm) (41) was used on the Axioimager. Quantification was performed based on iterative image processing of parasites with and without auxin in differential FBS conditions (0/1/10%) (42).

### **Western-blot analysis**

Parasites were harvested for western blot analysis following complete egress from their host. To remove any host cell debris the parasites were passed through a 3 µm filter, then counted by hemocytometer and solubilized in SDS buffer at equivalent cell numbers. Equal amount of protein was separated on a 4-12% gradient SDS-polyacrylamide (Life Technologies) and transferred to Nitrocellulose membrane using the XCellIII Blot Module (Invitrogen). Primary antibodies anti-HA (Rat, Roche) and anti-TOM40 (Rabbit) (Van Dooren et al. 2008) were used at a dilution of 1:200 and 1:1000, respectively. Secondary goat anti-mouse and anti-rabbit horse radish peroxidase (HRP) conjugated antibodies (Thermo Scientific) were used at 1:2000. Protein signal

was detected by chemiluminescence after membrane staining with Luminata Crescendo Western HRP detection kit (Millipore). The signal strength of protein was quantified using a BioRad Chemidoc imager (BioRad).

### **Phenotypic characterization:**

#### **Plaque assays in *Tg* mutants**

The extracellular parasites were harvested after filtration and counted by haemocytometer. Mutant parasites (~1000) were used to inoculate confluent HFF flask (25 cm<sup>2</sup>). The mutant parasites were grown for presence or absence of auxin (100 µM) for 7-10 days. After aspiration of the media cells were fixed with 100% ethanol for 5 min and plaque sizes were visualized by crystal violet staining (30-60 min) followed by 3 washes with phosphate-buffered saline. The average plaque areas (particles) were analysed using ImageJ software (n=3 independent experiments).

#### **Replication assay in *Tg* mutants**

In 4-well plates (with coverslips) equal numbers of parasites were allowed to invade confluent HFF cells for 2 h. After invasion, the plates were washed (X3) with ED1 (DMEM, 1% FBS) and parasites were grown for 24 h with or without auxin (100 µM). These coverslips were then processed for IFA and the number of parasites per vacuole were calculated (n=3 independent experiments).

### **Lipidomic analysis:**

Mutant parasites were grown on confluent monolayer HFF cells in 175 cm<sup>2</sup> flasks until host cells were fully lysed and then reinoculated and grown for a further 24 h with or without auxin (100 µM). Parasites were harvested as intracellular tachyzoites (at least  $1 \times 10^8$  cell equivalents per replicate) after syringe filtration with 3-µm pore size membrane. These parasites were metabolically quenched by rapid chilling in a dry ice-

ethanol slurry bath and then centrifuged down at 4°C. The parasite pellet obtained was washed with ice-cold PBS (X3), before transferring the final pellet to a microcentrifuge tube. GC-MS sample preparation and analyses were performed as described in Shunmugam and Dass *et al.*, (2021).

Then total lipids were extracted in chloroform/methanol/water (1:3:1, v/v/v) containing PC (C21:0/C21:0 at 10 nmol) and C13:0 or C15:0 (10 nmol) as internal standards for extraction. Polar and apolar metabolites were separated by phase partitioning by adding chloroform and water to give the ratio of chloroform/methanol/water as 2:1:0.8 (v/v/v). For lipid analysis, the organic phase was dried under N<sub>2</sub> gas and dissolved in 1-butanol to obtain 1 µl butanol/10<sup>7</sup> parasites.

#### Total lipid analysis:

The extracted total lipid sample was then added with

C15:0 or C13:0 (1 nmol), as an internal standard as stated before using TMSH for total fatty acid content. Resulting fatty acid methyl esters (FAME) were analysed by GC-MS as previously described (31,43). All FAMEs were identified by comparison of retention time and mass spectra from GC-MS with authentic chemical standards. The concentration of FAMEs was quantified after initial normalization to different internal standards and finally to parasite number.

#### Phospholipid and neutral lipid analysis:

For phospholipid analysis, the extracted total lipid extracted (as above) was separated with 1 nmol LPA (C17:0) or PA (C17:0/C17:0) (Avanti Polar lipids) by two-dimensional silica gel high-performance thin-layer chromatography (HPTLC, Merck). The solvent system used for the 1st and 2nd dimension was chloroform/methanol/28% ammonium hydroxide, 12:7:1.6 (v/v) and chloroform/acetone/methanol/acetic acid/water,

10:4:2:2.6:1 (v/v/v/v/v), respectively. For DAG, TAG, Free fatty acids (FFA) and cholesteryl ester (CE) analysis, total lipid fraction was separated by 1D-HPTLC using hexane/diethyl ether/formic acid, 80:20:2 (v/v/v) as solvent system. Then each lipid spot on the HPTLC plate was scraped off, and lipids were methanolized with 200  $\mu$ l 0.5 M methanolic HCl in the presence of 1 nmol pentadecanoic acid (C15:0) as internal standard at 85 °C for 4-4.5 h. The resulting FAMES were extracted with hexane and analysed by GC-MS (Agilent).

### **Lipid flux analyses via stable isotope metabolic labelling combined to lipidomics:**

#### **Tracking apicoplast FASII fatty acids:**

The mutant parasites were infected to a confluent monolayer of HFF in glucose-free-DMEM (1% FBS) supplemented with U- $^{13}\text{C}$ -glucose or U- $^{12}\text{C}$ -glucose at a final concentration of 800  $\mu\text{M}$ , with or without auxin (100  $\mu\text{M}$ ). The parasites were harvested up to 24 h post depletion and lipid were extracted as described above.  $^{13}\text{C}$  incorporation to each fatty acid was calculated as the percent of the metabolite pool containing one or more  $^{13}\text{C}$  atoms after correction for natural abundance and the amount of  $^{13}\text{C}$ -carbon source in the culture medium. The degree of the incorporation of  $^{13}\text{C}$  into fatty acids (% carbon incorporation) was determined by the mass isotopomer distribution (MID) of each FAMES. MID was obtained from the shift in isotopic mass dependent on the amount of  $^{12}\text{C}$  carbons compared to the integration of  $^{13}\text{C}$  carbon atoms. The total abundance of  $^{13}\text{C}$ -labeled fatty acids was obtained by calculating the concentration of all isotopomers of  $^{13}\text{C}$ -labeled FAMES and finally normalizing to authentic internal standards and parasite number.

### Tracking host-derived fatty acids

The HFF cells were grown to confluency in the presence of stable isotope U-13C-glucose at a final concentration of 800  $\mu$ M added to a glucose-free DMEM (1% FBS). After harvest and lipid extraction, similar calculations were performed as explained for FASII-derived lipids.

### **Statistics and reproducibility:**

The graphical data for this study was generated using GraphPad Prism software. Three biological replicates were used per experiment (at least  $n = 3$ , unless stated otherwise). The error bars are representative of the standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by unpaired  $t$  tests using GraphPad Prism. The range of statistical significance was signified as per the  $p$  value indicated in each graph.

## **Results**

### **Identification of a GPAT3 and a DGAT2 in *Toxoplasma* parasites**

Previously, we identified plant-like acyltransferases, *TgATS1* and *TgATS2* and an ER-localised *TgAGPAT* in the *T. gondii* genome (31,32). In addition, there are at least 4 other putative acyltransferases encoded in the *T. gondii* genome (44). To investigate ATs involved in the use or storage of FA, through the synthesis of LPA/PA and TAG, respectively, we searched for putative GPATs and DGATs amongst these putative candidates. Previous studies (31,32,37) and further searches on ToxoDB (37) revealed the presence of an animal-eukaryotic-like GPAT (*TGGT1\_256980*) and a DGAT (*TGGT1\_275600*). Protein sequence analyses by CLUSTAL multiple sequence alignment using the software MUSCLE (version 3.8) (45) was performed and

uncovered that these proteins fall under the GPAT3 and DGAT2 subfamilies of ATs, respectively (Figure ).

The putative *TgGPAT* (1002 aa, with 4 transmembrane domains) contained all 4 conserved motifs of this type of protein. The signature catalytic domain for GPATs is found in motif I (HXXXXD) and seems to also be conserved in other apicomplexans. Phylogenetic analyses of multiple types of GPATs (Fig. 1A) revealed that *TgGPAT* is like GPAT3-like ATs and clusters with the only two GPATs that exist in yeast. All motifs were predicted (Deep TMHMM) to be on the cytosolic side of the ER membrane like its human homolog *HsGPAT3* (16,26,39). As with other sequence studies, motif III and motif II of this enzyme family is the most and least conserved, correspondingly within apicomplexa models (16). Structure modelling of *TgGPAT* using *Cucurbita moschata* (squash or pumpkin) ATS1 (*CmATS1*, ID: Q9FEQ0) structure (40) as a threading template suggest that *TgGPAT* contains the putative G3P binding domain found in motif III (Fig. 1B). Only the region surrounding motif III seemed to align with the crystal structure of *CmATS1*. No membrane-bound AT crystal structure is available yet to perform structure modelling (46). This can explain the poor alignment to the other 4 motifs when designing this model, since ATS1 is plastid-derived and soluble. From these bioinformatic analysis, *TgGPAT* is predicted to be a putative AT required for the transfer of FA to form LPA in the parasite ER.

*TgDGAT2*, as expected showed similarity with DGAT2 type enzymes (30). *TgDGAT2* contains all DGAT2 motifs (I-VI) and has conserved amino acid sequence as seen in Figure 1B. This protein is predicted to be 533 aa and contains only 2 transmembrane domains. The catalytic DGAT domain is found in the cytosolic C-terminal end. As demonstrated Figure B, six regions with sequence conservation have been located by Cao (30). The most conserved regions include motif I, II and IV. Phylogenetic analyses

shows that *TgDGAT2*, as expected, clustered with other putative DGAT2 proteins in apicomplexa and is closely related to mammal and plants DGAT2 (Sup Figure 1C). Unlike the other apicomplexa proteins, the orthologue of *TgDGAT2*, a putative *P. falciparum* DGAT, did not align with the consensus sequences of DGAT2 and instead showed similarity to DGAT1 (47). Since no crystal structure has been published a DGAT2 family protein, putative 3D modelling of *TgDGAT2* was not preformed (46).

