INVESTIGATING THE PHOSPHATIDYLINOSITOL-4-PHOSPHATE SIGNALING AND THE PHOSPHOINOSITIDE SEC14-LIKE PITP-DEPENDENT REGULATION IN THE APICOMPLEXA PARASITE TOXOPLASMA GONDII

A Dissertation

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ABSTRACT

Phosphoinositides (PIPs) are a biologically essential class of phospholipids that contribute to organelle membrane identity, modulate membrane trafficking pathways, and are central components of major signal transduction pathways that operate on the cytosolic face of intracellular membranes in eukaryotes. Apicomplexans are obligate intracellular parasites that are important causative agents of disease in animals and humans and are experimentally tractable models for studying the deepest rooted common eukaryotic ancestor. As such, these organisms offer insights into the evolutionary origins of essential PIP signaling pathways. Herein, in this work it is reviewed and discussed the regulatory mechanisms that control the spatial and temporal regulation of PIP in the Apicomplexan parasites *Plasmodium* and *T. gondii* when compared with current eukaryotic models. Then, phosphatidylinositol-4-phosphate (PtdIns4P) – an essential potentiator of exocytic trafficking from the trans-Golgi network (TGN) to the plasma membrane in yeast and mammalian cells – is characterized in intracellular tachyzoites of *T. gondii*. PtdIns4P pools were detected in the Golgi/TGN and post TGN compartments and through high resolution imaging techniques it was confirmed that PtdIns4P regulates the formation of mature dense granules (DG). Contrary to current models, this finding indicated that the process of DG biogenesis is a multistep process that resembles those characterized for regulated secretory pathways in professional secretory cells. In addition, the description of the cohort of *T. gondii* Sec14-like PITPs led to the characterization of a candidate with potential sterol precursor-binding and transport to the parasite. Our data demonstrated that studying PIP signaling and Sec14-like PITPs-dependent regulation in Apicomplexa organisms offers crucial insight into the evolutionary origins of essential PIP signaling pathways.

DEDICATION

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NOMENCLATURE

AA	Arachidonic acid
ANTH	AP180 N-terminal homology
Akt	Ak strain transforming protein kinase (protein kinase B)
ATc	Anhydrotetracycline
ATG14L	Autophagy related 14
APT1	Apicoplast phosphate transporter 1
CDP	Cytidine diphosphate
CPL	Cathepsin protease L
DAG	Diacylglycerol
DG	Dense granule
ELC	Endocytic-like compartment
ENTH	Epsin N-terminal homology
EGFP	Enhanced Green Fluorescent Protein
ER	Endocytic reticulum
ESCRT	Endosomal sorting complexes required for transport
FAPP1	Four phosphate adaptor protein 1
FASII	Type II fatty-acid biosynthesis
FERM	4.1 protein/Ezrin/Radixin/Moesin
FRM2	Formin 2
FtsH 1	ATP-dependent zinc metalloprotease FtsH 1
FYVE	Fab1/YOTB/Vac1/EEA1

GRA	Dense granule protein
Hb	Hemoglobin
IMC	Inner membrane complex
INPP4A/B	Inositol Polyphosphate-4-Phosphatase Type IA and B
IPs	Inositol phosphates
LAP	Large Anterior Punctate
MIC	Microneme protein
MTM	Myotubularin
MVB	Multivesicular bodies
OSBP	Oxysterol binding proteins
ROP	Rhoptry protein
PDK1	Phosphoinositide-dependent kinase-1
PIP	Phosphoinositide
PH	Pleckstrin Homology
PHDOK5	PH domain of Downstream of tyrosine kinase 5
PIK	Phosphoinositide kinase
PI-PLC	PtdIns Phospholipase C
PI3K	Phosphoinositide 3-kinase
PI4K	Phosphoinositide 4-kinase
PIP5KI/II	Phosphatidylinositol-4-phosphate 5-kinases type I and II
PIPK	phosphatidylinositol kinases
PIPP	Proline-rich inositol polyphosphate 5-phosphatase
PITP	PtdIns transfer protein

PKC	Protein kinase C
PKG	cGMP-dependent protein kinase PM Plasma membrane
PLD	Phospholipase D
PROPPIN	β-propellers that bind polyphosphoinositides
Psd2	PtdSer decarboxylase 2
PtdCho	Phosphatidylcholine
PtdEt	Phosphatidylethanolamine
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PtdIns	Phosphatidylinositol
PtdOH	Phosphatidic acid
PtdSer	Phosphatidylserine
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
PX	Phox Homology
RFP	Red fluorescence protein
Sfh	Sec Fourteen homology Family
SHIP1/2	Src-homology 2 containing inositol phosphatases 1 and 2
SKIP	Skeletal muscle and kidney-enriched inositol phosphatase
START	StAR related lipid transfer domain
TGN	Trans-Golgi Network
UVRAG	UV Radiation Resistance Associated
YFP	Yellow fluorescence protein

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1. REGULATION OF PHOSPHOINOSITIDE METABOLISM IN APICOMPLEXAN PARASITES

1.1. Introduction

1.1.1. PIP signaling in eukaryotes

PIPs contribute to organelle identity, and regulate both membrane trafficking and other signal transduction events at eukaryotic cellular membranes (Figure 1.1). PIPs are produced by phosphorylation of the inositol headgroup of phosphatidylinositol (PtdIns). Diversity of PIP isoforms is greater in higher eukaryotes. For instance, yeast produces five distinct PIP isoforms: PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,5)P_2, and PtdIns(4,5)P_2. Whereas metazoan organisms generally produce seven isoforms: those found in yeast plus PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Each PIP isoform executes its own unique biological activities, and on this basis, these molecules are considered to create a signaling "code". This PIP code is the foundation of a major intracellular signaling system that interfaces with virtually all pathways that control and regulate cell growth, motility, division, and death in eukaryotic cells.

The PIP code is read by specific PIP-binding domains of effector proteins. The first such domain described was the Pleckstrin Homology (PH) domain from phospholipase C delta (PLCδ), which is a high affinity PtdIns(4,5)P_2-binding module (Garcia et al., 1995; Lemmon et al., 1995). This discovery paved the road for the identification of many other PIP-binding modules (e.g., FERM, FYVE, PX, ENTH/ANTH, PROPPIN domains, and others), that exhibit varying degrees of PIP-

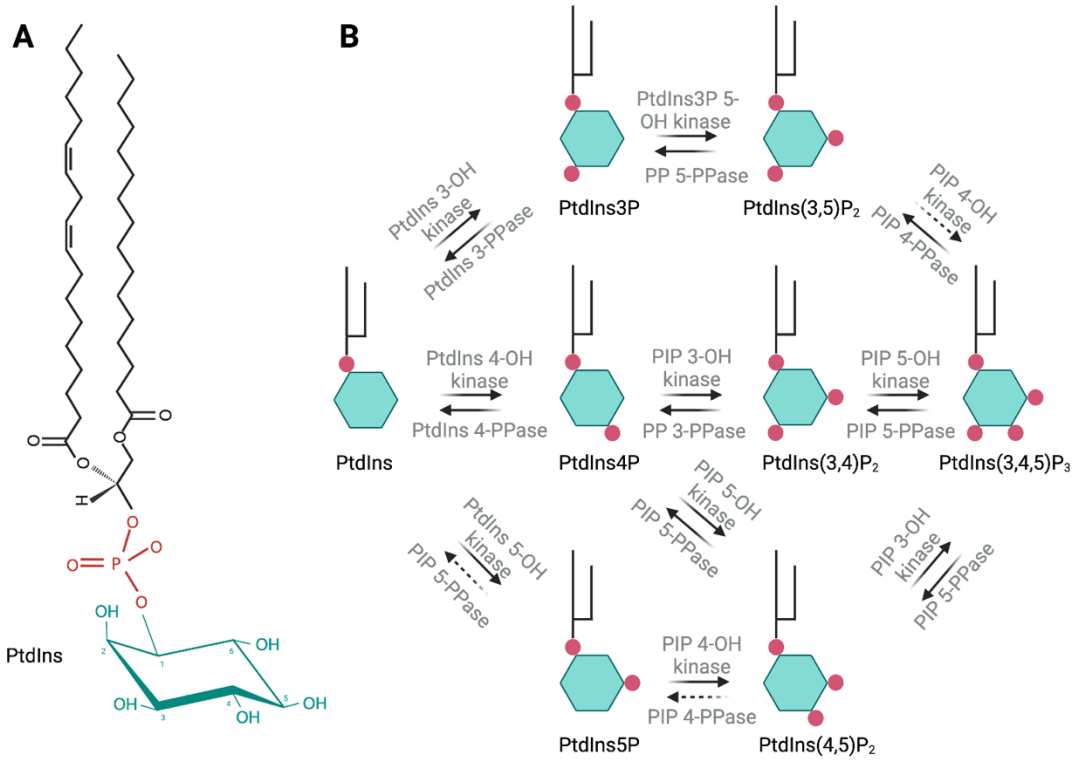

Figure 1. 1 Molecular structure of PtdIns and schematic representation of the seven PIP species and their synthesis in eukaryotic cells. Enzymatic activity involved in the synthesis or degradation of these species are indicated over the respective arrows. Dashed arrows indicate an enzymatic activity that has not been fully described.

binding specificity and affinity (Hammond et al., 2015). This chemical diversity allows intricate and combinatorial fine-tuning of PIP-dependent regulation of complex cell functions. In that regard, PIPs are uniquely eukaryotic phospholipids.

PIPs are not homogeneously distributed along the surface of intracellular membranes. Rather, these are organized into dynamic domains that impose spatial and temporal regulation of peripheral membrane protein binding and/or enzymatic reactions. Thus, PIP spatiotemporal location in a cell is tightly controlled. Cellular PIP levels are

regulated through the activity of PtdIns kinases (PIKs), phosphoinositide kinases (PIPK), and PIP phosphatases (Figure 1.1). PIKs catalyze the phosphorylation of PtdIns on the inositol ring at positions 3′, 4’ or 5’-OH. PIPK phosphorylate the inositol ring of PIPs at the 3’, 4’ or 5’-OH positions. Phosphatases catalyze the removal of a -PO4 group at 3′, 4’ or 5’-OH. Dysregulation of PIP homeostasis in human cells is strongly associated with cancer, primary immunodeficiencies, developmental disorders, and various other pathologies (Di Paolo and Camilli, 2006; Balla, 2013).

Organisms of the phylum Apicomplexa are primitive eukaryotes that exhibit a parasitic lifestyle. Recent advances in cellular and molecular studies of Apicomplexans demonstrate the role of phosphoinositide signaling networks at many key points of parasite biology. The unique biological niche occupied by Apicomplexa, and the resulting adaptations that come with it, identify these parasites as exceptional models for the study of novel and ancient aspects of phosphoinositide biology. In this review, we consider how phosphoinositide signaling is regulated in Apicomplexa, and how this information might be used to better understand both parasite biology and eukaryotic lipid signaling.

1.1.2. Apicomplexan parasites

Members of the phylum Apicomplexa are obligate intracellular parasites that infect both vertebrate and invertebrate hosts, and are significant disease-causing agents of both animals and humans. There are seven genera that infect humans. These include: (i) *Plasmodium* spp. which are the causative agents of malaria, (ii) the opportunistic

human pathogen *Toxoplasma gondii*, (iii) the malaria-like parasite *Babesia* spp., (iv) the animal parasite and opportunistic human pathogen *Cryptosporidium* spp., and (v) the human intestinal disease parasites *Isospora belli*, *Cyclospora cayetanensis*, and *Sarcocystis* spp., (Ackers et al., 1997; Chakraborty et al., 2017). Among the Apicomplexa, *Plasmodium* and *T. gondii* are the most experimentally tractable as these organisms can be cultured in vitro, can be studied in rodent models of infection, and are amenable to genetic manipulation. Thus, these two organisms represent the focus of this review.

Because the Apicomplexa are obligate intracellular parasites, these organisms must invade their respective host cells and establish a suitable intracellular environment that supports their replication and proliferation. In that regard, most Apicomplexans produce an elaborate intracellular compartment termed the parasitophorous vacuole (PV) through which the parasites scavenge nutrients from the host to power parasite cell division or differentiation. In the acute phases of infection, the parasites escape the PV and egress from the host cell in order to propagate the infection throughout the tissue. Apicomplexa have complex developmental cycles which include both acute and latent phases, and these organisms are able to pass through a variety of insect vectors and infect a variety of animal hosts.

1.1.3. The unique Apicomplexan endomembrane system

Apicomplexans possess highly specialized endomembrane structures that allow these parasites to carry out important functions associated with execution of their

complex life cycles. As members of the superphylum Alveolate, Apicomplexans have evolved a unique double membrane structure located below the plasma membrane. This inner membrane complex (IMC) is an alveolar network of flattened vesicles that covers the entire cell except its very basal and apical poles and the micropore/cytostome (an invagination or cup-shaped structure at the plasma membrane and PV membrane (PVM)). Protein profiling analyses in *Plasmodium* and *T. gondii* suggest the IMC executes a number of functions that include calcium storage, regulation of cell division, maintenance of structural stability during gliding motility, and serve as anchor for the actin-myosin motor complex required for parasite motility and invasion (Harding and Meisner, 2014).

The apicoplast is another unusual endomembrane compartment that represents a non-photosynthetic plastid-like organelle of red algal origin. It is currently thought that the apicoplast evolved into a dedicated metabolic compartment that houses anabolic activities indispensable for the parasite growth and viability. These include synthesis of heme and fatty acids via the FASII complex and isoprenoids compounds (Bergmann et al., 2019; Coppens et al., 2014).

Apicomplexa derive their name from a signature intracellular structure termed the 'apical complex'. This structure plays critical roles in interaction of the parasite with the host cell and the subsequent invasion process (Gubbels and Duraisingh, 2012). The 'apical complex' is a collection of specialized secretory organelles unique to Apicomplexa (i.e. micronemes, rhoptries and dense granules) along with the apicoplast. In some Apicomplexans the apical complex also includes an anterior-most conoid which

lies within a microtubule cylinder-like structure termed the polar ring. Micronemes, the smallest secretory organelles located at the apical pole of the cell, are required for parasite egress from host cells. These compartments also release effector proteins during invasion that interact with host cells receptors and trigger rhoptry secretion. Rhoptries are larger compartments that exhibit club-shaped morphologies with the slim end attached to the apical pole of the parasite. Release of rhoptry contents is required for formation of the moving junction, a ring-like protein structure that moves from the apical to the posterior end of the parasite and is required for host cell invasion.

Dense granules (DG) are secretory organelles located throughout the parasite cytoplasm and are released constitutively after invasion of the host. DG exocytosis is required to form and maintain the PVM. Moreover, DG cargo contribute to nutrient acquisition by the parasite and interfere with host cell signaling pathways (Gubbels and Duraisingh, 2012; Bai et al., 2018). The PVM is formed during parasite invasion via assembly of the moving junction at the interface of the parasite with the host plasma membrane. This unusual process incorporates parasite transmembrane proteins while preferentially excluding host plasma membrane proteins. The primary function of the PVM is to envelope the intracellular apicomplexan parasites and sequester them from the host cytoplasm to avoid intersection with the host endocytic pathway.

1.1.4. Apicomplexan life cycles

Members of the phylum Apicomplexa exhibit a wide range of morphologies, habitats, cell division processes, and life cycles. Although *Plasmodium* and *T. gondii*

both undergo both asexual and sexual reproduction, their cell cycles and life cycles differ. Malaria parasites belong to the class Aconoidasida based on the lack of conoid compartments in most of their life cycle stages. The conoid is a hollow barrel-shaped structure composed of tubulin fibers located in the apical pole of the parasite (Mehlhorn et al., 1980). *Plasmodium* parasites are transmitted to humans by the bite of an infected mosquito, which injects sporozoites into the blood stream (Figure 1.2A). Sporozoites subsequently infect liver cells and develop into merozoites through a replication process termed schizogony. This mode of cell replication involves multiple rounds of mitosis that yield a multinucleated syncytium. This syncytium is ultimately resolved by a synchronous assembly of multiple daughter cells (merozoites). Merozoites subsequently escape the liver and enter the blood stream to infect erythrocytes where they undergo asexual replication. This process involves merozoites adopting a ring-like morphology for further differentiation to trophozoites that subsequently divide via schizogony (schizont stage) to produce several merozoites. The liberation of merozoites after erythrocyte lysis leads to repeated infections of other erythrocytes, and this loop is the basis for the severe symptoms that accompany malarial disease. Some parasites differentiate into sexual erythrocytic stages (micro and macrogametocytes). These, in turn, are ingested by Anopheles mosquitoes during a blood meal. In the mosquito's stomach, macrogametocyte fertilization by a microgametocyte generates zygotes that develop into motile and elongated ookinetes. These ookinetes invade the midgut wall of the mosquito where they develop into oocysts and subsequently mature to produce

sporozoites in salivary glands of the mosquito (Read et al., 1993; Francia & Striepen, 2014).

T. gondii belongs to the subclass Coccidia of spore-forming organisms and infects the intestinal track of its definitive host -- the domestic cat (Figure 1.2B). Cats become infected directly by ingestion of sporulated oocysts wherein either sexual reproduction in the intestine, or asexual multiplication in peripherical organs, can take place (Ferguson et al., 1974; Francia & Striepen, 2014; and Carruthers, 2013). The feces of infected cats contain oocysts that sporulate in the environment and are accidentally ingested by humans and other vertebrates (i.e., the intermediate hosts). Shortly after ingestion, oocysts differentiate into tachyzoites and invade neural and muscle tissue. Once the parasites invade the host cell and establish a PV compartment, they develop into cyst bradyzoites. Bradyzoites represent a quiescent parasitic stage that retain the capability to differentiate back into tachyzoites and undergo successive rounds of asexual reproduction when the host becomes immunocompromised. Tachyzoite replication occurs via the unusual process of endodyogeny where genome duplication is followed by formation of a curved nucleus that couples two buds composed of nascent daughters. The daughter cells mature and ultimately bud out of the remnants of the mother cell. The internal budding occurs repeatedly until the PV is disrupted via the action of a *T. gondii* perforin-like protein. Host cell membranes burst quickly thereafter upon activation of the cell death receptor. The lytic cycle starts again when a tachyzoite invades and reproduces in a new host cell -- thereby propagating an acute infection of the affected tissue.

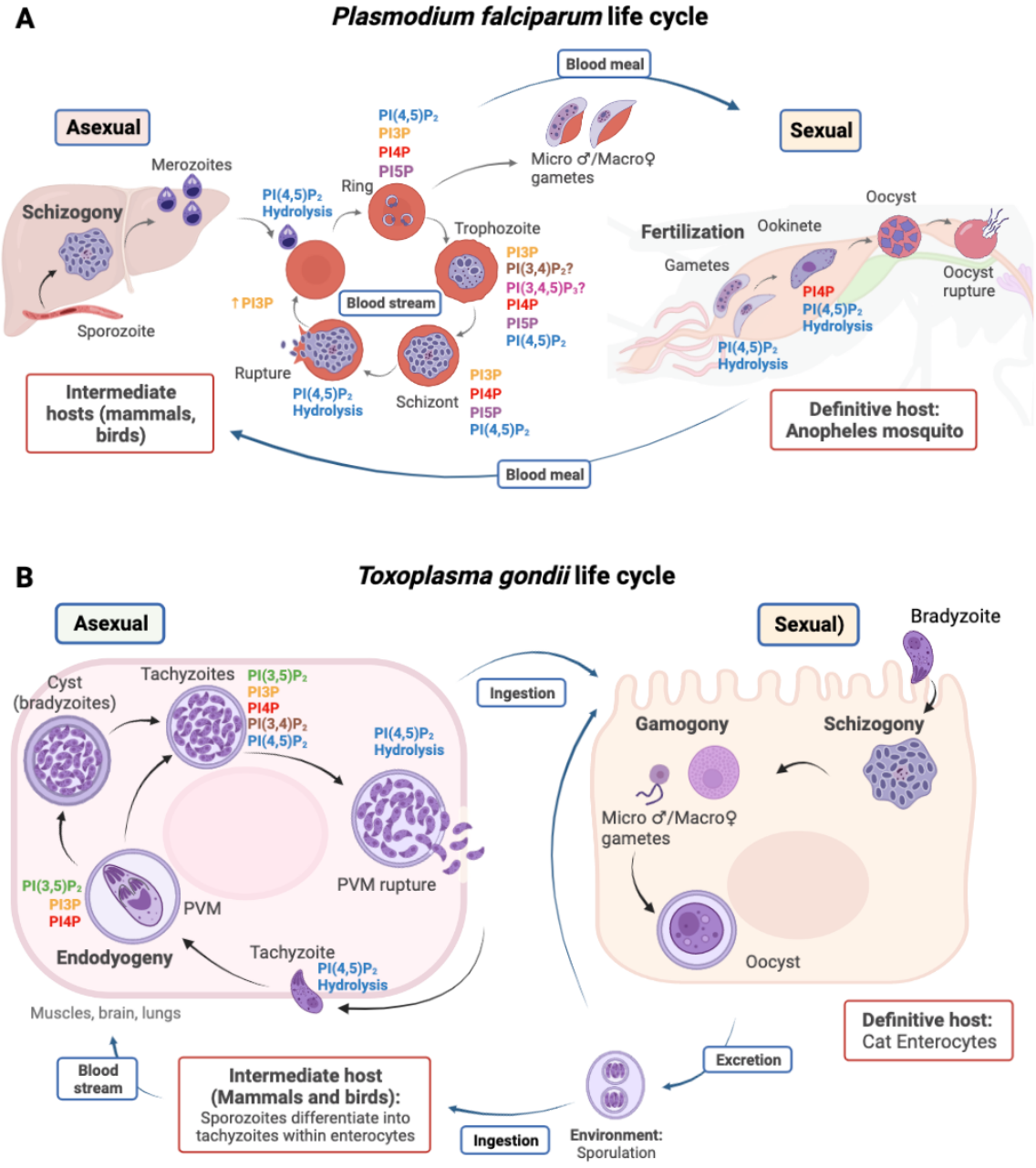

Figure 1.2 Life cycle of *P. falciparum* (A) and *T. gondii* (B). PIP detection and utilization is highlighted in specific stages during the parasite life cycles. Host types are outlined in red, and reproductive stages are outlined in blue.

1.1.5. PIP signaling in Apicomplexa

PtdIns represents a major class of membrane phospholipid in *T. gondii* and *Plasmodium*. It constitutes ~7% of the total of phospholipid content, and this value is similar to that found in mammalian cells (Welti et al., 2007; Vial et al., 2003). PtdIns metabolism has not been studied in detail in Apicomplexan organisms. The genomes of *T. gondii*, *Plasmodium*, and Eimeria all encode a PtdIns synthase (PIS) (Wengelnik et al., 2007; Séron et al., 2000; Kong et al., 2018). The *T. gondii* TgPIS is an essential enzyme that localizes to the Golgi network and consumes CDP-diacylglycerol (CDP-DAG) and myo-inositol as precursors in de novo PtdIns synthesis. The parasite is also capable of salvaging longer chain PtdIns molecular species (C38/C40) from the host cell (Ren et al., 2020).

The current cohort of Apicomplexan PIPs has been identified and localized by exploiting protein domains with high affinities and specificities for individual PIP species (PIP biosensors), and through parasite lipid profiling. The function of these PIPs has also been studied using controlled expression strategies targeting PtdIns and PIP kinases responsible for the synthesis of individual PIP species. As is the case in all other eukaryotes studied thus far, Apicomplexan PIPs are essential molecules for mediating important cell functions -- including the virulence of these organisms. For instance, PIPs are involved in apicoplast homeostasis, in host cell invasion by *T. gondii* (Daher et al., 2015; Bullen et al., 2016), in the transport and fusion of endosomes with lysosome-like compartments, and in motility and host cell egress processes in *Plasmodium* (Vaid et al., 2010; Brochet et al., 2014).

Understanding how PIP pools are formed and maintained in eukaryotes remains an intense area of study in contemporary cell biology. While much effort is invested in studying the enzymes that produce and consume PIPs, fundamental aspects of how PIP production is regulated and physically organized are still not completely understood. Moreover, most of the information on the metabolism and physiological roles of inositol lipids is derived from studies in a limited set of model organisms. Because the phylum Apicomplexa originated at a very early stage of eukaryotic evolution, i.e., prior to the branching of yeast and plants from the trunk of the eukaryotic tree (Escalante and Ayala, 1995), these primitive organisms provide a unique opportunity to study the action of rudimentary PIP signaling systems. Additionally, PIP signaling mechanisms in Apicomplexa offer intriguing possibilities for the development of potential drug targets to combat parasitic diseases.

Recently, two reviews focused on PIPs in Apicomplexa have been published. The first covers PIP distribution, cellular functions and studies of PtdIns and PIP-kinases in *T. gondii* and *Plasmodium* (Wengelnik et al., 2018). The second focuses on PIP-binding proteins involved in endocytic trafficking and autophagy in these parasites (Cernikova et al., 2019). The focus of this first chapter is to address regulatory mechanisms that control the spatial and temporal regulation of PIPs in *Plasmodium* and *T. gondii*, and to draw comparisons and distinctions of these mechanisms relative to those described for other eukaryotic systems – particularly mammalian cells and the model organism *S. cerevisiae w*hich we refer to as "yeast" in this first chapter.

1.2. PtdIns3P and its higher-order derivatives

1.2.1. The Apicomplexan PtdIns3P Kinases

PtdIns-3-phosphate (PtdIns3P) is produced by phosphorylation of the PtdIns inositol headgroup on the 3'-OH position. This reaction is catalyzed by class II and class III PtdIns 3-OH kinases (PI3Ks) in mammals (PIK3C2 and PIK3C3). Unicellular organisms express one PI3K -- the class III Vps34 (Vanhaesebroeck et al., 2010; Brown and Auger et al., 2011). Both *Plasmodium* and *T. gondii* encode a single PI3K Class III/Vps34 enzyme that is responsible for cellular all PtdIns3P synthesis: PfPI3K (PF3D7_0515300) and TgPI3K (TGME49_215700), respectively. These enzymes localize primarily to apicoplast, vacuolar and vesicular compartments, in both parasites where pools of PtdIns3P are produced (Figure 1.4B and C)). Both kinases exhibit a conserved PI3K Class III domain organization consisting of: (i) an N-terminal C2 domain, (ii) a PIK domain (PI3K family accessory domain that has been suggested to be involved in substrate presentation) and (iii) a C-terminal 3-OH kinase catalytic domain. Both PfPI3K and TgPI3K are essential for parasite survival (Brown and Auger, 2011; Daher et al., 2015; Vaid et al., 2010), and both enzymes are predicted to be substantially larger than any other PI3K described thus far (Figure 1.3).

PI3K class III/Vps34 is found in two principal complexes in mammalian cells: complex I and complex II (Fan et al., 2011; Rostislavleva et al., 2015). Complex I is composed of Vps34, Vps15, Beclin 1 (Vps30), an autophagy-related gene 14L (ATG14L), and this complex is responsible for generating PtdIns3P in the endoplasmic reticulum (ER). It is this pool that drives autophagosome preassembly machinery at the

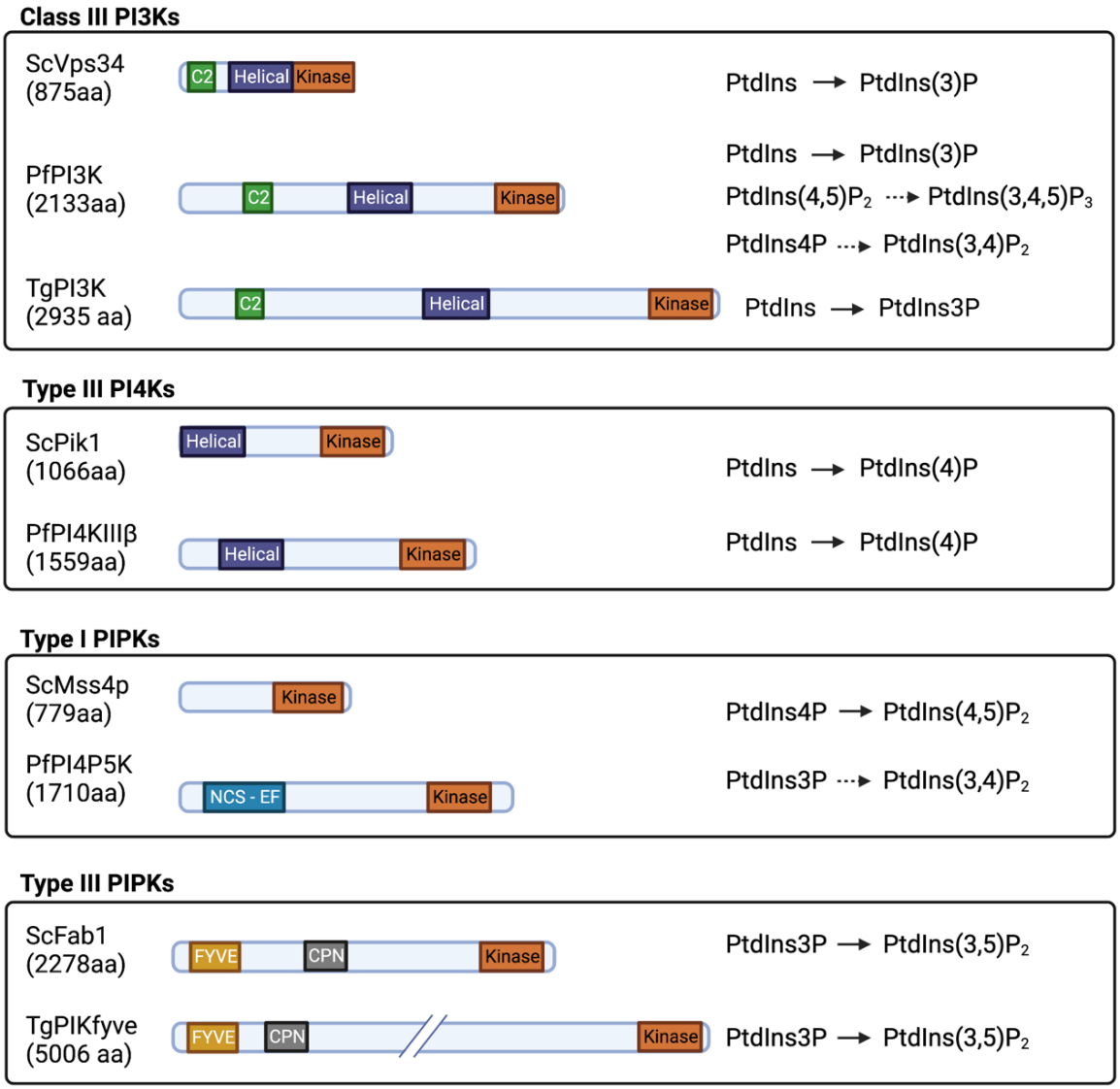

Figure 1.3 Domain organization and substrate specificity of current known Apicomplexan PIKs and PIPKs and their yeast homologs drawn to scale. Kinase: PI/PIP kinase domain; Helical: Helical domains; C2: C2 domain; FYVE: FYVE zinc finger domain; CPN: Chaperonin; and NCS-EF: NCS-like domain with EF hands.

omegasome (Mizushima et al., 2008). In complex II, ATG14L is replaced by UVRAG (Vps38 in yeast), which facilitates targeting of the complex to early endosomes to produce PtdIns3P. Recruitment of complex II occurs in a Rab5-dependent manner

(Figure 1.4A). This process potentiates maturation of early Rab5-positive vesicles to late Rab7-positive vesicles for further fusion with lysosomes (Vanhaesebroeck et al., 2010; Burke et al., 2018). Of these regulatory factors, only a candidate Vps15 ortholog is annotated by both *T. gondii* and *Plasmodium* genomes, and a prospective Vps30 is encoded by *T. gondii* (Supplementary Table S1.1) (PlasmoDB (v56); ToxoDB (v56)). The dedicated components that define complexes I and II (ATG14L and UVRAG, respectively) and aid in target membrane recognition have no obvious counterparts in these parasites. *Plasmodium* and *T. gondii* may express novel and specialized accessory proteins adapted for targeting Apicomplexan Vps34 enzymes to their unique endomembranous compartments.

1.2.2. PtdIns3P in starvation and autophagy during the Apicomplexan life cycle

Among the intraerythrocytic stages of *P. falciparum*, the highest levels of PtdIns3P are detected during transition from the ring to the schizont phase. The PfPI3K is localized to the limiting membranes of vesicular compartments and the food vacuole (Vaid et al., 2010). The food vacuole is the organelle where hemoglobin (Hb) is digested, and Hb represents the primary amino acid nutrient source for the parasite. Phosphoproteome profiling experiments reveal that PtdIns signaling and PfPI3K activity is elevated in extraerythrocytic merozoites relative to the schizont stage (Figure 1.2A). Moreover, ubiquitination and autophagy pathways are also upregulated in this stage (Lasonder et a., 2015). Upregulation of ubiquitination and autophagy is consistent with the observation that extraerythrocytic merozoites lack the food vacuole (Dluzewski et

al., 2008), thereby forcing the parasite to undergo autophagic digestion of organelles no longer required by extraerythrocytic merozoites as mechanism to combat starvation. Such observations reveal a potential role for PfPI3K in the regulation of starvation-induced processes during the extraerythrocytic stages (Lasonder et. al. 2015) (Figure 1.4B). Similarly, *T. gondii* TgPI3K activity stimulates mitochondrial fragmentation during mitophagy when the parasite encounters starvation conditions (Figure 1.4C) (Ghosh et al., 2012).

The role of autophagy as a life-sustaining process under conditions of starvation stress is well-established (Sinai et al., 2012; Qi et al., 2018). Elucidating the regulatory mechanisms that govern PtdIns3P synthesis is crucial for understanding how energy balance and cell homeostasis are maintained under starvation conditions in protozoan organisms. In that regard, genome-wide association studies report that PfPI3K/PtdIns3P levels are modulated by the action of an AKT ortholog (PfAKT) in *P. falciparum* as elevation of PfAKT expression in transgenic parasites induces a ~2-fold elevation of PtdIns3P levels (Mbengue et al., 2015). Akt is a master regulator of diverse cellular functions in mammals that include survival, growth, metabolism, migration, and differentiation (Vivanco et al., 2002; Sugiyama et al., 2019). In mammals, AKT activation can lead to diminished PtdIns3P production. For instance, activation of mTOR1 by the PI3K class I/Akt axis inactivates Vps34-containing complexes and suppresses autophagy (Marat and Haucke, 2016). A reaction where Akt stimulation increases PtdIns3P levels has not been well described in other organisms. One intriguing possibility is that the absence of the canonical components of Vps34

complexes in Apicomplexans generates an outcome for Akt-dependent PtdIns3P signaling that diverges from those classically observed in mammalian cells.

Downregulation of PtdIns3P signaling is executed by myotubularin 1 (MTM1) and MTM-related proteins that dephosphorylate PtdIns3P to PtdIns. MTM genes are nearly ubiquitously distributed throughout the Eukaryota (Vergne and Deretic 2010; Laporte et al., 1996). Thus far, *Cryptosporidium parvum* and *C. hominis* are the only Apicomplexans that encode an MTM-like protein. *Plasmodium* and *T. gondii* lack obvious MTM orthologs (Supplementary Table S1.1) Abrahamsen et al., 2004; Kerk and Moorhead, 2010; Laporte et al., 2003). Whether this activity is missing in *Plasmodium* and *T. gondii*, or whether it is fulfilled by a protein with no obvious homology to the myotubularins, remains to be determined.

1.2.3. PtdIns(3,5)P_2 synthesis in Apicomplexa

The basic mechanisms underlying the maintenance of PtdIns(3,5)P_2 levels are highly conserved. PtdIns3P 5-kinase (Fab1 in yeast and PIKfyve in mammals) phosphorylates PtdIns3P on the inositol ring at the 5'-OH position to produce PtdIns(3,5)P_2. PIKfyve and Fab1 both reside in large protein complexes that tightly regulate PtdIns(3,5)P_2 production. This complex comprises four more proteins in yeast: Fig4, Vac14, Vac7, and Atg18. PtdIns(3,5)P_2 turnover is proposed to be mainly catalyzed by Fig4, a 3,5-bisphosphate 5-phosphatase (Sac3 in mammals), which both positively and negatively regulates PtdIns(3,5)P_2 production. Vac14 (ArPIKfyve in mammals) functions as a scaffold protein that promotes PtdIns(3,5)P_2 synthesis. Vac7 is

an essential protein in yeast, but its function in Fab1 activation is not clear. Vac7 metazoan homologs have not been detected. Finally, Atg18 negatively regulates PtdIns(3,5)P_2 production -- likely through its interaction with Vac14. In yeast, all of these factors localize to the vacuolar membrane, whereas mammalian PIKfyve and ArPIKfyve localize to membranes of the endocytic system (Figure 4A) (Shisheva et al., 2008; Botelho et al, 2008; Gary et al, 2002; Jin et al, 2008; Hasegawa et al., 2017).