### ***TgGPAT* is essential for parasite growth and replication during tachyzoite stages in all nutrient conditions**

To determine the importance and function of *TgGPAT* and *TgDGAT2*, inducible KD parasite lines of *TgGPAT* and *TgDGAT2* were generated using auxin-inducible-degron system. These lines were generated with a C-terminal HA-tag in the Tir-RH parental line (48). The presence of the HA-tag and its locus were confirmed by PCR (Sup. Fig 2 and 3).

We first confirmed the *TgGPAT* inducible knockdown strain. Western blot analysis showed the *TgGPAT*-mAID protein migrated at its expected size of 110 kDa (Fig. 1A) and confirmed the downregulation of *TgGPAT*-mAID in the presence of 100  $\mu$ M auxin within 30 min of incubation (Fig. 1A). Immunofluorescence analyses (IFA) revealed that *TgGPAT*-HA signal localized to the cytosol, and more particularly to the periphery of the nucleus. Characteristic of a GPAT3-type AT, that localizes to the ER, we wanted to confirm if this was the case for *TgGPAT*. By performing a co-transfection with Der1-GFP, we found that *TgGPAT* partially localized to the ER and cytosol (Fig. 1B).

To determine the effect of the *TgGPAT* loss in the parasite's growth, plaque assays was performed. Furthermore, we wanted to determine if the nutrient availability played a role in the proteins function over parasite survival, so we preformed this viability

assay at varying FBS (foetal bovine serum) conditions (0-1% FBS). In the normal media condition, 1% FBS, there was already a significant decrease in the growth (Figure C, middle). Plaque assays performed in 0% and 10% FBS medium also demonstrated a significant decrease in parasite growth (Figure C, left and right). However, as the FBS content increased (0% to 10%) the difference in plaque area between treated and untreated cultures lessened, suggesting that even in the absence of *TgGPAT* the large availability of lipids from the host's environment allows the parasites to partially compensate. These data suggest that the role of *TgGPAT* is strongly associated to nutrient availability. The growth defect was also supported by a replication assay which showed a significant increase in small-numbered parasite vacuoles (1-2 parasites par vacuole) and a decrease in larger vacuoles (3-10 parasites par vacuole), which suggested impaired parasite development in the absence of *TgGPAT* (Figure D).

### ***TgDGAT1* and *TgDGAT2* are important to parasite growth under lipid starvation**

We then characterized the *TgDGAT2*-mAID inducible knock down strain. Western blot analysis showed that the *TgDGAT2*- mAID protein migrated around 60 kDa (Figure 2A), just above its loading control TOM40 (40 kDa). By western blot, we confirmed the complete protein degradation after 24 h under 100  $\mu$ M auxin treatment. IFA showed that *TgDGAT2*-mAID fluorescence localizes to the cytosol throughout the parasite cell. Under auxin treatment, *TgDGAT2*- mAID signal was not observed throughout the parasite cytosol, as expected, however a remaining fluorescent signal was observed condensed at or in close vicinity to the apicoplast CPN60 signal (Figure B, CPN60). This could suggest incomplete degradation of the protein, or a background signal seen from fluorescence in the apicoplast.

To determine the effect the loss of *TgDGAT2* protein had on parasite growth, a plaque assay was performed in different nutrient conditions in the same way as for *TgGPAT* (Fig. 3C). The loss of *TgDGAT2* by auxin treatment only led to a significant deficit in parasite progression when lipids/nutrients were retrained, specifically 0% FBS (Figure C, left). This suggests that *TgDGAT2* may have a nutrient dependent role, similar to *TgGPAT*. The lack of an effect on parasite growth at higher lipid availability (1-10% FBS) could be explained through a compensation by its other homologue, *TgDGAT1*. Previous studies were unable to generate a KO of this gene and the essentiality of this protein is still unclear (36).

To determine the possible compensating effect of these two DGATs, *TgDGAT1* was knocked out in the *TgDGAT2*-mAID strain to generate a  $\Delta TgDGAT1$ -*TgDGAT2*-HA-mAID line. The *TgDGAT1* gene was replaced with a GFP sequence, and IFA confirmed a disruption of the gene by a nuclear localization of the GFP signal. IFA analyses of  $\Delta TgDGAT1$ -*TgDGAT2*-HA-mAID revealed that in contrast to *TgDGAT2*-mAID, *TgDGAT2* has a perinuclear/ER like localization (Figure D).

We wanted to also determine if the loss of both paralogues of DGAT in the parasite influenced parasite growth, and more over if this growth was affected by nutrient availability. We performed a plaque assay similar to the *TgDGAT2*-mAID strain (Fig. 3E). There was a general trend of increased parasite growth when both *TgDGAT1* and *TgDGAT2* were absent, but this effect was only significant in 0% FBS conditions (low lipid environment). This phenotype mirrors the *TgDGAT2*-mAID parasite strain, in that absence of *TgDGAT2* (Fig. 3C) led to decreased parasite growth but disruption of both *TgDGAT1* and *TgDGAT2* led to an improvement of parasite development. This could suggest that at 0% FBS, these proteins are involved in controlling parasite growth.

## ***Tg*GPAT is neutral lipid and phospholipid metabolism**

To determine the nutrient-dependant roles of *Tg*GPAT and *Tg*DGAT2 in the lipid metabolism, we grew parasites in the presence or absence of auxin in 0-10% FBS to imitate nutrient (specifically lipids) starvation/excess and performed lipidomic analysis.

When the protein *Tg*GPAT was downregulated, the parasites had a lower abundance of lipids at 0% to 1% FBS but at 10% FBS, the degree of decrease was not significant suggesting that the parasite can, somewhat, compensate for the lack of *Tg*GPAT (Figure A). In the absence *Tg*GPAT, there were many changes in the relative abundance of each FA species. In Figure 4B, the ratio of the abundance of each FA species with/without auxin (without/with *Tg*GPAT) is plotted in a heatmap (where yellow is an increase and blue/purple is a decrease of abundance). We noticed that parasite lipid abundance of longer-chained FAs that are mostly host-derived such as C18:0, C20:0, C20:1, C20:4 and C20:5 is increased concomitantly to the increase of FBS (from 0% to 10% FBS). This suggests that with an increase in FBS levels, the absence of *Tg*GPAT leads to an accumulation of longer chained FA (possibly host-derived; Figure B and Sup. Fig 4A-C).

Since there were no changes at 10% FBS in the presence or absence of the protein (and that the parasite can somewhat compensate their lipid content for the lack of *Tg*GPAT at 10% FBS), we decided to focus our lipidomic analyses only at low lipid levels (0-1% FBS conditions, Figure A). We analysed the lipidomic changes the loss of this protein had on the proposed substrate FFA and other neutral/phospholipids. There was a significant increase in FFA levels and a decrease in PL (comprising of LPA too, the proposed product of *Tg*GPAT and simplest LPL) levels only at 1% FBS, considered to be low lipid availability (Figure C-D). The same changes were seen in 0% FBS but were not significant (Figure C).

Total TAG (0% FBS) levels were not affected, but the FA profile revealed that C14:0 (apicoplast-derived) decrease with an increase in stearic acid (C18:0), emphasizing the preference of this protein to longer chained FAs (Figure E). A similar change was seen for FFA levels at 0% FBS (Figure F), TAG (Figure G) and DAG at 1% FBS (Figure H). The protein's role in neutral lipid synthesis, specifically TAG/LD was highlighted with the decrease of LD volume at 1% FBS (Sup. Fig 4F).

### ***Tg*GPAT is involved in LPA formation**

In these limiting lipid conditions, the loss of *Tg*GPAT led to the decrease in the PL levels (Figure 4D and Sup. Fig. 4G/H) at 1% FBS. To investigate further we separated the total lipid extract by 2D-high performance thin layer chromatography (2D-HPTLC) to allow us to determine the levels individual PLs and LPLs (Fig. 4J). There was a significant decline of PC levels, which is the majority PL group in most eukaryotes. Importantly, we saw that LPA levels also decreased, which is in agreement with this LPL being the proposed product of *Tg*GPAT (Figure J).

### ***Tg*GPAT has a preference to using host-scavenged lipids**

To determine if the parasites have any substrate preference, for FASII-derived FA or host-derived FA, metabolic labelling using isotope-labelled U-<sup>13</sup>C-glucose were performed. To monitor the FASII-derived FA, the parasites were fed with U-<sup>13</sup>C-glucose, as previously described (31), under *Tg*GPAT degradation by auxin treatment. The incorporation rate of <sup>13</sup>C was determined for each FA species (Figure L). While FASII is fully active to produce the C14:0 fatty acid with fully <sup>13</sup>C labelled up M+14 (Fig. 4I), the abundance of FA generated from the FASII was much lower in the absence *Tg*GPAT protein (Figure K). We also determine the abundance of FA scavenged from the host by growing the host cell in the presence of <sup>13</sup>C-glucose prior to the inoculating the parasite to grow with or without auxin, as we previously described (7). As shown

in the Fig.4 L, the parasite FA-scavenging abilities seemed to also be hindered (Figure L), specifically that of oleic acid (C18:1) and eicosenoic acid (C20:1) which is exclusively derived from the host. This is further supported by changes in the mass isotopologue distribution of host-derived C14:0, C16:0, C16:1, C18:0 and C18:1 (Fig. 4J-N, increase specifically in M+0 of C18:0). This suggests that in the absence of *TgGPAT*, the parasite does in fact have a preference to host-scavenged lipids.

### ***TgDGAT2* is involved in neutral lipid metabolism, specifically DAG metabolism**

Thereafter, to determine the role of *TgDGAT2* in the lipid metabolism, we performed the same lipidomic analysis as for the *TgGPAT*-mAID. In contrast to *TgGPAT*, *TgDGAT2* degradation led to an overall increase in total lipid content at all FBS conditions (only significant at 0 and 10% FBS, Figure 4A). There was no difference in the relative abundance of FA in the mol% (Sup. Fig. 5A).

Then the abundance of DAG and TAG, the proposed substrate and product for the *TgDGAT2* was determined. There was an increase in the DAG at 0-10% FBS, specifically C16:0 (Figure B-D). However, the abundance of TAG was not affected.

To investigate the effect on TAG we performed lipid droplet (LD) analyses (Figure E). Lipid droplets were stained with Nile red and its fluorescence was determined by IFA to quantify the number of LD based on iterative image processing (42). The partial loss of *TgDGAT2* by auxin led to a significant decrease in the number of LD per parasites (0% and 1% FBS, Figure E) and volume (0% and 10% FBS, Figure D).

Since the FFA is also another putative substrate of *TgDGAT2*, its abundance was also investigated. As shown in the Fig. 5F and 5G, the relative abundance of C16:0 and C18:1 increased under 0% and 1% FBS when *TgDGAT2* was disrupted. This suggests that a presence of this protein may give parasites a preference towards longer chained

(possibly host-derived) FA species (Figure F/G). Since there is homologue *TgDGAT1*, it may compensate some part of the lipid metabolism thus the effect of the loss of *TgDGAT2* was not seen lipidomically, but it was clearly shown that *TgDGAT2* plays a role in LD formation.

## **Discussion**

### ***TgGPAT* is a GPAT3-type AT that has a nutrient dependant role in neutral lipid and phospholipid synthesis with a preference of host FAs**

We identified a GPAT3-type AT in *T. gondii* parasites by sequence analyses. The motif III of this protein has the highest similarity to the two yeast homologues (ScGPT2 and ScSCT1, Fig. 1 and Sup. Fig. 1A) rather than the human form, suggesting like with yeast that this protein may perform the roles of multiple GPAT-enzymes and that the parasite only houses two GPATs, *TgATS1* (*TGGT1\_270910*, plant/algal like) and *TgGPAT* (31,49,50). Additionally, GPAT3 type proteins are unique in that they can perform the roles of AGPAT/GPAT and unlike GPAT1/2 it specifically localizes to the ER and not the mitochondria of the cell, as we have displayed by IFA colocalization studies with a Der1-GFP plasmid (Fig. 2B)(16). Since *TgAGPAT* is not essential when conditionally knocked out, it could be proposed that the existence of a plant-like AGPAT (*TgATS2*) and a dual-functioning *TgGPAT* could compensate for the loss of this AGPAT activity (16,26,32). This could also explain why when *TgGPAT* is knocked-down the parasite is not able to survive as potentially GPAT and AGPAT activity is inhibited (Fig. 2).

We were unable to show full alignment (only motif 3) of a *TgGPAT* 3D model with the crystal structure of *CmATS1*, which is like *TgATS1* (31). Unlike plastid derived *TgATS1* and *CmATS1*, *TgGPAT* is predicted to be poorly soluble, and there is currently no crystal structure of a membrane bound AT (40,46,51).

This work shows that *TgGPAT* crucial to parasite growth and is involved in neutral lipid and PL metabolism (Figure ). The absence of a significant drop in lipid content level when the protein is absent in lipid restraining conditions, can suggest that *TgGPAT* is more important under nutrient restrictions. In support of this hypothesis, changes in the individual neutral lipid class were only observed at 1% FBS, with a similar lipidomic phenotype at 0% but which was not significant (Figure C/D). This can be misleading, but when we look at the FA profiles of TAG and FFA, we see a trend; the loss of *TgGPAT* causes an accumulation of longer FA chains (C18:0 and C18:1), suggesting a preferred substrate-binding affinity to oleate acid and other longer chains like the human homology (22) and *CmATS1* (40). This phenotype at low lipid conditions correlates a putative role in LD formation as LD predominantly form under low lipid levels (7,32). In other models, GPAT3 also possesses AGPAT activity which we did not investigate. It would be interesting to explore AGPAT activity using labelled LPA or enzymatic studies.

At 10% FBS (Fig. 4B), we see an exaggerated decline in C14:0 and an accumulation of FAs exclusively host-derived like eicosanoid acid (20:1) and arachidonic acid (20:4) (7). This is further supported through our metabolic host-labelled studies, which show a shutdown of parasite scavenging when *TgGPAT* is suppressed. This accumulation of FA substrate leads to a 'lipotoxic' effect whereby the parasite believes it no longer requires FAs as they can no longer be converted to PL, specifically the product LPA (Figure 4J), for membrane biogenesis. The effect this loss of LPA has on membrane integrity and curvature can be further investigated by electron microscopy as no visible membrane changes were noticed by phase-contrast microscopy.

These FAs are also not incorporated in neutral lipid synthesis (i.e. TAG/LD), as we see the loss of this protein leads to a suppression of LD formation at 1% FBS (Fig.

4E). Instead, these FAs accumulate in the parasite ultimately leading to cell death. The unexpected shutdown of the FASII (*de novo* FAS), can be explained by this fatal accumulation of FAs in the parasite (Figure L).

### ***Tg*DGAT2 has a nutrient dependant role in TAG formation**

Phylogenetic and evolutionary analyses show that the DGAT1 and DGAT2 families have asymmetrically evolved since the rise of eukaryotes and have undergone convergent evolution likely due to the importance of TAGs for all eukaryotic organisms (25). A DGAT1 isoform exists in the parasite (*TGGT1\_232730*) and the sequence motif analyses performed in this study, clearly indicated that *Tg*DGAT2 is not part of the same superfamily of ATs (36). In *P. falciparum* parasites previous studies show the existence of a single DGAT1 (no DGAT2), which could be detected by antibodies against *Tg*DGAT1, suggesting that *Pf*DGAT is the most likely the only candidate for plasmodial triacylglycerol synthesis in this parasite unlike *Toxoplasma* (47,52,53).

Following structural and sequence analyses of *Tg*DGAT2, it was surprising to find that despite having 2 transmembrane domains this protein seemed to localize evenly throughout the parasite (Figure B). Under auxin treatment, IFA revealed we were unable to completely knockdown the protein, which instead seemed to accumulate in the apicoplast. We suspect this is a default using the mAID system, even if auxin can cross all 4 membranes of the apicoplast, perhaps the recruitment of the SCF<sup>TIR1</sup> complex to the ubiquitinated protein in the apicoplast is not possible (48), resulting in a failure in protein degradation. Alternatively, this could be a background signal of the apicoplast since western blot analyses revealed complete protein loss (Figure A). The IFA analyses of the double mutant line (*Tg*ΔDGAT1-DGAT2-iKD, Fig. 3D) further supported the predicted membrane-bound nature of *Tg*DGAT2, as it has a perinuclear/ER cellular localization like the DGAT1 isoform (36).

Previous work by Quittnat *et al.*, (2004), hypothesized *TgDGAT1* to be essential due to failed attempts to knock-out this gene, granted in a time before CRISPR and other current advances in gene editing. We now show that *TgDGAT1* can be deleted (Figure 3D) and that both paralogues seem to be non-essential for parasite survival in high lipid conditions (Figure C/D). Instead, we see that absence of both DGATs in this parasite under lipid scarcity leads to a favourable increase in parasite growth, this could suggest that these proteins are involved in controlling parasite growth when resources are limited. In this case the TAG levels are decreased (so LD cannot be formed) and we can hypothesize that accumulating FFA are instead channelled to bulk PL synthesis facilitating membrane biogenesis and division (7,31,32). We initially thought there would be function redundancy under these conditions (36), unlike in other eukaryotic models where DGAT1 and DGAT2 have preferences to exogenous and endogenous FAs, respectively (28,54). This can further be investigated by metabolic labelling studies. *TgDGAT2* seemed to be crucial for parasite development at 0% FBS, imitating lipid starvation (Figure C), again suggesting another protein that is dependent on nutrient availability.

Using the *TgDGAT2*-mAID line, we were able to show that protein loss led to an increase in DAG levels, the substrate, but no changes in TAG levels were lipidomically observed. LD analyses showed that despite this the number of LD were diminished under *TgDGAT2* loss (Fig. 5E), suggesting a crucial role in LD biogenesis. This process is highly dynamic and known to sequester several proteins for complex formation (55). Understanding this complex in apicomplexa versus humans, would be crucial in conceptualizing a novel therapeutic strategy against the parasites. This can be done by large-scale protein association studies and biochemical techniques (MS-MS, NMR etc.).

The nutrient-dependency of these proteins provides insight into the parasite's nutrient flexibility in that they can adapt to different environmental conditions rendering proteins that were once non-essential, essential, or vice-versa.

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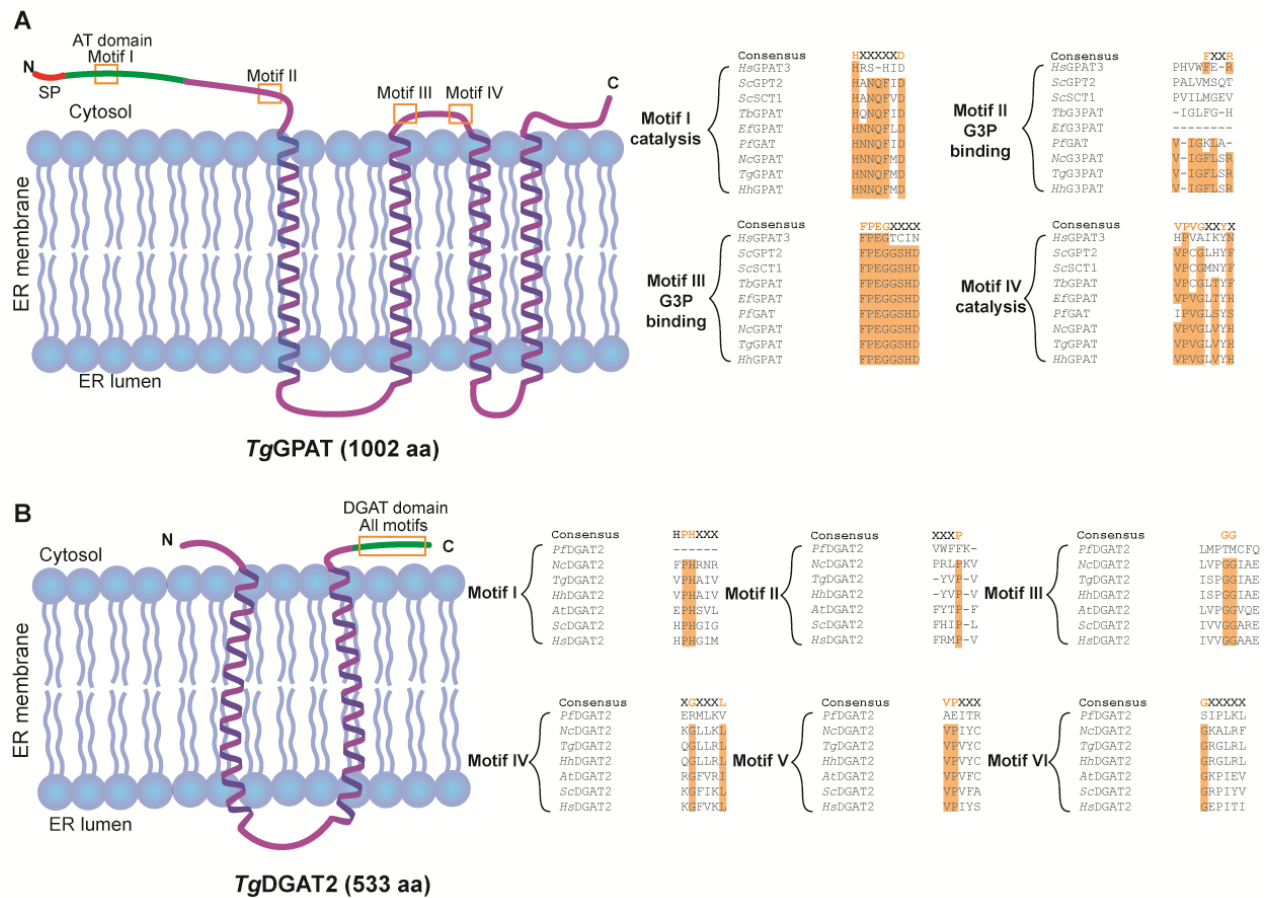
## Tables