PtdIns(3,5)P_2 in *T. gondii* is localized to a population of uncharacterized vesicles distributed throughout the cytoplasm of tachyzoites (Figure 4C), and its synthesis is associated with apicoplast biogenesis/inheritance (Daher et al., 2016; Daher et al., 2015). PtdIns(3,5)P_2 has not yet been reported in *Plasmodium*. Whether this reflects a genuine absence of PtdIns(3,5)P_2 in this parasite, or very low levels that fall below available thresholds of detection, is not clear. Putative homologs of Fab1/PIKfyve, Vac14 and Atg18 have been described and are essential for parasite growth in *T. gondii* (Daher et al., 2015; Bansal et al., 2017). Depletion of TgPIKfyve (TGME49_256920), TgArPIKfyve (TGME49_244040) or TgAtg18 result in similar apicoplast biogenesis/inheritance disruption phenotypes as those previously observed in strains where PtdIns3P signaling is blocked (Tawk et al., 2011). In independent studies, colocalization of TgPIKfyve and TgAtg18 with the otherwise uncharacterized PtdIns(3,5)P_2-containing vesicular structures was also observed (Daher et al., 2015; Bansal et al., 2017). These data suggest *T. gondii* employs a conserved PtdIns(3,5)P_2 regulatory complex to control vesicle trafficking to the apicoplast (Figure 4C).

The only component of the PtdIns(3,5)P_2 regulatory complex described thus far in *Plasmodium* is the Atg18 ortholog PfAtg18 (Bansal et al., 2017; Sudhakar et al., 2021). PfAtg18 and TgAtg18 bind PtdIns3P in vitro. TgAtg18 also binds PtdIns(3,5)P_2, but PfAtg18 does not. While these data are consistent with the notion that PtdIns(3,5)P_2 is absent in *Plasmodium*, candidates for a PIKfyve lacking the FYVE finger domain and ArPIKfyve and Fig4 homologs are listed in the *Plasmodium* database (PlasmoDB v56) (Supplementary Table S1.1). Prospective *P. berghei* PIKfyve- and ArPIKfyve-like proteins score as essential activities in an in vivo screen that monitores growth rate phenotypes of strains individually deficient in each of 2,578 *P. berghei* genes (Bushell et al., 2017). The available data suggest that *Plasmodium* PIKfyve is not involved in PtdIns3P binding and its conversion to PtdIns(3,5)P_2. Further characterization of these protein candidates, along with implementation of more sensitive PtdIns(3,5)P_2 detection methods, is required to determine whether or not *Plasmodium* produce PtdIns(3,5) P_2.

1.2.4. PtdIns3P and PtdIns(3,5)P_2 in Apicomplexa are not restricted to endosome trafficking pathways.

In mammalian and yeast cells, PtdIns3P localizes primarily to membranes of early endosomes, multivesicular bodies (MVB) and MVB transport intermediates where it regulates membrane receptor sorting and intralumenal vesicle formation (Gillooly et al., 2000; Petiot et al., 2003). Vsp34 complex II is responsible for producing PtdIns3P in these compartments, and PtdIns(3,5)P_2 is the signature marker for late endolysosomal membranes in higher eukaryotes and for the vacuole in yeast (Figure 1.4A).

PtdIns(3,5)P_2 synthesis is associated with endomembrane homeostasis via regulation of homotypic and heterotypic fusion and fission events involving early and late endosomes and MVB membranes (Ikonomov et al., 2006: Nicot et al., 2006; Whitley et al., 2009; Duex et al., 2006).

Unlike in most eukaryotic cells, endocytosis and exocytosis in Apicomplexan parasites take place within the context of a host eukaryotic cell. Hence, specialized endocytic structures (micropore/cytostome) and secretory organelles (rhoptries, micronemes and dense granules) are employed to carry out these processes. For instance, endocytosis of Hb by intraerythrocytic *Plasmodium* parasites takes place at the cytostome. When the cytostome matures and pinches off, the resulting endosomal structures containing erythrocytic material are trafficked to the food vacuole where Hb is metabolized to the non-toxic product hemozoin (Milani et al., 2015; Lazarus et al., 2008). PtdIns3P is detected in subsets of these erythrocytic material-containing endosomal structures in the cytoplasm and in the food vacuole membrane (Figure 1.4B) (Tawk et al., 2010). Moreover, PtdIns3P synthesis is linked to transport of Hb-loaded vesicles to the food vacuole for degradation (Vaid et al., 2010).

PtdIns3P levels in *P. falciparum* are regulated via proteolytic degradation of PfPI3K by PfKelch13, an adapter for E3 ubiquitin ligases involved in cytostome biogenesis and/or function (Mbengue et al., 2015: Yang et al., 2019). PfKelch13 shows homology to the mammalian kelch-like ECH-associated protein 1 (Keap1) which

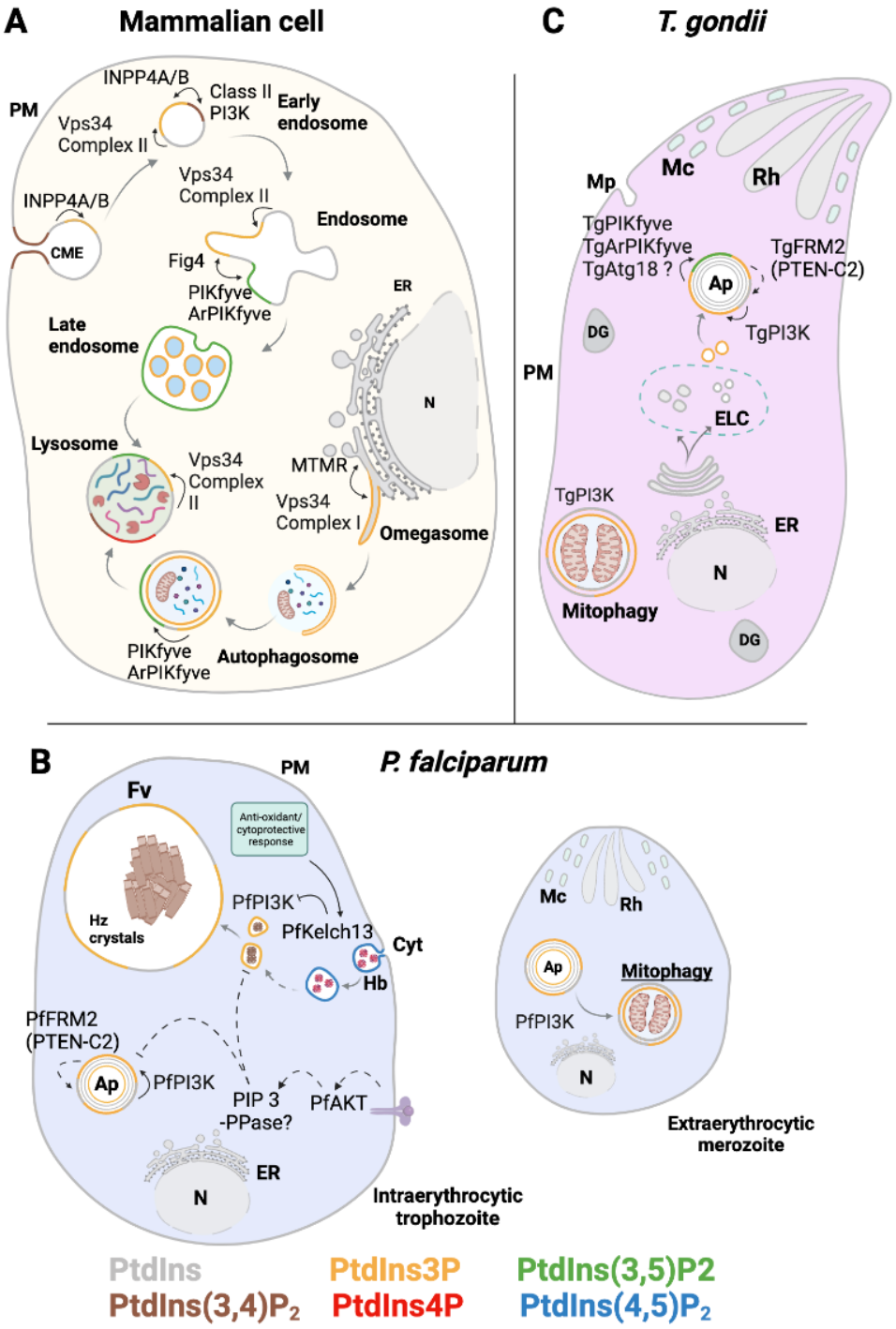

Figure 1.4 PIPs involved in the endocytic system and autophagy. (A) Mammalian: in preformed CME vesicles PtdIns(3,4)P_2 is dephosphorylated by 4-phosphatases (INPP4A/B) to form PtdIns3P. In early endosomes PtdIns3P is also generated from PtdIns by the class III PI3K Vps34 complex II. Maturation to late endosomes involves

Figure 1.4 Continued. PtdIns(3,5)P_2 synthesis. PtdIns3P synthesis by class III PI3K Vps34 complex II is required for initiating autophagy at the omegasome. Autophagosome maturation and fusion with the lysosome depend on conversion of PtdIns3P to PtdIns(3,5)P_2 by PIKfyve kinase. (B) *P. falciparum*: PtdIns(4,5)P_2 is detected in the cytostome and cytostomal vesicles. Maturation of cytostomal vesicles is accompanied by reduction of their size and enrichment of PtdIns3P by PfPI3K activity. PtdIns3P is also observed in the food vacuole membrane, autophagosomes and at the apicoplast. Negative regulation of PtdIns3P levels in these compartments might include the PTEN C2-like domain in PfFRM2, the activation of PfAKT signaling, and PfKelch13-dependent PfPI3K proteolysis. At the extracellular merozoite stage, PfPI3K activity regulates autophagy-related processes such as mitophagy. (C) *T. gondii*: PtdIns3P is produced by TgPI3K at the outer and inner membranes of the Apicoplast and at neighboring vesicles. PtdIns(3,5)P_2 production from PtdIns3P by the complex TgPIKfyve, TgArPIKyve, and possibly TgAtg18 is key to apicoplast maintenance. TgPI3K activity is also involved in mitophagy during parasite starvation. The PTEN-C2 domain of TgFRM2 may negatively regulate PtdIns3P at the apicoplast. Solid line: Proposed reaction in previous publications; Dashed line: Proposed reaction. Abbreviations: PM: plasma membrane; ER: endoplasmic reticulum; N: nucleus; Cyt: cytostome; Mp; micropore; Mc: microneme; Rh: Rhotry; Fv: food vacuole; Hz crystals: hemozoin crystals; Ap: apicoplast; DG: dense granule; ELC: endocytic-like compartment.

controls the adaptative response to oxidative stress. A mutation in the PfKelch13 propeller domain affects substrate affinity with a consequent decrease in PfPI3K proteolysis and elevation in bulk intracellular PtdIns3P (Ariey et al., 2013; Mbengue et al., 2015). PfKelch13 localizes to the ER, to Rab5A/ 5B/ 6/ 7/ 11A-positive vesicles and to structures adjacent to the cytostome. Co-immunoprecipitation analyses reveal PfKelch13 physically associates with multiple factors that regulate vesicle trafficking and endocytosis in eukaryotes -- i.e., members of the Rab family of GTPases (Sar1, Sec23 and others) -- but not with PfPI3K (Gnädig et al., 2020). The precise function of PfKelch13 remains to be determined. The subcellular localization and structural similarity of PfKelch13 with human Keap1 suggests PfKelch13 promotes ubiquitination

of PfPI3K as part of an antioxidant/cytoprotective response induced during conditions of vigorous digestion of Hb.

PtdIns3P in *P. falciparum* also localizes to the apicoplast (Figure 1.4B), where it might act as a targeting signal for membrane transport (Tawk et al., 2010). In *T. gondii*, PtdIns3P is not associated with Rab5-positive vesicles or endosome-like structures. Rather, the major PtdIns3P-containing subcellular compartments in *T. gondii* are the apicoplast outer membrane and adjacent vesicles (Figure 1.4C) (Tawk et al., 2011). The distinctions in PtdIns3P localization in these two parasites likely reflect differences in their endocytic trafficking pathways. Unlike *Plasmodium*, endocytosis in *T. gondii* occurs during intracellular and extracellular parasite stages and it intersects with exocytic trafficking pathway(s) (Gras et al., 2019; McGovern et al., 2018; Duo et al., 2014). This model posits that endocytosis occurs at membrane invaginations distinct from the micropore (a structure similar to the cytostome) and that endocytosed material is sorted in an endocytic-like compartment (ELC). The ELC is marked by Rab5 and Rab7 (Tomavo et al., 2013), and with plant-like vacuole proteins (Parussini et al., 2010). The ELC also represents the maturation center for microneme (Harper et al., 2006) and rhoptry secretory proteins (McGovern et al., 2018). Further analyses of how PtdIns3P pools are specified in the *T. gondii* endocytic system are required for understanding how endocytic and exocytic pathways have been repurposed in these parasites and how they intersect.

The function of PtdIns3P in the *T. gondii* apicoplast outer membrane and surrounding vesicles was analyzed by studying the effects of prolonged expression of the

PtdIns3P biosensor 2xFYVE domain of the mammalian Hrs protein. Sustained PtdIns3P pool sequestration results in loss of the apicoplast, and this event ultimately leads to parasite death. Those data suggest that PtdIns3P promotes the fusion of vesicles containing nuclear-encoded apicoplast proteins with the outermost membrane of the apicoplast (FtsH1 and APT1) (Tawk et al., 2011). Furthermore, ablation of TgPI3K, TgPIKfyve and TgArPIKfyve activities recapitulate apicoplast loss and associated cell death -- suggesting that both lipid kinases act in the same biosynthetic pathway where PtdIns3P serves as a precursor for the synthesis of PtdIns(3,5)P_2 (Daher et al., 2015). These findings reveal novel roles for PtdIns3P and PtdIns(3,5)P_2 in intracellular vesicular trafficking with the apicoplast as target organelle.

1.3. The case for higher-order PtdIns3P derivatives in Apicomplexa

In vivo functions for PtdIns(3,4,5)P_3 have primarily been studied in mammalian cells, where it exists at very low levels in quiescent cells. This PIP functions to recruit proteins with PtdIns(3,4,5)P_3 - selective PH domains to the plasma membrane. Such effectors include the Akt protein kinase (protein kinase B) and phosphoinositide-dependent kinase-1 (PDK1). This process promotes cell proliferation and survival, and regulates cytoskeleton dynamics, motility, membrane trafficking and apoptosis (Toyoshima et al., 2007; Cantley, 2002; Tengholm et al., 2009). PtdIns(3,4,5)P_3 synthesis is triggered by stimulation of tyrosine kinase- and/or G-protein coupled receptors that lead to activation of class I PI3K (Figure 1.5A). The activated kinase catalyzes phosphorylation of PtdIns(4,5)P_2 in the plasma membrane and elevates

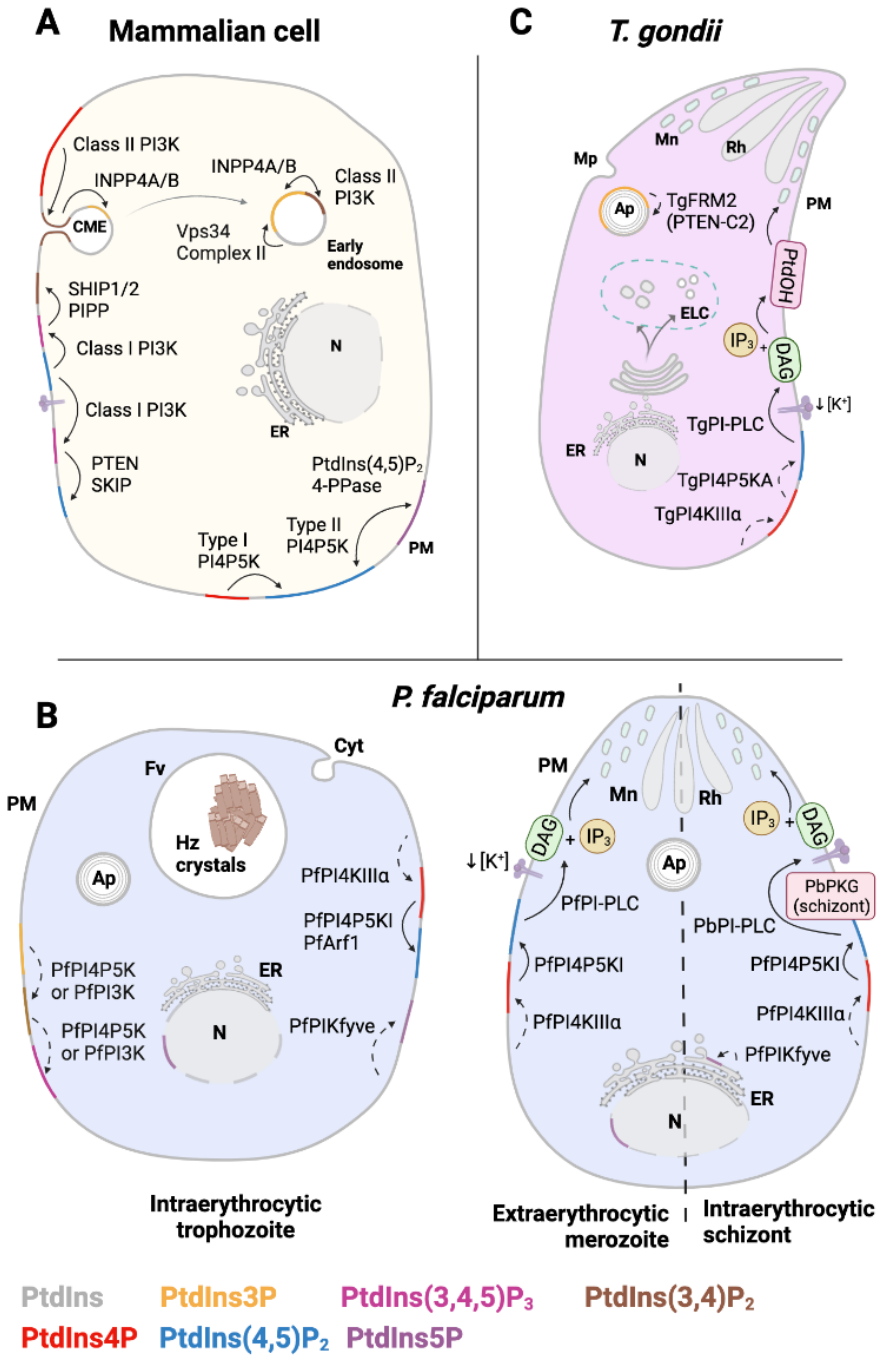

Figure 1.5 PIP regulation in the plasma membrane PM. (A) Mammalian: PtdIns(4,5)P_2 is mainly produced at the PM by phosphorylation of PtdIns4P by Type I PI4P5K. Stimulation of tyrosine kinase- and/or G-protein coupled receptors lead to class I PI3K activation and conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Dephosphorylation of PtdIns(3,4,5)P_3 by PTEN forms PtdIns(4,5)P_2 and turns off the signaling relay, while dephosphorylation of PtdIns(3,4,5)P_3 by SHIP1/2 generates PtdIns(3,4)P_2 to promote

Figure 1.5 Continued. clathrin-mediated endocytosis. PtdIns5P is found at the PM after dephosphorylation PtdIns(4,5)P_2 by a PIP_2-4-phosphatase. (B) *P. falciparum*: PtdIns(4,5)P_2 in PM is generated from PtdIns4P by type I PI4P5K after recruitment/activation by PfArf1. PtdIns(4,5)P_2 is hydrolyzed by PfPI-PLC to produce secondary messengers DAG and IP_3. In *P. berghei*, positive regulation of PbPI-PLC occurs after activation of PbPKG and/or exposure to specific environmental queues (i.e., decrease of extracellular [K^+]) to trigger microneme secretion. PtdIns(3,4,5)P_3 may be generated by consecutive phosphorylation of PtdIns(3,4)P_2 at OH-4 and OH-5 positions by PfPI4P5K. PtdIns5P, found at PM and nucleus in trophozoites, as well as at the ER in schizonts, may be formed by PfPIKfyve-dependent PtdIns phosphorylation. (C) *T. gondii*: TgPI-PLC hydrolyzes PtdIns(4,5)P_2 to generate DAG and IP_3. DAG is phosphorylated to PtdOH and triggers microneme exocytosis. Solid line: Proposed reaction in previous publications; Dashed line: Proposed reaction. Abbreviations: PM: plasma membrane; ER: endoplasmic reticulum; N: nucleus; Cyt: cytostome; Mp; micropore; Mc: microneme; Rh: Rhotry; Fv: food vacuole; Hz crystals: hemozoin crystals; Ap: apicoplast; DG: dense granule; ELC: endocytic-like compartment.

PtdIns(3,4,5)P_3 levels as much as 100-fold (Vanhaesebroeck et al., 2001; Tengholm et al., 2009). An alternative pathway to generate PtdIns(3,4,5)P_3 has been described in human platelet cells, mouse fibroblasts and plant cells (Cunningham et al., 1990; Brearley et al., 1993). This pathway involves PIP5KII-dependent phosphorylation of PtdIns3P to form PtdIns(3,4)P_2 with subsequent PIP5KI-dependent phosphorylation of PtdIns(3,4)P_2 to PtdIns(3,4,5)P_3 (Zhang et al., 1997).

PtdIns(3,4,5)P_3 has not been detected in Apicomplexans (Ebrahimzadeh et al., 2018). However, studies in *P. falciparum*-infected erythrocytes show an incremental increase of PtdIns(3,4,5)P_3 in erythrocytic infected states (Figure 1.2A) (Tawk et al., 2010). The sole PfPI3K forms PtdIns(3,4,5)P_3 from its precursor PtdIns(4,5)P_2 in vitro (Vaid et al., 2010). Primary structure comparisons show PfPI3K shares some structural features with the catalytic and helical domains of the human class I PI3K PIK3CG. These comparisons suggest that PfPI3K might produce PtdIns(3,4)P_2 using a PtdIns4P

substrate and PtdIns(3,4,5)P_3 from a PtdIns(4,5)P_2 precursor in vitro -- in addition to producing PtdIns3P (Vaid et al., 2010). However, when parasites are treated with wortmannin, a PI3K inhibitor, only levels of PtdIns3P in infected erythrocytes are diminished – a result that suggests that PfPI3K produces exclusively PtdIns3P in vivo (Tawk et al., 2010). Thus, Tawk and colleagues (2010) propose that *P. falciparum* may harbor a similar PtdIns(3,4,5)P_3 production pathway to the one described in the fission yeast *Schizosaccharomyces pombe* where PtdIns(3,4,5)P_3 synthesis is mediated by consecutive phosphorylation at the D4 and D5 positions of PtdIns3P by a PI4P5K (Figure 1.5B) (Mitra et al., 2004). As mentioned previously, this route corresponds to the so-called alternate synthesis pathway in multicellular organisms. This pathway might not only support PtdIns(3,4,5)P_3 production in *P. falciparum*-infected erythrocytes, but it also suggests the form of an ancient pathway for PtdIns(3,4,5)P_3 synthesis that precedes functional diversification of the PI3K family.

PtdIns(3,4,5)P_3 signals are terminated via dephosphorylation of the D3-position to form PtdIns(4,5)P_2. Among these negative regulators of the PI3K axis in mammals are the enzymes PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SKIP (skeletal muscle and kidney-enriched inositol phosphatase) (Figure 1.5A) (Ono et al., 2001; Ijuin and Takenawa, 2003). By contrast, 5-phosphatases that generate PtdIns(3,4)P_2, such as the Src-homology 2 containing inositol phosphatases 1 and 2 (SHIP1 and SHIP2), and the poorly characterized proline-rich inositol polyphosphate 5-phosphatase (PIPP), do not suppress PtdIns(3,4,5)P_3 signaling (Astle et al., 2006; Pedicone et al., 2021; Tengholm et al., 2009).

The PTEN-C2 domain has only been described as an accessory domain in the formin 2 proteins of *P. falciparum*, *P. cynomotgi, P. knowlesi, P. vivax,* and *T. gondii* (Pathak et al., 2019; Stortz et al., 2018). Formin is a multidomain protein that is an important regulator of actin polymerization in eukaryotes. The PTEN-C2 domain-containing formin 2 (FRM2) localizes adjacent to the apicoplast in both *P. falciparum* and *T. gondii* (Figure 1.4B and C). Conditional disruption of the gene encoding PfFRM2 and TgFRM2 results in structurally aberrant apicoplasts, loss of cell viability in *P. falciparum* (the only case studied thus far), deranged F-actin network dynamics, and impaired cytokinesis (Stortz et al., 2018). These phenotypes recapitulate those that accompany diminutions in PtdIns3P and PtdIns(3,5)P_2 synthesis in both Apicomplexans (Tawk et al., 2010; Daher et al., 2015). These collective observations raise interesting questions regarding whether PfFRM2 and TgFRM2 function is linked to apicoplast biogenesis/maintenance by PTEN-C2 domain-dependent regulation of PtdIns(3,4,5)P_3, although the presence of PtdIns(3,4,5)P_3 in this compartment has not been confirmed. Alternatively, the PTEN-C2 domain of PfFRM2 and TgFRM2 might sense PtdIns3P and PtdIns(3,5)P_2. PlasmoDB(v56) and ToxoDB(v56) databases identify common homologs for SHIP1, SHIP2, SKIP and PIPP phosphatases: PF3D7_0705500 and TGME49_238400, respectively (Supplementary Table S1.1). Both genes are essential for parasite viability and both are annotated as putative inositol polyphosphate-related phosphatases. These represent candidate 5-phosphatases that regulate PtdIns(3,4,5)P_3 signaling in both of these organisms.

As in the case of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 has only been detected in *P. falciparum*-infected erythrocytes, and its production via phosphorylation of PtdIns4P by the sole PfPI3K has only been demonstrated in vitro (Vaid et al, 2010; Tawk et al., 2010). In *T. gondii*, the PtdIns(3,4)P_2-selective PH domain of human TAPP1 localizes to the plasma membrane in intracellular tachyzoites (Arabiotorre et al., 2023). This result indicates existence of a persistent PtdIns(3,4)P_2 pool in this compartment. Apicomplexan genome database annotations do not identify other candidate class II and class III PI3-kinases, which produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in higher organisms (Brown and Auger, 2011). Thus, as suggested previously, PtdIns(3,4)P_2 might be produced by phosphorylation at D4 of PtdIns3P by a PI4P5K (Mitra et al., 2004; Tawk et al., 2010).

In mammals, downregulation of PtdIns(3,4)P_2 signaling occurs primarily via the action of members of the 3- and 4-phosphatase protein families also involved in PtdIns(3,4,5)P_3 degradation. Candidate orthologs for specific PtdIns(3,4)P_2 phosphatases such as the 4-phosphatases INPP4A and INPP4B are not present in Apicomplexa. Hence, there is little evidence for the existence of regulatory proteins involved in producing or consuming PtdIns(3,4)P_2 in the Apicomplexa.

1.4. Apicomplexan PtdiIns-4-OH Kinases

PtdIns4P regulates membrane trafficking through the TGN/endosomal system, lipid homeostasis, autophagy, cytokinesis, and actin dynamics in mammals and yeast (Rivas et al., 1999; Hama et al., 1999; Walch-Solimena et al., 1999 Jović et al., 2012; D'Angelo et al., 2007; Wang et al., 2015; Sechi et al., 2014). In this section, we discuss

synthesis of PtdIns4P, mechanisms of its regulation, and its role in signaling processes in Apicomplexa. PtdIns4P is produced by PtdIns 4 OH kinases (PI4Ks) and is degraded by PtdIns4P phosphatases. PI4Ks are classified in two structural classes: (1) type II kinases represented by PI4KIIα and PI4KIIβ in mammals and Lsb6 in yeast; and (2) type III kinases represented by PI4KIIIα and PI4KIIIβ in mammals and their respective yeast orthologs Stt4 and Pik1 (Strahl et al., 2007; Clayton et al., 2013; Han et al., 2002). Candidate PI4Ks are listed in both *P. falciparum* and *T. gondii* genome databases (Wengelnik et al., 2018). There are three PI4K candidates in *P. falciparum*: PfPI4KII, PfPI4KIIIα and PfPI4KIIIβ. *T. gondii* encodes three orthologs of the *P. falciparum* proteins (TgPI4KII, TgPI4KIIIα and TgPI4KIIIβ) and four putative "PI3K/PI4K-like" proteins. It is likely that these "PI3K/PI4K-like" proteins are not true PtdIns kinases. In all four cases, the human and yeast proteins with the highest similarity represent protein kinases. These include Tor2/mTOR (homologous to the product of TGME49_316430). Thus, we will not further discuss those kinases in this review.

Type II PI4Ks are dispensable for viability in yeast and metazoa, yet these enzymes carry out specific cellular functions. In mammals, PI4KIIα and PI4KIIβ are associated with endosomal compartments (Jović et al., 2012; Balla et al., 2002; Wei et al., 2002). In yeast, Lsb6 regulates endosome motility in a manner that is apparently independent of its catalytic activity (Chang et al., 2005). Phylogenetic analyses of PIP lipid kinases indicate that *Plasmodium* type II PI4K is more closely related to the plant type II PI4Ks than to metazoan or yeast isoforms. This relationship likely reflects lateral gene transfer from a red algal endosymbiont (Brown and Auger et al., 2011; McFadden

et al., 2011). Genes annotated to encode type II PI4K-like proteins are listed in *Plasmodium* and *T. gondii* genomes. Although these proteins appear to confer fitness to these organisms, their activities and functions remain uncharacterized. For these reasons, we focus discussion on the Type III PI4Ks (Bushell et al., 2017 ; Sidik et al., 2016).

1.4.1. PtdIns4P synthesis in the apicomplexan secretory pathway

PfPI4KIIIβ (PF3D7_0509800) is the sole apicomplexan enzyme directly demonstrated to have PI4K activity (McNamara et al., 2013; Stenberg and Roepe, 2020). Sequence alignment and secondary structure predictions suggest that PfPI4KIIIβ domain organization is similar to that of human PI4KIIIβ (UniProt Q9UBF8) -- including helical N- and C-lobes and a conserved catalytic domain. PfPI4KIIIβ is twice as large its human ortholog due to expansion of sequences of unknown significance that separate the conserved domains (Figure 1.3). The lipid kinase activity of the PfPI4KIIIβ catalytic domain and the C-terminal portion of the N-lobe (KmATP = 72.5 μM) is comparable with the lipid kinase activities of human PI4KIIIα and PI4KIIIβ (70 μM and 90 μM, respectively) (Sternberg and Roepe, 2020). This kinase likely executes essential functions at all stages of the parasitic life cycle in vertebrate hosts (McNamara et al., 2013; Bushell et al., 2017). The *P. berghei* PI4KIIIβ (PBANKA_1109400) is phosphorylated and potentially regulated by the protein kinase PbPKG (Figure 1.6B) and has a role in parasite gliding motility. However, it is not known if the lipid kinase activity is required in this context. A mutant strain lacking consensus PKG-dependent

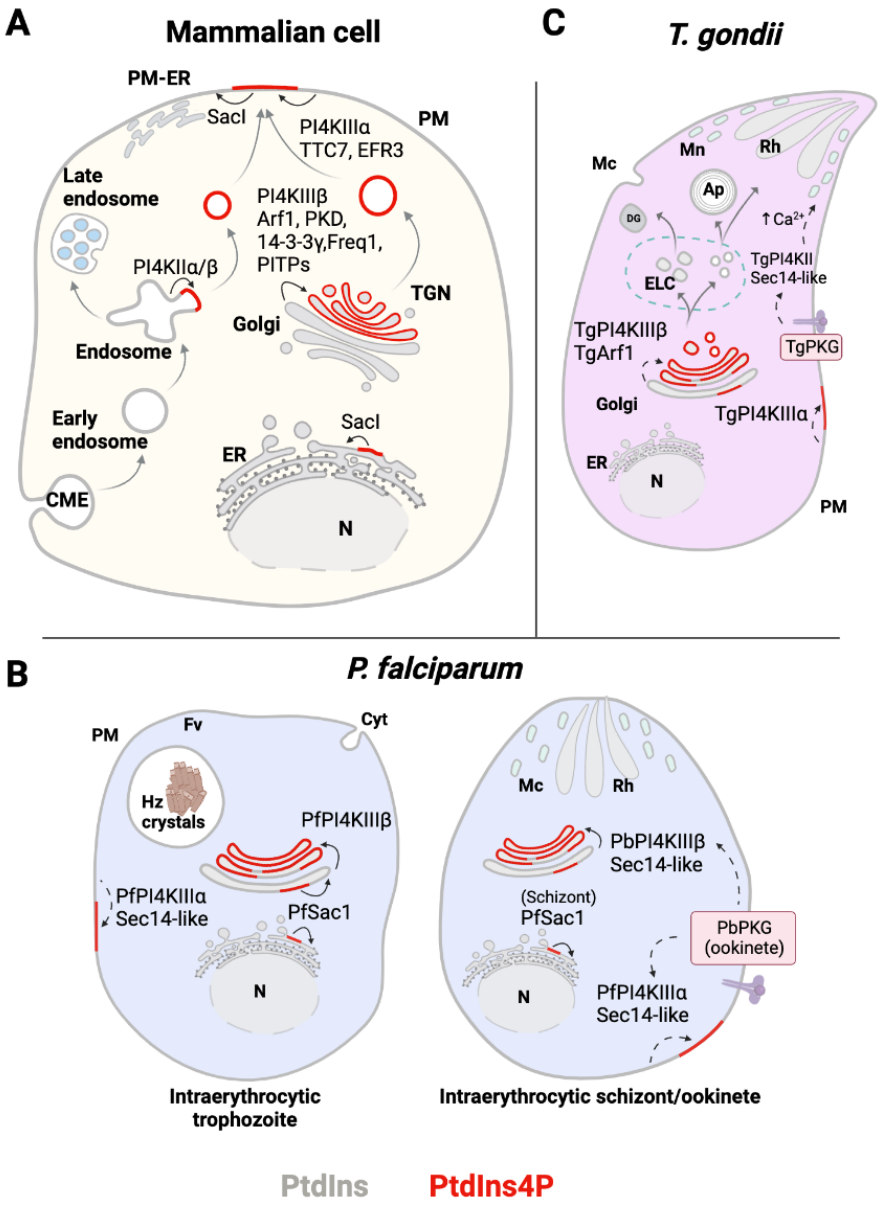

Figure 1.6 PIPs involved in exocytosis. (A) Mammalian: PtdIns4P is produced in the TGN and the PM by PI4KIIIβ and PI4KIIIα, respectively. PI4KIIIα is recruited to the PM and activated by TTC7 and EFR3. At the TGN, PI4KIIIβ is recruited by Freq1 and activated by PKD and 14-3-3γ, while PITPs improve PI4KIIIβ inefficient activity. Recycling of molecules from endosomes through exocytosis follows synthesis of PtdIns4P by PI4KIIα/β at the endosomes. Degradation of PtdIns4P occurs through activity of the phosphatase Sac1 in the PM and at the Golgi and ER membranes. (B) *Plasmodium*: PtdIns4P is produced at the Golgi by PfPI4KIIIβ, where it regulates membrane trafficking events during trophozoite and schizont stages. PbPKG positively regulated PfPI4KIIIβ in ookinetes. PfSacI negatively regulated PtdIns4P levels occurs at

Figure 1.6 Continued. the ER. (C) *T. gondii*: PtdIns4P is expected to be found at the Golgi and potentially generated by TgPI4KIIIβ and TgArf1. Solid line: Proposed reaction in previous publications; Dashed line: Proposed reaction in this review. Abbreviations: PM: plasma membrane; ER: endoplasmic reticulum; N: nucleus; Cyt: cytostome; Mp; micropore; Mc: microneme; Rh: Rhotry; Fv: food vacuole; Hz crystals: hemozoin crystals; Ap: apicoplast; DG: dense granule; ELC: endocytic-like compartment.

phosphorylation sites in PbPI4KIIIβ (pi4kS534A and pi4kS534A/S538A) exhibits a significant decrease in gliding speed compared with control lines (Brochet et al., 2014).

PfPI4PKIIIβ is distributed throughout the cytosol in trophozoites and is enriched at the apical ends of developing daughter merozoites (McNamara et al., 2013). PtdIns4P pools have been detected at the Golgi by colocalization with the marker ERD2 (ER Retention Defective protein 2) and at the plasma membrane throughout the erythrocytic cycle of *P. falciparum* (Figures 1.6B and 1.2A) (Ebrahimzadeh et al., 2018; McNamara et al., 2013). Synthesis of Golgi PtdIns4P pools is sensitive to imidazopyrazine, a PfPI4KIIIβ inhibitor, suggesting that this pool is generated by PfPI4KIIIβ. Specificity of imidazopyrazine was determined by comparing its effects with quinoxaline, a known inhibitor of human PI4KIIIβ. Both inhibitors induce essentially the same schizont-stage arrest. Moreover, imidazopyrazine- and quinoxaline-resistant parasites carry missense substitutions either in the PfPI4KIIIβ coding gene or in genes encoding proteins whose activity is regulated by the kinase. These resistant lines show comparable cross-resistance against both inhibitors. Taken together, these data make a convincing case that PfPI4KIIIβ is the direct cellular target of imidazopyrazine (McNamara et al., 2013).

Trafficking of post-Golgi vesicles produced in a PI4KIIIβ-dependent manner target to the plasma membrane and are involved in promoting cell division and cytokinesis in yeast and higher eukaryotes (Audhya et al., 2000; Schorr et al., 2001; Sechi et al., 2014). A recent study reported PtdIns4P pools in the *T. gondii* Golgi/TGN system and in post-TGN compartments. Moreover, perturbation of PtdIns4P signaling by expression of high affinity PtdIns4P binding domains evoked defects in dense granule biogenesis (maturation?) and exocytosis (Arabiotorre et al., 2023). Those results are not easily reconciled with current models that envision a process where mature secretion-competent dense granules bud directly from the TGN (Griffith et al., 2022). Rather, the data indicate a role for PtdIns4P signaling in a multi-step dense granule maturation pathway that resembles those characterized for regulated secretory pathways in professional secretory cells (Figure 1.7).

How are PtdIns4P signals read? TgRab11A is an ortholog of the ras-like GTPase Rab11 – a known PtdIns4P effector in higher eukaryotes – and this protein plays a key role in dense granule exocytosis and protein trafficking to the plasma membrane (Venugopal et al., 2020). Additional evidence for Rab11 in promoting downstream PtdIns4P signaling comes from studies in *P. falciparum*. In a manner similar to the case of cytokinesis in Drosophila spermatocytes, where deposition of new membrane at the midzone requires PI4KIIIβ-dependent Rab11 localization at Golgi and midzone membranes (Polevoy, et al., 2009), PfPI4KIIIβ-PfRab11A-regulated membrane trafficking facilitates cytokinesis during the intraerythrocytic stage (McNamara et al., 2013). The association of PfRab11A with PfPI4KIIIβ activity, and the coordination of

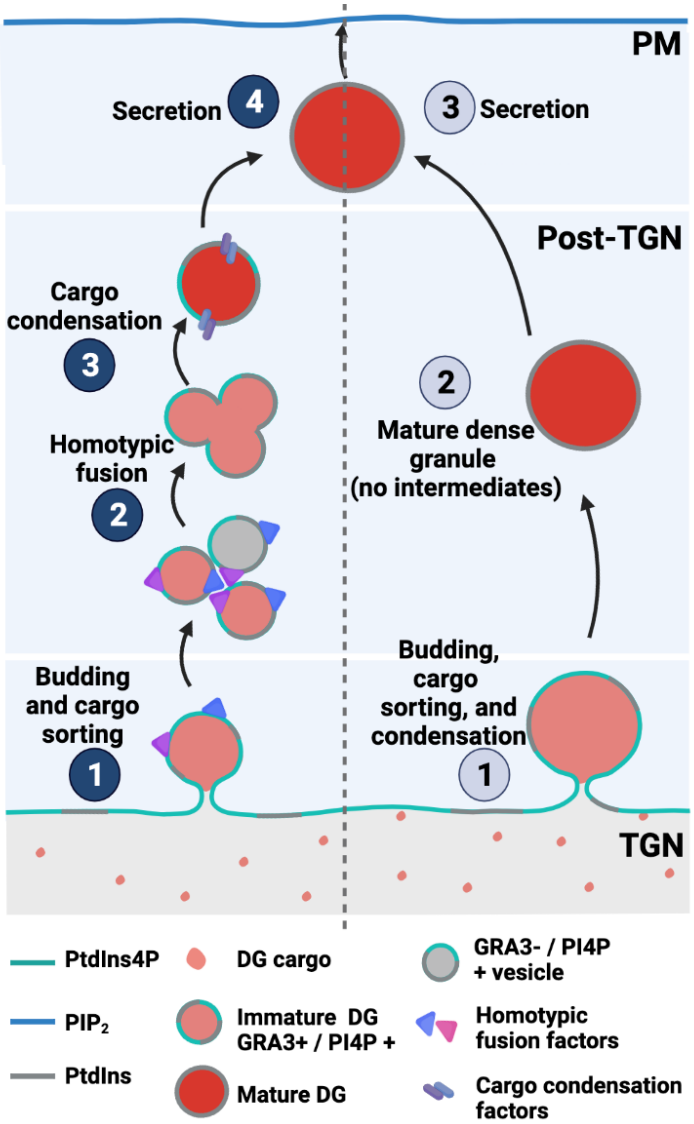

Figure 1.7 PtdIns4P-dependent dense granule multi-step biogenesis/maturation model (left) compared with direct budding models (right) in *T. gondii*.

Rab11A and its effectors by PI4KIIIβ on PtdIns4P-enriched membranes in Drosophila and humans (Polevoy, et al., 2009; Burke et al., 2014), suggests apicomplexan PI4KIIIβ-mediated PtdIns4P production effects proper membrane recruitment of Rab11A for purposes of membrane trafficking control in *P. falciparum* and *T. gondii*.

In yeast and mammals, recruitment of Pik1 and PI4KIIIβ to the Golgi is facilitated by the small GTPase Arf1, the plasma membrane protein NCS-1 (Frq1 in yeast) and GGA2 (Figure 1.6A) (Zhao et al., 2001; Daboussi et al., 2017 Highland and Fromme, 2021). Among these regulatory components, an Arf1 homolog has been identified in apicomplexans and is associated with trafficking processes -- although a role for the Arf1 homolog in PI4KIIIβ recruitment to the apicomplexan Golgi system has not yet been described. *T. gondii* Arf1 (TgArf1) localizes to Golgi membranes. In a permeabilized cell secretion assay, TgArf1 stimulated secretion of preformed dense granules (Liendo et al., 2001). TgArf1 may promote PtdIns4P synthesis at the Golgi and subsequently potentiate membrane trafficking from this compartment as is the case for yeast and mammalian Arf1 orthologs (Figure 1.6C). *P. falciparum* Arf1 (PfArf1), however, has not yet been associated with PtdIns4P synthesis. PfArf1 localizes to the ER and is required for *Plasmodium* export element (PEXEL)-dependent and -independent protein export pathways from this compartment (Taku et al., 2021).

In mammalian cells, activation of PI4KIIIβ at the Golgi/TGN occurs via phosphorylation of Ser294 by protein kinase D (PKD) (Hausser et al., 2005). This modification is maintained by interaction of PI4KIIIβ with the 14-3-3γ adaptor protein and sustains the PtdIns4P production that supports membrane fission events (Hausser et al., 2006). In *P. falciparum* and *T. gondii*, the closest ortholog to PKD1/2 is CDPK7. In *T. gondii*, CDPK7 activity is essential for correct positioning and partitioning of the centrosomes during parasite division (Morlon-Guyot et al., 2014). Moreover, genes encoding potential 14–3-3 orthologs have been identified in P. falciparum, P. knowlesi

and *T. gondii*. However, their precise functions remain to be elucidated (Al-Khedery et al., 1999; Koyama et al., 2001; del Mar Siles-Lucas and Gottstein, 2003). Quantitative phospho-proteomic profiling in *T. gondii* parasites lacking TgCDPK7 show that TgRab11A is a primary substrate for modification by TgCDPK7 and that TgCDPK7-dependent phosphorylation of TgRab11A is critical for protein trafficking, TgRab11A localization and parasite division (Bansal et al., 2021). The association of apicomplexan Rab11A with PI4KIIIβ regulators supports the likelihood that conserved mechanisms for PtdIns4P synthesis exist in the Golgi/TGN system of apicomplexans.

1.4.2. PITP-dependent regulation of PtdIns4P signaling in the secretory pathway

Studies in yeast reveal that PtdIns4P kinases are biologically insufficient enzymes, i.e., the levels of PtdIns4P these produce fall below the threshold required to sustain PtdIns4P signaling and maintain cell viability. This functional barrier is overcome by the activity of phosphatidylinositol transfer proteins (PITPs) which are more accurately referred to as PtdIns exchange proteins. Sec14, the major yeast PITP exchanges PtdCho for PtdIns on membrane surfaces. It is this heterotypic exchange cycle that stimulates PI4K to phosphorylate its substrate, likely because the PtdIns headgroup becomes exposed from the membrane bilayer and available for kinase action (Schaaf et al, 2008; Bankaitis et al., 2010; Sugiura et al., 2019). In the yeast TGN/endosomal system, the affected kinase is Pik1 (PI4KIIIβ in mammals). Upon stimulation by the Sec14 lipid exchange cycle, Pik1 produces sufficient PtdIns4P to

potentiate membrane trafficking (Rivas et al, 1999; Hama et al., 1999; Schaaf et al., 2008; Bankaitis et al., 2010).

PITPs are evolutionarily conserved proteins that fall into two structurally distinct families: the Sec14-like PITPs, of which Sec14 is the founding member, and StAR-related lipid transfer (StART) PITPs (Sha et al., 1998; Schaaf et al., 2008; Yoder et al., 2001). These PITP families are structurally unrelated, but both include members that regulate PtdIns 4-OH kinase activities through the heterotypic lipid exchange reaction between PtdIns and a secondary ligand (Schaaf et al., 2008; Bankaitis et al., 2010; Xie et al., 2018). The identity of the secondary ligand serves as signaling input into the PITP exchange lipid cycle. For example, PtdCho is the secondary ligand of Sec14, and one of PtdCho biosynthetic pathways is represented by the CDP-choline pathway which consumes DAG. Moreover, adequate levels of DAG in TGN/endosomal membranes are necessary to initiate vesicle budding and exocytosis. When sufficient DAG levels are available for vesicle biogenesis at the TGN/endosomes -- and to be used as substrate for PtdCho production -- Sec14 senses PtdCho production levels as metabolic information to drive PtdIns4P synthesis.

Apicomplexans encode both Sec14- and StART-like proteins (Supplementary Table S1.1). *Plasmodium* and *T. gondii* encode 4 and 10 proteins containing the Sec14 domain (also referred as the CRAL-TRIO domain with a N-CRAL-TRIO region), respectively. The protein architecture and sizes of *T. gondii* Sec14-like proteins are more diverse than what is observed in yeast and in *P. falciparum* (Figure 1.8). At least three of these candidates are multidomain proteins of some 2000 amino acids. Yeast encodes six

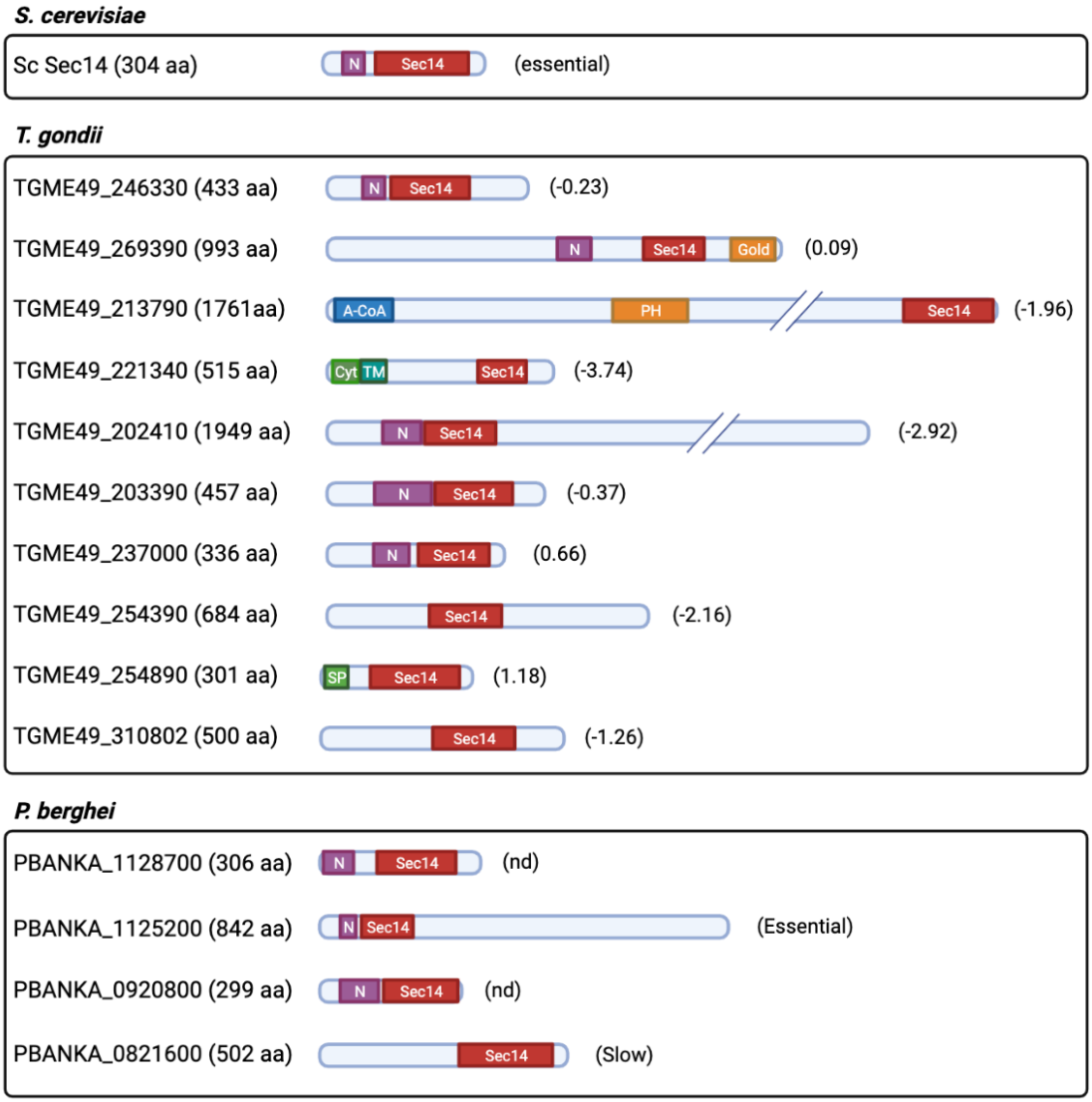

Figure 1.8 Prediction of protein architecture of Sec14-like protein candidates in *T. gondii* and *P. falciparum*. ScSec14p is depicted as the founding member of the Sec14-like protein family. Domain architecture of *T. gondii* and *P. falciparum* protein candidates that were selected upon prediction of the CRAL-TRIO domain through the Interpro domain finding tool in PlasmoDB (v56) and ToxoDB (v56). N: N-term CRAL-TRIO; Gold: Golgi dynamics domain; A-CoA: Acyl-CoA binding domain; PH: Pleckstrin Homology domain; Cyt: Cytoplasmic domain; TM: Transmembrane domain; SP: Signal peptide; (#): gene phenotypic score obtained from Sidik et al., 2016 for *T. gondii*; and relative growth rate score obtained from Bushell et al., 2016 for *P. falciparum* gene candidates (Essential: essential for growth; Slow: significant slow growth rate; and nd: not determined).

single domain Sec14-like proteins of similar size (Wu et al., 2000; Li et al., 2002; Routt et al., 2005; Ren et al., 2011; Kotomura et., 2018), and only Sec14p is essential for cell viability (Bankaitis et al., 1989). The biological requirement of Sec14-like genes in *T. gondii* is also distinct as 5 of 10 of these genes are required for parasite survival (Sidik et al., 2016). These observations suggest that Sec14-like proteins are involved in diverse functions in *T. gondii* parasites.

Although apicomplexan Sec14-like proteins have yet to be functionally characterized, there are indirect indications that some of these likely mediate PtdIns4P metabolism. For instance, mutations in a putative PfPI4KIIIα (PF3D7_0419900) and PfSec14-like (PF3D7_0626400) proteins were the primary contributors to positive fitness in *P. falciparum* kelch13 loss-of-function mutants (Cerqueira et al., 2017). As described above, PtdIns3P levels are regulated by PfKelch13 (Mbengue et al., 2015). These results suggest that PfPI4KIIIα (PF3D7_0419900) and PfSec14-like (PF3D7_0626400) candidates normalize PtdIns3P metabolism/signaling in parasites lacking functional PfKelch13. An independent phosphoproteomic study in *P. berghei* ookinetes linked PbPI4KIIIα (PBANKA_0722000), PbSec14-like protein (PBANKA_1125200) and PbPI4KIIIβ (PBANKA_1109400) to a phosphoinositide (PtdIns4P/PtdIns(4,5)P_2?) signaling pathway that responds to an endogenous cyclicGMP-dependent protein kinase (PKG) involved in intracellular Ca^{2+} mobilization (Brochet et al., 2014). In addition, two independent *T. gondii* phosphoproteomic analyses reported that a Sec14-like protein (TGME49_254390) (Nofal et al., 2022) and a putative TgPI4KII (TGME49_276170) (Herneisen et al., 2022) are phosphorylated

targets of Ca^{2+}-dependent protein kinases (TgCDPK) and TgPKG, respectively. Collectively, these data suggest the intriguing possibility that Sec14-like protein-dependent PtdIns4P production is part of a cGMP/Ca^{2+} pathway that modulates microneme secretion and parasite egress in apicomplexans.

A StART-like PITP has been described in *P. falciparum*. This essential PfStART-like protein (PF3D7_0104200) is present in the PV (in trophozoite stages) and in small compartments surrounding the edge of the parasite (in mature schizonts). Lipid transfer assays show that PF3D7_0104200 bind and transfers PtdIns and PtdCho between membranes in vitro at levels comparable to those measured for rat PITPα. It was suggested this protein is involved in acquisition of phospholipids from the host cell (van Ooij., 2013; Hill et al., 2016). However, the possibility that it might stimulate PIP synthesis more directly via a heterotypic exchange cycle has not been addressed. *T. gondii* also encodes a single candidate StART-like PITP (TgME49_289570), which is predicted to represent a multi-domain protein that contains a N-terminal PITP domain, a PH domain and a C-terminal OSBP domain. This gene has not been studied directly, but it serves as a likely essential gene in a CRISPR-based phenotypic screen (Sidik et al., 2016).

1.4.3. Regulators of PtdIns4P production at the plasma membrane

PtdIns4P can be delivered to the plasma membrane by vesicular carriers from the Golgi complex and from recycling endosomes (Dickson et al., 2014). PtdIns4P is also directly produced in the plasma membrane by PI4KIIIα isoforms, e.g., Stt4 in yeast and

the mammalian ortholog PI4KIIIα (Figure 1.6A). Recruitment and activation of these kinases in the plasma membrane occurs via interaction with soluble TTC7 and transmembrane EFR3 proteins that represent homologs of the yeast Ypp1 and Efr3 proteins, respectively (Audhya et al., 2000; Nakatsu et al., 2012; Batrouni et al., 2021). Both *Plasmodium* and *T. gondii* encode single candidate PI4KIIIα-like proteins (PF3D7_0419900 and TGGT1_228690) (Wengelnik et al., 2018). However, no obvious candidate orthologs are annotated for any of the known PI4KIIIα/Stt4 accessory proteins. As described above, PtdIns4P was detected at the plasma membrane throughout the erythrocytic cycle of *P. falciparum* (Ebrahimzadeh et al., 2018). Hence, recruitment of PI4KIIIα to the apicomplexan plasma membrane, and production of PtdIns4P at that site, might occur via a novel mechanism.

1.4.4. Downregulation of PtdIns4P signaling in Apicomplexa

Downregulation of PtdIns4P signaling is primarily executed by the highly conserved PtdIns4P-phosphatase Sac1. This enzyme shows substrate promiscuity in vitro but primarily dephosphorylates the D-4 position of the inositol ring to produce PtdIns at the expense of PtdIns4P in vivo (Guo et al., 1999; Rivas et al., 1999). Sac1 is enriched at the Golgi and ER membranes of budding yeast and metazoan organisms and is proposed to establish a PtdIns4P gradient across the Golgi complex with the TGN harboring the highest level of PtdIns4P (Wood et al., 2012; Piao and Mayinger, 2012; Venditti et al., 2016; Del Bel and Bril, 2018). In addition, Sac1 is proposed to degrade PtdIns4P derived from the plasma membrane at ER-plasma membrane contact sites

(Figure 1.6A) (Chung et al., 2015). A *P. falciparum* Sac1 (PfSac1: PF3D7_1354200) contains the conserved CX5R(T/S) motif essential for catalytic activity (Liu and Bankaitis, 2010; Thériault et al., 2017). This PfSac1 is expressed in all asexual blood stages and is likely required for survival during the erythrocytic cycle. PfSac1 also localizes to ER and Golgi membranes (Figure 1.6B), whereas localization to both organelles becomes less apparent during early schizont stages. Yeast and mammalian Sac1 shuttle between the ER and Golgi in a growth phase-dependent manner. Whereas Sac1 concentrates at the ER in dividing cells, it translocates to the Golgi in resting cells or under starvation conditions (Whitters et al., 1993; Blagoveshchenskaya et al., 2008). On that basis, it is suggested that PfSac1 disengages from the Golgi system in schizonts and distributes preferentially to transitional ER (Thériault et al., 2017). Whether PfSac1 localization is subject to regulation similar to that observed in yeast, perhaps to control expansion of PtdIns4P-rich domains in the Golgi during progression of parasite erythrocytic life cycle, remains to be determined.

In yeast, Sec14-dependent PtdIns4P signaling is also downregulated by one of the seven members of the oxysterol binding protein (OSBP) superfamily -- Kes1/Osh4 (Fang et al., 1996). Kes1/Osh4 is proposed to act as a sterol-regulated `brake' of TGN/endosomal trafficking via its ability to clamp/sequester Pik1-generated Golgi PtdIns4P from its effectors (Mousley et al., 2012). Genomic annotations predict one OSBP/ORP homolog in *Plasmodium* and three in *T. gondii* (Supplementary Table S1.1). However, these proteins have not been characterized. As described in the previous section, the single candidate StART-like PITP in *T. gondii* is a predicted multi-domain

protein with a C-terminal OSBP-like domain. Although neither the PITP nor the OSBP activities of these domains have been confirmed, this architecture presents a physical coupling of two domains that exhibit functional antagonism in regulating PtdIns4P signaling. This curious point suggests that the relationship between PITP activity and OSBP antagonism described in yeast might have evolved in the most ancient eukaryotes. Whether that antagonism operates through regulation of PI4P signaling in *T. gondii* remains unknown.

Two OSBP/ORP proteins have been identified and characterized in *C. parvum* (CpORP1 and CpORP2). Cholesterol is not a binding ligand for either protein, whereas PtdOH and PtdIns monophosphates (PtdIns3P, PtdIns4P and PtdIns5P) bound with high affinity. CpORP1 localizes to the PVM – suggestive of a lipid transport role across the interface between the parasite and the host intestinal lumen (Zeng et al., 2006). Alternatively, CpORP1 may be involved in PIP sensing at the PVM.

Few factors associated with synthesis and regulation of PtdIns4P levels in Plasmodium have been described, and there is a complete lack of information regarding PtdIns4P metabolism in apicomplexan organisms. As reviewed, PtdIns4P distributes to the Golgi where it executes important regulatory roles in vesicle trafficking and secretion, and to the plasma membrane. Contrary to protein regulators associated with PtdIns4P synthesis in the plasma membrane, almost every such regulator associated with PtdIns4P in the Golgi (discussed in this section) has at least one candidate homologue in *Plasmodium* and *T. gondii* (Supplementary Table S1.1). Hence, addressing the link between PtdIns4P metabolism in Golgi and trafficking of specialized Apicomplexa

secretory proteins (microneme, rhoptry and dense granule proteins) identifies an important step in understanding ancient and conserved intracellular trafficking and cell secretion processes.

1.5. Regulation of PtdIns(4,5)P_2 in Apicomplexa

PtdIns(4,5)P_2 is primarily concentrated at the plasma membrane of eukaryotic cells and contributes to many activities that involve the plasma membrane. These include actin cytoskeleton assembly, cell polarization, cell migration, endocytic vesicle formation and scission, exocytic vesicle fusion to the plasma membrane, and ion channel and transporter activation. Upon receptor stimulation, PtdIns(4,5)P_2 is metabolized to produce second messengers such as PtdIns(3,4,5)P_3, inositol trisphosphate (IP_3 or I(1,4,5)P_3), DAG, phosphatidic acid (PtdOH), arachidonic acid (AA) and lysophospholipids that further propagate and amplify signal transduction (Di Paolo and Camilli, 2006; Wen et al., 2021; De Craene et al., 2017). In mammals, the major pathway for producing PtdIns(4,5)P_2 in the plasma membrane is by phosphorylation of PtdIns4P by a type I PI4P5K (PIP5K1-α, PIP5K1-β and PIP5K1-γ) (Figure 1.5A). In multicellular organisms, a second type II PI5P4K phosphorylates PtdIns5P to produce PtdIns(4,5)P_2 in the Golgi. In yeast, the type I PI4P5K (Mss4p) is the only PIP kinase that generates PtdIns(4,5)P_2, and it is essential for cell viability (Ishihara et al., 1998; Hinchliffe and Irvine, 2010; Desrivières et al., 1998; Rameh, 2010).

PtdIns(4,5)P_2 is by far the most abundant PIP in *P. falciparum* (Tawk et al., 2010). PtdIns(4,5)P_2 is detected at the plasma membrane throughout all stages of the cell

cycle (Figure 1.2A) and, during ring stages, in membranes of large vesicles that likely represent endocytic compartments (Figure 4B) (Ebrahimzadeh et al., 2018). A putative bifunctional PIP5K was identified in *P. falciparum* (PF3D7_0110600) that contains a C-terminal type I PI4P5K domain with catalytic specificity for PtdIns4P, and an N-terminal domain with potential helix-loop-helix EF-hand-like motifs found in members of the NCS family (NCS-EF domain) (Figure 1.3). This PfPI4P5K preferentially phosphorylates PtdIns4P and is stimulated by myristoylated Arf1 in vitro (Leber et al. 2009). The physical association of type I PI4P5K and NCS-EF domains is unique to Apicomplexa. This property could reflect an exclusive modulation of the PtdIns(4,5)P_2 synthesis in response to changes in intracellular calcium concentrations within the parasite. PtdIns(4,5)P_2 in *T. gondii* has not been directly described. Unlike *Plasmodium*, which only encodes a single putative PI4P5K isoform, *T. gondii* encodes two isoforms of PI4P5K termed TgPI4P5KA and TgPI4P5KB (TGME49_230490 and TGME49_245730 respectively) (Wengelnik et al., 2018). Only TgPI4P5KA is likely to be essential for parasite viability (Sidik et al., 2016), and this enzyme exhibits an EF-hand motif. This organization recommends this isoform as the true PfPI4P5K ortholog and that it is involved in crosstalk between Ca^{2+} signaling and PIP metabolism. In that regard, PtdIns(4,5)P_2 pools have been detected at the *T. gondii* plasma membrane using a 2xPHPLCδ reporter (Arabiotorre et al., 2023).

1.5.1. Regulation of PtdIns(4,5)P_2 levels in Apicomplexa

PtdOH is a potent stimulator of type I PI4P5Ks in mammals and is generated in part by phospholipase D (PLD)-mediated degradation of PtdCho. PLDs in mammals are involved in a complex inter-regulatory relationship with type I PI4P5K (Jones et al., 2000; Divecha et al., 2000; Hinchliffe and Invine, 2010). However, PfPI4P5K is not stimulated by PtdOH in vitro (Leber et al. 2009). PLD-like proteins have not been identified in either *T. gondii* or in *Plasmodium* genome databases – a finding consistent with the lack of regulation of PfPI4P5K by PtdOH. Perhaps PtdOH-dependent stimulation of type I PI4P5K was acquired in multicellular organisms later in the evolutionary process.

In mammals and yeast, PtdIns(4,5)P_2 steady-state levels are subject to negative regulation by synaptojanin-like proteins (5-phosphatases) that degrade the PtdIns(4,5)P_2 and downregulate signaling on internal membranes. In metazoans, these enzymes regulate the synaptic vesicle cycle and, in yeast, are linked to endocytosis and actin dynamics (Verstreken et al., 2003; Stefan et al., 2002). Proteins homologous to synaptojanins 1 and 2 are encoded in *Plasmodium* and *T. gondii* genomes (Supplementary Table S1.1). These proteins have not been extensively studied in Apicomplexa. However, one and two of these putative homologs in *Plasmodium* and *T. gondii*, respectively, play essential roles in infective stages (Bushell et al., 2017 and Sidik et al., 2016).

The role of PtdIns(4,5)P_2 in parasite invasion, egress and motility

The hydrolysis of PtdIns(4,5)P_2 by phospholipases generates a set of secondary messengers that transduce highly specific intracellular signals (Bunney and Katan, 2011). For instance, hydrolysis of PtdIns(4,5)P_2 by phospholipase C (PLC) produces plasma membrane DAG and soluble IP_3. DGA binds and activates C1 domain-containing proteins such as protein kinase C (PKC) family at the plasma membrane, while IP_3 diffuses into the cytosol to interact with the sarco-ER Ca^{2+} release channel (SERCA) (Streb et al., 1983; Berridge et al., 1984). In mammals, there are six families of PLC enzymes (PLCβ, γ, δ, ε, ζ, η), whereas only PLCδ is present in single cell eukaryotes (Bunney and Katan, 2011; Koyanagi et al., 1998). *P. falciparum* and *P. berghei* harbor essential genes that encode putative phosphoinositide-specific PLC (PI-PLC) proteins: PfPI-PLC and PbPI-PLC (PF10_0132 and PBANKA_121190, respectively). These proteins bear the signatures of ancestral δ-type PLCs. Both versions contain all domains found in PLCδ: (i) the lipid binding pleckstrin-homology (PH) domain, (ii) the calcium-binding motif EF-hand, (iii) the catalytic domain consisting of an X- and a Y-domain, and (iv) the calcium/lipid- binding C2-domain. Nonetheless, *Plasmodium* PI-PLCs are twice as large as their mammalian orthologues due to large insertions of unknown function (Raabe et al., 2011a). *T. gondii* also encodes a single essential gene for PI-PLC (TgPI-PLC; TGME49_248830) that bears a domain structure similar to those of *Plasmodium* PI-PLCs (Bullen et al., 2016).

How PI-PLC activity is regulated in Apicomplexans is not well understood. In *P. berghei*, PtdIns(4,5)P_2 hydrolysis occurs in the plasma membrane, and the resulting IP_3

triggers Ca^{2+} mobilization in the cytosol during gametocyte activation. These responses are blocked by a PI-PLC inhibitor (U73122) (Raabe et al., 2011b). Exposure of *P. falciparum* merozoites to the low levels of K^+ normally found in human plasma elevate intracellular Ca^{2+} levels that, in turn, stimulate protein release from micronemes (Figure 1.5B). This pathway of protein exocytosis from micronemes is inhibited by U73122. In addition, interaction of released microneme proteins with erythrocytic receptors restores basal cytosolic Ca^{2+} levels in the parasite and triggers rhoptry secretion. This sequential discharge of micronemes and rhoptry contents in merozoites is essential for erythrocyte invasion (Singh et al., 2010). TgPI-PLC localizes to punctate structures in the cytosol, to the plasma membrane and to the apex of intracellular and extracellular tachyzoites. While the enzyme shows preferential substrate specificity towards PtdIns over PtdIns(4,5)P_2 in vitro (Fang et al., 2006), the most likely in vivo substrate is PtdIns(4,5)P_2. That an extracellular drop in K^+ levels activates TgPI-PLC-dependent intracellular Ca^{2+} mobilization reveals similar regulatory mechanisms with *Plasmodium* PI-PLC isoforms (Figure 1.5C) (Bullen et al., 2016).

P. falciparum PKG-mediated sensing of cGMP (PF3D7_1436600) is associated with Ca^{2+} mobilization-dependent processes, e.g., inhibition of PfPKG disrupted exoneme and microneme release and compromized egress of merozoites (Collins et al., 2013). Moreover, in *P. berghei*, a putative PKG (PBANKA_1008200) controls activation of PbPI4KIIIβ and peripheral localization of PbPI4P5K via phosphorylation of these enzymes in ookinetes. Additionally, PKG activity is critical for maintaining high cytosolic Ca^{2+} levels in gliding ookinetes. There is a direct link between PKG, PIP

metabolism and Ca^{2+} signaling as evidenced by the demonstration that PKG activation leads to rapid PtdIns(4,5)P_2 hydrolysis and elevations in intracellular Ca^{2+} in *P. falciparum* schizonts. This is consistent with a role for PKG in regulating Ca^{2+} mobilization-dependent processes during this life cycle stage. In gliding ookinetes (where Ca^{2+} levels are high) PKG inhibition elicits the opposite effect. PtdIns levels increase while PIP and PtdIns(4,5)P_2 levels decrease. These data suggest that PKG regulates the rate limiting conversion of PtdIns to PtdIns4P in the ookinete stage via phosphorylation of PI4KIIIβ (Brochet et al., 2014).

In *T. gondii*, increases in intracellular Ca^{2+} can trigger the microneme discharge required for parasite invasion, motility, and egress (Carruthers and Sibley, 1999; Lourido et al., 2012). *T. gondii* encode multiple PKG isoforms and only isoform I (TGME49_311360) is essential for parasite invasion, motility, and for triggering microneme secretion (Wiersma et al., 2004; Brown et a., 2017). As in *Plasmodium* PKG, TgPKG isoform I-mediated sensing of cGMP induces Ca^{2+} release. Nonetheless, serum albumin dependent-microneme release, which is mediated by TgPKG activation, does not require Ca^{2+} mobilization (Brown et a., 2016). Although TgPKG and its direct involvement with PtdIns(4,5)P_2 metabolism has not been addressed, it is suggested that TgPKG-dependent Ca^{2+} mobilization occurs upon upregulation of PtdIns4P and PtdIns(4,5)P_2 synthesis – as shown in *P. falciparum* schizonts (Brochet et al., 2014; Bisio and Soldati-Favre, 2019). Also, it is possible that an alternate pathway for stimulation of microneme secretion regulated by TgPKG that does not requires

intracellular Ca^{2+} mobilization and is triggered by serum albumin exists (Brown et a., 2016).

1.5.2. Inositol phosphates (IPs) in Apicomplexa

IPs are highly polar and energy-rich molecules species that regulate important cellular signaling functions in Eukaryotes (Dong et al., 2019; Sahu et al., 2020). IP_3 also serves as precursor of Inositol hexakisphosphate (IP_6) and its inositol pyrophosphates derivatives PP IP_5 (or IP_7) and $(PP)_2$-IP_4 (or IP_8) (Mtchell, 2018). A phylogenetic study detected presence of genes coding for two out of the four Eukaryotic kinase families involved in IP_3 phosphorylation and of its derivatives in *P. falciparum* and *C. parvum* -- the inositol polyphosphate kinase (IPK) and diphosphoinositol pentakisphosphate kinase (PPIP5K) (Laha et al., 2021). This finding suggests limited production of inositol pyrophosphates in Apicomplexans.

In Apicomplexa the IP_3 content has been detected and measured after addressing its involvement in the activation of Ca^{2+} mobilization following rapid PIP_2 hydrolysis in *Plasmodium* (Raabe et al., 2011b). Interestingly, genes encoding members of the IP3 receptor (IP_3R) family are absent in this phylum (Naganume et al., 2006; Ladenburger et al., 2009), however, addition of IP_3 and known IP_3R competitive inhibitors stimulate and decrease Ca^{2+} release in *T. gondii* in vitro, respectively (Chini et al., 2005). Moreover, IP_3 production is reduced following pharmacological inhibition of ryanodine receptor (RyR)-like channels, suggesting that RyR mediated Ca^{2+} release stimulates sustained PbPI-PLC activity (Raabe et al., 2011b). This indicates that Apicomplexans have unique

IP_3R-like receptors with similar regulation instructions described in mammalian cells (Berridge et al., 2003).