Table 1: Primers used in this study

Primer name	5'→3'
Forward primer <i>TgGPAT</i>	TCGCTCCTCTTCAAATGAAGAGCCTCATGAA CAACGCAGAGGAAATTCTCGCTAGCAAGGGC TCGGGC
Reverse primer <i>TgGPAT</i>	TATATATACATATATATATATATATATATGCATAC ATGACATGAGAAATGCCATAGGGCGAATTGG AGCTCC
Guide primer <i>TgGPAT</i>	GCGCAAATAGAGGGGAAAGGA
Forward primer <i>TgDGAT2</i>	TTGTGGAATCGAAGAGGAAGGAAAACGGGG GGTCCGGTCCTCCCGTGAGTCGTCCTCAC
Reverse primer <i>TgDGAT2</i>	ACGCATGGAAGCATTTTTGATCTAGCCAGGT CAAGCTCTATGCCAGAACACTTGTCAACCG
Guide primer <i>TgDGAT2</i>	GCAGCGGGATTGCCCAAAGC
Forward primer <i>TgDGAT1</i>	TTGTGGAATCGAAGAGGAAGGAAAACGGGG GGTCCGGTCCTCCCGTGAGTCGTCCTCAC
Reverse primer $\Delta TgDGAT1$	ACGCATGGAAGCATTTTTGATCTAGCCAGGT CAAGCTCTATGCCAGAACACTTGTCAACCG
Guide primer $\Delta TgDGAT1$	GCAGCGGGATTGCCCAAAGC
<b>Screening primers:</b>	
<i>SSTg35</i>	ACTCAGCGAATGAATCTCCA
<i>SSTg38</i>	CGCCATGCATGGATGTATCC
<i>SSTg39</i>	TCGTGATGGTCGAGGTGACG

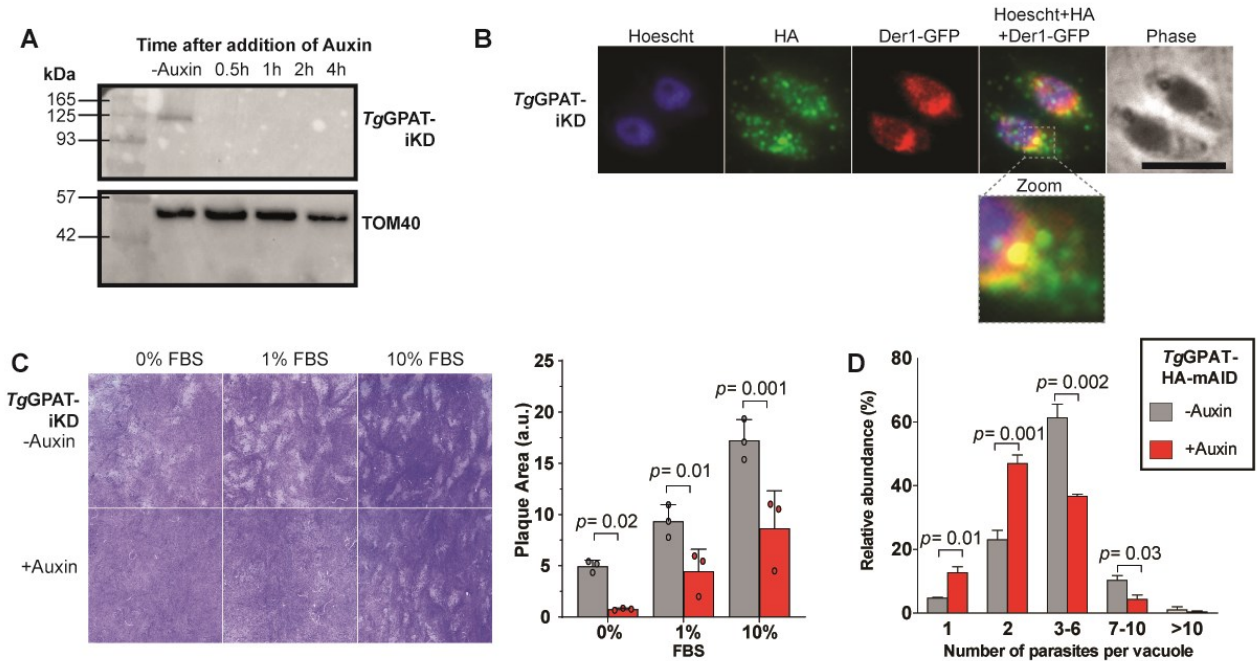
SST <sub>g</sub> 48	ATACATTCGCAAATCAATCTTTCGCAGGTACG GC
SST <sub>g</sub> 49	TTGAAGTCGTAGCAGCAACCAACGA

## Figures



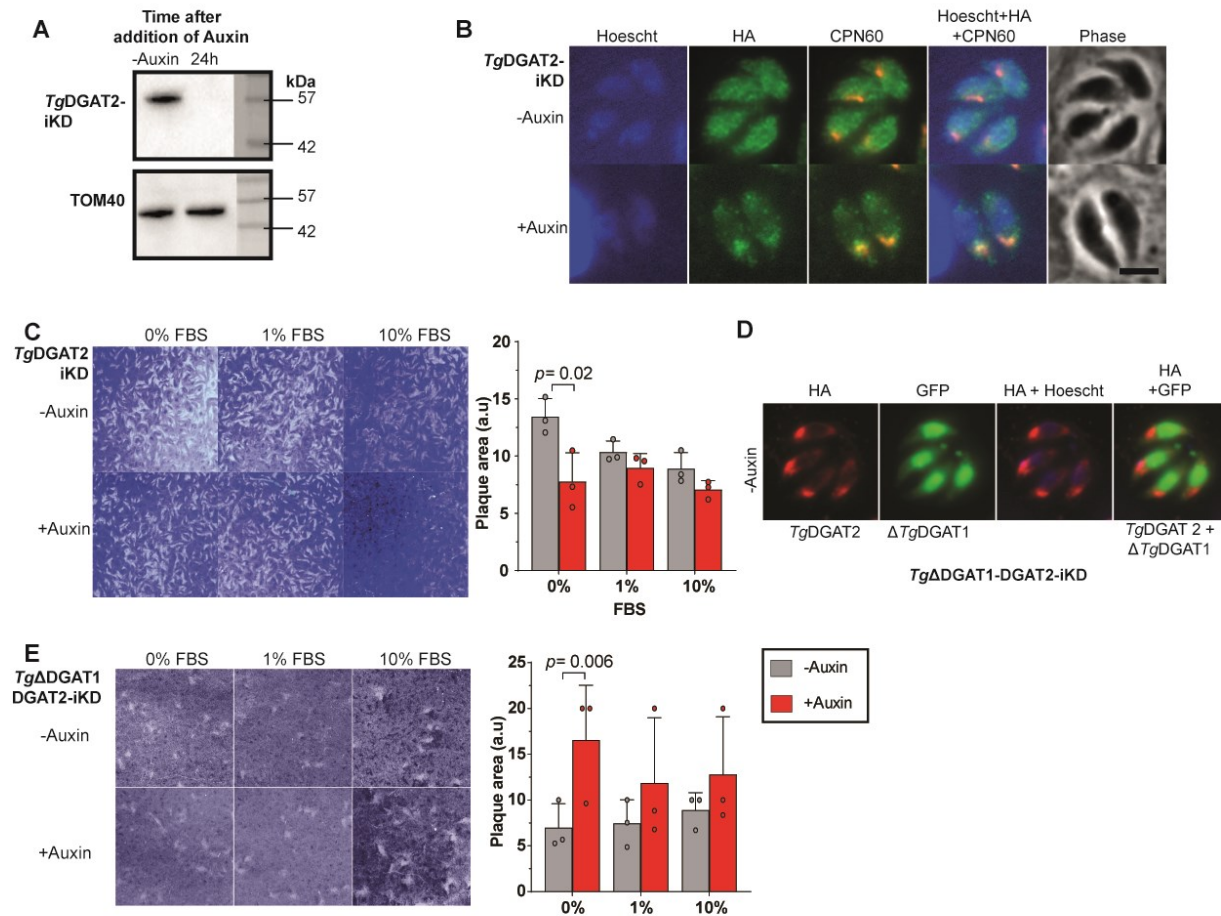
**Figure 1: Protein sequence analysis of acyltransferases in *Toxoplasma gondii* parasites.**

Schematic representations of putative A) glycerol-3-phosphate (G3P) acyltransferase (GPAT) and B) diacylglycerol acyltransferase 2 (DGAT2). *TgGPAT* (TGTT1\_256980) and *TgDGAT2* (TGTT1\_275600) have 4 and 2 transmembrane domains respectively, with the catalytic domains (AT-acyltransferase or DGAT in green) suggested to be on the cytosolic side of the ER membrane. The signal peptide (SP) is indicated in red. The consensus sequence of each motif for each protein is indicated on the right of each diagram with identical sequences highlighted in orange. The number of amino acid (aa) per protein are indicated below. Genes used for sequence analyses: GPAT3- *Homo sapiens* GPAT3 (Q53EU6), *Saccharomyces cerevisiae* GPT2 (P36148), *Saccharomyces cerevisiae* SCT1 (P32784), *Trypanosoma brucei* GPAT (TbG972.10.3920), *Eimeria falciformis* GPAT (ASN64451.1) *Plasmodium falciparum* GAT (PF3D7\_1212500), *Neospora caninum* GPAT (NCLIV\_029980), *Hammondia hammondi* GPAT (HHA\_256980) and for DGAT2- *Homo sapiens* DGAT2 (Q96PD7), *Arabidopsis thaliana* DGAT2 (Q9ASU1), *Saccharomyces cerevisiae* DGAT2 (Q08650), *Plasmodium falciparum* DGAT1 (PF3D7\_0322300), *Neospora caninum* DGAT2 (NCLIV\_029090) and *Hammondia hammondi* DGAT2 (HHA\_275600).