1.6. PtdIns5P In Apicomplexa

PtdIns5P in metazoa is proposed to be involved in cytoskeleton organization, modulation of transcriptional activity, T cell signaling, and regulation of endosomal protein sorting and autophagocytotic events. However, its metabolism and cellular functions remain poorly understood (Niebuhr, et al., 2002; Gozani et al., 2003; Guittard et al., 2009; Boal et al., 2015; Vicinanza et al., 2015). PtdIns5P is produced in multicellular organisms as well as in wild-type *S. cerevisiae* at relatively small levels (Zolov et al., 2012). The phylogenetic distribution of proteins implicated in the regulation of PtdIns5P metabolism and of its precursors in the Eukarya reveal four groups of co-evolving proteins (Lecompte et al., 2008). These groups (or triads) comprise a phosphatase, a kinase, and a regulator. Examples include the following: (1) the Vps15p– class III PI3K– enzymatically active MTM (aMTM) complex that regulates PtdIns3P turnover; (2) the Vac14–PIKfyve–Fig4 complex that regulates the balance between PtdIns3P and PtdIns(3,5)P_2; (3) the class I PI3K–catalytically dead MTM (dMTM)–aMTM complex initially hypothesized to regulate the interconversion between PtdIns(3,5)P_2 and PtdIns5P, and (4) the PI4P5KII–PtdIns(4,5)P_2 4-phosphatase complex that converts PtdIns5P to PtdIns(4,5)P_2 (and vice versa) found exclusively in the Metazoa (Figure 1.5A) (Lecompte et al., 2008). PIKfyve is the major activity for

producing PtdIns5P from PtdIns in mammals (Sbrissa et al., 2012; Zolov et al., 2012; Shisheva et al., 2013).

As reviewed in previous sections, some components from these complexes are either poorly conserved in *T. gondii* and *Plasmodium* (PIK3R4–PIK3C3–aMTM and VAC14–PIKfyve–FIG4) or not conserved at all (PIK3C–dMTM–aMTM and PIP5K2–PtdIns(4,5)P_2 4-phosphatases) (Supplementary Table S1.1). Nonetheless, PtdIns5P is inferred to be present in *P. falciparum* on the basis of the membrane recruitment of the PtdIns5P-binding PH domain of mammalian PHDOK5 (Downstream of tyrosine kinase 5). By this criterion PtdIns5P is present in the plasma membrane and in the cytoplasm in the early stages of the intraerythrocytic cycle (Figure 2A). During trophozoite maturation, PtdIns5P is transiently detected in the nucleus (Figure 1.5B). During the schizont stage, PtdIns5P adopts a profile similar to that of PfSec13p (a marker of the transitional ER) (Ebrahimzadeh et al., 2018). These localization data suggest that the functions and regulation of PtdIns5P in *P. falciparum* and other Apicomplexans diverge from those of other eukaryotic organisms studied thus far.

1.7. Regulation of host PIPKs by Apicomplexan organisms

Apicomplexan parasites modulate host cell physiological processes to persist in host cells. One such strategy involves manipulation of host PI3K class I activation at the host cell plasma membrane to promote parasite invasion. *C. parvum* induces accumulation and activation of host PI3K class IA at the host-cell parasite interface during invasion of biliary epithelial cells (Chen et al., 2004). Activation of host PI3K

class IA leads to host cell actin remodeling at the host cell-parasite interface that potentiates the invasion process. How *C. parvum* activates PI3K in this process remains unclear. A potential mechanism involves *C. parvum* host cell attachment-dependent phosphorylation of host PI3K class IA. This hypothesis is supported by elevated tyrosine phosphorylation of the host PI3K p85 subunit in *C. parvum*-infected cells. Such PI3K class IA activation is expected to nucleate Cdc42 accumulation and activation at the host cell-parasite interface with subsequent actin remodeling at that site (Chen et al., 2004).

Strategies that control host cell survival upon parasite infection are also linked to parasite-mediated host PI3K activation. *P. berghei* induces early release of hepatocyte growth factor (HGF) during sporozoite invasion (Leirião et al., 2005). Interaction of HGF with its receptor c-MET promotes survival of uninfected hepatocytes (Carrolo et al. 2003). This interaction is amplified by sporozoite migration through the hepatocyte, and this process induces cell wounding and enhanced release of HGF. HGF/MET signaling produces an anti-apoptotic response at early time points of infection via activation of the PI3-kinase/Akt pathway (Leirião et al., 2005). Theileria parva, the causative agent of East Coast fever, a particularly problematic disease of cattle in Africa, also promotes host cell immortalization that requires irreversible activation of class I PI3K. T. parva-transformed B cells further augment their proliferative potential via a granulocyte-monocyte colony-stimulating factor (GM-CSF) autocrine loop that sustains host class I PI3K activity and thereby stimulates activation of the transcription factor AP-1 (Activator protein 1) (Baumgartner et al., 2000; Dessauge et al. 2005).

Lastly, an important strategy that enables Apicomplexa to invade and survive within the host cell environment is the ability to evade destruction by the host. This capability is well documented for *T. gondii* and involves activation of the PI3K/AKT pathway. During infection, *T. gondii* release excreted/secreted antigens (TgESAs) that interact with cell surface receptors of macrophages and dendritic cells and thereby modulate the immune response (Wang et al., 2017). Such upregulation of host PI3K/AKT pathway by TgESAs results in adverse pregnancy outcomes in mice (Chen et al., 2019), and also results in negative modulation of Forkhead box-p3 (Foxp3) expression. Foxp3 is a transcription factor whose function is critical for activation of CD4+CD25+ regulatory T cell (Treg) differentiation (So and Croft, 2007). The result of functional suppression of Fox3p is insufficiency of the Treg response to *T. gondii* infection. Moreover, TgESA-treated dendritic cells show elevated PI3K/AKT pathway activity that leads to diminished production of exoge¬nous and endogenous reactive oxygen species (ROS). Downregulation of ROS production creates a host environment that favors *T. gondii* proliferation (Choi et al., 2020). Among the TgESAs, microneme proteins 3 and 6 (MIC3 and MIC6) are epidermal growth factor receptor ligands that activate the PI3K/Akt signaling pathway and dampen autophagy protein LC3 expression and parasitophorous vacuole-lysosomal fusion. This circuit protects the parasite from clearance by the host autophagy machinery (Muniz-Feliciano et al., 2013; Wang et al., 2016; Zhu et a., 2019). These examples show that Apicomplexa not only have machinery to regulate their endogenous PIP levels but have also developed the means for

regulating host PIP levels involved in signaling pathways that activate/inactivate host responses to the benefit of the parasite.

1.8. Concluding remarks

Studies focused on PIP metabolism and its regulation in Apicomplexa are in their infancy. Among the seventeen prospective PtdIns and PIP kinase classes identified in *Plasmodium* and *T. gondii*, only five have been characterized in any detail (PfPI3K, TgPI3K, PfPI4KIIIβ, PfPI4P5K, and TgPIKfyve). Reports of PIP phosphatases and regulatory factors that mediate PdtIns/PIP synthesis and catabolism in Apicomplexa are similarly sparse. This lack of information is reflected in the underdevelopment of drugs targeting PI/PIP signaling pathways -- especially in *T. gondii* and other members of the phylum. An understanding of the shape of the PIP signaling landscape in Apicomplexa holds the potential to reveal the ancestral blueprint for this major intracellular signaling system, and how it has been adapted to more complicated regulatory roles in higher eukaryotes.

The presence and compartmental distribution of most PIP species has been mainly addressed in the intraerythrocytic stages of *P. falciparum* parasites. Few PIP species have been detected and mapped in *T. gondii* or other members of the phylum, however. Expansion of PIP studies to Apicomplexa with life cycles diverged from those of *Plasmodium* will further inform how the built-in plasticity of PIP signaling networks is suited for regulation of complex developmental differences in evolutionarily related organisms.

Synthesis and spatial arrangement of PIPs are tightly controlled during division in higher eukaryotic cells and yeast (Cauvin et al., 2014; Schorr et al., 2001). PIP synthesis regulation throughout the cell cycle in specific life cycle stages has been poorly explored in Apicomplexa. Complexities of this phylum -- two-host life cycle, unconventional cell division phases and remarkably fast multiplication – make Apicomplexa an ideal model to understand how eukaryotes can adjust or recycle PIPs during progression of cell division.

Factors that regulate Apicomplexan PIP signaling are often found by virtue of their orthology to mammalian/yeast PdtIns/PIP kinase regulators. Several of these factors are seemingly absent in *Plasmodium* and *T. gondii*. Expansion on studies addressing uncharacterized Apicomplexan-specific proteins would inform fundamental aspects of how PIP production is regulated in ancient eukaryotic organisms.

Finally, PIP pathways in single-celled eukaryotes have proven potent and specific drug targets, e.g., the imidazopyrazine compounds that target *Plasmodium* PI4KIIIβ activity, or multiple small molecule inhibitors of Sec14 in yeast (McNamara et a., 2013; Nile et al 2014; Filipuzzi et al., 2016; Khan et al., 2021). In this review we listed undescribed essential orthologs of mammalian and yeast proteins involved in regulation of PIP synthesis in *Plasmodium* and *T. gondii*. Characterization of these proteins, specifically ones that diverge from their counterparts in higher organisms promise to reveal effective strategies for designing antiparasitic drugs with high target selectivities.

1.9. References

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2. PHOSPHATIDYLINOSITOL-4-PHOSPHATE REGULATES THE FORMATION OF DENSE GRANULES IN *TOXOPLASMA GONDII*

2.1. Introduction

Phosphoinositides (PIPs) are phosphorylated derivatives of the glycerol-based phospholipid phosphatidylinositol (PtdIns). PIPs are chemically distinguished by positionally specific phosphorylations at the 3-OH, 4-OH and 5-OH positions on the inositol ring that constitutes the headgroup of these lipids (Carpenter and Cantley, 1990). The relatively low chemical complexity of the PIP cohort notwithstanding (mammals express seven PIPs while yeast produce four) these molecules regulate literally hundreds of distinct biological outcomes (reviewed in Di Paolo and Camilli, 2006; Balla, 2013). Among the various cellular events regulated PIPs, maintenance of organelle identity and regulation of membrane trafficking events is of direct relevance to this study. In yeast and higher eukaryotes, PIP-regulated membrane trafficking processes include: (1) endocytosis and the formation of PtdIns(3)P-positive vesicles from the PM and its fusion with the endocytic system (Gillooly et al., 2000; Petiot et al., 2003; Ikonomov et al., 2006: Whitley et al., 2009; Duex et al., 2006), (2) the PtdIns(3)P-dependent formation of autophagosomes and PtdIns(3,5)P_2-dependent fusion with lysosomes (Mizushima et al., 2008), and (3) formation of exocytic vesicles at the TGN in a PtdIns4P-dependent manner and their subsequent transport to the plasma membrane (Rivas et al., 1999; Hama et al., 1999; Walch-Solimena et al., 1999 Jović et al., 2012). Considerable effort has been productively invested in the identification and characterization of protein

factors that function as downstream effectors of PIP signaling in these various membrane trafficking events – primarily in yeast and in mammalian cells (Rothman et al., 1994; Schekman et al., 1996; Olayioye et al., 2019). Very little is known about the relationship of PIP signaling and control of membrane trafficking in ancient eukaryotic systems, however.

Organisms of the phylum Apicomplexa are very primitive eukaryotes that exhibit an obligate intracellular parasitic lifestyle that comes with unique adaptations. Given the deep roots of apicomplexan parasites in the eukaryotic evolutionary tree (Escalante et al., 1995; Levine et al., 2018), these parasites offer exceptional models for understanding the primordial design of PIP signaling systems. *T. gondii* is arguably the most experimentally tractable apicomplexan parasite. This organism can be cultured in vitro, is amenable to genetic manipulation, and rodent models have been established for studying *T. gondii* infection (Pittman et al., 2014; Jacot and Soldati-Favre, 2020). Like other apicomplexans, *T. gondii* harbors unique endomembrane organelles that are dedicated to the execution of activities essential for completion of their complex parasitic life cycles. For instance, a set of specialized late secretory organelles – micronemes, rhoptries, and dense granules (DGs) – are used to store and exocytose cargos required for successful invasion, intracellular survival, and egress from host cells (Carruthers et al., 1999; Nichols et al., 1983; Bai et al., 2018). Although some factors involved in the biogenesis and function of micronemes and rhoptries have been characterized, very little is known concerning mechanisms of DG biogenesis. Moreover,

the roles of PIPs in the formation of, and cargo trafficking from, these organelles remain unstudied.

PIP binding domains have been successfully used to localize and analyze intracellular PIP pools applied in many cell types ranging from yeast to plants and to mammals. Such approaches have also been applied to *T. gondii* to visualize intracellular distributions of PtdIns3P and PtdIns(3,5)P_2 in this parasite. Those studies revealed important roles for the 3-OH phosphoinositides in the biogenesis and/or inheritance of the apicoplast (Tawk et al., 2011; Daher et al., 2015). However, the localization of and function of the 4'-OH phosphorylated PIPs, particularly PdIns4P, has not been studied in detail in neither *T. gondii* nor in Apicomplexa in general (McNamara et al., 2013). Herein, we use the PtdIns4P-binding domains of mammalian GOLPH3, FAPP1 and bacterial SidM (P4M) proteins to obtain spatial and functional information regarding PtdIns4P signaling in intracellular tachyzoites of *T. gondii*. Our data demonstrate the presence of PtdIns4P pools in the Golgi/TGN system and post-TGN compartments and provide evidence for cell-cycle-dependent regulation of PtdIns4P signaling. Moreover, we show that compromised PtdIns4P signaling results in structural perturbation of DGs with accompanying deficits in exocytosis of DG cargo. Taken together, the data not only demonstrate that PtdIns4P itself is a key regulator of DG biogenesis and exocytosis, but also suggest that the biogenic pathway for DG formation in *T. gondii* is more complex than simple budding from the TGN -- as is presently proposed (Griffith et al., 2022). Finally, the collective results argue for a primordial role for PtdIns4P signaling in

membrane trafficking through the late stages of the secretory pathway – a role that has been preserved throughout the Eukaryota from yeast to primates.

2.2. Results

2.2.1. Visualization of *T. gondii* PtdIns4P pools by genetically encoded biosensors.

To visualize pools of PtdIns4P and its higher-order derivatives PtdIns(4,5)P_2 and PtdIns(3,4)P_2 in *T. gondii*, fluorescent biosensors based on PIP binding domains of known specificity were expressed in the parasite and imaged. A tandem human PLCδ PH domain fused to enhanced green fluorescent protein at the C-terminus (2xPHPLCδ-EGFP; (Várnai et al., 1998), and a PH domain of human TAPP1 fused to YFP at the N-terminus (YFP-TAPP1PH; Hogan et al., 2004), were used to monitor distribution of PtdIns(4,5)P_2 and its regioisomer PtdIns(3,4)P_2, respectively. Expression of these biosensors was driven by the tubulin (Tub1) promoter. The corresponding reporter genes were transfected into *T. gondii* RH tachyzoites and their profiles recorded under a transient transfection regime. Wide-field fluorescence imaging performed 24 hours post-transfection (hpt) reported recruitment of both biosensors to the plasma membrane of intracellular parasites although diffuse cytoplasmic signals were also detected in both cases (Figure 2.1A). These data suggest both PIP_2 species localize predominantly to the plasma membrane of *T. gondii*. These profiles are consistent with those reported in other eukaryotes (Hammond et al., 2012; Halet et al., 2002; Heo et al., 2006).

PtdIns4P is a metabolic precursor for both PtdIns(4,5)P_2 and PtdIns(3,4)P_2 and serves intrinsically essential signaling functions in all eukaryotic cells. To monitor PtdIns4P distribution in *T. gondii*, three independent biosensors were designed that exploit distinct PtdIns4P-specific binding modules. These included: (i) the N-terminus of human GOLPH3, (ii) the PH domain of human FAPP1, and (iii) the P4M domain of the

Legionella pneumophilia SidM protein (Wood et al., 2009; Dowler et al., 2000, Del Campo et al., 2014). These reporters were chosen because of their abilities to detect distinct, but overlapping, pools of PtdIns4P in secretory pathway organelles of mammalian cells and yeast (Levine and Munro, 2002; Orii et al., 2021; Xie et al., 2018; Hammond et al., 2014). Moreover, although these domains are all specific for PtdIns4P binding, these differ in their affinities for PtdIns4P. For example, the GOLPH3 module exhibits a relatively modest PtdIns4P-binding affinity ($K_D = 2.6 \pm 0.2$ μM) (Wood et al., 2009), whereas the SidM P4M domain has the highest ($K_D = 3.8 \pm 2.7$ nM) (Del Campo et al., 2014). The human FAPP1PH domain exhibits an intermediate binding affinity that is sensitive to pH (K_D = 200 nM at pH 6.5 and 460 nM at pH 7.4; He et al., 2011). The transfection and imaging regime for these experiments was as described above for the PtdIns(4,5)P_2 and PtdIns(3,4)P_2 biosensors.

In describing the results, all transiently transfected RH-WT parasites are heretofore referred to by the convention of RH followed by the designation of the corresponding PtdIns4P-binding domain -- i.e., RH-GOLPH3, RH-FAPP1PH and RH-SidM-P4M. Unless otherwise stated, all cells analyzed had not yet initiated the process of cell division by endodyogeny and are broadly described as G1/S cells. Wide-field fluorescence imaging reported multiple PtdIns4P distribution profiles in G1/S cells as a function of the specific biosensor used to image PtdIns4P profiles (Figure 2.1B). For example, the RH-GOLPH3 biosensor highlighted a single juxta-nuclear stack and punctate structures that showed a predominantly apical distribution. These apical

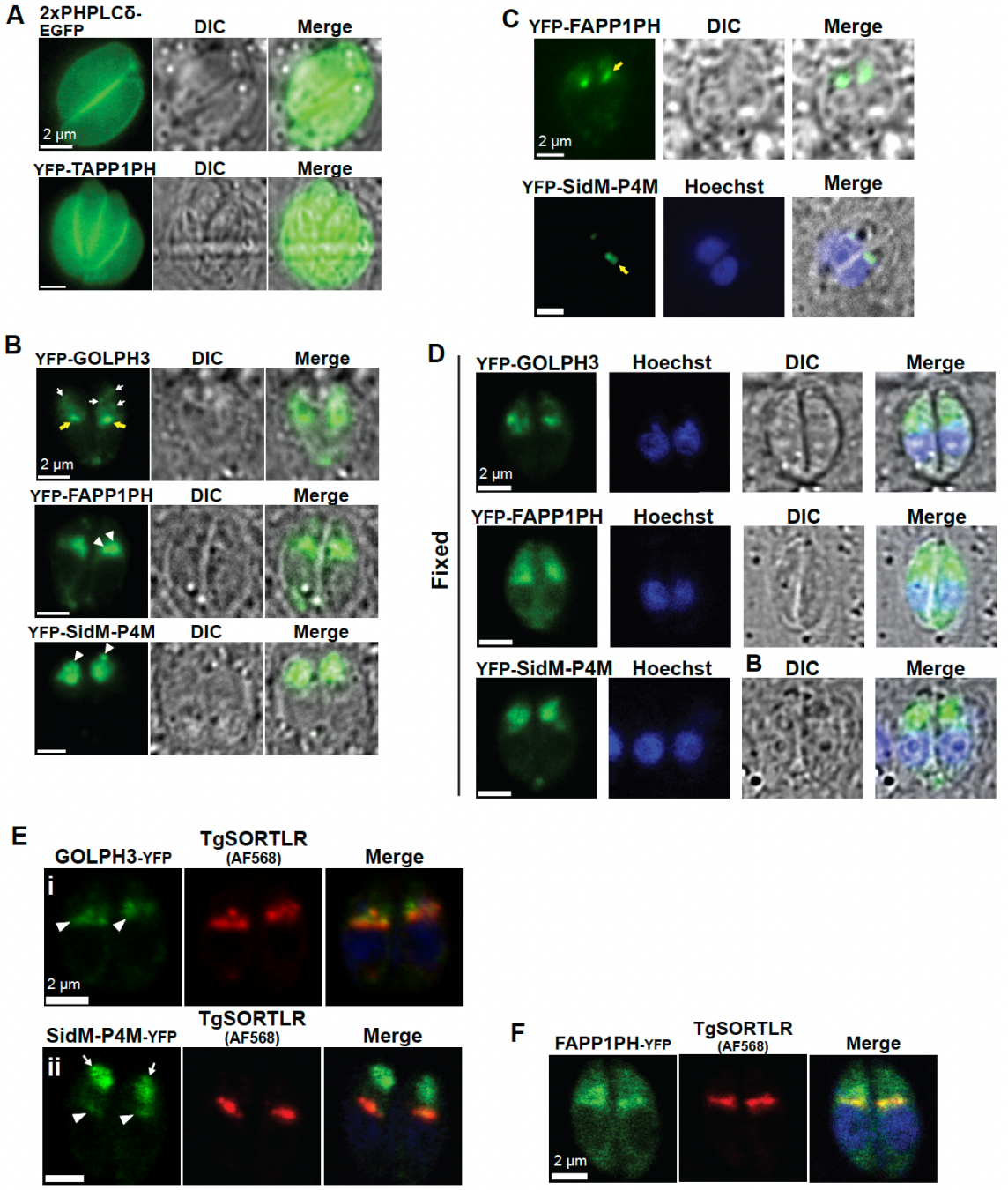

Figure 2.1 Imaging of PtdIns(4,5)P_2 and PtdIns(3,4)P_2 at the plasma membrane and PtdIns4P pools at Golgi/TGN and post-TGN compartments using specific biosensors. (A-B) Widefield fluorescence microscopy of live intracellular parasites expressing the indicated PIP biosensors are shown. (A) PtdIns(4,5)P_2 (2xPHPLCδ-EGFP) and PtdIns(3,4)P_2 (YFP-TAPP1PH) localize to the plasma membrane. (B) PtdIns4P pools in RH-GOLPH3 cells localize to a single stack-like structure (yellow

Figure 2.1 Continued. arrows) and scattered anterior small vesicles (white arrows); PtdIns4P pools in RH-FAPP1PH and RH-SidM-P4M strains localize to the LAP body (arrowheads). (C) RH-FAPP1PH and RH-SidM-P4M cells expressing low levels of the respective biosensors show PtdIns4P in a single stack-like structure (yellow arrows). (D) Fluorescence images of biosensor localization in RH-GOLPH3, RH-FAPP1PH and RH-SidM-P4M strains. In RH-GOLPH3 and RH-SidM-P4M parasites. The localization of the biosensor is conserved following fixation, while RH-FAPP1PH cells show a more diffused pattern when compared to the profiles seen in live cells. Parasite DNA is visualized using DAPI (blue). (E-F) Confocal images of immunofluorescence analysis (IFA) for the identification of Golgi/TGN (using anti-TgSORTLR immunoglobulin) in parasites during G1/S phase. (E) GOLPH3-YFP and SidM-P4M-YFP colocalize with TgSORTLR (arrowheads). The LAP body forms in a post-Golgi/TGN compartment in RH-SidM-P4M cells (arrows), but not in RH-GOLPH3 cells. (F) The LAP body forms in a post-Golgi/TGN compartment in RH-FAPP1PH cells.

structures were visualized as a single large and intensely stained puncta in both RH-FAPP1PH and RH-SidM-P4M parasites -- especially in RH-SidM-P4M parasites (Figure 2.1B, arrowheads). For the remainder of this work, we refer to these apical structures as LAP bodies (Large Anterior Punctate body). That the appearance of LAP bodies reflected the affinity of the PtdIns4P biosensor was indicated by the following observations. First, expression of the higher affinity FAPP1PH- and SidM-P4M-based biosensors resulted in appearance of LAP bodies, whereas expression of the lower affinity GOLPH3-based biosensor did not. Second, parasites expressing lower fluorescence intensities for the FAPP1PH and SidM-P4M reporters showed distributions that recapitulated those of GOLPH3 biosensor-expressing parasites (Figure 2.1C). We also noted that all three PtdIns4P biosensors marked small cytoplasmic puncta that were frequently in the vicinity of the residual body in tachyzoites that had completed the first round of cell division post-infection.

2.2.2. Golgi/TGN and post-TGN compartments host PtdIns4P pools.

The identities of PtdIns4P-containing organelles were determined in double-label immunofluorescence analyses using antibodies directed against established markers for specific parasite compartments. In these efforts, we first assessed whether the localization of PtdIns4P biosensors in parasites fixed with 4% paraformaldehyde (PFA) recapitulated the profiles observed in live cells. Indeed, the localization profiles of GOLPH3 and SidM-P4M domains in fixed parasites were consistent with those observed in live cells (Figure 2.1D). However, only the expected juxta-nuclear compartment localization was detected in fixed RH-FAPP1PH parasites. The LAP body observed in living parasites was absent and replaced by a diffuse cytosolic signal (Figure 2.1D). This fixation artifact had been previously noted in mammalian cells for the FAPP1PH domain (Schmiedeberg et al., 2009). Therefore, further use of the RH-FAPP1PH was limited to live parasite experiments whereas the RH-SidM-P4M reporter was deployed in immunofluorescence studies using fixed parasites.

As significant pools of PtdIns4P are present in Golgi/trans-Golgi network (TGN) membranes in mammalian and yeast cells (Clayton et al., 2013; Rivas et al., 1999; Hama et al., 1999), immunofluorescence approaches were used to assess RH-GOLPH3 and RH-SidM-P4M co-localization with the *T. gondii* TGN marker TgSORTLR (Sortilin-like Receptor; Sloves et al., 2012). Both PtdIns4P biosensors colocalized with varying degrees with TgSORTLR. The GOLPH3-derived PtdIns4P sensor localized to the Golgi/TGN membranes (Pearson's correlation coefficient ± SEM = 0.62 ± 0.03; Figure 2.1Ei, arrowheads). Recruitment of SidM-P4M to the Golgi/TGN compartment was less

efficient but nonetheless detectable (Pearson's correlation coefficient ± SEM = 0.14 ± 0.04; Figure 2.1Eii, arrowheads). This relatively low incidence of colocalization was likely due to SidM-P4M being strongly recruited to a distinct PtdIns4P pool that persists in a post-TGN compartment intracellular compartment – i.e. the LAP body (Figure 2.1Eii, arrows).

Although colocalization of FAPP1PH with Golgi/TGN membranes was not quantified due to the fixation artifacts described above, we noted this biosensor highlighted both Golgi/TGN membranes and the LAP body in a manner similar to that observed for the SidM-P4M biosensor (Figure 2.1F). Taken together, these data confirm that the stacked juxta-nuclear compartment to which the PtdIns4P biosensors localized represented the parasite Golgi/TGN system. The data further identified a distinct PtdIns4P pool that resided in a second intracellular compartment (the LAP body) characterized by its large punctate profile and its localization to the apical region of the cell.

2.2.3. The LAP body is a post-Golgi compartment that harbors GRA3 secretory cargo.

What is the nature of the LAP body that houses the second PtdIns4P pool identified by the SidM-P4M biosensor? The most attractive possibility was that this structure represented a secretory organelle. To examine this possibility in further detail, we assessed co-localization of RH-SidM-P4M with the microneme marker MIC3 and with the genetically-encoded rhoptry marker ROP1-RFP (Figure 2.2A). As illustrated by

the images in Figure 2.2B, the SidM-P4M biosensor failed to colocalize with either the microneme or the rhoptry marker. Moreover, the morphologies of these secretory organelles were not obviously disturbed in SidM-P4M-expressing parasites when compared to RH-WT stained for MIC3 and to RH-ROP1-RFP parasites, respectively.

By contrast, whereas immunostaining for the DG marker GRA3 in RH-WT parasites showed small DG puncta that were dispersed throughout the cytoplasm (arrows in Figure 2.2C), RH-SidM-P4M strains exhibited two distinct DG profiles that associated with the level of biosensor expression. RH-SidM-P4M parasites with low fluorescence signal, i.e. cells inferred to exhibit low PtdIns4P biosensor expression, displayed DGs with morphologies and intracellular distributions that were similar to those of WT parasites. Moreover, only an infrequent colocalization of GRA3 with the PtdIns4P biosensor was detected (Pearson's correlation coefficient ± SEM = 0.337 ± 0.013; Figure 2.2D upper panel). The predominant population of RH-SidM-P4M parasites exhibited stronger fluorescence signals, however, and those cells were inferred to exhibit robust expression of SidM-P4M-YFP. In these parasites, strong co-localization of SidM-P4M-YFP with GRA3 was observed at the LAP body (Pearson's correlation coefficient ± SEM = 0.893 ± 0.015; Figure 2.2D lower panel). These results were recapitulated in RH-FAPPP1H parasites (Figure 2.2E). Interestingly, the intracellular distributions and the morphologies of GRA3-positive DGs in RH-GOLPH3 parasites were similar to those of RH-WT (Figure 2.2F). However, RH-GOLPH3 parasites that exhibited GRA3 co-localization with LAP bodies with profiles similar to those of RH-

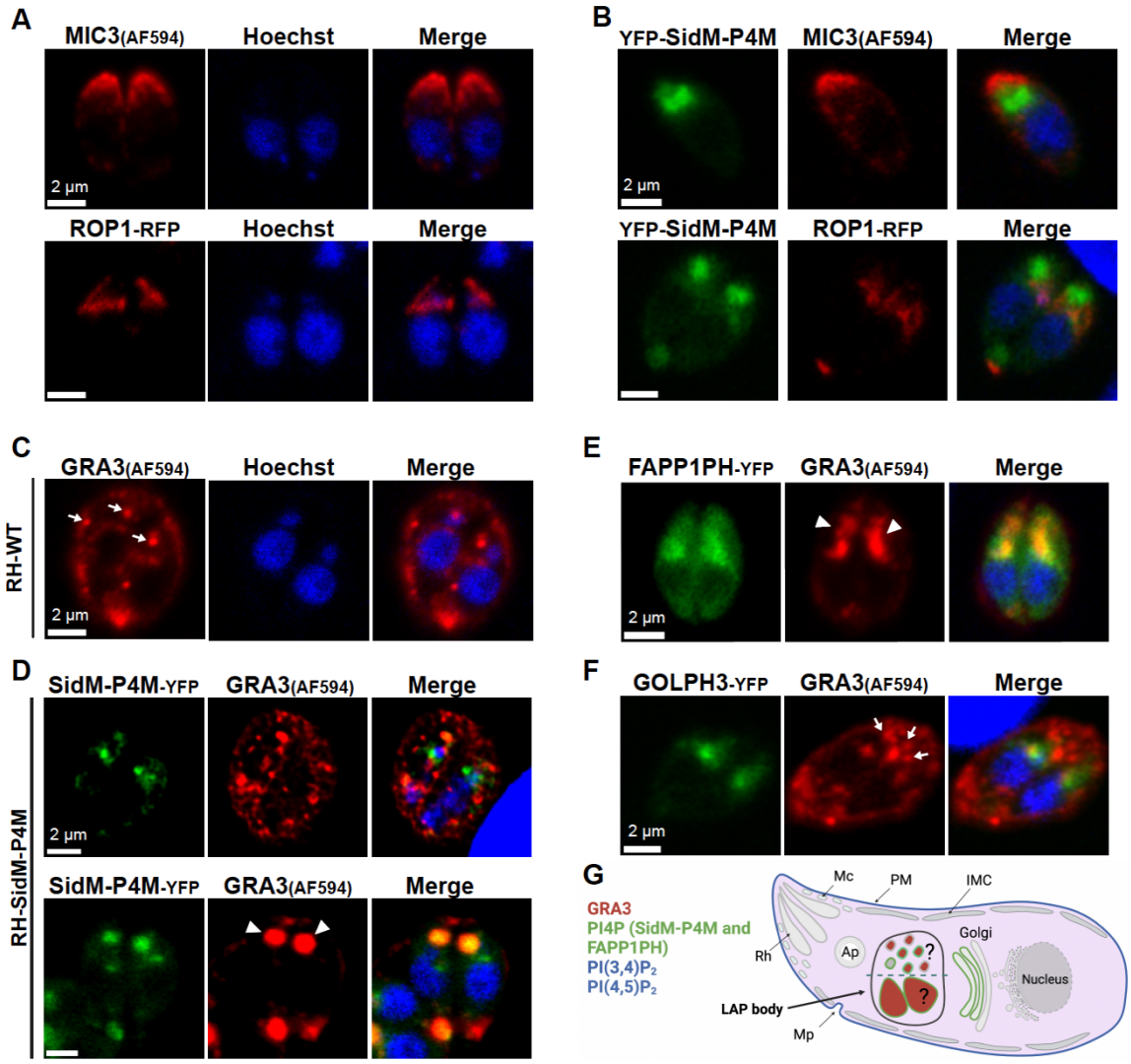

Figure 2.2 Specific derangement of DG cargo-carrying compartments in parasites expressing high affinity-PtdIns4P biosensors. Confocal microscopy images of fixed RH-WT and transgenic strains are shown. Parasite DNA is visualized using Hoechst (blue). (A) The microneme marker (IFA with anti-MIC3) and the rhoptry marker (ROP1-RFP) are normally distributed in RH-WT and RH-ROP1 strains, respectively. (B) Distribution of microneme (MIC3) and rhoptry (ROP1-RFP) markers are not affected by expression of YFP-SidM-P4M. (C-F) IFA of DG using GRA3 as marker (identified using anti-GRA3 imunoglobulin) during G1/S phase. (C) RH-WT contain scattered DGs of consistent dimensions in the cytoplasm (arrows). (D) Cells with a lower fluorescence signal of YFP-SidM-P4M do not form a LAP body and show WT-like GRA3 distributions (upper panel). The LAP body in RH-SidM-P4M parasites is stained with anti-GRA3 antibodies (arrowheads in lower panel). (E) RH-FAPP1PH parasites show a LAP body stained with anti-GRA3 antibodies (arrow heads). (F) DG

Figure 2.2 Continued. marker distribution is typically not affected in RH-GOLPH3 strains (arrows). (G) Summary schematic representation of PIP pools described in this manuscript. PtdIns(4,5)P_2 and PtdIns(3,4)P_2 localize to the parasite plasma membrane. SidM-P4M and FAPP1PH biosensors localize PtdIns4P pools to the Golgi/TGN and the LAP body. The LAP body is only formed upon expression of these PtdIns4P biosensors and is accompanied by GRA3 localization to this structure.

SidM-P4M cells were occasionally observed. YFP-GOLPH3 expression was particularly robust in those less frequent cases. These collective results identified the LAP body as a post-Golgi compartment that houses DG cargo (Figure 2.2G), and indicated a role for PtdIns4P biosensor expression in the induction of LAP body formation.

2.2.4. Expression of high-affinity PtdIns4P-binding domains induces altered intracellular distribution of multiple DG cargo.

As the GOLPH3 biosensor harbors a domain with a lower PtdIns4P binding affinity than does the SidM-P4M biosensor (Wood et al., 2009; Del Campo et al., 2014), the data raised the possibility that it was sequestration of PtdIns4P from its effectors by high-affinity PtdIns4P-binding domains that induced morphological derangement of compartments involved in DG biogenesis into LAP bodies. To further evaluate and quantify LAP body formation upon sequestration of intracellular PtdIns4P pools, two genetically-encoded reporters for DG cargo were generated -- GRA3-RFP and GRA2-RFP. These constructs allowed vital monitoring of pools of DG cargo synthesized after parasite transfection and subsequent infection of host cells. Reporter constructs for each DG cargo were transiently co-transfected into RH-WT cells with an appropriate PtdIns4P biosensor construct. Live parasites were subsequently imaged. Fifty

parasitophorous vacuoles (PVs) were analyzed in three independent biological replicates for each experimental condition, and DG intracellular distribution was scored as: no visible vesicles in the cytoplasm (DG-less), dispersed vesicles of typical morphology (Normal), or LAP body containing (LAP body) (Figure 2.3).

The control condition represented by RH-GRA3 or RH-GRA2 strains presented both normal and absent DG distribution phenotypes but no LAP body formation (Figure 2.3A). In cells expressing the high affinity FAPP1PH and SidM-P4M PtdIns4P-binding domains, the DG cargo reporters redistributed into LAP bodies (Figure 2.3B, large). Quantification of those images reported a significant increase in this redistribution phenotype for both the GRA3 (mean % of PVs with LAP bodies ± SEM = 36.4% ± 3.2 for FAPP1PH and 58.5% ± 14.8 for SidM-P4M; Figure 2.3C) and the GRA2 reporters (mean % of PVs with LAP bodies ± SEM = 48% ± 5.2 for FAPP1PH and 64% ± 3 for SidM-P4M; Figure 2.3D). Thus, LAP body formation reflected altered intracellular distribution of at least two DG cargos.

That the DG cargo redistribution phenotype resulted from diminished PtdIns4P signaling was demonstrated by the fact that expression of the mutant SidM-P4M^{K568A} and FAPP1PH$^{K7A/R18L}$ biosensors induced no such effect (Figure 2.3C-E). These respective mutant domains are defective for PtdIns4P-binding (Del Campo et al., 2014; Jung et al., 2002) and, consistent with those defects, both mutant biosensors exhibited primarily cytoplasmic profiles (Figure 2.3E). These results demonstrate that localization

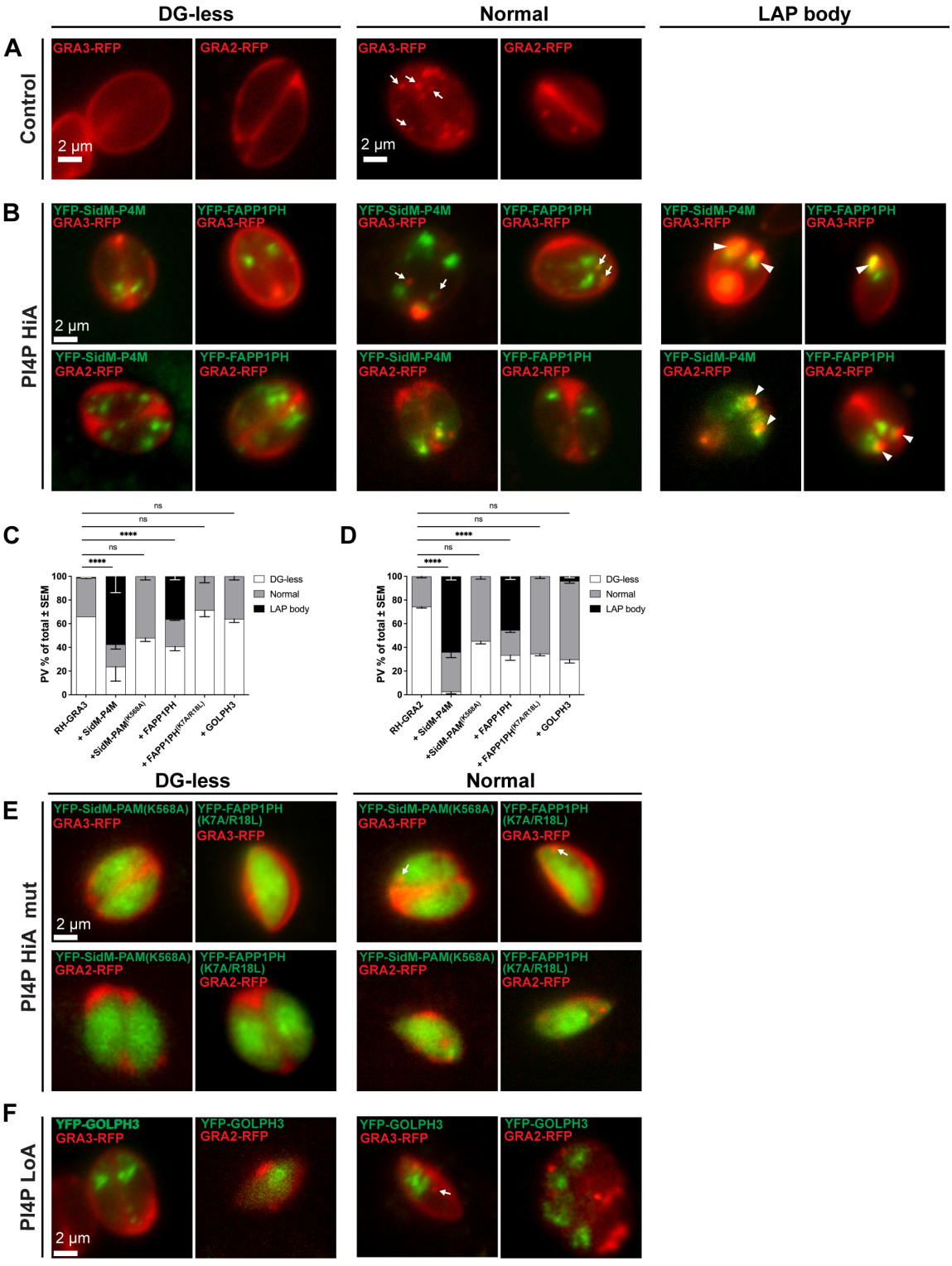

Figure 2.3 LAP body formation is induced by high affinity-PtdIns4P biosensor expression. (A-C) Widefield fluorescence microscopy of live intracellular parasites

Figure 2.3 Continued. expressing the indicated PIP biosensors, classified by the DG reporter distribution phenotype: DG-less, normal (arrows) or LAP body (arrow heads). (A) Control reporter strains RH-GRA3 and RH-GRA2 exhibit either DG-less or normal cytoplasmic puncta profiles. (B) Parasites transiently co-expressing GRA3-RFP or GRA2-RFP in the face of high-affinity SidM-P4M- or FAPP1PH-based PtdIns4P biosensor co-expression (PI4P HiA) form a LAP body (arrow heads). (C-D) Quantification of PVs exhibiting the (C) GRA3-RFP or (D) GRA2-RFP phenotypes (DG-less, normal, LAP body) in the face of co-expression with the indicated biosensor (n=50 PVs). Data show mean ± SEM of three independent experiments. Statistical analyses compared the control reporter strain (RH-GRA3-RFP or RH-GRA2-RFP) and parasites co-expressing the DG reporter and the appropriate PIP biosensor with regard to LAP body phenotype. Statistical significance was calculated using two-way ANOVA followed by Dunnet's multiple comparison test; $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****). (E) Expression of the SidM-P4M^{K568A} and FAPP1PH$^{K7A/R18L}$ biosensor PtdIns4P-binding mutants (PI4P HiA mut) does not affect GRA3-RFP or GRA2-RFP distribution. (F) Parasites co-expressing the GRA3-RFP or GRA2-RFP reporter with the lower affinity (LoA) PtdIns4P YFP-GOLPH3 biosensor do not form LAP bodies.

of the FAPP1PH- and SidM-P4M-based biosensors to the parasite Golgi/TGN system, and the morphological derangement of DG cargo-positive compartments in the form of LAP bodies, was dependent on the PtdIns4P-binding properties of these biosensors. By contrast, and consistent with DG cargo distribution representing a PtdIns4P-dependent process, expression of the lower affinity YFP-GOLPH3 biosensor failed to significantly alter the intracellular distribution of DG cargo relative to wild-type controls (Figure 2.3F).

2.2.5. Altered DG morphologies report a specific PtdIns4P requirement.

PtdIns4P is the metabolic precursor of the bis-phosphorylated phosphoinositides PtdIns(4,5)P_2 and PtdIns(3,4)P_2. To determine whether redistribution of DG cargo into LAP bodies reflected an intrinsic defect in PtdIns4P signaling, or some indirect

downstream effect of PtdIns(4,5)P_2 and/or PtdIns(3,4)P_2 limitation under conditions of compromised PtdIns4P availability, the effects of high-affinity PtdIns4P-binding domain expression on parasite PtdIns(4,5)P_2 and PtdIns(3,4)P_2 pools were assessed. To that end, *T. gondii* cells transiently co-expressing FAPPPH1-RFP along with either the 2xPHPLCδ-EGFP or YFP-TAPP1PH biosensors were analyzed. Decreased production of these PIP_2 species as a consequence of sequestration of intracellular PtdIns4P by FAPPPH1 was expected to induce release of the corresponding PIP_2 biosensors from the plasma membrane. This prediction was not borne out by the data, however. FAPPPH1-RFP expression failed to compromise association of either PIP_2 biosensor with the plasma membrane (Figure 2.4A).

As independent approach, the intracellular distributions of both the GRA2-RFP and GRA3-RFP DG cargo reporters were visualized in parasites expressing either of the two PIP_2 biosensors. In neither case was distribution of the DG cargo reporters altered by PIP_2 biosensor expression relative to unperturbed wild-type controls (Figure 2.4B-D). Taken together, these data report an intrinsic signaling role for PtdIns4P in the biogenesis and/or function of intracellular compartments that participate in DG cargo trafficking – an involvement distinct from the role of PtdIns4P as metabolic precursor for PtdIns(4,5)P_2 or PtdIns(3,4)P_2 signaling.

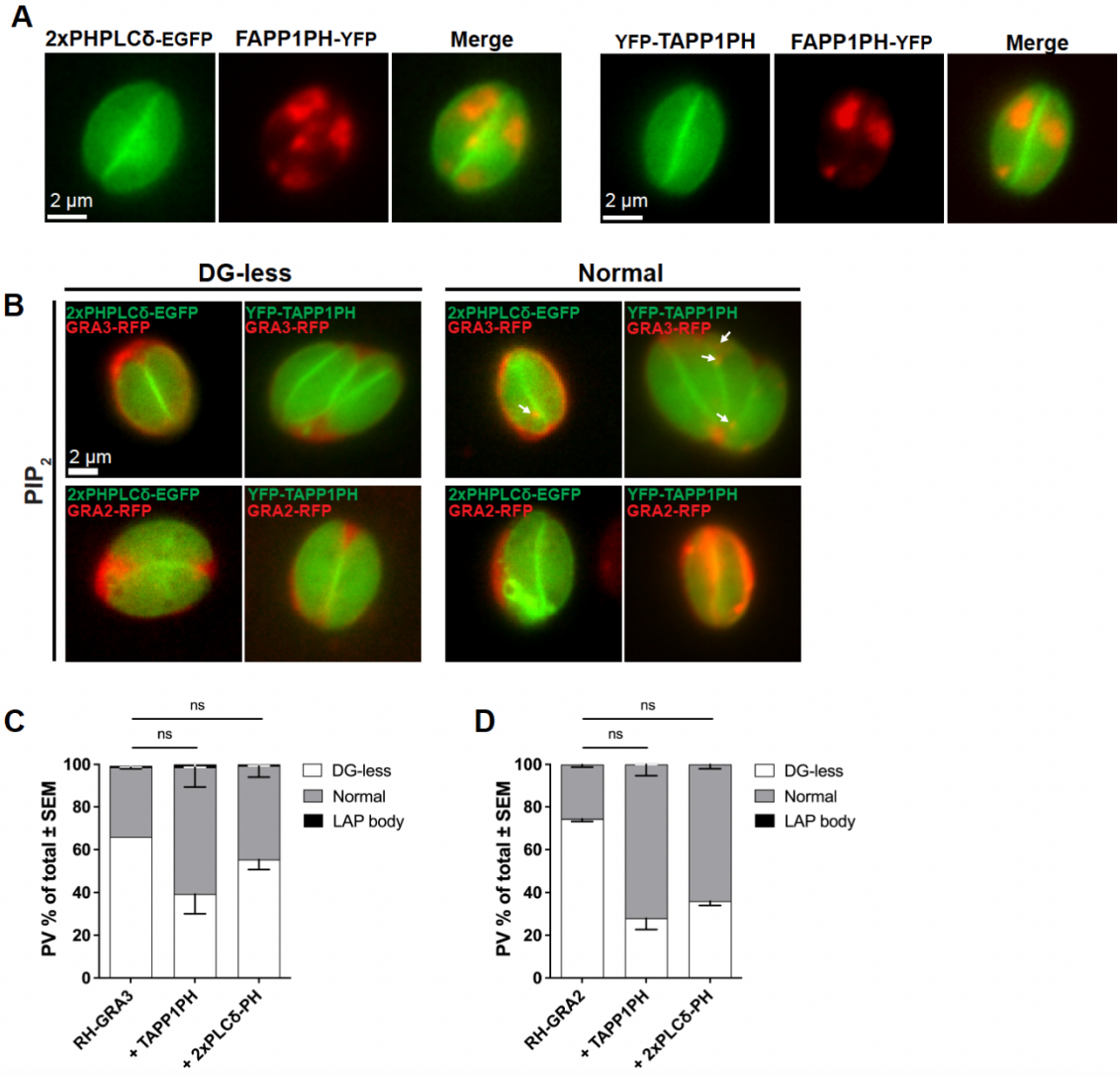

Figure 2.4 Expression of PI(4,5)P_2 and PI(3,4)P_2 biosensors does not induce LAP body formation. (A) Widefield fluorescence microscopy of live intracellular parasites co-expressing the indicated PIP biosensors: FAPP1PH-RFP and 2xPHPLCδ-EGFP or FAPP1PH-RFP and YFP-TAPP1PH. The distribution of neither PIP_2 biosensors was affected by PtdIns4P pool sequestration at the Golgi-TGN and post-TGN compartments. (B) Widefield fluorescence microscopy of live intracellular parasites expressing the indicated PIP biosensors, classified by the DG reporter distribution phenotype: DG-less, normal (arrows) or LAP body (arrow heads). Expression of 2xPHPLCδ-EGFP or YFP-TAPP1PH in reporter strains RH-GRA3-RFP or RH-GRA2-RFP did not result in LAP body formation. (C-D) Quantification of PV phenotypes as reported by (C) GRA3-RFP or (D) GRA2-RFP cargo (DG-less, normal, LAP body) as a function of co-expression with the indicated biosensor (n=50 PVs). Data are represented as mean ± SEM of three independent biological replicates. Statistical analysis compared the control reporter

Figure 2.4 Continued. strain (RH-GRA3-RFP or RH-GRA2-RFP) with parasites co-expressing the DG reporter and the appropriate PIP biosensor with regard to LAP body phenotype. Two-way ANOVA followed by Dunnet's multiple comparison test was used to determine statistical significance; $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

2.2.6. Ultrastructure of dense granule compartments under conditions of PtdIns4P stress.

To gain a more precise description of LAP body structure two independent approaches were employed. First, LAP bodies were imaged using confocal microscopy coupled with an Airyscan detector that increased spatial resolution some 1.7X and an increase in signal to noise ration of up to 8X after linear deconvolution analysis (Huff et al., 2015). This enhanced resolution is accurate down to 140 nm in the xy-plane – thereby allowing quantification of alterations in DG morphologies. In G1/S RH-WT parasites, GRA3-positive DGs were distributed throughout the cell (Figure 2.5A), and z-projections of confocal images indicate these DGs exhibited a mean diameter of 273 ± 7.5 nm. Interestingly, the LAP bodies of RH-YFP-SidM-P4M-expressing parasites were not simple homogeneous structures. Rather, the LAP bodies consisted of a 'clustered' network of smaller DGs (Figure 2.5B). These smaller DGs (sDG) exhibited a mean diameter of 140 ± 1.6 nm that was approximately half of that exhibited by the DGs of wild-type parasites (Figure 2.5C). Overlay of the GRA3 and RH-YFP-SidM-P4M profiles confirmed colocalization of these two reporters (Pearson's correlation coefficient = 0.77 ± 0.04; black arrows in Figure 2.5B). A striking feature of the

reconstructed images was that the PtdIns4P biosensor was not isotropically distributed throughout the LAP body. Rather, the biosensor, and by inference PtdIns4P, was

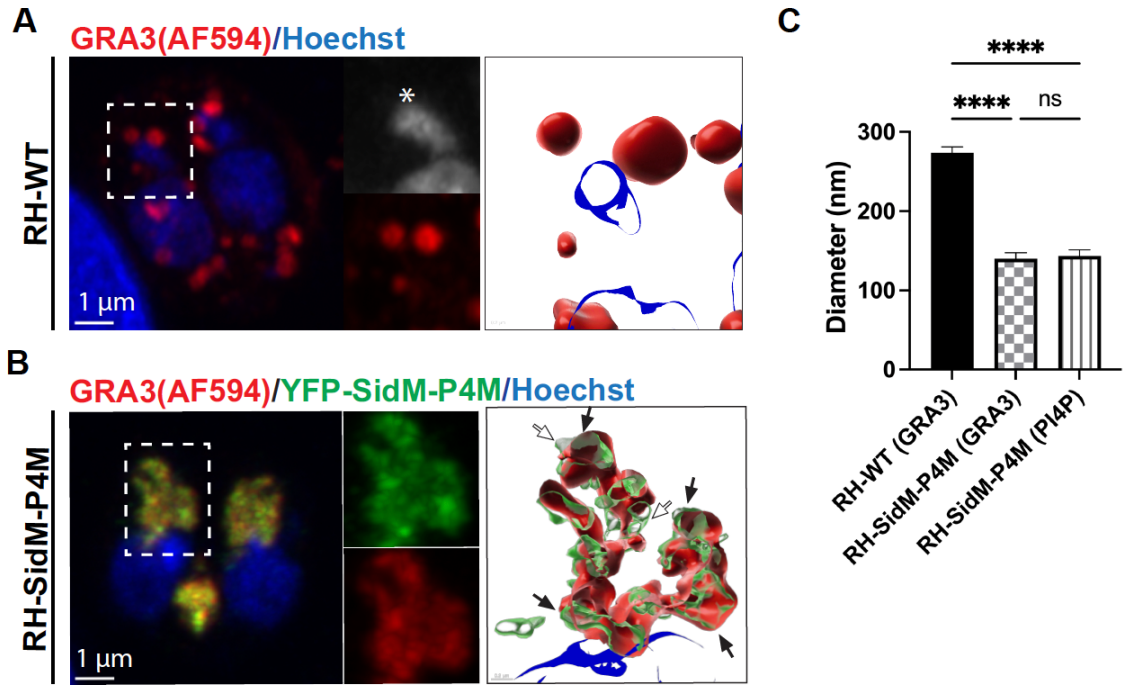

Figure 2.5 The LAP body is comprised of a network of small GRA3- and PtdIns4P-containing vesicle. (A-B) Z-projections of Airyscan acquisitions of RH-WT and RH-SidM-P4M parasites stained with anti-GRA3 immunoglobulin. Parasite DNA is visualized using Hoechst (blue). (A) Control RH-WT parasites showing DG cargo (GRA3) distributed in discrete puncta throughout the cytoplasm and PVM. G1/S phase was identified by a single undivided apicoplast in the cells of interest (asterisk). 3D-Reconstructions show the absence of a LAP body in the subapical area (dashed square). (B) RH-SidM-P4M parasites during G1/S phase present a LAP body that consists of numerous sDG enriched with GRA3 and PtdIns4P (dashed square). 3D-Reconstruction of the LAP body confirms spatial co-localization of GRA3 and PtdIns4P reporters (black arrows). The PtdIns4P biosensor is concentrated in distinct domains (white arrows). (C) Quantification of the diameter (nm) of sDGs in RH-WT parasites (control) and sDGs of RH-SidM-P4M parasites (n = 11 for both cases). Mean values ± SEM are shown. Statistical analyses used one-way ANOVA followed by Tukey's multiple comparison test to assess significance; $p > 0.05$ (ns); $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

concentrated in discrete domains throughout the sDG network (white arrows in Figure 2.5B). PtdIns4P-positive structures devoid of GRA3 were also noted -- suggesting different classes of cargo-carrying vesicles are present within the LAP body.

As an independent means of probing LAP body structure with higher precision, we employed correlative light electron microscopy (CLEM). Electron microscopy analysis required confident and unbiased identification of YFP-SidM-P4M-expressing parasites in the electron microscopy grid -- a requirement made difficult by: (i) the low efficiency with which the biosensor construct is transfected into parasites, and (ii) our inability to create stable expression cell lines for the high affinity PtdIns4P biosensors. As such, CLEM was employed to specifically target YFP-SidM-P4M-expressing parasites for further EM imaging. To that end, human foreskin fibroblasts were seeded onto gridded glass-bottom coverslip dishes, infected with RH-SidM-P4M parasites, and fixed parasites were stained with antibodies directed against GRA3. The positions of either RH-WT or RH-SidM-P4M parasites were registered in the coordinate system of the gridded coverslip by confocal microscopy. RH-SidM-P4M parasites in which LAP bodies were evident by confocal microscopy and identified as regions of interest (ROI) and specifically selected for CLEM imaging. The grids were then processed for transmission EM (TEM) and the ROIs visualized by transmission electron microscopy. In excellent agreement with the results of the Airyscan imaging experiments, the TEM acquisitions reported well dispersed mature DGs (Figure 2.6A). Again, consistent with the Airyscan imaging data, RH-SidM-P4M-expressing parasites presented the LAP body as a single large cluster of electron dense vesicles in the apical region of the cell

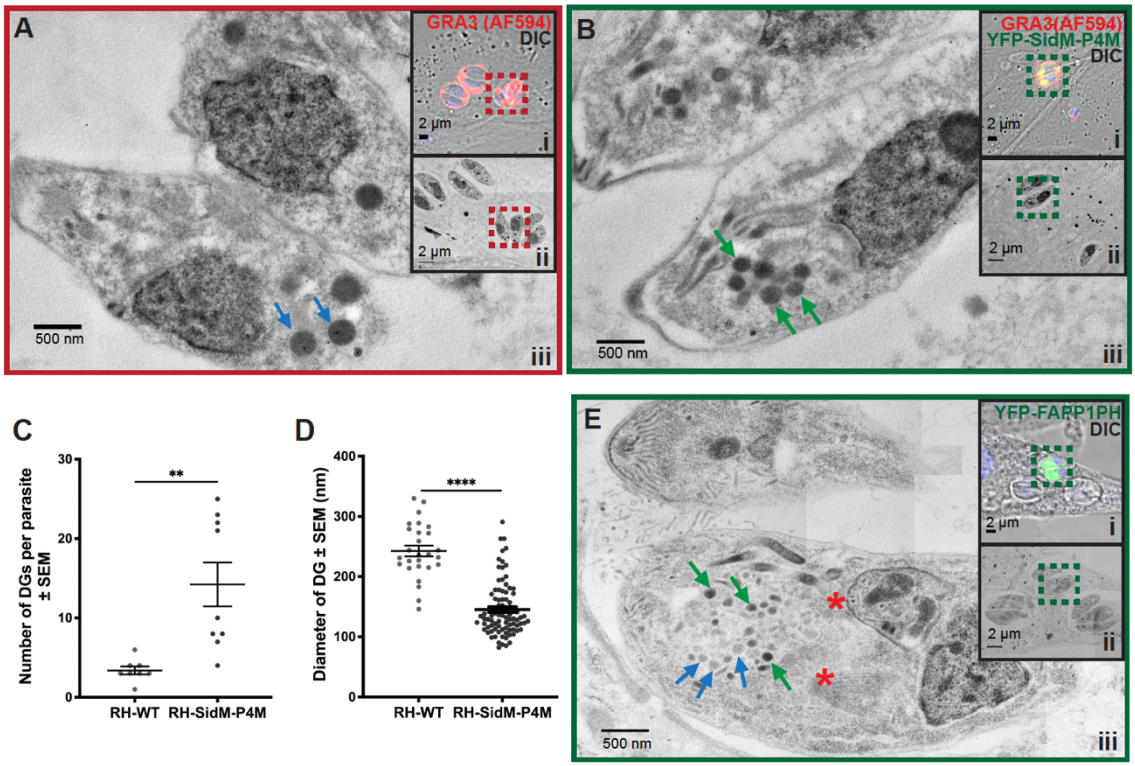

Figure 2.6 High resolution imaging of LAP body structure by correlative light electron microscopy. (A, B and E). CLEM acquisitions of stained RH-WT or transgenic parasites involved the following steps: (i) targeting and recognition of ROIs (dashed square) was performed in a confocal microscope and selected for imaging by TEM at (ii) low and (iii) high magnification. (A) RH-WT show scattered distribution of mature highly electro-dense DGs distributed throughout the cytoplasm (red arrows). (B) RH-SidM-P4M show a pool of DG-cargo carrying vesicles in the subapical area that are of smaller size (sDGs) relative to WT DGs (green arrows). (C) Quantification of DG number per tachyzoite and per EM section (n = 8 for WT and SidM-P4M). Data represent the mean ± SEM. (D) Quantification of the average DG diameter (nm) per tachyzoite and per EM section (n =8 for WT and SidM-P4M and n=3 for FAPP1PH). Data are represented as mean ± SEM, and statistical significance was determined using the Mann-Whitney t-test; $p > 0.05$ (ns); $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****). (E) An RH-FAPP1PH parasite undergoing endodyogeny (red asterisks indicate position of daughter cells) presents a LAP body consisting of an ‘clustered’ sDG network (green arrows) that resides in the subapical region of the mother cell. sDGs with reduced electron density are indicated by blue arrows.

(green arrows in Figure 2.6B). The electron dense vesicles in RH-SidM-P4M were approximately 5X more numerous than the DGs of wild-type parasites (mean sDG count per EM section ± SEM = 3 ± 0.5 for RH-WT and 14 ± 3.7 for SidM-P4M; Figure 2.6C), and were also about half the dimension of those observed in RH-WT parasites (mean DG diameter ± SEM = 242 ± 8.7 nm for RH-WT and 145 ± 4.7 nm for SidM-P4M; Figure 2.6D). Morphometric values for DGs obtained in the wild-type strain are in excellent agreement with previous measurements (Dubey et al., 1998). CLEM analyses of RH-FAPP1PH parasites yielded comparable results and, in this case, the reduced electron densities of the 'clustered' sDGs that comprise the LAP body were particularly noteworthy (Figure 2.6E). Thus, data from two independent high resolution imaging approaches converge on the conclusion that the LAP body formed upon parasite expression of high affinity PtdIns4P binding domains represented a 'clustered' network of small vesicles that carry DG cargo.

2.2.7. PtdIns4P stress compromises DG cargo exocytosis.

Deranged organelle morphology is often associated with compromised function. Thus, we tested whether expression of high-affinity PtdIns4P-binding domains compromised exocytosis of DG cargo. To quantify exocytosis, RH-WT and RH-SidM-P4M parasites were immunostained for GRA3 and the intensities of exocytosed GRA3 signal in the PV membrane (PVM) were ratioed to total GRA3 intensity in the PV as a measure of DG exocytosis efficiency. For RH-SidM-P4M cells, only parasites with

GRA3-containing LAP bodies were analyzed. Acquisition parameters were normalized across all samples to minimize technical bias.

The data show a ca. 58% reduction in normalized GRA3 signal at the PVM of SidM-P4M-dependent LAP body-containing parasites in comparison with RH-WT (Figure 2.7A and B). That the observed DG exocytic defects were the consequences of reduced PtdIns4P signaling was indicated by the fact that the PVM GRA3/total GRA3

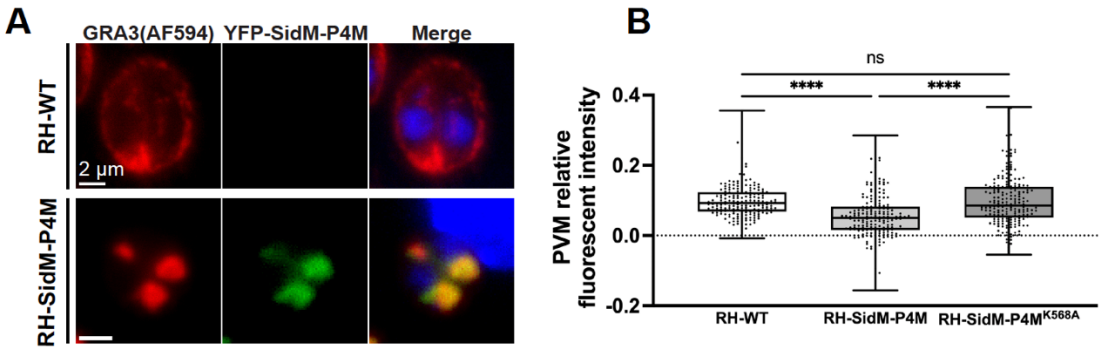

Figure 2.7 Sequestration of PtdIns4P inhibits secretion of GRA3 to the PVM. (A) Widefield fluorescence microscopy of fixed intracellular parasites immunostained with anti-GRA3 immunoglobulin in the absence (upper panel) or presence (lower panel) of SidM-P4M-YFP expression. In RH-WT cells, GRA3 localizes to the PV and PVM. In RH-SidM-P4M cells, GRA3 is mainly retained in the LAP body in the intracellular apical region and is absent from the PVM. Parasite DNA is visualized using Hoechst (blue). (B) Quantification of fluorescence intensity of GRA3 in the PVMs of parasites exhibiting SidM-P4M-dependent LAP body formation is significantly reduced compared to that of PVMs of RH-WT and SidM-P4M^{K568A}– expressing strains. The box and whisker plot shows the average of indicated individual measurements ± SEM per experimental condition. Individual measurements were collected from two independent biological replicates (n=196). Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparison test; $p > 0.05$ (ns); $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

ratios measured for parasites expressing the PtdIns4P-binding mutant of SidM-P4M (RH-SidM-P4M^{K568A} parasites) were not significantly diminished compared to those of RH-WT (Figure 2.7B). Taken together, these collective data demonstrate that PtdIns4P stress in *T. gondii* results in: (i) altered DG biogenesis, and (ii) compromise of the normally efficient exocytosis of DG contents.

2.2.8. PtdIns4P biosensor distribution through the parasite cell cycle.

PtdIns4P distribution profiles were also followed throughout the parasite cell division cycle. For those experiments, an inner membrane complex (IMC) reporter was constructed where the IMC marker IMC1 was fused to RFP (IMC1-RFP) to landmark cell cycle stage in proliferating intracellular parasites – as has been done previously (Ouologuem and Ross, 2014). Live parasites expressing IMC1-RFP and YFP-SidM-P4M were imaged by widefield fluorescence microscopy during various stages of the cell cycle. Representative images collected for each cell cycle stage are shown in Figure 2.8A. In G1/S parasites, YFP-SidM-P4M localized primarily to the LAP body in agreement with the various data documented above. At initiation of cell division, a prominent recruitment of YFP-SidM-P4M to a split juxta-nuclear stacked compartment was observed (white arrows in Figure 2.8Ai) and, to a lesser extent, to apical vesicles (yellow arrows in Figure 2.8Ai). Quantification of parasites with YFP-SidM-P4M distribution to the LAP body demonstrated that, contrary to what was observed in G1/S parasites or for cells in the next division stage (elongation 1), only ~8.5% of cells at the initiation phase exhibited positive signal at the LAP body. The remaining cells exhibited

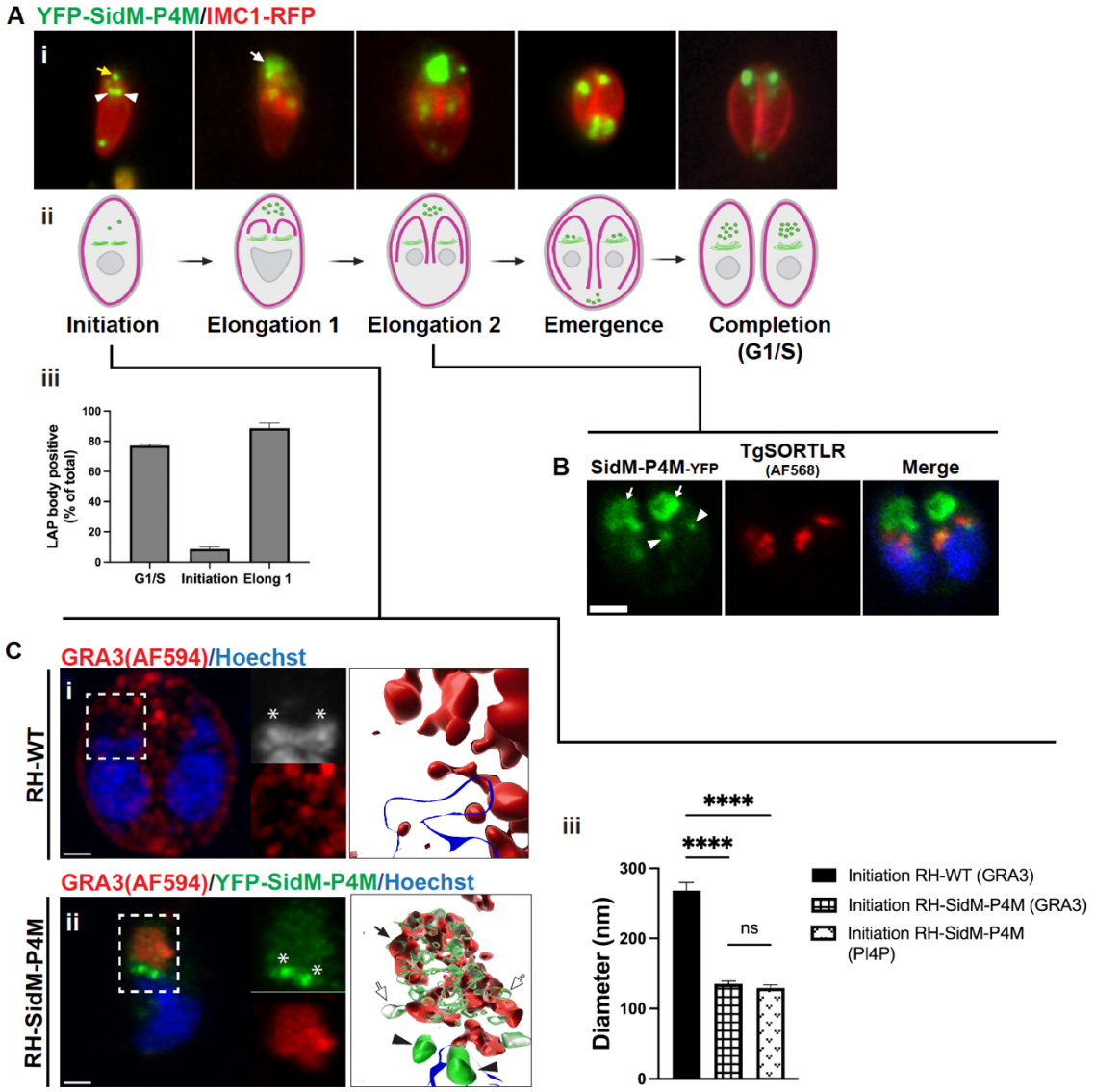

Figure 2.8 PtdIns4P biosensor relocates away from the LAP body during the initial stages of parasite cell division. (Ai) Widefield fluorescence microscopy images of live cells co-expressing YFP-SidM-P4M and cell cycle marker IMC1-RFP at each step of the cell cycle are shown. A dividing Golgi system marks initiation of endodyogeny (arrowheads). YFP-SidM-P4M targets to the LAP body during most stages of the cell cycle (white arrows). During the initiation stage, however, YFP-SidM-P4M redistributes to the dividing Golgi system (arrowheads) and to apical vesicles (yellow arrow). (Aii) Schematic representation of the relative positions of the IMC (magenta) and of PtdIns4P pools (green) at the Golgi/TGN and post-TGN compartment at each cell cycle stage (initiation, elongation (1 and 2), emergence and completion/G1). (Aiii) Quantification of parasites showing recruitment of YFP-SidM-P4M to the LAP body at the indicated stages of endodyogeny. Quantification data were obtained from two independent

Figure 2.8 Continued. experiments (n ≤ 15 parasites per stage). Data show mean ± SEM. (B) Confocal IFA images identifying Golgi/TGN (TgSORTLR) in a dividing RH-SidM-P4M parasite during the elongation stage of cell division. YFP-SidM-P4M targets to the dividing Golgi/TGN compartment (arrowheads) and the LAP body (arrows). DNA is visualized using DAPI (blue). (C) Z-projection of Airyscan acquisitions of parasites immunostained with anti-GRA3 immunoglobulin during initiation of endodyogeny. This stage is identified by the division of apicoplast in RH-WT (DNA visualized with Hoechst; asterisks) and fission of the Golgi/TGN system in RH-YFP-SidM-P4M cells (asterisks). (Ci) Control RH-WT parasites show GRA3 distributed in discrete puncta throughout the cytoplasm and PVM, while the RH-YFP-SidM-P4M strain displays a GRA3 positive-LAP body with low YFP-SidM-P4M signal (Cii). Rather, YFP-SidM-P4M targets to the recently divided Golgi/TGN. 3D reconstruction demonstrates two regions within the LAP body: GRA3+/PtdIns4P+ (black arrows), and GRA3-/PtdIns4P+ (white arrows). Parasite DNA is visualized using Hoechst (blue). (Ciii) Quantification of the sDG diameter (nm) in dividing RH-WT (control) vs RH-YFP-SidM-P4M parasites (n = 11 for both cases). Data are presented as mean ± SEM, and statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test; $p > 0.05$ (ns); $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

profiles that reported redistribution of YFP-SidM-P4M to the split juxta-nuclear stacked compartment (Figure 2.8Aii and iii). During cell elongation stages, that stacked compartment completed fission and each daughter cell inherited a copy. Thus, the split juxta-nuclear compartment exhibited the features expected of a dividing Golgi system (Pelletier et al., 2002). That this stacked compartment indeed represented the Golgi/TGN was confirmed by the fact that it was marked with TgSORTLR (Figure 2.8B).