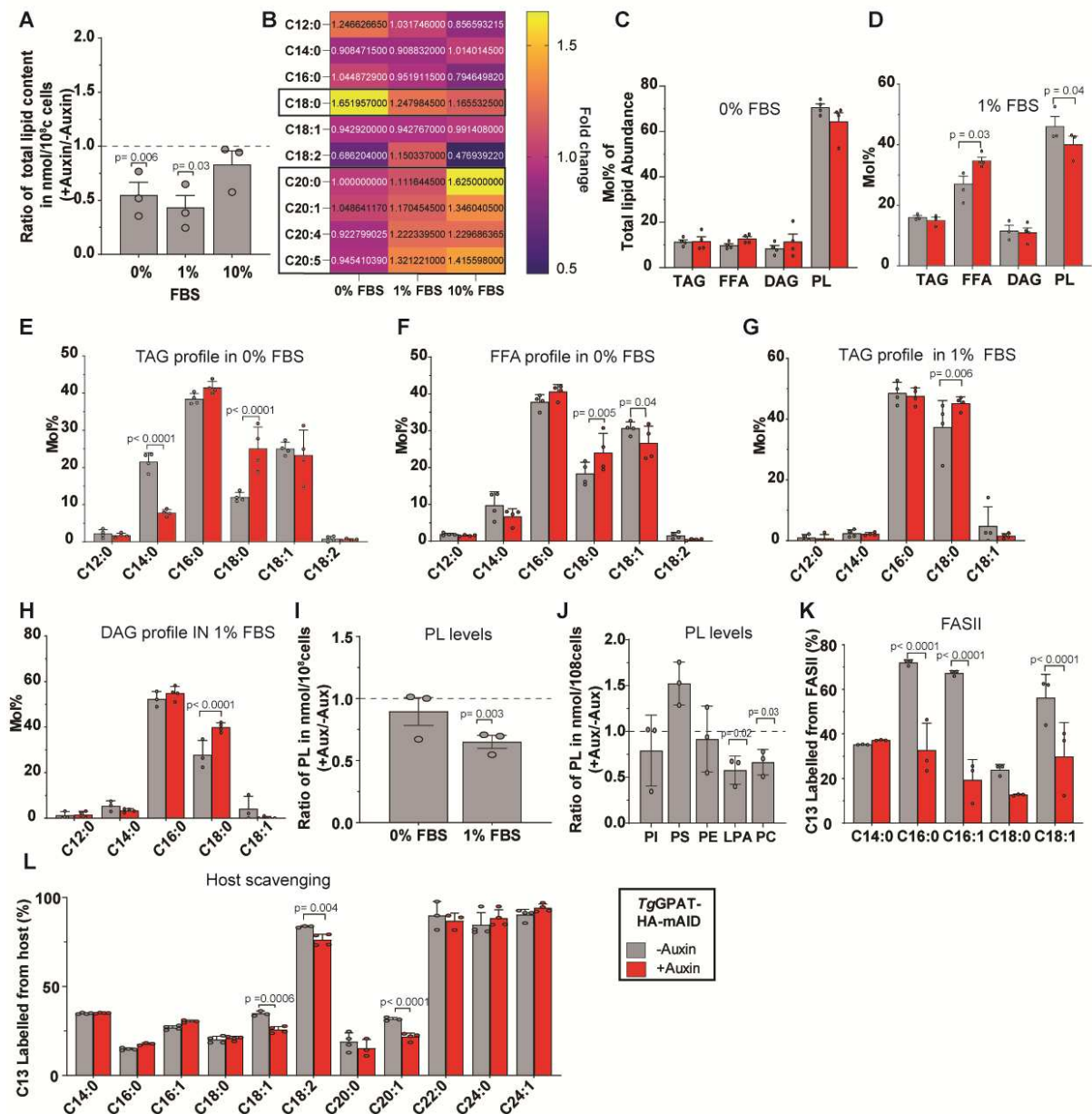
**Figure 2: Cellular phenotypic characterisation of *TgGPAT*.**

A) Western blot shows *TgGPAT* downregulation in *TgGPAT*-mAID iKD parasite line, in less than 30min after adding 100 $\mu$ M Auxin. *TgGPAT* (120 kDa) was probed with anti-HA antibody and *TOM40* (40 kDa) was used as the loading control. B) IFA showing *TgGPAT* (in green) in close vicinity of ER marker *Der1* (in red). C) Plaque assays performed to determine changes in parasite growth after depletion of *TgGPAT*, showed significant effects in the presence of 0%, 1% and 10% FBS. The plaque area percentage (right) of *TgGPAT*-mAID iKD (+Auxin) declined by 4-5% in 0% and 1% FBS and even more in 10% FBS, depicting a strong growth defect. There is a statistical significance between the treated cultures in conditions of 0% vs 1% FBS and 0% vs 10% FBS, suggesting a 'rescue effect' in a high lipid-nutrient environment. D) A replication assay revealed significant difference between the relative abundance of each number of parasites of vacuoles between treated and untreated *TgGPAT*-mAID iKD cultures, depicting a growth defect in the absence of the protein. Scale bars= 2  $\mu$ M. The error bars are representative of standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by t-test using GraphPad Prism. Range of statistical significance was signified as per the *p* value indicated in each graph.



**Figure 3: Cellular phenotypic characterization of *TgDGAT2***

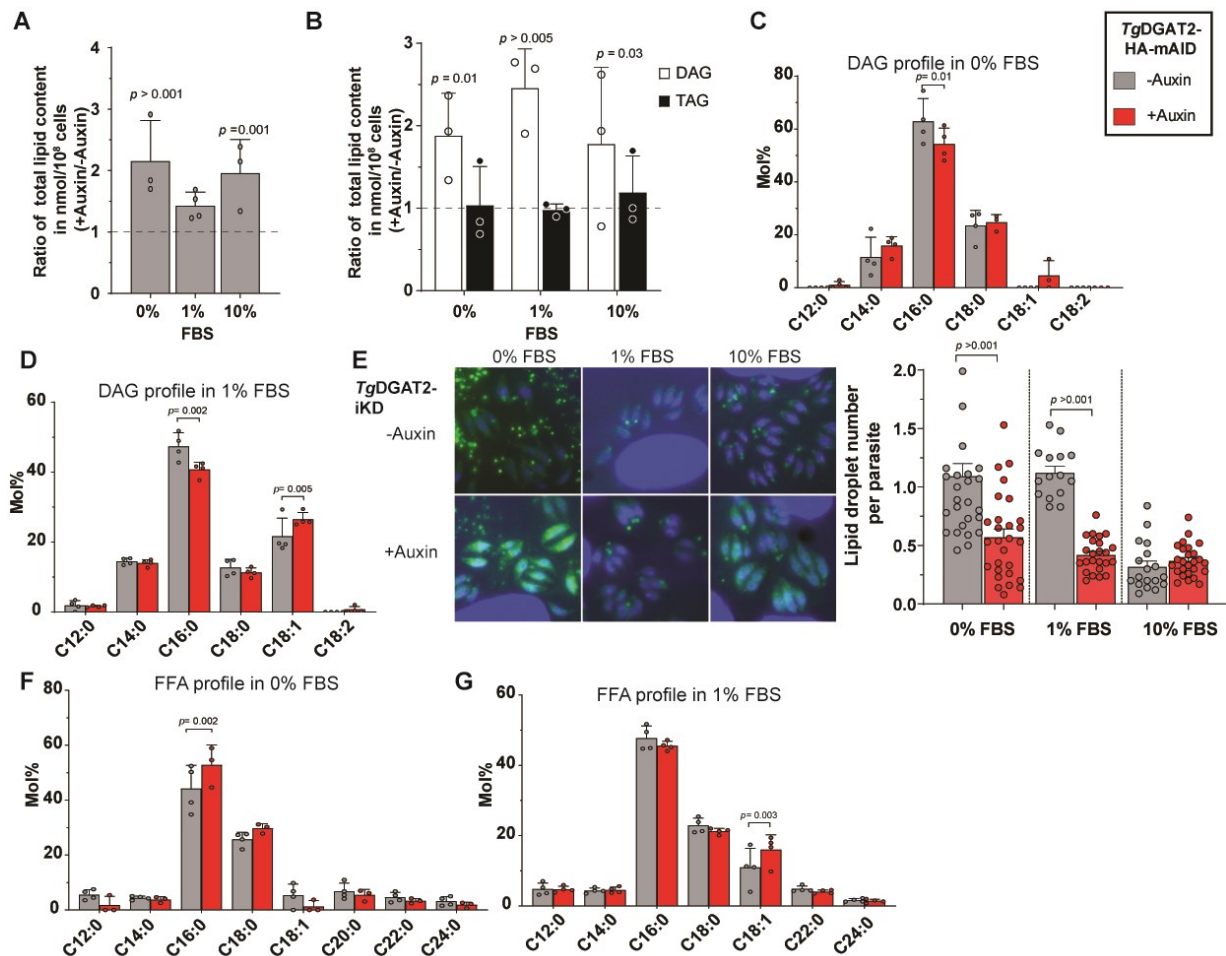
A) Western blot shows *TgDGAT2* downregulation in *TgDGAT2*-mAID iKD parasite line, after of 100 μM auxin treatment. *TgGPAT* (120 kDa) was probed with anti-HA antibody and TOM40 antibody (40 kDa) was used as the loading control. B) IFA showing down regulation of *TgDGAT2* (in green) under the same conditions. Even with auxin treatment there is an accumulation of immunofluorescence signal in the apicoplast (anti-CPN60 in red). C) Plaque assays performed to determine changes in parasite growth after depletion of *TgDGAT2* under 0%, 1% and 10% FBS. The plaque area percentage (right) of *TgDGAT2*-mAID iKD (+Auxin) declined by 5% in 0% FBS, depicting a strong growth defect under nutrient stress (a.u.-arbitrary units). D) IFA showing down regulation of *TgΔDGAT1*-DGAT2-iKD, with *TgDGAT2* being perinuclear and the *ΔTgDGAT1*-GFP signal in the nucleus as expected. Scale bars= 2 μm. The error bars are representative of standard error of mean (SEM) for each study. Statistical significance was determined for each experiment by t-test using GraphPad Prism. Range of statistical significance was signified as per the *p* value indicated in each graph.



**Figure 4: Lipidomic analyses of *TgGPAT*-mAID-iKD**

A) A ratio of the total lipid content without/with *TgGPAT* in parasites under 0-10% FBS conditions, *p*-values are compared to 1 (unchanged levels). B) A heatmap displaying a ratio of fatty acid (FA) abundance in nmol/parasite (10<sup>8</sup>) without/with *TgGPAT* in parasites under 0-10% FBS, yellow and purple indicate a fold increase or decrease in FA species, respectively. Mol% of each FA species found in triacylglycerol (TAG), free fatty acids (FFA), diacylglycerol (DAG) and phospholipids (PL) found in the total lipid content of parasites in C) 0% and D) 1% FBS with (grey, -auxin) and without (red, +auxin) *TgGPAT*. Mol% of FA species of E) TAG and F) FFA levels at 0% FBS with (grey) and without (red) *TgGPAT*. Mol% of FA species of G) TAG, H) FFA and I) DAG levels at 1% FBS with (grey) and without (red) *TgGPAT*. J) A ratio of the total PL content without/with *TgGPAT* in parasites under 0-1% FBS conditions and K) the same ratio of individual PL (phosphatidylinositol-PI, phosphatidylserine-PS, phosphatidylethanolamine-PE, lysophosphatidic acid-LPA, and phosphatidylcholine-PC) at 1% FBS, *p*-values are from an unpaired of the ratio vs 1 (unchanged levels). Percentage of <sup>13</sup>C labelled FA species that are L) FASII-

derived and M) host-derived.  $n = 3$ , unpaired  $t$  test  $p$  values are shown on graphs. Data are all presented as mean values  $\pm$  standard error of mean.



**Figure 5: Lipidomic investigation of *TgDGAT2***

A) A ratio of the total lipid content without/with *TgDGAT2* in parasites under 0-10% FBS conditions,  $p$ -values are from an unpaired of the ratio vs 1 (unchanged levels). B) A ratio diacylglycerol-DAG (white) and triacylglycerol-TAG (black) without/with *TgDGAT2* in parasites under 0-10% FBS conditions,  $p$ -values are from an unpaired of the ratio vs 1 (unchanged levels). Mol% of each FA species found in DAG at C) 0% and D) 1% FBS with (grey, -auxin) and without (red, +auxin) *TgDGAT2*. E) Immunofluorescence analyses of lipid droplet in parasites with and without auxin using Nile red (in green) and Hoechst (DNA staining) under 0-1% FBS conditions, with an accompanying graph (left) with the average lipid droplet number per parasite quantified by iterative image processing. Mol% of each FA species found in FFA at F) 0% and G) 1% FBS with (grey, -auxin) and without (red, +auxin) *TgDGAT2*.

**A**

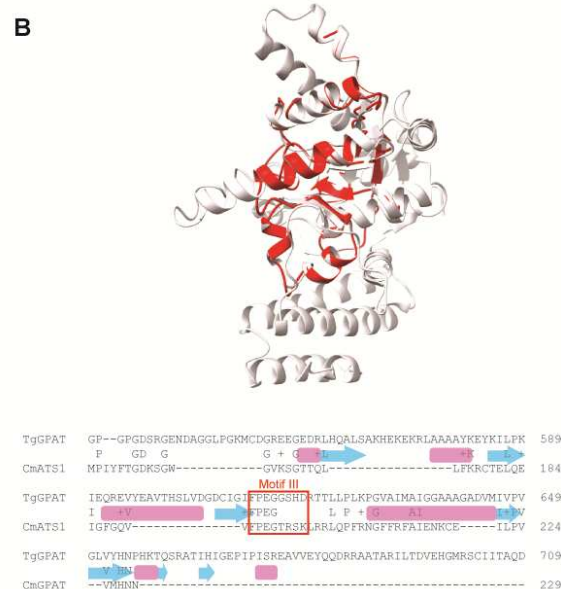
Arabis thaliana GPAT3  
Arabidopsis thaliana GPAT5  
Arabidopsis thaliana GPAT9  
Homo sapiens GPAT4  
Rattus norvegicus GPAT3  
Rattus norvegicus GPAT4  
Homo sapiens GPAT3  
Saccharomyces cerevisiae LPAAT  
Cucurbita mosata GPAT  
Chlorella variabilis GPAT  
Cryptosporidium parvum GPAT  
Escherichia coli plS  
Cyanobacterium stanieri GPAT  
Trypanosoma brucei GPAT  
Eimeria falciformis GPAT  
Plasmodium falciparum GPAT  
Neospora caninum GPAT  
Toxoplasma gondii GPAT  
Hammondia hammondi GPAT  
Saccharomyces cerevisiae GPT2  
Saccharomyces cerevisiae SCT1  
Galdieria sulphuraria GPAT  
Polytomella parva GPAT  
Nicotiana tabacum GPAT  
Homo sapiens GPAT2  
Homo sapiens GPAT1  
Sus scrofa GPAT1  
Escherichia coli pls B  
Chromera velia plsC1  
Chromera velia plsC2

Plants  
Mammals  
Apicomplexa  
GPAT1/2-like (mitochondrial)  
GPAT3/4-like (microsomal)

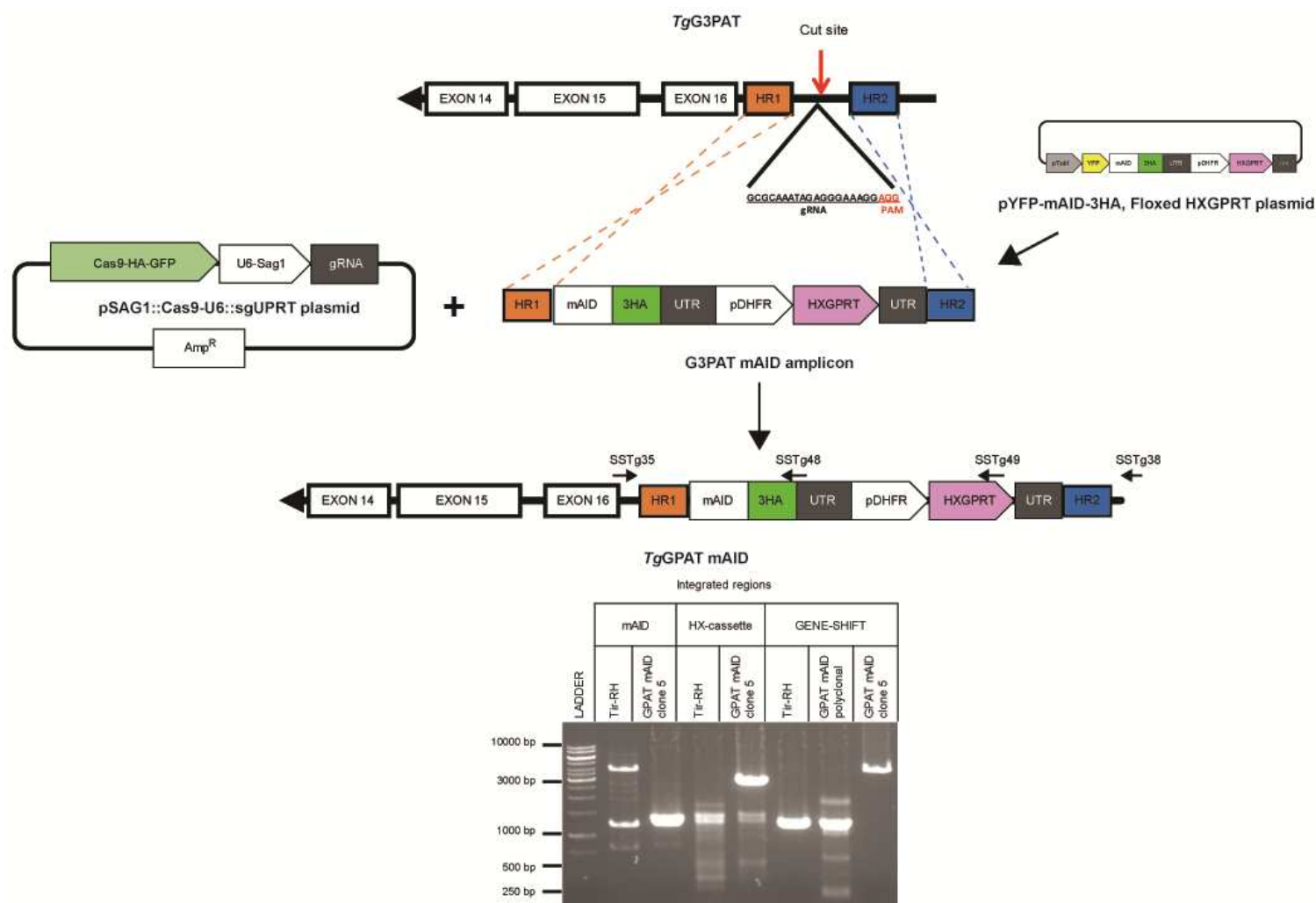
**C**

Brassica napus DGATa  
Brassica napus DGATb  
Plasmodium falciparum DGAT1  
Babesia bovis DGAT  
Saccharomyces cerevisiae DGAT2  
Homo sapiens DGAT2  
Rattus norvegicus DGAT2  
Sus scrofa DGAT2  
Arabidopsis thaliana DGAT2  
Nicotiana tabacum DGAT2  
Neospora caninum DGAT2  
Toxoplasma gondii DGAT2  
Hammondia hammondi DGAT2  
Chromera velia DGAT2  
Mycobacterium tuberculosis DGAT2a  
Mycobacterium tuberculosis DGAT2b  
Vitrella brassicaformis DGAT2  
Cryptosporidium parvum DGAT2