By contrast to the Golgi system, the LAP body was neither divided nor inherited. At elongation stages of the cell cycle, YFP-SidM-P4M partially re-localized back to the LAP body of the mother cell and, upon daughter cell emergence, the LAP body migrated towards the basal pole of mother cell body remnants -- potentially to the residual body. At completion of the cell cycle (marked by entrance of daughter cells into G1), YFP-SidM-P4M redistributed primarily to a LAP body profile. Thus, the biosensor data report

a mobilization of the PtdIns4P biosensor from the LAP body to a newly dividing Golgi system upon initiation of endodyogeny, and relocalization of the biosensor back to the LAP body in subsequent stages of the cell cycle.

2.2.9. PtdIns4P biosensor redistributes away from the LAP body in dividing parasites.

The apparent 'disappearance' of the PtdIns4P-positive LAP body in parasites initiating cell division, suggested two possibilities: (i) the LAP body is disassembled during initiation of endodyogeny and subsequently reforms in later stages of the cell cycle, or (ii) the biosensor relocates away from an otherwise intact LAP body. To distinguish between these possibilities, LAP body structure was examined in dividing parasites by high resolution Airyscan confocal imaging with the modification that GRA3 replaced YFP-SidM-P4M as LAP body marker. In RH-WT parasites, GRA3-positive DGs were distributed throughout the cell during the initiation of mitosis as landmarked by the elongation of the apicoplast (asterisk in Figure 2.8Ci; Martins-Duarte et al., 2021). By contrast, parasites expressing YFP-SidM-P4M during the initiation of cell division exhibited a GRA3-positive LAP body (Figure 2.8Cii). Whereas the RH-WT DGs exhibited a mean diameter of 268 ± 11.5 nm – a value consistent with that measured for DGs of RH-WT cells in G1/S phase (273 ± 7.5 nm; see above) -- the sDGs that comprised the LAP body in those dividing parasites presented dimensions similar to those of YFP-SidM-P4M-expressing parasites in G1/S phase (mean ± SEM in G1/S phase: 140 ± 7.6 nm vs in mitosis: 135 ± 3.8 nm) (Figure 2.8Ciii). This GRA3-positive

structure remained prominent even though SidM-P4M PtdIns4P biosensor localization to the LAP body was strongly diminished during the initiation of cell division (Pearson's correlation coefficient 0.39 ± 0.05; white arrows in Figure 2.8Cii, compared to 0.77 ± 0.04 for G1/S phase cells). The redistribution of YFP-SidM-P4M from the LAP body to the Golgi/TGN system at the initiation of cell division, and its restored targeting to the LAP body in subsequent stages of the cell cycle, suggests a complex and previously unappreciated regulation of PtdIns4P signaling in DG cargo-containing compartments upon initiation of parasite cell division.

2.3. Discussion

Current models envision formation of DGs to result via direct budding from the *T. gondii* TGN as mature structures with appropriately sorted and condensed cargo (Griffith et al., 2022). Thus, DGs are not considered to undergo the types of more complex maturation processes that are hallmarks of dense core vesicle compartments that form the basis of regulated exocytosis systems of other eukaryotes – e.g. dense core granules of neuroendocrine cells or insulin secretory granules (ISGs) of pancreatic β-cells. Direct budding models are based on imaging studies that document the presence of morphologically uniform mature DGs with no recognizable maturation intermediates (Karsten et al., 1998; Coppens et al., 1999). Direct budding mechanisms leave unanswered questions, however. Although there is considerable evidence to suggest that the DG exocytic pathway is a default pathway in *T. gondii* (Heaslip et al., 2016; Striepen et al., 1998), these models require that DG cargo be segregated from other secretory cargo and condensed – presumably at the site of DG budding from the TGN surface. Moreover, proteins marked for retention in the TGN must either be excluded from loading into the nascent DG vesicle or otherwise retrieved from a DG vesicle after scission from TGN membranes. How appropriate cargo is condensed into these electron dense granules remains an unresolved question in the field of apicomplexan secretory trafficking. Mechanisms of cargo quality control are also not understood in detail. These questions are of particular interest as efficient trafficking and exocytosis of virulence factors is fundamental to host cell invasion by the parasite, its intracellular proliferation,

and ultimate egress of the parasite from the exhausted host cell to initiate successive rounds of infection.

2.3.1. Reconsidering ideas for DG biogenesis

While existing imaging data argue for direct DG budding models based onf the lack of morphological evidence for discrete steps in the DG biogenic process, our findings indicate that DG formation in *T. gondii* is a more complex process than that envisioned by simple direct budding models – one perhaps more in line with the pathways of dense core vesicle and secretory granule biogenesis in other systems. Using neuroendocrine cells as example, dense core granules mature in a stage-specific progression of homotypic fusion and retrograde trafficking of missorted cargo back to the TGN (Merighi, 2018). Moreover, the maturation process is typically slow in systems where immature DG-like structures are visible under the electron microscope. For example, ISG biogenesis in mammalian pancreatic β-cells is not only a high flux pathway, but ISG maturation requires three hours to complete (Davidson et al., 1988; Omar-Hmeadi et al., 2021). The lack of information regarding discrete stages of DG biogenesis in *T. gondii* is potentially a reflection of the process being too rapid and/or of insufficient flux to permit confident visualization of transient biogenic intermediates by electron microscopy – i.e. the only method with the resolution necessary to recognize such intermediates.

As demonstrated in other systems (Zhang et al., 2017; Tandon et al., 1998), either in vitro reconstitution or in vivo stage-specific inhibition approaches are required

to systematically dissect DG biogenesis to arrive at a detailed description of the process in *T. gondii*. We argue that it is from the latter perspective that the expression of high affinity PtdIns4P protein domains in *T. gondii* is particularly informative. Our description of the LAP body as a 'clustered' network of small PtdIns4P-decorated vesicles loaded with DG cargo argues for reconsideration of direct budding models for DG biogenesis in *T. gondii*. Furthermore, the fact that LAP body formation is induced by high affinity PtdIns4P biosensor expression demonstrates an important and previously unappreciated role for PtdIns4P signaling in DG biogenesis. Finally, the morphological properties of the LAP body itself offer other insights into this process. In Figure 2.9, we outline a speculative model for DG biogenesis that takes these insights into account.

2.3.2. Functional interpretation of the LAP body

How do we interpret the LAP body and how does this structure relate to DG biogenesis? We favor the idea that the LAP body represents an exaggerated compartment of nascent DGs arrested at a late stage(s) of biogenesis that we loosely refer to as maturation. The morphological data indicate that the small DG cargo-containing vesicles that comprise the LAP body network have completed budding from the TGN (Figure 2.9, step 1). Those data further suggest these have largely completed the TGN (Figure 2.9, step 1). Those data further suggest these have largely completed the process of cargo segregation (Figure 2.9, step 2). These conclusions are supported by demonstrations that the small LAP body vesicles are loaded with multiple DG cargo, are of homogeneous dimensions and are devoid of detectable quantities of TGN resident

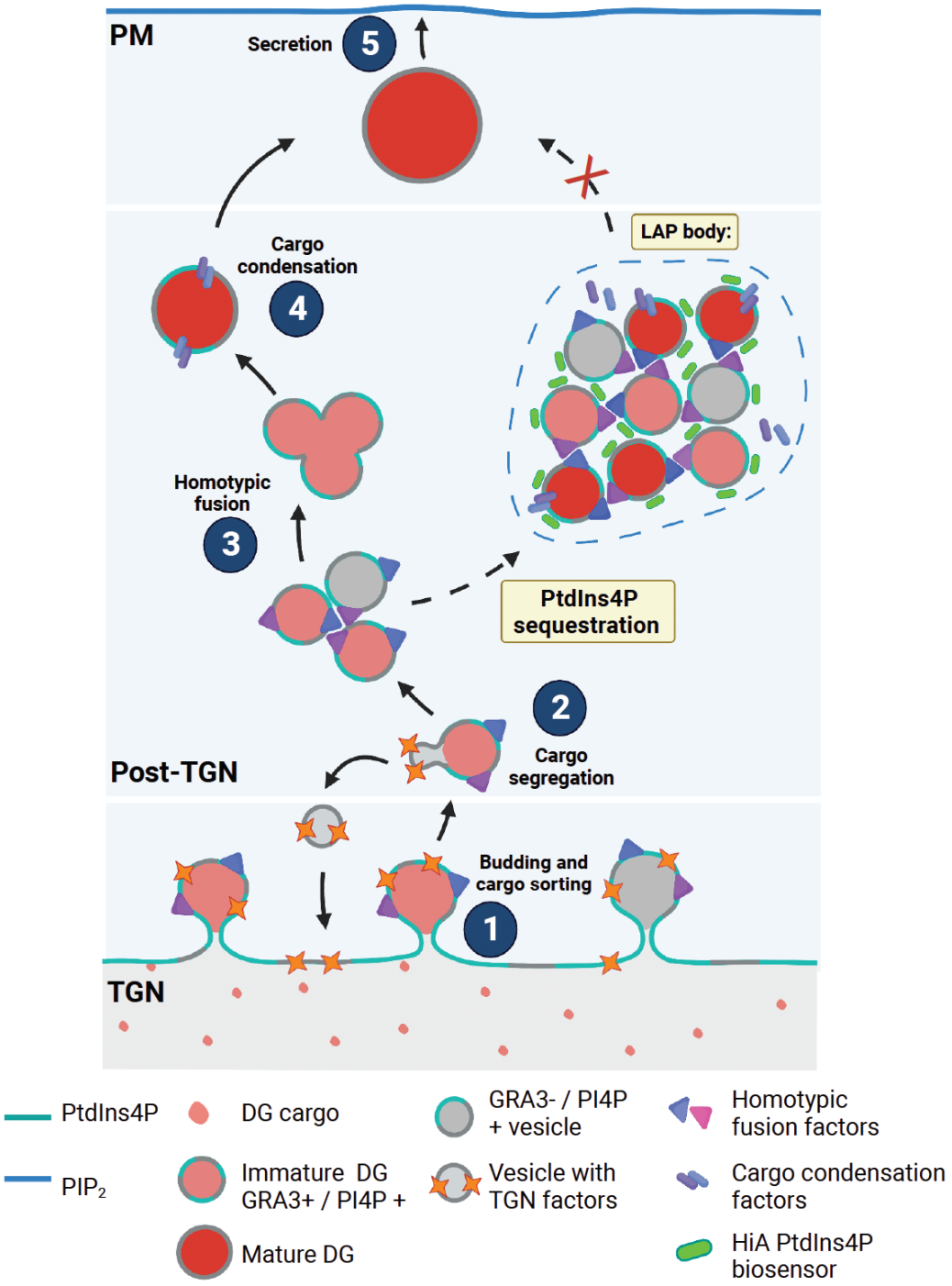

Figure 2.9 Model for DG biogenesis, maturation and exocytosis. Solid arrows mark proposed model for DG biogenesis and maturation in *T. gondii*. Budding and cargo sorting from PtdIns4P-enriched domains of the TGN (step 1) are followed by a further round(s) of cargo segregation (step 2) to generate immature DGs. Maturation of these

Figure 2.9 Continued. structures at a post-TGN compartment is characterized by the PtdIns4P-dependent homotypic fusion (step 3) – interference of which results in LAP body formation. Subsequent steps involve cargo condensation in the maturing DGs (step 4). Removal of PtdIns4P from mature DGs membrane marks completion of the maturation process and acquisition of secretion competence (step 5).

proteins (e.g. TgSORTLR). These data demonstrate the small vesicles are not a result of TGN fragmentation induced by expression of high affinity PtdIns4P binding modules. The fact that these vesicles are considerably smaller and (in some circumstances) less electron dense than the mature DGs of WT parasites suggests a late maturation step(s) is perturbed. In turn, these perturbations compromise exocytosis of DG cargo.

2.3.3. A role for PtdIns4P in DG biogenesis/maturation

As LAP body formation requires expression of high affinity PtdIns4P-binding modules, we interpret LAP body formation as a consequence of biologically insufficient PtdIns4P signaling in the process of DG biogenesis/maturation. The small dimensions of the clustered LAP body vesicles relative to mature DGs of WT cells suggests that homotypic fusion of the small clustered DGs is a discrete PtdIns4P-requiring step in the formation of the larger mature DGs (Figure 2.9, step 3). That PtdIns4P distribution within the LAP body and on the small vesicle surfaces is not isotropic suggests the existence of discrete PtdIns4P domains on clustered DGs. It is tempting to speculate that these domains might be concentrated in vesicle factors required for homotypic tethering and/or fusion. In that regard, mammalian COPII coated vesicles derived from the endoplasmic reticulum engage in homotypic tethering to form vesicular/tubular clusters

(VTCs; Yu et al., 2006). Although not known to be a PtdIns4P-dependent process in the case of COPII vesicles, the general morphological similarities in the VTCs and LAP bodies are notable. Alternatively, given that the clustered LAP body DGs are in some circumstances less electron dense than the mature DGs of WT parasites, PtdIns4P might also be required for the optimal activity of factors required for cargo condensation (Figure 2.9, step 4). For example, cargo condensation in dense core granules of other systems require recruitment of proton pumps into the maturing DG to acidify the compartment and potentiate cargo packing into crystalline forms (Rhodes et al., 1987; Schoonderwoert et al., 2000). The existence of PtdIns4P-positive structures in the LAP body devoid of GRA3 cargo suggest different classes of vesicles (as defined by cargo) are present in the network. Fusion of immature DGs with these vesicles might contribute to delivery of factors that regulate DG cargo condensation.

2.3.4. Signals for completion of DG maturation and competence for exocytosis.

Models positing a Ptdins4P signaling involvement in DG maturation must account for how completion of maturation is recognized. In that regard, LAP-body DGs are marked with both PtdIns4P and DG cargo, whereas mature DGs do not recruit high affinity PtdIns4P biosensors. We infer from those data that mature DGs are either devoid of, or present very low amounts of, PtdIns4P on their surfaces. These results raise the intriguing possibility that loss of PtdIns4P from the cytosolic face of the DG membrane marks completion of the maturation process and acquisition of competence for exocytosis (Figure 2.9, step 5). Such a strategy applies to the maturation pathway of

constitutive post-Golgi secretory vesicles in yeast where the PtdIns4P loaded onto the nascent vesicle during the budding process is degraded as secretory vesicles approach sites of exocytosis – a lipid remodeling event that reprograms the small GTPase specificity of vesicle-associated nucleotide exchange factors (Ling et al., 2014). PtdIns4P pools on ISG surfaces must also be degraded by the PtdIns4P-phosphatase Sac2 prior to mature granule association with the plasma membrane (Olsen et al., 2003, Nguyen et al., 2019).

With regard to regulation of PtdIns4P levels, we observed that high affinity PtdIns4P biosensors significantly redistributed away from the LAP body to the dividing Golgi system in parasites initiating endodyogeny. The simplest interpretation of those results is that LAP body PtdIns4P levels are reduced during initiation of endodyogeny and that the biosensors return to what is typically a PtdIns4P-enriched compartment. Such remodeling might reflect a shift in emphasis in specificity of cargo exocytosis at this cell cycle stage, or perhaps PtdIns4P levels increase in the Golgi system at this stage to promote division of this compartment. In any event, as the reduction of PtdIns4P on the surfaces of LAP body vesicles was not sufficient to induce their exocytosis, we interpret the LAP body as an exocytic ‘backwater’ that is not subject to efficient chase to an exocytosis-competent intermediate. Perhaps this is why the structure migrates towards the residual body of the mother cell during endodyogeny.

2.3.5. Primordial functions for PtdIns4P signaling

Apicomplexa are ancient eukaryotes that serve as excellent model organisms for the study of root mechanisms of eukaryotic cell function and evolution. In that regard, the collective data identify a role for Ptdins4P in the maturation of DGs and ultimate exocytosis of DG cargo in *T. gondii*. The similarities to aspects of both constitutive and regulatory secretory pathways in other eukaryotes are noteworthy as described above. This is perhaps not surprising as *T. gondii*, given its parasitic lifestyle, can be considered a professional secretory cell – one for which there is evidence for both constitutive and regulated DG secretory pathways (Chaturvedi et al, 1999; Coppens et al., 1999; Griffith et al., 2022).

The collective data now raise key questions regarding: (i) which PtdIns 4-OH kinase(s) produces the PtdIns4P pool(s) whose sequestration induces LAP body formation and defects in DG exocytosis, and (ii) how is synthesis of this PtdIns4P pool(s) regulated? With regard to the latter point, the LAP body phenotypes largely recapitulate those recently described in mammalian pancreatic β-cells where various stages of ISG formation, maturation and regulated exocytosis are PtdIns4P-dependent processes whose execution is perturbed upon functional ablation of PITPα – a PtdIns/phosphatidylcholine transfer protein (PITP) that stimulates PtdIns4P production in pancreatic β-cell TGN membranes (Yeh et al., 2022). Similarly, the major yeast PITP Sec14 plays an essential role in potentiating PtdIns4P-dependent membrane trafficking through the yeast TGN/endosomal system (Bankaitis et al., 1990; Hama et al., 1999; Rivas et al., 1999; Schaaf et al., 2008; Bankaitis et al., 2010; Grabon et al., 2019).

Does *T. gondii* utilize a PITP in a similar manner for PtdIns4P-dependent DG activities? This seems probable as *T. gondii* potentially encodes ten Sec14-like PITPs and one mammalian PITPα-like ortholog (ToxoDB (v56); http://ToxoDB.org). Moreover, time-resolved phosphoproteome analyses identified a Sec14-like protein (TGME49_254390) and a putative PtdIns 4-OH kinase (TGME49_276170) in a cohort of lipid signaling proteins modified by phosphorylation upon induction of egress -- a process dependent on phosphatidic acid and phospholipase C (TgPI-PLC) signaling (Herneisen et al., 2022, Nofal et al. 2022). Yet another of the *T. gondii* Sec14-like PITPs (TGME49_213790) is appended to a PH domain -- as is the single PITP α-like ortholog (TGME49_289570). Such domain architectures recommend these putative PITPs as attractive candidates for engaging the PtdIns4P pool(s) identified in this work. Functional dissection of these activities promises to add additional layers of complexity to the mechanism of PtdIns4P signaling in the regulation of DG biogenesis, maturation and exocytosis in apicomplexan parasites.

2.4. Materials and methods

2.4.1. Cell culture and *T. gondii* strains

The human foreskin fibroblast-1 (HFF-1) cell line was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO2 in a humidified atmosphere. The *T. gondii* RH strain was maintained by serial passage using HFF-1 monolayers as host cells. Parasites were cultured to egress and purified from host cells by disrupting the host cells via serial passage through 18- and 25-guage needles. Parasites were subsequently collected from the host cell lysate in the pellet after centrifugation of the crude lysate at 900 x g for 5 minutes.

2.4.2. Generation and selection of stable transgenic parasites

RH strain was transfected by electroporation as described previously (Soldati and Boothroyd, 1993). Using an electroporation cuvette as vessel, 107 tachyzoites were mixed with 25 μg of plasmid in transfection buffer (120mM KCl, 150μM CaCl2, 5mM MgCl2, 2mM EDTA, 25mM HEPES KOH, 10mM potassium phosphate, pH 7.6). In co-transfection experiments, 12.5 μg of each plasmid was used. A single electrical pulse (1.3kV, 25uF) was applied using the Bio-Rad Gene Pulse II electroporation apparatus. Parasites were allowed to recover after plating on an HFF-1 monolayer in the absence of drug for 24 hours. When appropriate, selection of stable transgenic parasites was carried out in the presence of a chloramphenicol (20 μM) selection. Stable clones were isolated by limiting dilution under conditions of continuous drug selection.

2.4.3. Plasmid constructs

All *T. gondii* expression vectors expressing fluorescently-labeled organelle markers were based on the pTUB-CAT (Chloramphenicol acetyltransferase) system (Kim et al., 1993). Gene expression is driven by the tubulin promoter. In all cases, the DNA restriction fragments carrying the gene of interest were subcloned into the BglII/AvrII sites of pTUB. These constructs were engineered such that these carried a Myc-tag and a fluorescent protein gene between flanking the cloning site. Additional details regarding constructs of interest are described in the Figure legends. The primers used for cloning all *T. gondii* biomarkers are listed in Supplementary Table S2.1.

2.4.4. Immunofluorescence analysis (IFAs)

Confluent HFF-1 host cells grown in 35mm glass-bottom coverslip dishes were infected with tachyzoites for 24 hrs, washed 1X with phosphate buffered saline (PBS) 1X and then fixed with 4% PFA (v/v) for 15 min at room temperature and washed 3X (all washing incubations were performed for 10 min with PBS 1X solution). Cells were permeabilized with 0.2% Triton-X (v/v) for 4 min at RT, blocked with 2% BSA in PBS 1X overnight at 4°C, incubated with organelle-specific primary antibodies diluted in blocking buffer for 1 hour. Details regarding the primary and secondary antibodies used in this study are listed in Supplementary Table S2.2. Coverslip were washed with PBS three times, incubated with conjugated secondary antibodies in blocking buffer for 1 hour, and washed again with PBS three times. Cell DNA was stained with Hoechst

solution (1:5000) for 5 min. After three washes with PBS, coverslips were flooded with PBS and stored at 4˚C for no longer than seven days.

2.4.5. Image acquisition

Confocal images were collected in a NikonA1R microscope (CFI Plan Apo lambda 60x/1.4 oil objective) and Zeiss LSM 780 NLO multiphoton microscope (Plan-Apochromat 63x/1.4 oil DIC M27 objective). Imaging processing was performed using ImageJ (FIJI) (NIH). Pearson's correlation coefficients were calculated using the colocalization analysis plugin JaCoP in FIJI (ImageJ) and with the colocalization analysis tool in ZEN Blue 2.3. Super-resolution fluorescence imaging was performed with a Zeiss LSM 780 NLO multiphoton microscope equipped with an Airyscan detector (Plan-Apochromat 63x/1.4 oil DIC M27 objective). Images obtained with Airyscan detector were deconvoluted using ZEN 2.3 (blue edition) software, and gamma values were increased or decreased in the order of 0.1-0.8 units). Pearson's correlation and colocalization coefficients of regions of interest (subapical areas of individual tachyzoites) were calculated using ZEN blue software. 3D reconstructions were generated using the 3D view module in Imaris 9.8v.

2.4.6. Definition and quantification of GRA3 profiles

Transgenic parasites were generated by electroporation as described above. Two plasmid pTUB constructs were used for co-transfection: 12.5 µg of pTUB-Myc-GRA3-RFP and 12.5 µg of pTUB constructs expressing YFP-FAPP1PH, YFP-SidM-P4M,

YFP-GOLPH, YFP- SidM-P4M^{K568A}, FAPP1PH$^{K7A/R18L}$, 2xPHPLCδ-EGFP, or TAPP1PH-YFP as appropriate. Transfected tachyzoites were plated onto confluent HFF-1 cells in 35mm glass-bottom coverslip dishes with 2 ml of supplemented DMEM. Plates were incubated for 24 hours. Only parasites expressing the constructs of interest were counted using an epifluorescence microscope (Nikon Eclipse Ti). The criteria used for binning parasite populations based on GRA3 and GRA2 phenotype was as follows: 1) Each unit was considered as a PV that contained one or more tachyzoites; 2) up to 50 PVs were counted per experiment; 3) PVs were binned as displaying large or small puncta phenotype when at least one of the tachyzoites in the vacuole presented one of these phenotypes. In cases where both small and large puncta pattern were observed in the same PV, the PV was classified as displaying large puncta. All experimental results represent the data from three independent biological replicates.

2.4.7. Ratiometric measurement of DG exocytosis efficiency

Wild-type and transiently transfected parasites were grown for 24 h, fixed with 4% PFA and stained with anti-GRA3 as mentioned previously. Imaging and counting of 100 PVs per experimental and control conditions was performed in epifluorescence mode. Camera settings such as format (no binning), exposure time (10 msec) and dynamic range (11-bit and Gain 4) were fixed for the TRITC filter channel to collect unbiased imaging data for all experimental conditions. To calculate GRA3 secretion efficiency as a function of transient YFP-SidM-P4M expression, we adopted the ratiometric approach of Li et al (2022). Regions of interests (ROI) were drawn around

the PVM region (PVM), the intracellular region occupied by the nucleus (nuclear region), the area surrounding the entire PV and parasites (Total *Toxoplasma*), and a background region outside the PV. The mean intensities for each region were measured in ImageJ (FIJI) and the PVM relative intensity was obtained as:

$$PVM\ relative\ intensity = \frac{(mean\ PVM - mean\ nuclear\ region)\ x\ PVM\ area}{(mean\ total\ Toxoplasma - mean\ background)\ x\ area\ total\ Toxoplasma}$$

2.4.8. Correlative light electron microscopy

Mattek glass-bottom coverslip dishes (P35G-1.5-14-C-GRID) were used to seed and infect HFF-1 cells with transiently transfected parasites that express pTUB-YFP-FAPP1PH-Myc or pTUB-YFP-SidM-P4M-Myc. At 24 hours post inoculation coverslips were fixed with 4% PFA and 0.025% glutaraldehyde (GA) in PBS for 15 min and washed 3X with PBS with a 5 min incubation per wash. Parasites were permeabilized with 0.2% Triton-X (v/v) in PBS for 4 min, washed 1X with PB (0.1 M phosphate buffer pH 7.2), incubated with primary mouse anti-GRA3 antibody secondary antibody directed against Alexa fluor 594 (A11032) (see Supplementary Table S2.2 for used dilutions), stained with Hoechst solution (1:5000) for 5 min, and washed 3X with PB with a 10 min incubation per wash. Intracellular parasites were identified using a confocal NikonA1R microscope outfitted with a CFI Plan Apo lambda 60x/1.4 oil objective (to acquire z-sections of parasites with a LAP body to create a map of the parasite location), and a CFI Plan Apo lambda 20x/0.75 objective (to identify the

position of parasites with a LAP body within the coordinate system in the glass-gridded coverslip).

Coordinates of interest in the grid were marked with nail varnish on the base of the coverslip. Cells were fixed again with 4% PFA and 0.5% GA in PB overnight at 4 ºC, washed 3X with distilled water (10 min each), post-stained with 1% osmium (OsO4) for 15 min and with filtered 2% uranyl acetate for 20 min (water washes between each step – 3X and 10 min incubation each). The samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96%, 100%, 100%, 100%) with incubations of 10 min at each step. Cells on coverslips were embedded in a modified Quetol/Spurr's resin mixed 1:1 with ethanol for 2 hours, followed by resin alone for 4 hours. To limit embedding to the targeted region of the glass-gridded coverslip, a plastic capsule (both ends open) was placed up-side-down on the coverslip over the varnish mark and incubated overnight at 60°C (Hanson et al., 2010). Immobilized capsules were filled with resin and incubated at 60°C for 48 hours. Resin blocks were detached by heating the coverslip with a passing flame for 7 sec under the bottom of the coverslip (Loussert et al., 2012). Serial ultrathin sections (~100 nm) were collected using a Leica UC7 ultramicrotome and mounted on formvar slot grids. Sections were stained with lead citrate for 7-10 min and imaged using an FEI Morgagni 268 transmission electron microscope at 70 KeV with a MegaView III CCD camera and iTEM image acquisition software. Image analyses were performed with ImageJ. Data were collected from eight parasites per experimental condition.

2.4.9. Statistical analyses

Data obtained from at least three independent biological replicates were presented as mean ± standard error of the mean (SEM). Two-way ANOVA followed by Dunnett's multiple comparison test was used to compare two or more experimental groups to a single control group. One-way ANOVA followed by Tukey's multiple comparison test was used to compare two or more experimental groups to every other group. T-test (Mann-Whitney test) was performed to compare the means of two groups. Data were calculated and illustrated using GraphPad Prism 6 software. A P value < 0.05 was set as threshold for statistical significance.

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3. IDENTIFICATION OF A *TOXOPLASMA GONDII* STEROL PRECURSOR-EXCHANGER PROTEIN OF THE SEC14-LIKE PITP FAMILY

3.1. Introduction

Lipids are essential components of cell signaling in eukaryotic cells. A major example is PtdIns and its phosphorylated derivatives – the PIPs. These membrane compounds are key secondary messengers that contribute to multiple cell signaling pathways. PIPs are produced by the phosphorylation of the inositol headgroup of PtdIns at the 3-OH, 4-OH, and 5-OH positions by specialized PtdIns/PIP kinases (Carpenter and Cantley, 1990). The phosphorylation diversity of PtdIns allows the formation of seven species of PIPs that act as spatiotemporal barcodes to recruit and activate specific protein effectors (Wang et al., 2018). Understanding how specific PIP pools are formed and maintained to establish diverse and precise cell internal communications, as well as how cell signaling is diversified by the use of a limited number of PIPs, is a major problem in contemporary cell biology. Although much effort is invested in understanding the enzymes that produce and consume PIPs (Vanhaesebroeck et al., 2010; Nakatsu et al., 2012; Burke et al., 2014), the discovery of factors that regulate PIP metabolic processes defines unappreciated gaps in our understanding. New insights that fill these knowledge gaps come from studies of PITPs. The functional principle was described in the founding member of the Sec14 superfamily – *S. cerevisiae* Sec14. ScSec14 positively regulates PtdIns4P production at the TGN to activate vesicle trafficking to the plasma membrane in two consecutive steps. First, ScSec14 binds

PtdCho to sense levels of DAG, which is consumed in the CDP-choline pathway to produce PtdCho. Moreover, DAG is required for vesicle biogenesis at the TGN/endosomes. In the second step, when sufficient levels of DAG are detected, a heterotypic exchange cycle of PtdIns and PtdCho by ScSec14 takes place and serves as a presentation mechanism of PtdIns to the biologically insufficient PI4K, Pik1. In this model, the identity of the secondary ligand, e.g., PtdCho, serves as signaling input into the PITP exchange lipid cycle to activate PIP signaling (Schaaf et al., 2008; Bankaitis et al., 2010; Graham and Burd, 2011; Sugiura et al., 2019).

PITPs are evolutionarily conserved proteins that fall into two distinct families: the Sec14-like PITPs, of which Sec14 is the founding member, and the StAR-related lipid transfer (StART) PITPs (Schaaf et al., 2008; Yoder et al., 2001). These PITP families are structurally unrelated but share a common biochemical activity: the heterotypic lipid exchange of PtdIns with a secondary ligand on a membrane surface (Schaaf et al., 2008; Bankaitis et al., 2010; Xie et al., 2018). Currently, functional information on the regulation of PIPs through Sec14-like PITPs is mainly derived from studies of mammals and yeast, but to understand the evolution of PIP signaling, we can turn to an earlier eukaryotic lineage. An appropriate model to study ancient cellular mechanisms is the Apicomplexa phylum, which originated more than a billion years ago, while macroscopic multicellular life only appeared about 600 million years ago (Escalante et al., 1995; Zhu et al., 2016). The model organism in this study is the parasite *T. gondii*, which belongs to this early diverged taxonomic group and is a leading pathogen causing foodborne illness in the United States. Although Sec14-like PITP

proteins in *T. gondii* have not yet been characterized, candidates have been identified as possible mediators of PtdIns4P metabolism in wide phosphoproteomic analyses (Herneisen et al., 2022; Nofal et al., 2022).

Herein, we report an organized search of Sec14-like PITP candidates in *T. gondii* that led to the selection and characterization of a candidate. This candidate is identified as TgSfhA and showed the highest protein sequence conservation and folding homology to the yeast Sec14-like PITP members and the human Sec14-L2 protein. In silico and biochemical characterization showed that TgSfhA is a non-canonical Sec14-like PITP with potential activity to bind/exchange steroid precursor(s) or a steroid molecule. TgSfhA is the first described Sec14-like PITP in an Apicomplexa organism and provides the potential to uncover primitive and conserved Sec14-like PITP regulatory mechanisms.

3.2. Results

3.2.1. TgSfhA is the most closely related to the yeast Sec14-like PITPs

Ten single reading frames annotated in the ToxoDB (v56) have a predicted translation product that contains a Sec14-like Sfh PITP (or CRAL-TRIO) domain (Panagabko et al., 2003; Bankaitis et al., 2010). These genes are referred to in this report with the acronym Sfh – for Sec Fourteen homology– followed by a unique alphabetic letter. The protein architecture and sizes of these proteins differ from those of the essential ScSec14 (Bankaitis et al., 1989; Figure 3.1A). At least three *T. gondii* candidates are multidomain proteins (TgSfhB, TgSfhC, and TgSfhD), and four are composed of more than 500 amino acids (TgSfhB, TgSfhC, TgSfhE, and TgSfhH). At least half of the *T. gondii S*ec14-like candidates (TgSfhC, TgSfhD, TgSfhG, TgSfhH, and TgSfhI) contain predicted transmembrane regions (TM), suggesting these are insoluble proteins. On the contrary, all yeast Sec14-like family members are confirmed to be soluble proteins (Tripathi et al., 2019). A phylogenetic analysis of all *T. gondii* Sec14-like PITP protein sequences demonstrated the existence of three clades: the TgSfhJ/H, TgSfhG/E, and TgSfhC/B/F/A/I/D clades (Figure 3.1B). The Sec14-like PITP sequence diversification in three clades suggests multiple and distinct functions of this protein family in the parasite. It is worth noting that all four candidates containing multiple domains are clustered in the same clade (SfhC/B/F/A/I/D). Multiple modules in Sec14-like proteins have been associated with the integration of relatively newly evolved specific-lineage cellular functions (Montag et al., 2020).

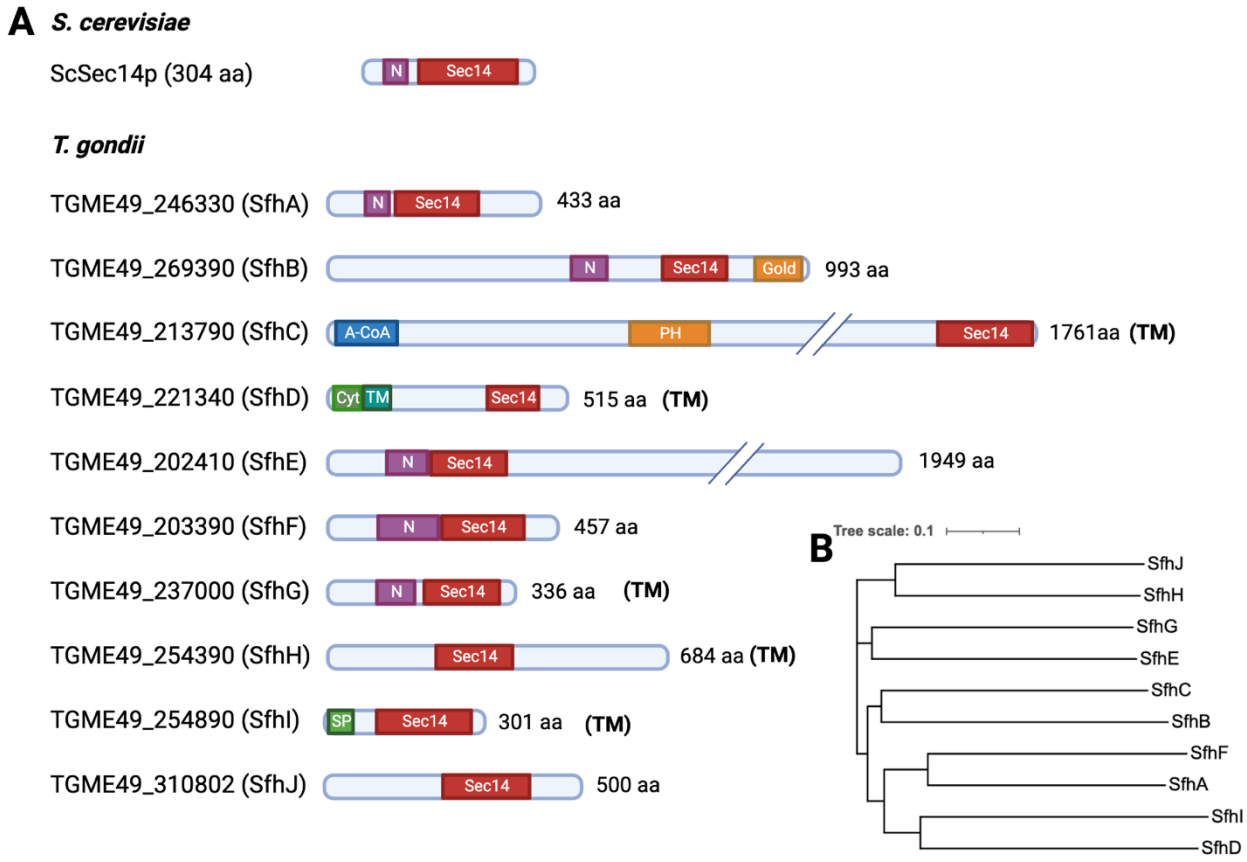

Figure 3.1 Prediction of protein architecture of Sec14-like protein candidates in *T. gondii*. (A) ScSec14 is depicted as the founding member of the Sec14-like protein family. Domain architecture of *T. gondii* proteins that were selected upon prediction of the CRAL-TRIO domain through the Interpro domain finding tool in PlasmoDB (v56) and ToxoDB (v56). N: N-term CRAL-TRIO; Gold: Golgi dynamics domain; A-CoA: Acyl-CoA binding domain; PH: Pleckstrin Homology domain; Cyt: Cytoplasmic domain; TM: Transmembrane domain; SP: Signal peptide and (*TM): Predicted transmembrane region (Hebditch et al., 2017). (B) Phylogenetic relationships of *T. gondii* Sec14-like PITPs generated with full-length amino acid sequences.