Most probably DGAT1  
Mammals  
Plants  
Apicomplexa



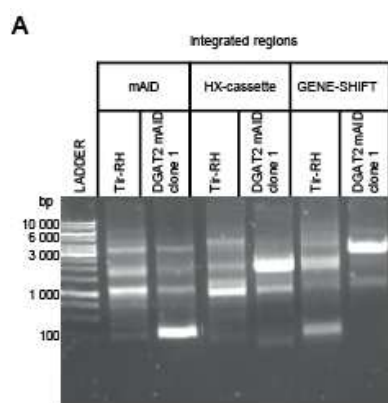
A) Phylogenetic tree of glycerol-3-phosphate acyl transferases (GPAT) in different species of organisms namely plants, mammals, unicellular organisms including apicomplexa parasites. This analysis shows that *TgGPAT* is like GPAT type 3/4 microsomal ATs usually found near the endomembrane system. B) Structure modelling of *TgGPAT* (in red) using *Cucurbita moschata* (squash or pumpkin, in white) ATS1 (*CmATS1*, ID: Q9FEQ0) structure (40) as a threading template suggest that *TgGPAT* contains the putative G3P binding domain found in motif III. Pink areas indicate  $\alpha$ -helixes and blue arrows show  $\beta$ -pleated sheets. C) Phylogenetic tree of diacylglycerol acyltransferase (DGAT) in different species of organisms namely plants, mammals, unicellular organisms including apicomplexa parasites. This shows that *TgDGAT2* is more related to DGAT2 in mammals, plants and are unique from DGAT1 proteins (like *PfDGAT*, *BbDGAT*).



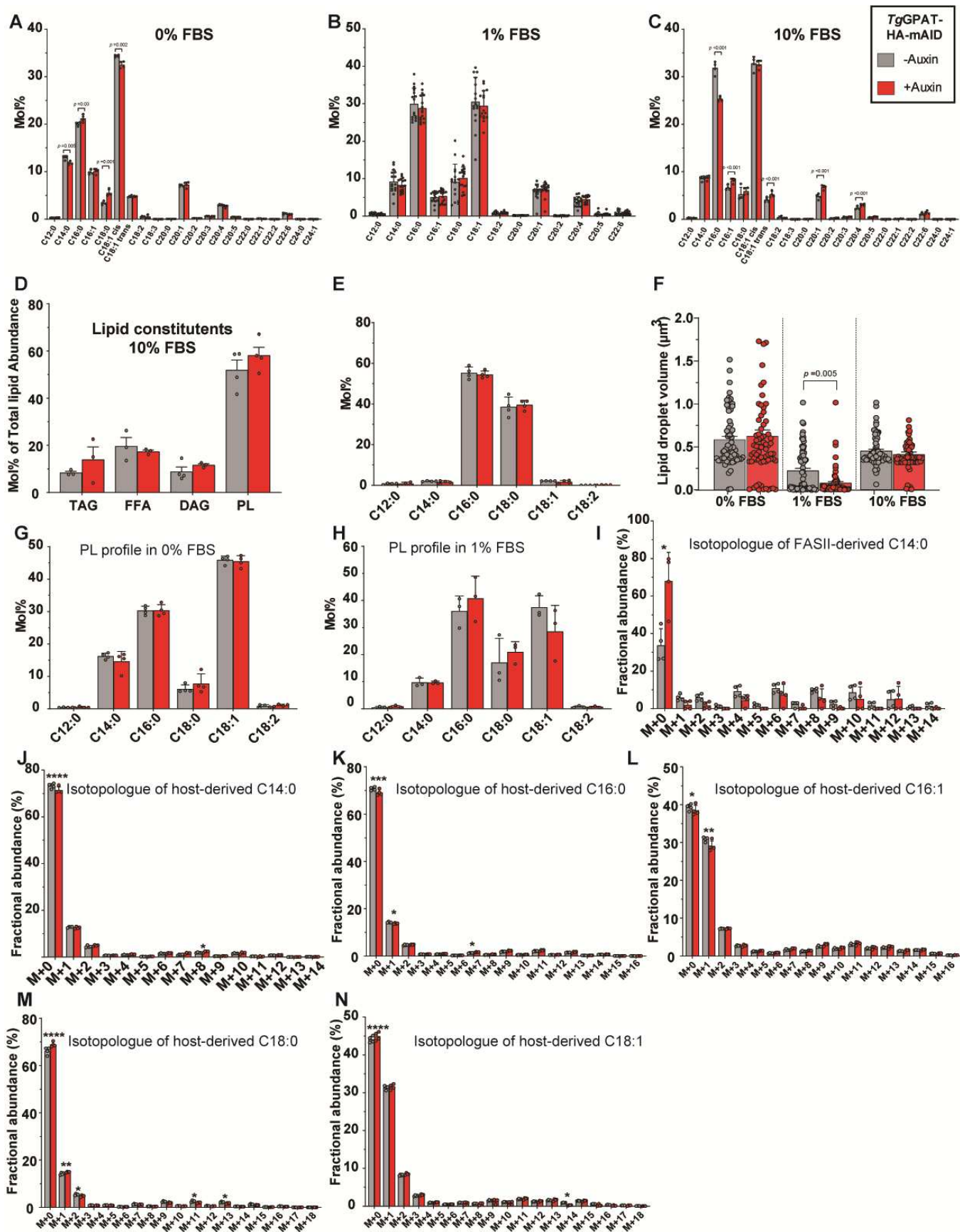
**Sup. Figure 2: CRISPR-Cas9 strategies used generate an auxin inducible mutant of *TgGPAT* in *Toxoplasma* parasite.**

A C-terminally tagged HA line with inducible post-translational protein degradation was generated using the Auxin-inducible Degron system (AID). Homology regions of the C-terminal end of *TgGPAT*/*TgDGAT2* (not shown here) excluding the stop codon were amplified using the primers found in *Tables*

*Table 1*. PCR (Table 1) were preformed using mutant parasite DNA to show correct integration of the mAID sequence, the HX drug cassette and correct gene insertion.

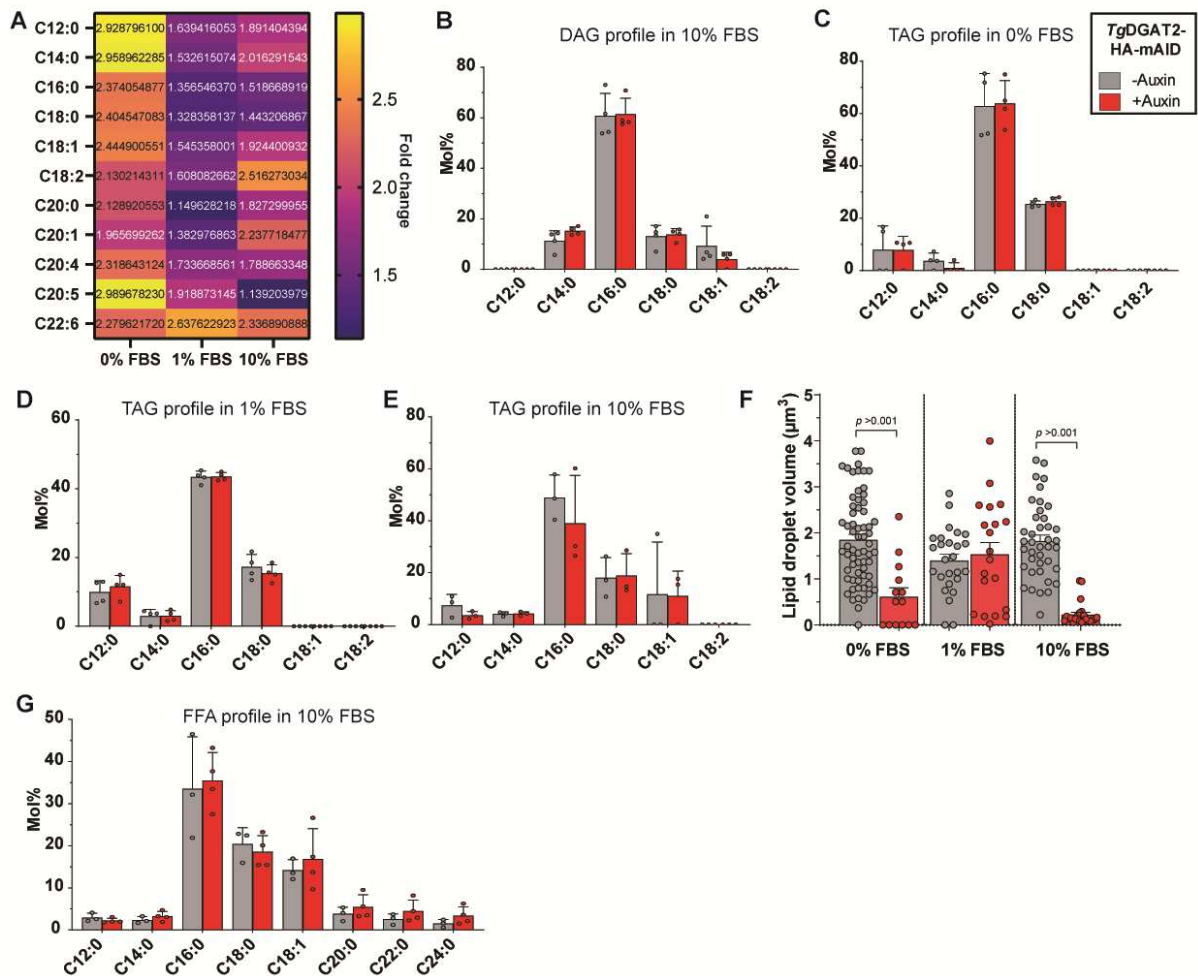


**Sup. Figure 3: PCRs showing correct integration of auxin-inducible knock down system of *TgDGAT2*.**



#### **Sup. Figure 4: Supplementary lipidomic data of *TgGPAT*-HA-mAID**

A-C) Fatty acid (FA) profile total lipid content with (grey) and without (red) *TgGPAT* in 0-10% FBS. D) Mol% of each FA species found in triacylglycerol (TAG), free fatty acids (FFA), diacylglycerol (DAG) and phospholipids (PL) found in the total lipid content of parasites in 10% FBS. E) Lipid droplet analyses of the lipid droplet volume of parasites with (grey) and without (red) *TgGPAT* in 0-10% FBS. F) FA profile of PL content without/with *TgGPAT* in parasites under G) 0% and H) 1% FBS conditions. Parasite lipid mass isotopologue distributions in 1% FBS for I) FASII-derived C14:0 and host-derived J) C14:0, K) C16:0, L) C16:1, M) C18:0 and N) C18:1.  $n = 3$ , unpaired t test  $p$  values represented in the figures as follows:  $p > 0.05$ ;  $*p < 0.05$ ;  $**p < 0.01$ . Data are all presented as mean values  $\pm$  standard error of mean.