Primary sequence BLAST analysis of the *T. gondii* candidate Sec14-like binding domains against ScSec14 revealed that TgSfhA (TGGT1_246330) shares the highest protein sequence homology with ScSec14 of the *T. gondii* candidates, according to its low E-value score (6e-17). The next most homologous are TgSfhB and TgSfhG, with

much lower probability values (Table 3.1). The canonical heterotypic exchange reaction performed by ScSec14 requires binding to PtdIns and PtdCho (Sugiura et al., 2019). Residues involved in lipid binding by ScSfh1 (the closest ScSec14 homolog; Li et al., 2000) were described in Schaaf et al. (2008). These PtdIns- and PtdCho-binding barcodes were used as an alignment template to predict the conservation of lipid-binding

Table 3.1. Data collection from in silico analysis performed in *T. gondii* Sec14-like proteins. (Φ) E-values of BLAST analysis obtained from alignments against ScSec14. (ε) Number of residues that were reported to interact with specific phospholipid in the lipid-binding domain of ScSfh1 (Schaaf et al., 2008).

ToxoDB ID	**Lab ID**	**BLAST E-valueΦ**	**PI (8)ε**	**PC (10)ε**
TGGT1_246330	TgSfhA	6e-17	7	1
TGGT1_269390	TgSfhB	1e-9	6	1
TGGT1_213790	TgSfhC	2e-7	6	0
TGGT1_221340	TgSfhD	0.018	4	1
TGGT1_202410	TgSfhE	3e-5	4	0
TGGT1_203390	TgSfhF	8e-7	4	0
TGGT1_237000	TgSfhG	8e-9	4	2
TGGT1_254390	TgSfhH	6e-8	3	1
TGGT1_254890	TgSfhI	7e-4	3	2
TGGT1_310802	TgSfhJ	1e-4	5	0

specificity in the structural analysis of *T. gondii* Sfhs protein sequences. By this analysis, TgSfhA also showed the best match of lipid binding barcodes: 7/8 for the PtdIns- and 1/2 for the PtdCho-binding barcode (Table 3.1). Only the PtdIns-binding barcode is highly conserved in all members of the Sec14-like protein family, while the nature of the second "ligand" – PtdCho in the case of ScSec14 – varies among all these proteins, contributing to the functional diversification of Sec14-like PITPs. The conservation of the PtdIns-binding activity reflects an essential feature in the Sec14-like family (Ren et al., 2011; Ren et al., 2014; Li et al., 2000; Tripathi et al., 2019). The protein architecture and sequence analyses performed in the *T. gondii* Sec14-like cohort suggest that the lipid binding domain of TgSfhA is the most conserved among this group. Thus, TgSfhA was selected as a candidate to study Sec14-like functions in *T. gondii*.

3.2.2. TgSfhA is a predicted homolog of yeast ScSfh3 and human Sec14-L2

The function of Sec14-like PITP family members in the yeast *S. cerevisiae* (ScSec14, ScSfh1, ScSfh2, ScSfh3, ScSfh4, and ScSfh5) has been better defined in comparison to other organisms (Skinner et al., 1993; Ren et al., 2014; Routt et al., 2005; Desfougères et al., 2008; Wang et al., 2020; Mizuike et al., 2019; Khan et al., 2020). Thus, to predict the functional role of TgSfhA, yeast Sec14 family protein sequences were used as queries in BLAST analysis against TgSfhA. This revealed closer homology to ScSfh3 (also called PDR16) and ScSfh4 than to the founder protein of the family, ScSec14 (E-values: 4e-26 and 3e-26, respectively). Next, we predicted the structure of TgSfhA using the MOE 2013.08 modeling software. MOE utilized ScSfh3 (2.34 Å, PDB

code: 4fmm) and human Sec14-L2 (PDB: 4omk) as templates onto which to thread the TgSfhA sequence as these had a higher homology matching score than the structures for ScSec14 or ScSfh1.

Sec14-L2 and ScSfh3 are associated with sterol metabolism in eukaryotic cells. Sec14-L2 (also called human supernatant protein factor (SPF)) binds/exchanges precursor molecules of sterol synthesis, such as squalene and squalene-2,3-epoxide. In the sterol backbone synthesis pathway, squalene is converted to squalene-2,3-epoxide by the terbinafine target squalene 2,3-epoxidase (ERG1 gene product; Figure 3.2A). The binding of Sec14-L2 with these intermediates stimulates cholesterol biosynthesis in the cell (Porter, 2003; Shibata et al., 2001). Moreover, ScSfh3 is a PtdIns- and sterol-exchange protein that negatively modulates neutral lipid consumption during yeast meiosis (Ren et al., 2014).

The TgSfhA 3D homology model generated using Sec14-L2 as a template (similarity: 45%; Figure 3.2B) shows a Sec14-like moiety composed of a two-helix bundle (instead of a three-helix bundle like in Sec14-L2) and a hydrophobic binding pocket. TgSfhA in this model has two 'gating helices' instead of one, and three parallel stranded β-sheets instead of four when compared with the Sec14-L2 structure (Christen et al., 2015). The TgSfhA 3D homology model generated using ScSfh3 as a template had a lower similarity score (similarity: 27%; Figure 3.2C); however, a more comparable Sec14-like architecture was observed. TgSfhA folding was distinguished by a β-sheet floor composed by 5 parallel stranded β-sheets, 3 α-helices packed against one face of the sheet enclosing the hydrophobic binding pocket and a 'gating helix' region that

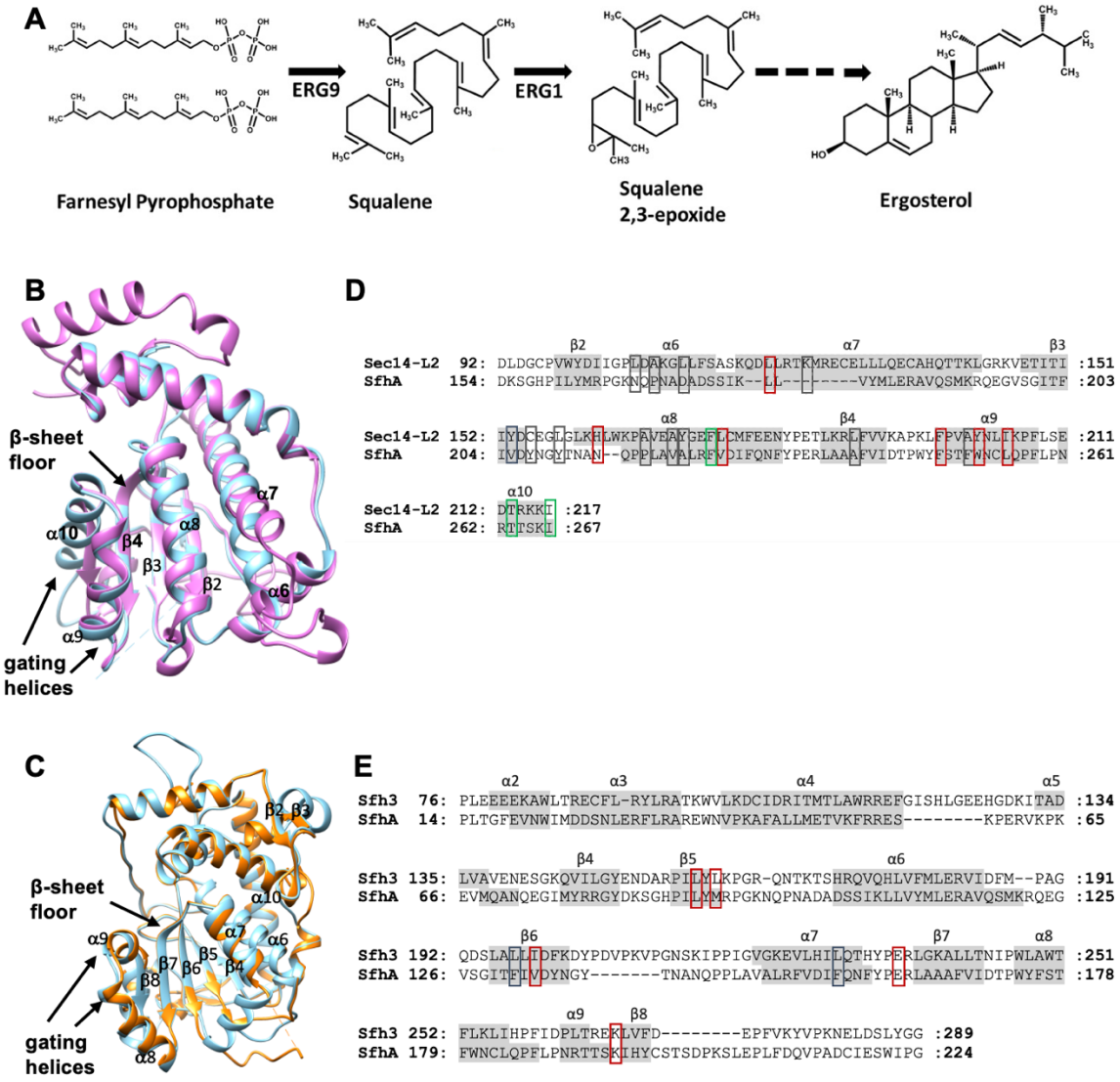

Figure 3.2 TgSfhA folding prediction using human Sec14-L2 and yeast ScSfh3 templates. (A) Squalene is a metabolic intermediate in the ergosterol biosynthesis pathway and is produced from farnesyl pyrophosphate by ERG9 gene product squalene synthase. Subsequently, squalene is converted to squalene 2,3-epoxide in a reaction catalyzed by the squalene 2,3-epoxidase (encoded by ERG1) that consumes molecular oxygen. (B) The CRAL-TRIO domain of TgSfhA (cyan) was modeled on the crystal structure of the CRAL-TRIO domain of human Sec14-L2 (PDB 4omk; pink). (C) The CRAL-TRIO domain of TgSfhA (cyan) was modeled using the crystal structure of the CRAL-TRIO domain of yeast ScSfh3 (PDB 4fmm; orange). (B and C) The protein structures were modeled in MOE 2013.08 and visualized in Chimera 1.16. (D) Protein sequence alignment of TgSfhA and Sec14-L2. Common squalene- and squalene-2,3-epoxide- interacting residues are indicated in red (conserved in TgSfhA) and blue (not

Figure 3.2 Continued. conserved in TgSfhA) squares. And specific squalene- (I217) and squalene-2,3-epoxide (F174 and I151)-binding residues are highlighted with green squares. (E) Protein sequence alignment of TgSfhA and ScSfh3. Sterol-binding residues in ScSfh3 are indicated in red (conserved in TgSfhA) and blue (not conserved in TgSfhA) squares. (D and E) α-helices and β-sheets are highlighted in grey.

controls access to the phospholipid-binding pocket, similar to ScSfh3 (Tripathi et al., 2019). Analysis of residue orientation was excluded from TgSfhA homology models as these had homology scores lower than 80%.

To investigate whether TgSfhA conserves the sterol and sterol-precursor barcode reported for ScSfh3 and Sec14-L2, respectively, a protein sequence alignment analysis was performed between these proteins and TgSfhA. First, squalene- and squalene-2,3-epoxide-binding residues in Sec14-L2 were used as references to identify a potential sterol precursor-barcode in TgSfhA. Sec14-L2 contains three regions that interact simultaneously with squalene- and squalene-2,3-epoxide also called common ligand-interacting regions: 1) the first region includes helices α6 and α7 (L106, A108, L111, L120 and K124); 2) the second region includes strand β3 and helix α8 (Y153, C155, L158, H162, A167, A170, Y171, L175 and L189); and 3) the third region includes helix α9 (F198, A201, Y202 and I205) (Christen et al., 2015; Figure 3.2D). TgSfhA seems to fully conserve only two of these eighteen residues: L120 and F198 (or L180 and F248 in TgSfhA) in the first region. However, four residues located in regions 2 and 3 (H162, L175, T202, and I205) are substituted with amino acids of similar functional groups in TgSfhA (N213, V225, W252, and L255). Although these analyses show no significant conservation of Sec14-L2 common ligand-interacting regions in TgSfhA, the specific

squalene- (T213 and I217) and squalene-2,3-epoxide- (F174 and I151) binding amino acids are more conserved, especially for squalene (residues in TgSfhA for squalene: T263 and I267; and for squalene-2,3-epoxide: F224; green squares in Figure 3.2D).

Next, the ability of TgSfhA to bind sterol was predicted by sequence comparison to the ScSfh3 sterol-binding barcode. Structural modeling of ergosterol, cholesterol, dihydroergosterol, and cholestatrienol docking in the ScSfh3 hydrophobic cavity was confirmed experimentally (Tripathi et al., 2019). The sterol-barcode consists of L158 and L160 on β5, L197 and I199 on β6 sheet, and Leu229 on helix α7. These residues are involved in the alignment of the hydrophobic sterol planar ring system in the hydrophobic pocket. The alignment analysis in Figure 3.2E shows significant conservation in the positioning of most of these sterol-binding residues in TgSfhA. Only L158 is fully conserved in TgSfhA (L89), while L160 and I199 are replaced with amino acids of the same functional group (M91 on β5 and F130 and V132 on β6). Moreover, L197 and L229 are not conserved. ScSfh3 residues E235 and K267 were predicted to interact with the inositol headgroup of PtdIns and with the 3-hydroxyl group of the sterol molecule, and these are fully conserved in the TgSfhA sequence (E163 and K194).

Overall, the examination of TgSfhA folding shows better score correlation with Sec14-L2; however, higher conservation of the ScSfh3-sterol-binding topography is evident and indicates that an intermediate in the sterol synthesis pathway may be a TgSfhA ligand.

3.2.3. TgSfhA binds/exchanges squalene in vitro

To determine whether TgSfhA binds and transfers cholesterol or squalene, radiolabeled lipid transfer assays were performed. Purified TgSfhA was subjected to an in-vitro lipid-transfer assay that quantifies mobilization of [^{3}H]-squalene or [^{3}H]-cholesterol from a donor vesicle to an acceptor bovine heart mitochondria (BHM) (Figure 3.3A). Transfer activity is expressed as a fraction of the ratio of [^{3}H]-lipid in the acceptor vesicle fraction to total input [^{3}H]-lipid in donor vesicle fraction × 100%, after subtracting the background. Purified ScSfh3 was used as a positive control to verify the exchange of [^{3}H]-cholesterol. ScSfh2, a squalene binding/exchanger protein belonging to the yeast Sec14-like cohort was used as a positive control to query [^{3}H]-squalene transfer activities (Tripathi et al., 2019). TgSfhA displayed a very low capacity to transfer [^{3}H]-cholesterol (max 36% of ScSfh3) (Figure 3.3B). TgSfhA [^{3}H]-squalene transfer activity was comparable to the positive control (35.5%) only at protein levels 30-fold higher than the basal concentration of positive control ScSfh2 (26.3%) (Figure 3.3C). To verify that low transfer levels of TgSfhA were not caused by protein degradation or misfolding, purified TgSfhA was quantified and subjected to quality control analysis. SDS-PAGE analysis revealed a single clean band of the expected size (~ 48 kDa) indicating that the protein was not degraded. Mass spectrometry of the TgSfhA protein suspension revealed the existence of four different charged species of the same protein (+11, +12, +13, and +14) bound to the Escherichia coli phospholipid phosphatidylglycerol. This data suggested that TgSfhA folding was not affected in the *E. coli* BL21 expression system and that this protein is capable of interacting with a lipid ligand.

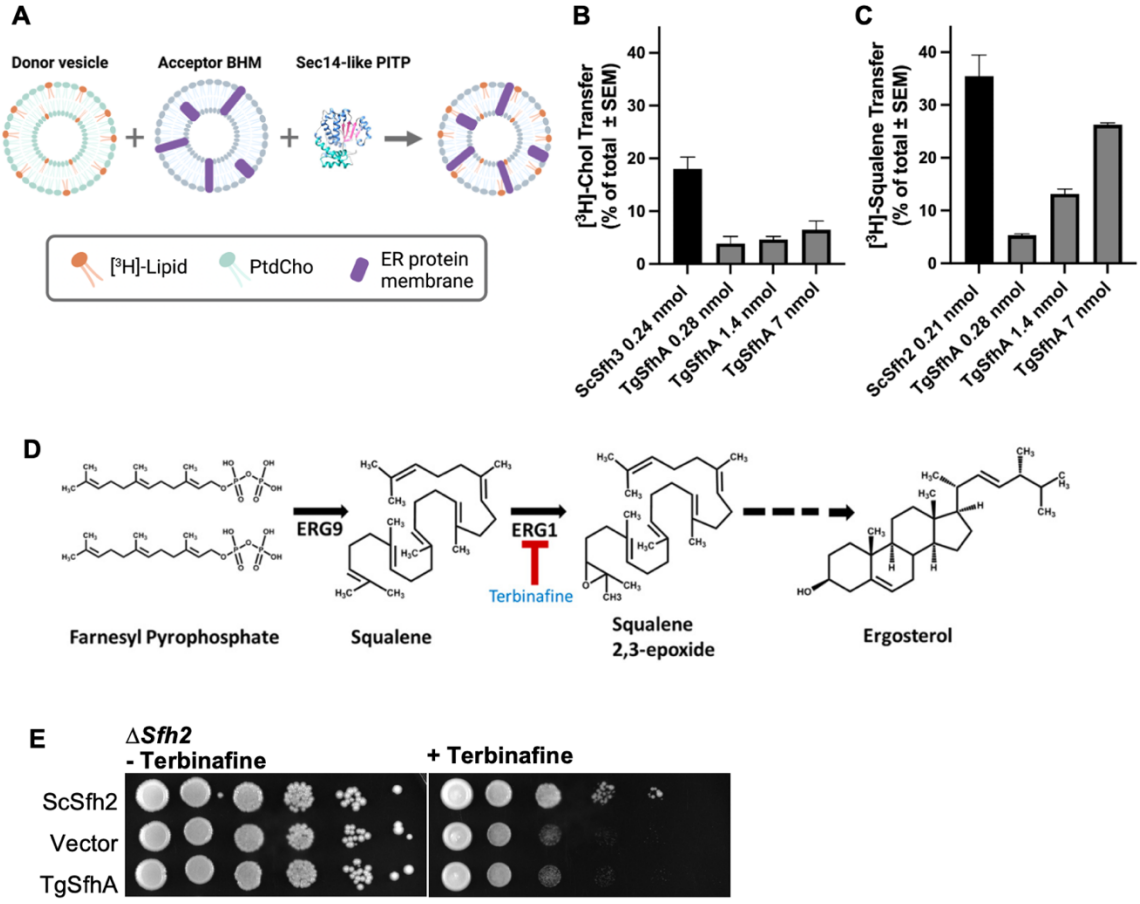

Figure 3.3 TgSfhA can exchange squalene between membranes. (A) Overview of the radiolabel lipid transfer assay illustrates the composition of donor vesicles with [^{3}H]-lipid (orange) and PtdCho (blue) and its incubation with acceptor BHM (grey) and a PITP (at 37 ºC for 30 min). Further separation of the fraction containing BHM takes place to measure the radioactivity of the transferred [^{3}H]-lipid. (B) [^{3}H]-cholesterol input in this assay ranged from 12 to 16 x103 cpm, and background ranged from 4 to 8 x103 cpm. The amount (nmol) of protein assayed is indicated on the *x*-axis. Transfer values are the mean of three independent assays performed in triplicate. (C) [^{3}H]-squalene input in this assay ranged from 7 to 19 x10^3 cpm, and background ranged from 1 to 4 x10^3 cpm. The amount (nmol) of protein assayed is indicated on the *x*-axis. Transfer values are the mean of three independent assays performed in triplicate. (D) Squalene 2,3-epoxidase is inhibited by the synthetic allylamine terbinafine. Scheme obtained from Tripathi and col. (2019). (E) ScSfh2-deficient yeast cells exhibit increased sensitivity to terbinafine. *ΔScsfh2* yeast strains transformed with a mock vector or vectors carrying *ScSFH2* or *TgSFHA* genes were spotted in 10-fold dilution series onto YPD plates without or with the squalene 2,3-epoxidase inhibitor terbinafine (250 µM), as indicated. Plates were incubated at 30 °C for 72 h.

A second biochemical analysis was implemented to analyze the ability of TgSfhA to transfer/bind squalene in vivo. Terbinafine is a drug that inhibits the biosynthesis of ergosterol in yeast at the level of squalene epoxidase, causing the toxic accumulation of intracellular squalene (Figure 3.3D) (Darkes et al., 2003). Expression of *ScSFH2* overcomes this lethal effect, while ScSfh2-deficient cells (Δ*Scsfh2* strain) are sensitized to the terbinafine challenge (Tripathi et al., 2019). Figure 3.3E shows that expression of *TgSFHA* fails to rescue the ScSfh2-dependent growth effect in the presence of terbinafine and indicates that TgSfhA binding activity to intracellular squalene is not equivalent to ScSfh2 binding activity (lower panel). This data confirms the relatively low affinity of TgSfhA towards squalene detected in the previous in vitro assay. Overall, these data suggest that TgSfhA might bind a lipid of the sterol family, but it is unlikely that the main ligand is squalene or cholesterol.

3.2.4. TgSfhA is a non-canonical PtdIns-binding Sec14-like PITP

To assess functional similarities between ScSec14 and TgSfhA, TgSfhA was assayed for the ability to carry out the PtdIns exchange cycle, in which ScSec14 stimulates PtdIns kinase activity through exchange of PtdIns for another lipid at the membrane interface (Bankaitis et al., 1989; Bankaitis et al., 2010; Schaaf et al., 2008). The in vitro lipid transfer analysis revealed that TgSfhA exchanges [^{3}H]-PtdIns between membranes with low efficiency compared to ScSec14. TgSfhA transferred only 8.2% of total lipid at 7 nmol of protein assayed, whereas 0.28 nmol of ScSec14 (positive control) had a robust [^{3}H]-PtdIns transfer activity of 32.4 % (Figure 3.4A). As the heterotypic

exchange cycle of ScSec14 utilizes PtdCho as the counter-ligand, the ability of TgSfhA to transfer [^{3}H]-PtdCho was also measured, however, no [^{3}H]-PtdCho-transfer activity was detected (Figure 3.4B). In a second in vivo biochemical assay, TgSfhA was tested for the ability to stimulate PI4K to generate PtdIns4P at the TGN . The *sec14-1^{ts}* mutant yeast strain was used to test whether this ScSec14-dependent physiological phenotype can be rescued by exogenous expression of *TgSFHA* during non-permissive temperatures (37 ºC) (Schaaf et al., 2008). Expression of a wild-type *ScSEC14* (positive control) rescues growth at 37 °C. However, expression of *TgSFHA* did not restore growth at the restrictive temperature (Figure 3.4C). This indicates that PtdIns exchange by TgSfhA is not sufficient to stimulate ScSec14-dependent PtdIns4P production at the TGN in yeast.

Furthermore, due to a relatively high protein sequence homology between TgSfhA and ScSfh4 detected in previous in silico analyses, TgSfhA was tested for its ability to function in an ScSfh4-dependent context. ScSfh4 regulates the production of phosphatidylethanolamine (PtdEtn) at the phosphatidylserine (PtdSer) decarboxylation step via a PtdIns4P homeostatic mechanism that influences PtdSer accessibility to PtdSer decarboxylase 2 (Psd2). The deletion of *ScSFH4* and *PSD1* disturbs PtdEtn production through the Psd1- and Psd2-dependent pathways, conferring ethanolamine (Etn) auxotrophy on this mutant strain (Wang et al., 2020). *TgSFHA* expression in the Δ*Scsfh4*Δ*psd1* yeast strain did not reverse the Etn auxotrophy compared to (Figure 3.4D). Thus, TgSfhA cannot function in the ScSfh4-dependent context in vivo, despite the relatively high homology.

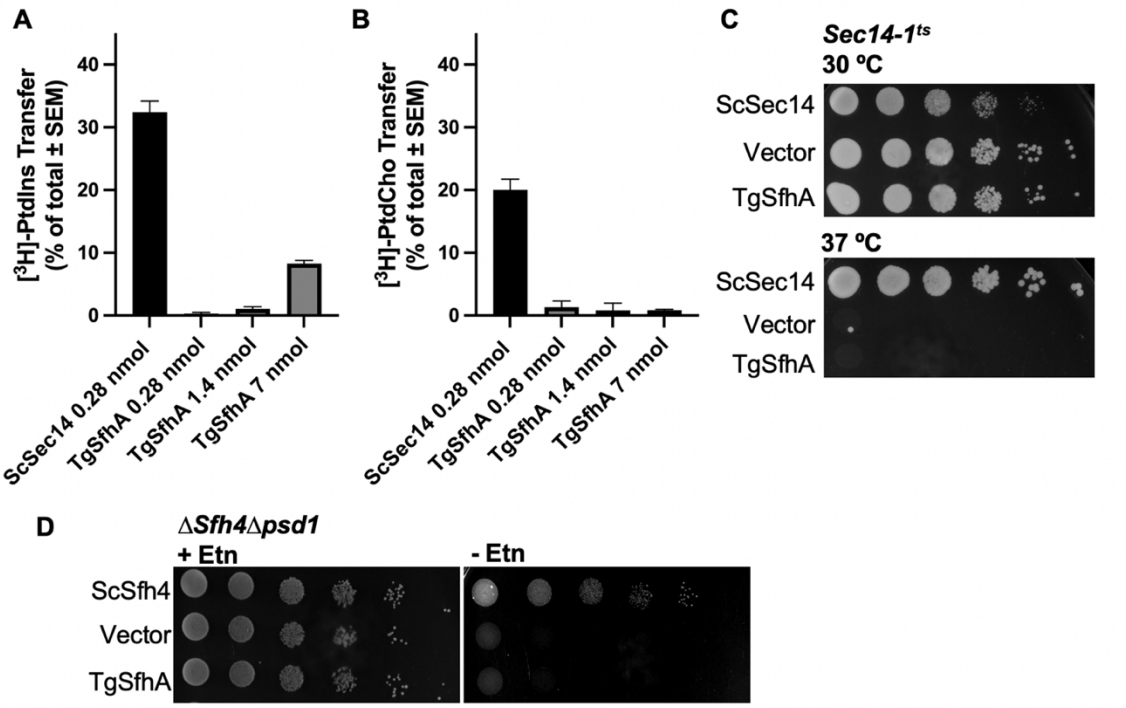

Figure 3.4 PtdIns-binding activity of Sec14-like PITPs is not conserved in TgSfhA. (A) Purified TgSfhA protein was assayed for PtdIns transfer from rat liver microsomes to acceptor vesicles. [^{3}H]-PtdIns input in this assay ranged from 8 to 15 x10^3 cpm, and background ranged from 6 to 8 x10^2 cpm. Values represent averages from three independent experiments plotted as mean ± SEM. (B) TgSfhA protein was assayed for PtdCho transfer from acceptor vesicles to BHMs. [^{3}H]-PtdCho input in this assay ranged from 8 to 13 x103 cpm, and background ranged from 0.8 to 2 x102 cpm. Values represent averages from three independent experiments plotted as mean ± SEM. (C) *sec14-1^{ts}* yeast cells transformed with a mock vector or vectors carrying *ScSEC14* or *TgSFHA* were spotted in 10-fold dilution series on YPD agar medium and incubated at the permissive and restrictive temperatures of 30°C and 37°C, respectively. Growth at 37°C signifies that the protein of interest is competent for PtdIns binding/exchange. (D) Δ*Scsfh4*Δ*psd1* yeast cells transformed with a mock vector or vectors carrying *ScSFH4* or *TgSFHA* were spotted in 10-fold dilution onto uracil-free agar medium either supplemented with Etn (2 mM) or not as indicated. Failure to grow in the absence of Etn identifies failure in Psd2 pathway activity.

3.2.5. TgSfhA is registered in a subapical single puncta structure in *T. gondii*

To localize TgSfhA in *T. gondii*, a fluorescence reporter (YFP-TgSfhA) was designed and used to transiently transfect RH-WT. Confocal images of intracellular tachyzoites (Figure 3.5A, left panel) show that TgSfhA localizes to the cytoplasm and as well as to a single punctate structure in the subapical area of the tachyzoite (arrow). Co-expression of YFP-TgSfhA with the inner membrane complex 1 protein (RFP-IMC1) showed that in some parasites the TgSfhA-positive structure is positioned at the periphery of the parasite, possibly at the plasma membrane (Figure 3.5A, right panel). This is indicative of a mobile TgSfhA-enriched structure that relocates from the plasma membrane to the subapical cytoplasm or vice versa.

To further elucidate the localization of YFP-TgSfhA, co-localization experiments were performed using markers against known apical intracellular compartments. Cells stably expressing YFP-TgSfhA (referred to from now on as RH-YFP-TgSfhA) were successfully selected and used in the localization analysis. Figure 3.5B (panels i, ii, and iv) shows that the TgSfhA single structure does not colocalize with markers for secretory compartments – such as rhoptries (ROP1-RFP), micronemes (anti-MIC3) and dense granules (GRA2-RFP). The apicoplast, a compartment also located in this subapical area was shown to be distinct from the TgSfhA-enriched structure (Figure 3.5B, panel iii).

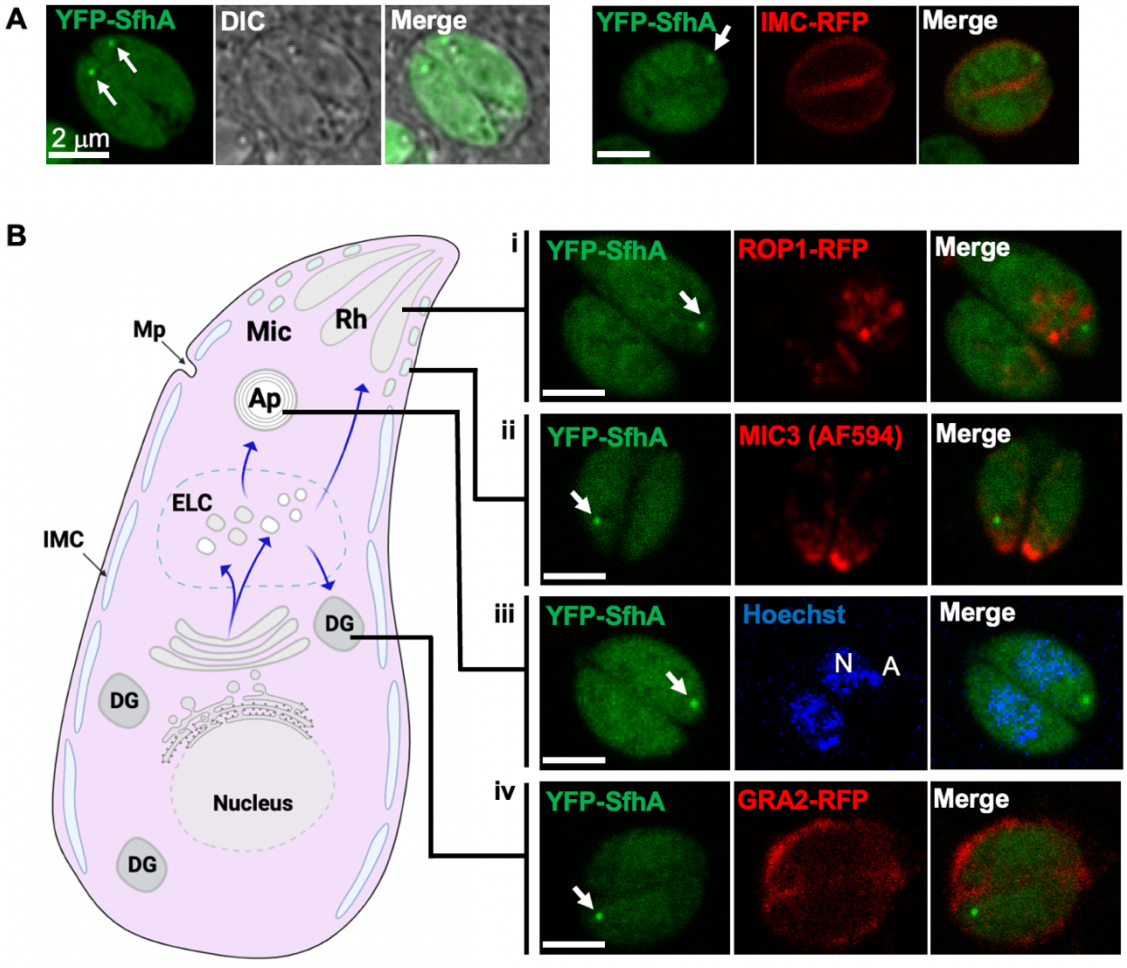

Figure 3.5 Localization of TgSfhA in intracellular tachyzoites. (A) Fluorescence confocal images of RH strain expressing YFP-TgSfhA (left panel) and co-expression with IMC-1-RFP (right panel). (B) Representative images of parasites stably expressing YFP-TgSfhA and stained for, or co-expressing, apical compartment markers: (i) ROP1-RFP; (ii) anti-MIC3; (iii) Hoechst and (iv) GRA2-RFP. Mp; micropore; Mic: microneme; Rh: Rhotry; Ap: apicoplast; DG: dense granule; ELC: endocytic-like compartment; IMC: inner membrane complex.

3.2.6. TgSfhA partially localizes to the endocytic system in extracellular parasites

Other compartments that are found in the apical area of the parasite are associated with endocytic trafficking. The endocytic system is active throughout the cell

cycle of the parasite; however, few cellular markers have been identified to label endocytic vesicles (Gras et al., 2019; McGovern et al., 2018). Recently ingested material reaches the lysosome-like or vacuolar compartment in *T. gondii*, as such it is recognized as an endocytic structure (McGovern et al., 2017). The cathepsin-like protease L (CPL) is a marker for this compartment, though staining against CPL is better visualized in extracellular tachyzoites (Figure 3.6A) (McGovern et al., 2018). Extracellular parasites expressing RH-YFP-TgSfhA were stained with anti-CPL and imaged in a widefield fluorescence microscope. TgSfhA localizes to multiple vesicular structures that in some parasites are CPL-positive (Figure 3.6B upper panel). These results suggest that TgSfhA localizes to the vacuolar or lysosome-like compartment of extracellular parasites.

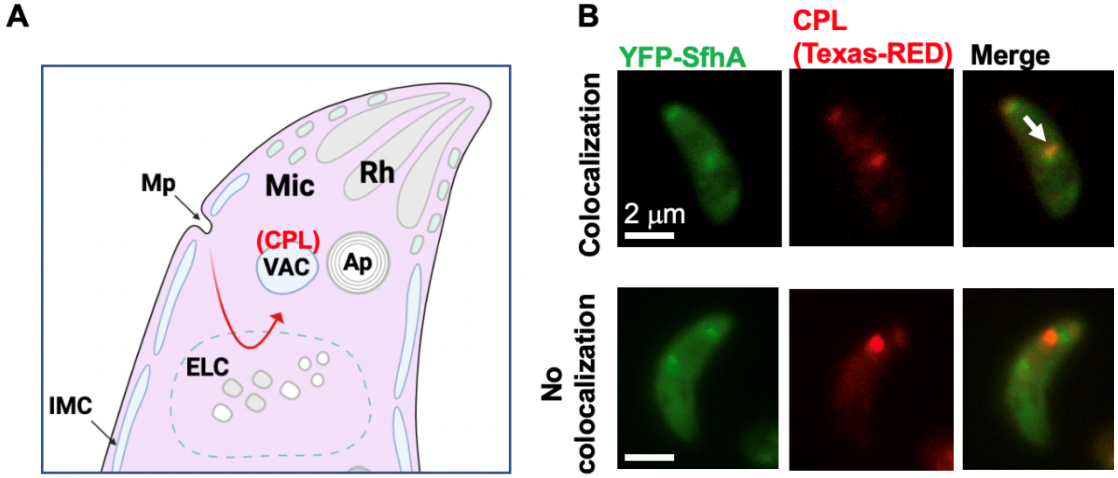

Figure 3.6 TgSfhA is detected in vacuolar compartment of extracellular tachyzoites. (A) Cartoon of a *T. gondii* tachyzoite showing trafficking of endocytosed material (red arrow) through the ELC and reaching the vacuolar compartment. (B) Extracellular RH-TgSfhA showing co-localization (arrow in upper panel) and no colocalization (lower panel) with anti-CPL.