### Sup. Figure 5: Supplementary lipidomic data of *TgDGAT2*-HA-mAID

A) A heatmap displaying a ratio of fatty acid (FA) levels in nmol/parasite ( $10^8$ ) without/with *TgDGAT2* in parasites under 0-10% FBS. B) Mol% of FA species of diacylglycerol (DAG) at 10% FBS with (grey) and without (red) *TgDGAT2*. Mol% of FA species of triacylglycerol (TAG) at C) 0%, D) 1% and E) 10% FBS with and without *TgDGAT2*. F) Lipid droplet analyses of the lipid droplet volume of this parasites line with and without auxin in 0-10% FBS. G) Mol% of FA species of free FA (FFA) this parasites line with and without auxin in 10% FBS.  $n = 3$ , unpaired t test  $p$  values are shown on graphs. Data are all presented as mean values  $\pm$  standard error of mean.

## CHAPTER 7: CONCLUSION AND PERSPECTIVES

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Apicomplexan parasites depend on lipids for survival. These biomolecules play an indispensable role by regulating metabolic flux, acting as signalling molecules, storage fuels and structural building blocks of membranes. Collectively the three studies presented in my thesis work have contributed to our understanding of the lipid metabolism of apicomplexan parasites. These parasites are unique in that they can rely on lipid scavenging and *de novo* synthesis, making their lipid mechanisms complicated. We know from this and previous work that these parasites can metabolically reprogram and adapt to changing environments. This metabolic capacity comes in handy seeing that apicomplexa have complex lifecycles and invade a large range of different cells in different vectors.

My PhD project focused on the processes involved in parasite nutrient adaptation when it comes to PL synthesis and lipid storage formation. I did this by investigating three protein families which I hypothesized are important in these mechanisms:

- Acyl-CoA synthetases (ACSs)
- Phospholipases
- Acyltransferases (ATs)

### ***Deciphering the putative peroxisomal metabolome in Apicomplexa:***

Our phylogenetic work now postulates that 7 ACSs exist in *Toxoplasma* parasites. A large scale full lipidomic/metabolic study of these highly diverse proteins would contribute to understanding the parasites complex lipid metabolism. I studied an unusual ACS, *TgACS1*, which can activate FAs derived from TAG under nutrient stress. This protein contains a peroxisomal targeting sequence (PTS), which has been characterized before as an essential tripeptide for correctly localization to the peroxisome (Ding, Clayton and Soldati, 2000; Kaasch and Joiner, 2000; Pratt *et al.*,

2013; Ludewig-Klingner *et al.*, 2017; Moog, Przyborski and Maier, 2017). This protein is peroxisomal too and activates FAs destined for energy generation via  $\beta$ -oxidation, which instead of the mitochondria could possibly exist in a peroxisomal-like organelle. The close association of peroxisomes and LDs in other eukaryotic model, emphasize that this could also occur in the parasite (Kohlwein, Veenhuis and van der Klei, 2013). Additionally, I show that this process is most probably only induced under certain circumstances such as extracellular conditions or when nutrients are scarce. It is crucial to identify the metabolites of the  $\beta$ -oxidation pathway to understand when, why and even if this process occurs in Apicomplexa.

The protein needed for the correct localization of FAs into the mitochondria to allow for FA oxidation (CPT1a) is absent in *Toxoplasma* and *Plasmodium*, hence why peroxisomal FA oxidation seems more probable (Danne *et al.*, 2012). The lack of peroxisomal import proteins and enzymes in plasmodial parasites suggest that the occurrence of conventional peroxisomes is unlikely (Mcintosh, Elliott and Joiner, 2005).

Some proteins involved in the biogenesis of peroxisomes (PEX proteins) also exist in *Toxoplasma* and *P. falciparum* parasites. To date, there is still no microscopic evidence of this organelles and we do not know how they are involved in metabolic processes (Ludewig-Klingner *et al.*, 2017; Moog, Przyborski and Maier, 2017). Additionally, the nutrient conditions of previous work (which were predominantly bioinformatical) were often overlooked and it is apparent that the lipid/nutrient availability can drastically change the metabolic choices made by the parasite (Shunmugam *et al.*, 2021; Shunmugam, Arnold, *et al.*, 2022; Walsh *et al.*, 2022).

Future work should entail deciphering the metabolome of these parasites under conditions known to induce  $\beta$ -oxidation such as excess FAs or glucose/amino acid

starvation. Electron microscopy would also be necessary to identify peroxisomal like vesicles and what other organelles they associate with (i.e. lipid droplets). Alternatively, the metabolome/lipidome of the parasite's unique mitochondrion should be characterized. The loss and repurposing of certain proteins could suggest that a  $\beta$ -oxidation process that is different from the host, can potentially be targeted through therapeutic strategies.

### ***Lipid mechanisms of sexual conversion:***

The discovery that LPC regulates sexual stage conversion in *P. falciparum* parasite was a turning point in the field Apicomplexa biology (Brancucci *et al.*, 2017). This work stressed how important the balance of PL and lyso-PL is in parasite development and disease propagation. Previous work has also highlighted this by characterizing a phospholipase, an enzyme responsible for the hydrolysis of a FA from the phosphoglycerol backbone of PC resulting in LPC (Singh *et al.*, 2019; Flammersfeld *et al.*, 2020). This specific group of phospholipases A2 enzymes (PLA2) are known to mediate membrane remodelling and vesicle secretion in organisms. Numerous phospholipases exist in *Plasmodium* which may or may not have redundant function. My thesis focused on the investigation of a phospholipase that is highly expressed in schizonts and sexual stage parasites and is critical when lipid deprived.

Lipidomic analyses show that *Pf*PNPLA2 is responsible formation of LBPA from PG. LBPA is a lipid normally localized to the plasma membrane, hence this protein's importance for membrane biogenesis and parasite division. LBPA (BMP) and PG seems to have an indirect relationship. Previous work show that PG levels are lowered and LBPA levels are heightened upon sexual commitment in red blood stages (Gulati *et al.*, 2015; Tewari *et al.*, 2020). My thesis work only focussed on asexual stages. Future perspectives would include viability and metabolic studies on sexual stages to

determine if indeed this protein plays a role in sexual conversion. Heterologous complementation and protein kinetics could be used to confirm enzyme function.

Additionally, the role of this protein in nutrient deprivation must be further studied as the limitation on parasite survival in these conditions resulted in poor sample yield for lipidomic analyses. It would be important to understand the lipidic mechanisms of sexual conversion, since targeting this process can ultimately hinder disease transmission.

### ***How ATs play an indispensable role in parasite lipid droplet biogenesis and lipid detoxification:***

Investigating the role of two acyltransferases in *Toxoplasma* parasites was the final part of my thesis work. Both *TgGPAT* and *TgDGAT2* have not been previously characterized and the results gathered here are novel to the field. These proteins have a strongly nutrient dependant role in the formation of either LPA or TAG, both crucial products of the glycerolipid pathway which is essential for membrane biogenesis and lipid detoxification (LD formation).

Unlike their hosts, *Toxoplasma* parasites have evolved to contain 2 sets of ATs (GPAT, AGPAT and DGAT) involved in the either an ER or apicoplast-based glycerolipid process. *TgGPAT* like its plastid paralogue, *TgATS1* is essential under lipid starvation and enrichment (Amiar *et al.*, 2016). This protein is essential to parasite development and its loss results in deleterious effects due to an accumulation of FAs followed by a lipotoxic death.

The sequential ATs, *TgAGPAT* and *TgATS2* (plastid-origin), are not similar since their absence have no significant effects on parasite survival. Therefore, I hypothesize that *TgGPAT*, which we now know is a GPAT3-type AT, may act as both a GPAT and

AGPAT (Sukumaran *et al.*, 2009). The exact enzymatic mechanisms of this protein should be investigated by complementation studies or enzymatic assays.

Throughout my thesis works, there is lipid group that is consistently affected, TAGs. This lipid group is the major component of LDs and seems to be crucial in the detoxification of lipids and prevents parasite death (Shunmugam *et al.*, 2021). Host-lipid droplet biology is deeply studied (considering they are larger and more numerous) in these parasites than their own LD biogenesis mechanisms (Nishikawa *et al.*, 2005; Romano *et al.*, 2013; Nolan, Romano and Coppens, 2017b; Nolan *et al.*, 2018; Pernas, Bean and Boothroyd, 2018; Vallochi *et al.*, 2018). Our lab is starting to unravel certain concepts about parasite LD biology, and this can be achieved by identifying and characterizing putative LD association proteins and other ATs.

Solubility issues with *TgGPAT*, a membrane bound AT, may pose certain limitations when attempting to isolate the protein (Shabits, 2017). Instead, complementation assays using an optimized codon sequence in a hybrid yeast mutant may be a solution. This protein is extremely important to parasite survival, and it would be worth investigating its druggability.

*TgDGAT2*, on the other hand seems to only have two-transmembrane domains and may be more soluble than *TgGPAT*. This protein and its paralogue *DGAT1*, are essential in LD biogenesis (in other eukaryotic models) and are usually in association with other proteins like *SEIPIN*, *MOSPD*, *VAP-B* etc., but binding partners/interaction are completely unknown in these parasites (Liang *et al.*, 2008; Zhang and Reue, 2017; Bhatt-Wessel *et al.*, 2018b; Choudhary *et al.*, 2018; Combot *et al.*, 2022).

My PhD work showed the protein essentiality in this parasite is highly dynamic and strongly dependant on nutrient availability. I show how this capability can influence

metabolic reprogramming (i.e. inducing  $\beta$ -oxidation), parasite stage conversion and lipid detoxification (LD vs membrane biogenesis). Collectively this brings novel data on how plastic and metabolically different these parasites are from their hosts. This work contributes to developing novel therapeutics in combating these diseases.

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