3.2.7. TgSfhA is not essential

A genome-wide genetic screen assessed the contribution of each gene to parasite fitness during infection of human fibroblasts (Sidik et al., 2016). of the fitness score for *TgSFHA* is - 0.23, just above the cut-off value to classify a gene as essential (< -1.5). In order to determine whether function of TgSfhA is essential in tachyzoites, the *TgSFHA* endogenous promoter was replaced with a Tet-repressible promoter to control *TgSFHA* expression (Jacot and Soldati-Favre, 2020) (Figure 3.7A). This strain is referred to as *Tgsfhai* from now on. In addition to promoter replacement, the genomic insertion also fuses the endogenous *TgSFHA* with a 5' Myc tag sequence, allowing for detection of the resulting protein by western blotting. The replacement of the *TgSFHA* endogenous promoter was confirmed by the successful disruption of TgSfhA expression after the addition of ATc in two different *Tgsfhai* mutant strains (Figure 3.7B). Parasite growth was examined in a plaque assay 7 days post-infection with 50 tachyzoites of *Δku80* (control), *Tgsfhai1* and *Tgsfhai2* mutant strains. As shown in Figure 3.7C and 3.7D no significant difference in the number of plaques formed per condition was observed in *Tgsfhai* parasites in the +ATc condition compared to either the -ATc condition or to *Δku80* cells. These data confirm that expression of *TgSFHA* is not essential for parasite survival and growth.

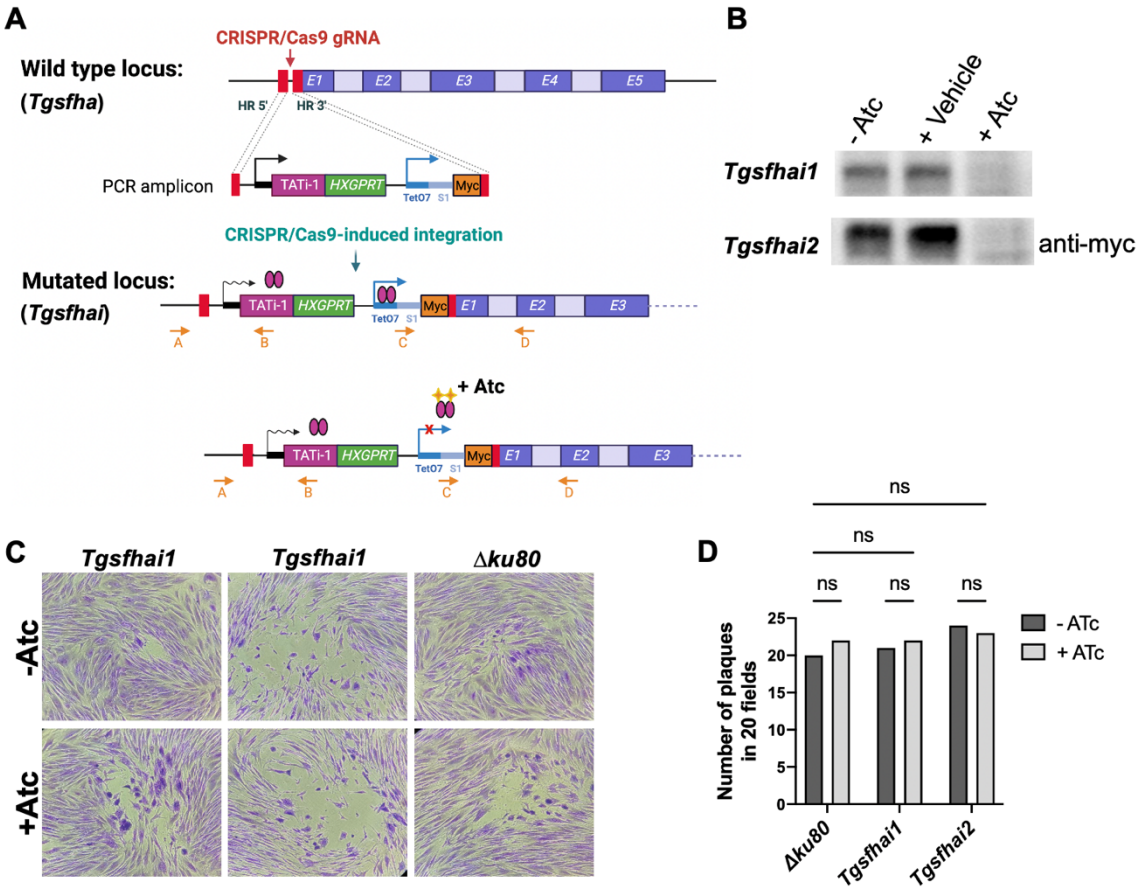

Figure 3.7 Conditional knockout of *TgSFHA* does not affect parasite growth. (A) Schematic representation of the tetracycline repressor-based inducible knockdown strategy. A PCR amplicon that contains all the elements for transactivation and permits N-terminal myc tagging of TgSfhA was inserted upstream of the start codon by double homologous recombination assisted by CRISPR/Cas9. The new tetO regulatable promoter is only deactivated in the presence of Anhydrotetracycline (ATc) which releases TATi-1 from its physical interaction with the promoter. (B) Diagnostic immunoblot of *TgSFHA* expression in *Tgsfhai* parasites (clone 1 and 2) 48 hr post-addition of ATc with antibody against the Myc tag. (C) Growth assay was performed in *Δku80* (control) and *Tgsfhai* parasites (clone 1 and 2) with or without ATc. (D) Quantification of the number of plaques formed in 20 microscopic fields (10x objective) for *Δku80* (control) and *Tgsfhai* mutants (clone 1 and 2) in the absence and presence of ATc. Data show single experiment; $p > 0.05$ (ns), Two-Way ANOVA.

3.3. Discussion

Much of what is known about PITP-dependent regulation of PIP signaling is based on studies performed in current eukaryotic model organisms, e.g., mouse, , yeast, and plants. These models belong to the animals (Metazoa), fungi lineages, and land plants, respectively, which emerged at least 500 million years after protozoa, e.g., Apicomplexa, originated in the eukaryotic tree of life (Sebé-Pedrós et al., 2017). As a result, the current bodies of work describe relatively recent evolutionary events in PI/PIP signaling and PITP functional mechanisms.

The principle of how Sec14-like PITPs regulate PIP signaling was first described in ScSec14, the founding member of the Sec14-like PITP protein family. ScSec14 binds PtdIns and PtdCho to establish a heterotypic exchange cycle on membrane surfaces. This activity exposes PtdIns to the inefficient PtdIns 4-OH kinase to stimulate PtdIns4P synthesis. The identity of the ScSec14 secondary ligand serves as signaling input into the PITP exchange cycle (Bankaitis et al., 2010; Schaaf et al., 2008; Sugiura et al., 2019). The phospholipid binding/exchange reaction based on a two-ligand priming mechanism is an evolutionarily conserved feature among Sec14-like proteins (Huang et al., 2016). However, the recent expansion in the characterization of new Sec14-like PITP family members has led to the discovery of "non-canonical" Sec14-like proteins that either potentiate PtdIns 4-OH kinase activities only through physical interactions with the kinase (Wang et al., 2020) or those bind non-lipid ligands such as heme (Khan et al., 2020). Many questions have been raised in response to these observations, such as: did typical Sec14-like proteins appear first? Or did canonical and non-canonical Sec14-like

proteins originate in the same primitive organism or lineage? How has evolution shaped the ligand binding specificity of each Sec14-like PITP type? These questions are especially important for deciphering PIP signaling diversification and the emergence of PIP regulatory mechanisms in eukaryotes. The use of an early divergent eukaryotic model such as Apicomplexa to characterize Sec14-like proteins is an opportunity to answer key questions about the evolution of this protein family, such as: was stimulation of PI4P synthesis the original role of Sec14 proteins? Is the PtdIns-binding barcode invariably conserved?

3.3.1. The diversity of Sec14-like multidomain proteins in *T. gondii*

The protein architecture and sizes of *T. gondii* Sec14-like candidates described in this Section are more diverse than those described in the yeast *S. cerevisiae*. In *T. gondii,* at least four out of the ten candidates have additional modular structures or domains, e.g., the GOLD, PH, and Acetyl-CoA binding domains. Multidomain Sec14-like PITPs are often encoded in high eukaryote organisms, such as humans and plants (Curwin and McMaster, 2008; Montag et al., 2020). A phylogenetic study in land plants showed that the occurrence of multidomain Sec14-like-PITPs increased in more evolved green species, as did the protein modular complexity. This association was linked to more recently evolved species that require additional mechanisms to integrate developmental and environmental signals to control Sec14-like-PITP-mediated membrane identity (Montag et al., 2020). Although *T. gondii* evolved from a primordial eukaryotic cell at an earlier stage than yeast and plants and is often considered a

hypothetical model for the last eukaryotic common ancestor (White and Suvorova, 2018), this parasite has overcome constant adaptations to new environments, specifically the infection of multiple emerging host species. *T. gondii* has a wide range of host species, including all warm-blooded animals (intermediate hosts) and felines, which are the definitive hosts for sexual reproduction. Thus, the enrichment of Sec14-like PITP candidates in the parasite genome, in addition to the size diversity and multiple domain configurations of these proteins, may reflect increase in the complexity of Sec14-like PITP function and mechanisms. Possibly, the need for the development of unique lipid binding specificities and protein-membrane associations was required for the establishment of new host–parasite interactions. Furthermore, half of these *T. gondii* Sec14-like PITP candidates are reported to be essential during the tachyzoite stage of the life cycle, the pathogenic stage responsible for toxoplasmosis (Sidik et al., 2016). This is contrary to what has been observed for the yeast Sec14-like PITPs, where the only reported essential member is ScSec14, the founder protein of the family. These observations indicate that multiple and indispensable cellular processes rely on the activity of Sec14-like proteins in *T. gondii* parasites.

In the parasite Sec14-like phylogenetic tree, TgSfhA falls into the third clade (TgSfhC/B/F/A/I/D) along with candidates containing complex protein architectures. This shows that TgSfhA is more closely related to these multidomain candidate. As previously indicated the emergence of multidomain PITPs is associated with new functions in an organism (Montag et al., 2020). Thus, location of TgSfhA in this multidomain cluster would indicate that TgSfhA is one of the last single-domain Sec14-

like candidates that emerge in the parasite. This may explain why TgSfhA is the closest *T. gondii* ortholog to Sec14-like proteins in more recently divergent eukaryotic organisms, like yeast.

3.3.2. A non-canonical Sec14-like protein in *T. gondii*

The inability of TgSfhA to stimulate PtdIns4P signaling at the TGN in yeast indicated that TgSfhA does not function like the canonical ScSec14. The BLAST analysis revealed that the closest orthologs of TgSfhA are the yeast ScSfh4 and ScSfh3 proteins. Both yeast proteins transfer PtdIns in vitro (with ~30% that of ScSec14) and stimulate basal PtdIns4P levels in yeast (Ren et al., 2014; Wu et al., 2000). However, these are considered non-canonical Sec14-like PITPs, due to the fact that ScSfh3 exchange activity antagonizes the classical ScSec14-dependent PtdIns4P signaling at the TGN (Ren et al., 2014), and in the case of ScSfh4, its PtdIns-binding activity is dispensable for stimulating the specific biological outcome it is linked to – Psd2-dependent PtdSer decarboxylation (Wang et al., 2020). The ability of TgSfhA to bind/exchange PtdIns between membranes was detected at 25-fold higher protein levels than those of ScSec14. And like ScSfh3, TgSfhA showed ~30% of the PtdIns-transfer rate for ScSec14. The fact that elevated concentrations of TgSfhA never induced PtdCho transfer demonstrated that the transfer of PtdIns by TgSfhA was not the result of secondary effects associated with protein overload in the reaction, suggesting that the low PtdIns transfer activity is nonetheless real. Possibly optimization of the in vitro lipid exchange assay to more closely mimic *T. gondii* membrane composition might allow

TgSfhA to transfer PtdIns more readily. Although *T. gondii* possesses the neutral and polar lipid species generally found in eukaryotic cells, it also produces unique lipids (Coppens et al., 2000; Coppens et al., 2014). Thus, it is tempting to speculate that the presence of Apicomplexan-unique lipids or proteins is required to generate a specific membrane physicochemical environment to allow recruitment of TgSfhA to vesicles in order to establish robust PtdIns exchange events.

Although mass spectroscopy analysis of purified TgSfhA from *E. coli* indicated that this protein was bound to an abundant *E. coli* membrane phospholipid, the correct protein folding should be confirmed through the analysis of the TgSfhA circular dichroism spectra (Greenfield et al., 2006). Furthermore, the TgSfhA-PtdIns binding interaction must be validated by resolving the TgSfhA crystal structure after incubating the protein with PtdIns to determine protein-lipid interaction (Sha et al., 1998).

3.3.3. Does TgSfhA sense or stimulate cholesterol metabolism in the host cell?

The significant homology between TgSfhA and two known PITPs that act as modulators of cholesterol metabolism – Sec14-L2 and ScSfh3 – was an important observation as *T. gondii* does not produce cholesterol, although it has the enzymatic machinery to produce steroid precursor molecules (Ling et al., 2007). Cholesterol is essential for the replication and development of the parasite upon host cell infection, thus, cholesterol must be scavenged from the host cell (O'Neal et al., 2020; Coppens et al., 2000; Coppens et al., 2014). *T. gondii* relies on ATP-binding protein transporters to translocate cholesterol molecules through the parasitophorous vacuole membrane (PVM)

(Ehrenman et al., 2010). Sec14-like PITPs exchange lipids without requiring ATP hydrolysis. Could TgSfhA play such a role in parasite sterol metabolism? Lipid transfer assays showed that the primary ligand is probably not squalene or cholesterol as these lipids were inefficiently exchanged between membranes. Then what is the ligand for TgSfhA? Considering the higher folding protein homology score with Sec14-L2 and relative higher rate of squalene transfer among the tested lipids in this work, a potential ligand may be another intermediate molecule of the cholesterol synthesis pathway, the mevalonate pathway, or possibly a species of sterol other than cholesterol (Goldstein and Brown., 1990). In extracellular parasites, TgSfhA localizes to the vacuolar compartment (or lysosome-like organelle) (Lamarque et al., 2008; McGovern et al., 2017). As such, TgSfhA is poised to interact with intermediate sterol molecules that are internalized from the host cell through endocytosis. Could TgSfhA be involved in sensing intermediates of cholesterol that are obtained from endocytosis of host cell content? This can be tested by tracking a neutral lipid dye like Nile Red (Greenspan et al., 1985) in parasites expressing YFP-SfhA. Further, the identification of the primary and secondary lipid ligand should be determined though mass spectral analysis of purified TgSfhA from a eukaryotic expression system, ideally from the parasite itself or the mammalian host.

Alternatively, in a mechanism similar to that of Sec14L2 or ScSfh3, TgSfhA may bind an as-of-yet unidentified steroid as part of a presentation mechanism to trigger conversion of another signaling molecule that is involved in the regulation of cell signaling pathways in the parasite such as lipid metabolism, cell cycle, egress, motility,

and others (Ren et al., 2014; Zingg et al., 2014). Collection of biological evidence is required to test this possibility. The experiments to identify the cell signaling pathways in which TgSfhA functions could utilize the *Tgsfhai* strain for transcriptomic analysis. Two experimental setups are informative: (1) during TgSfhA downregulation (*Tgsfhai*, +ATc) under normal host cell conditions, and (2) during wild-type TgSfhA conditions (*Tgsfhai*, -ATc) in the absence of cholesterol intermediates, by infecting host cells with deprived cholesterol synthesis but supplemented with cholesterol. Transcriptomic read outs in the first experimental condition will inform which cellular pathways overall are associated with TgSfhA function. The correlation of this data with the second transcriptomic analysis condition would test whether absence of the prospective lipid ligand "cholesterol intermediate" also identifies similar pathways. These questions can also be addressed using protein-protein interaction assays such as the APEX-catalyzed biotinylation (Hung et al., 2016).

One of the roles of Sec14-L2 is to potentiate a late-stage reaction of cholesterol synthesis, specifically the oxygen-dependent epoxidation of squalene to form 2,3-oxidosqualene (Friedlander et al., 1980; Shibata et al., 2006). Therefore, another prospective role for TgSfhA involves the modulation of host cell cholesterol metabolism. Levels of cholesterol in *T. gondii*-infected host cells have not been directly measured (Coppens et al., 2000), however, *Eimeria bovisi* (proliferation stage)-infected cells have high rates of cholesterol synthesis (Tauber et al., 2018). The mevalonate pathway takes place in the ER in mammalian cells, and in infected cells, the host ER is physically associated with the PVM (Kaplan and Simoni, 1985; Sinai et al., 1997).

Could TgSfhA be secreted and trafficked to the host ER to manipulate this pathway? An important caveat of this model is that there is no evidence of secretion from *T. gondii* lysosome-like organelles, where TgSfhA partially localizes. Furthermore, localization of YFP-tagged TgSfhA to the host ER was no observed, but immunofluorescence against the endogenously-tagged protein would inform whether or not TgSfhA indeed associates with the host ER. Furthermore, staining with a neutral lipid dye such as Nile Red would test whether TgSfhA localizes to neutral lipid rich regions.

The occurrence of redundant cholesterol acquisition mechanisms in *T. gondii* reveals how critical cholesterol uptake is for this organism. Besides ATP-binding protein transporters that translocate cholesterol to the parasite vacuole, unidentified factors in *T. gondii* increase expression of host cholesterol biosynthetic enzymes (Blader et al., 2001). The presence of these multiple mechanisms may explain the dispensable role of TgSfhA observed in the knockdown experiments presented in this work, if it is indeed associated with hijacking or regulating cholesterol from host cell.

3.3.4. The variety of steroid molecules in mammalian cells as potential lipid ligands of TgSfhA

Sec14-L2 is also well known to bind the steroid molecules Vitamin E or α-tocopherol (αT) and its phosphorylated derivative (αTP) (Zingg et al., 2014). Because TgSfhA specifically, though inefficiently, binds squalene, it's possible that the true ligand is a related molecule. Sterol derived molecules are mainly produced in animal cells or consumed by these organisms; thus, these compounds should be considered as

prospective lipid ligand of TgSfhA and be tested in transfer lipid assays. Some of these include glucocorticoid and mineralocorticoid hormones, cholecalciferol or vitamin D and neurosteroids. These molecules are important regulators of gene expression in mammalian cells and control multiple cell physiological processes (Falkenstein et al., 2000).

3.3.5. The study of TgSfhA and the functional adaptations of Sec14-like PITPs in the evolution of eukaryotic cells

Initially, PITPs were described as lipid carriers, however, it is now clear that Sec14 PITP-like family members regulate PtdIns kinase activities and the metabolism of diverse lipids in the cell. The multiple biological functions associated with each Sec14 PITP-like member are based on the lipid presentation mechanisms and on physical associations with other lipid-binding enzymes. The diverse Sec14-like-dependent biological functions described in the current model organisms (yeast and higher eukaryotes) have been associated only with modulation of their own cell signaling relay and lipid metabolism. The characterization of TgSfhA in this Section is the first report of a PITP encoded in an Apicomplexa pathogen. Investigating the role of TgSfhA in the parasite/host cell and its association with cholesterol metabolism and cell signaling holds the potential to uncover primitive and conserved Sec14-like PITP regulatory mechanisms. According to phylogenetic analysis, TgSfhA is a relatively recent member of the *T. gondii* Sec14-like cohort, thus characterization of this candidate would also reveal the capacities of *T. gondii* to take advantage host lipid metabolism – specifically

of sterol related-molecules – and evolve developmental and physiological mechanisms to adapt to new environments.

3.4. Materials and methods

3.4.1. Generation of plasmid constructs

Plasmids are listed in Supplementary Table S3.1, and a comprehensive list of the primers used in this study is provided in Supplementary Table S3.2.

3.4.2. Yeast strain and media

Yeast strains utilized are described in detail elsewhere (Bankaitis et al. 1989, Wang et al., 2020.; Tripathi et al., 2019), and included: *CTY1-1A* (*MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts*), *PYY19* (*MATa ura3-52 lys2-801 his3-Δ200 Δsfh4::URA3 Δpsd1::kanMX*) and Δ*sfh2* (*MATa ura3-52 lys2-801 his3-Δ200* Δ*sfh2::G418*). Media included YPD (1% yeast extract, 2% bactopeptone, and 2% glucose) and minimal SD (0.67% nitrogen base, 2% glucose with amino acids supplemented).

3.4.3. Protein purification

pET28b-His8-TgSfhA was transformed into the E. coli BL21 system (RIL/DE3; New England BioLabs Inc, Ipswich, MA). Transformed cells were cultured overnight at 37°C and subcultured into 3L of LB media supplemented with 50 μg/mL kanamycin and 35 μg/mL chloramphenicol. Cells were grown for 4 hrs at 37°C, then induced with IPTG (100μM final concentration) and incubated again for 6 – 8 hrs at 37°C. Cultures were harvested and cells lysed by a French Press cell Press (Thermo). Clarified lysates were incubated with TALON metal affinity beads (Clontech, Mountain View, CA), and bound proteins were eluted using a 25–200 mM imidazole step gradient regime. Protein

concentration and protein mass was estimated by SDS-PAGE and Coomassie staining using BSA titration series as standards.

3.4.4. Lipid transfer assays

Transfer assays measured [^{3}H]-PtdIns transport from rat liver microsomes to PtdCho liposomes. And [^{3}H]-PtdCho, [^{3}H]-Cholesterol or [^{3}H]-Squalene from liposomes (98-99 mol% PtdCho, 1-2mol% [^{3}H]-lipid) to bovine heart mitochondria (BHM) (Aitken et al., 1990; Bankaitis et al., 1990; Schaaf et al., 2008; Tripathi et al., 2019).

3.4.5. Bioinformatic analysis and Homology modeling

T. gondii Sec14-like homologs and its protein architecture were identified using Interpro database tool in the ToxoDB (v60) (https://toxodb.org/toxo/app/). All protein sequence alignment were performed with protein-protein BLAST algorithm (https://blast.ncbi.nlm.nih.gov/). Protein structural alignments were carried out within the MOE software package version 2013.08. Homology model of TgSfhA was generated with the MOE 2013.08 modeling package. The target TgSfhA sequence was threaded on the dimeric ScSfh3 crystal structure (2.34 Å, PDBe code: 4fmm) in an open conformation and bound with PtdIns

3.4.6. Cell culture and *T. gondii* strains

ATCC human foreskin fibroblast-1 (HFF-1) cell line was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), incubated at 37°C with

5% CO2 in humidified air. The *T. gondii* RH, *Δku80*, *Tgsfhai1* and *Tgsfhai2* strains were maintained by serial passage in HFF-1 monolayers. Parasites were cultured to egress and purified from host cell lysate by passage through 18G and 25G needles and pelleting at 2K rpm for 5 minutes.

Generation of transgenic parasites selection of stably transgenic parasites RH strain was transfected by electroporation as described by (Soldati and Boothroyd, 1993) with *T. gondii* expression vectors containing an organelle fluorescent marker utilized the pTUB-CAT (Chloramphenicol acetyltransferase) system. In an electroporation cuvette 107 tachyzoites were mixed with 25 μg of plasmid in transfection buffer (120mM KCl, 150μM CaCl2, 5mM MgCl2, 2mM EDTA, 25mM HEPES KOH, 10mM KPO4). For co-transfections, 12.5 μg of each plasmid were used. Then, an electrical pulse of 1.3kV and 25uF was applied using the Bio-Rad Gene Pulse II electroporation apparatus. Parasites were allowed to recover on HFF-1 monolayer in the absence of drug for 24 hours. When needed, selection of stable transgenic parasites was carried out in the presence of a selection drug: chloramphenicol (20 μM); mycophenolic acid (25 μg/ml) with xanthine (25 μg/ml). Stable clones were isolated by limiting dilution under drug selection.

3.4.7. Immunofluorescence analysis (IFAs) and western blot

Confluent HFF-1 host cells grown in 35mm glass-bottom coverslip dishes were infected with tachyzoites for 24 hrs, washed 1X with phosphate buffered saline (PBS) 1X and then fixed with 4% PFA (v/v) for 15 min at room temperature and washed 3X

(all washing incubations were performed for 10 min with PBS 1X solution). Cells were permeabilized with 0.2% Triton-X (v/v) for 4 min at RT, blocked with 2% BSA in PBS 1X overnight at 4°C, incubated with organelle-specific primary antibodies diluted in blocking buffer for 1 hour. Details regarding the primary and secondary antibodies used in this study are listed in Supplementary Table S3.3. Coverslip were washed with PBS three times, incubated with conjugated secondary antibodies listed in Supplementary Table S3.3 in blocking buffer for 1 hour, and washed again with PBS three times. Cell DNA was stained with Hoechst solution (1:5000) for 5 min. After three washes with PBS, coverslips were flooded with PBS and stored at 4˚C for no longer than seven days.

Intracellular parasite extract was obtained from HFF-1 cells infected for 24 - 29 hours. Cells were scrapped, collected, and lysed to release parasites using a 22G needle. Parasites were centrifuged 5 min at 2000 rpm. Supernatant was discarded, and pellet was resuspended in 100 µl of lysis buffer (NaCl 150 Mm, TX-100 1%, sodium deoxycholate 0.5%, SDS 1%, and Tris pH8 50 Mm). Lysis of parasites was carried out by 3 rounds of sonication set at 20% for 10 seconds. Lysate suspensions were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 2% BSA in TBST (Tris buffer saline solution, 0.1% Tween 20) at room temperature for 1 hour and then incubated with primary antibody and then incubated with secondary antibody (Details regarding the primary and secondary antibodies used in this study are listed in Supplementary Table S3.4). Membranes were revealed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo

34095) and detection of chemiluminescence was performed with Bio-Rad ChemiDocTM XRST Imaging system.

3.4.8. Image acquisition:

Confocal images were collected in a NikonA1R microscope (CFI Plan Apo lambda 60x/1.4 oil objective). While imaging processing was performed with ImageJ (FIJI) (NIH).

3.4.9. Assessment of Parasite Growth Using Plaque Assay

Monolayers of HFF, grown in six-well plates, were infected with 50 tachyzoites per well. After 7 days of incubation at normal growth conditions (37 ºC, 5% CO2) in the presence or absence of ATc. Cells were fixed 5 min with - 20 ºC methanol 100%, stained with crystal violet solution (2% [wt/vol], pH 7.4) for 10 min at room temperature, and washed once with PBS. The number of plaques per 20 fields using an 10X objective was used as a measure of the parasite growth capacity.

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4. FINAL DISCUSSION

4.1. Overview

The functional roles, and mechanisms of synthesis and maintenance of PIP pools in Apicomplexan organisms are poorly studied. The present work aimed to uncover functional roles of uncharacterized PIPs and PIP signaling regulation based on Sec14-like-dependent mechanisms.

To accomplish these objectives, an initial review (Section 1) was presented to compare PIP signaling regulation between current eukaryotic models, e.g., mammalian and yeast cells, with tractable Apicomplexan organisms. Reports on PtdIns and PIP kinases, PIP phosphatases and regulatory factors in Apicomplexa are sparse. Most of the information was collected in the intraerythrocytic stages of *P. falciparum* parasites and it is evident that fewer PIP species have been detected and mapped in *T. gondii* and in other members of the phylum, e.g., *Cryptosporidium* spp. For example, the biological function of 4'-OH phosphorylated PIPs, notably PdIns4P, has not been thoroughly investigated in *T. gondii* or Apicomplexa in general. Moreover, formation and trafficking of secretory vesicles in higher eukaryotes and yeast is regulated by PtdIns4P, and Apicomplexans are considered professional secretory organisms. Hence, the first major objective of this work was to characterize PtdIns4P in *T. gondii* and address its role in membrane trafficking of secretory organelles (Section 2). To elucidate PtdIns4P distribution with high sensitivity, three independent biosensors with distinct PtdIns4P-specific binding modules led to the identification of PtdIns4P pools at the Golgi/TGN

and post TGN, and the appearance of a LAP body only detected when using relatively high affinity PtdIns4P biosensors. The exclusive recruitment of DG cargo to the LAP body demonstrated that accessibility of post-TGN PtdIns4P pools was required for the complete formation and maturation of DGs. Strikingly, these findings were incompatible with current models for DG formation, which are based on direct budding of DG from TGN without intermediate immature DG vesicles. These models derive from the lack of morphological evidence for discrete steps in DG biogenesis. The identification of such an intermediate, trapped by PtdIns4P sequestration as described in Section 2, indicates that DG formation in *T. gondii* is a more complex process than that envisioned by simple direct budding models. The functional role of PtdIns4P in *T. gondii* therefore appears conserved with respect to the regulatory function of PtdIns4P in the secretory pathway of higher eukaryotes and in yeast. This observation raised a key question: are regulatory mechanisms of PIP signaling in mammals and yeast also conserved in Apicomplexa? Canonical or typical Sec14-like PITPs regulate PtdIns4P signaling at the TGN by presentation of PtdIns to a PI4K. Thus, the second major objective in this work was to characterize a close *T. gondii* Sec14-like PITP homolog to the yeast ScSec14 (Section 3) and analyze conservation of canonical PIP signaling regulation. Prior to this work, members of the Sec14-like PITP family in *T. gondii* have not been described. In silico analysis approaches revealed diversity of protein architecture in the cohort of *T. gondii* Sec14-like PITPs. This unexpected trait in an ancient unicellular organism indicates evolutionary complexity probably associated with adaptation and acquisition of new functions. Moreover, the characterization of TgSfhA – the candidate with the highest

protein sequence conservation to ScSec14 – revealed this is a non-canonical or atypical Sec14-like PITP. TgSfhA is most similar by primary sequence to the human Sec14-L2 and yeast ScSfh3 proteins. TgSfhA has some capacity to bind to a sterol precursor and may act as a modulator of sterol metabolism in the host cell. This is therefore the first indication of a PITP encoded by a pathogen that may potentially influence the PIP signaling and/or lipid metabolism of a host cell.

Finally, the aim of this Section 4 is to integrate the collected findings, to interpret the implications and value of exploration of PIPs in Apicomplexa and the regulatory mechanisms associated with Sec14-like PITP proteins, and to further understanding of the link between cell signaling and metabolism in primordial eukaryotes.

4.2. Apicomplexa as a model organism to explore evolutionary origins of PIP synthesis

The ever-expanding list of human disorders associated with defects in PIP-metabolizing enzymes serves as the finest illustration of the significance of PIPs in cell physiology and dynamic membrane remodeling (Nicholson et al., 2011; Madsen et al., 2018; Narkis et al., 2007). How do PIPs affect so many different cellular functions given that these makes up less than 1% of the entire phospholipid pool in mammalian cells? First, multiple PIP species – up to seven distinct species in eukaryotic cells – are generated from the phosphorylation of the inositol ring, which allows for specific protein interactions, with either cytosolic or intermembrane proteins, and further signaling diversification. Second, the low abundance and subcellular localization of these PIP

species establish biochemical identities for each cellular compartment. Third, these seven species can be interconverted resulting in a metabolic network with multiple interdependencies. The tight spatiotemporal control of these PIP interconversions – or PIP synthesis and turnover – is critically mediated by numerous and specific phosphatases and phosphoinositide lipid kinases (PIKs) that are activated and recruited upon unique cellular cues. All these evolutionary advantages have allowed PIPs to enable rapid cellular rewiring of membrane flux and metabolism to adapt to changing external and internal environmental conditions.

PtdIns/PIP production and utilization as a key component of membrane lipid signaling most likely occurred in an archaeal-eukaryote ancestor. The rationale is that phosphorylated derivatives of PtdIns have only been registered as membrane components in eukaryotic cells. Later, functions for PI and PIPs grew substantially after eukaryotes evolved and as they diversified (Michell, 2008). Apicomplexan lineages diverge from a proto-apicomplexan ancestor more than a billion years ago, probably soon after the appearance of the first eukaryotic ancestor, making these unicellular organisms older than the three multicellular kingdoms of animals, plants, and fungi (Escalante and Ayala, 1995). The understanding of the shape of the PIP signaling landscape in Apicomplexa (*T. gondii* and *Plasmodium*) holds the potential to reveal the ancestral blueprint for this major intracellular signaling system, and how it has been adapted to more complicated regulatory roles in higher eukaryotes.

4.3. The latest PIP discoveries in *T. gondii* and the contribution to deciphering the origins of endomembranous compartments in eukaryotes

The current endocytic system model in *T. gondii* states that a post-TGN compartment called the endosomal-like compartment (ELC) is a sorting hub that intersects TGN-delivered secretory cargo and ingested host cytosolic material. Further trafficking of the endocytosed material is directed to the lysosome-like structure or plant-like vacuole (McGovern et al., 2018). The ELC is labelled with vertebrate markers of early and late endocytic vesicles (Rab5 and Rab7, respectively), however, these factors only regulate the maturation of secretory material, such as microneme and rhoptry proteins, before reaching its respective storing organelles (Kremer et al., 2013; Parussini et al., 2010). The genes encoding other "classic" endocytic factors, like the ones forming the protein sorting complex or ESCRT machinery, are absent in the parasite (Leung et al., 2008). While only one putative clathrin chain gene is detected (CHC1) in *T. gondii*, it is associated with Golgi transport and not with endocytosis (Pieperhoff et al., 2013). PtdIns3P – a main recruiter of the ESCRT machinery to the endocytic membranes – is detected at the apicoplast and has no association with the endocytic pathway (Tawk et al., 2011). Due to this observation, some authors have suggested that the endocytic compartment in *T. gondii* has been repurposed to function in the secretory pathway due to a probable loss of "classical" resident proteins e.g., ESCRT machinery (Tomavo et al., 2014; Leung et al., 2008). However, there is not enough phylogenetic and biological evidence to confirm this evolutionary hypothesis (Spielmann et al., 2020). A reanalysis of this model might interpret the endocytic and

secretory compartments merging as a simplified or primitive version of the "classical" segregated membrane trafficking system described in vertebrate cell models. Interestingly, the fusion of endocytic and exocytic compartments at the TGN/post-TGN compartment is also observed in land plant cells (Zhuang et al., 2015), which arose earlier than metazoans. Regardless, ESCRT-related components associated with MVB, PtdIns3P- and clathrin-mediated endocytosis have been reported in plants (Kale et al., 2010; Chen et al., 2011; Zhuang et al., 2015). Endocytic mechanisms in plans and metazoans are more complex and more diverse than in *T. gondii* and other Apicomplexans, as these systems implement phagocytosis, pinocytosis, hormone receptor endocytosis and receptors recycling, just to mention a few (Field et al., 2007; Gáborik and Hunyady, 2004; Martins et al., 2015). While in *T. gondii* only endocytosis-mediated modification of the plasma membrane during motility and endocytosis at the micropore have been observed (Gras et al., 2019; Nichols et al., 1994); hence, a less complex endocytic pathway seems to be suitable for *T. gondii*. The synthesis of PtdIns3P at endocytic membranes appears to have contributed to the emergence and anchoring of multiple factors involved in the development of sophisticated compartment arrangements, which drove the evolution of the eukaryotic cell.

The compartments that constitute the conventional secretory pathway are highly conserved in eukaryotic cells (Zhuang et al., 2015; Schekman, 1982; Ellgaard et al., 1999; Becker and Melkonian, 1996). The general organization and flow of secretory proteins consists of the synthesis of secretory proteins in the rough ER, budding of protein-loaded vesicles from the smooth ER and fusion to the cis-Golgi, trafficking of

proteins to the TGN, budding of secretory vesicles, transport, maturation, and fusion with the plasma membrane (Rothman and Orci, 1992). The synthesis of PtdIns4P at the Golgi/TGN regulates vesicle trafficking from the TGN in yeast, mammalian, and plant cells (Wood et al., 2012; Wang et al., 2003; Ischebeck et al., 2008). Section 2 confirmed that PtdIns4P is present in the secretory pathway of *T. gondii* at the TGN and in an additional pool at a post-TGN compartment, and sequestration of these pools negatively affects DG maturation, biogenesis, and secretion. Contrary to the endocytic system, the secretory pathway in *T. gondii* conserves its "classical" Golgi phospholipid membrane marker – PtdIns4P. The Apicomplexan parasite *P. falciparum* also conserves PtdIns4P synthesis at the Golgi with a potential membrane trafficking regulatory role (McNamara et al., 2013). An interpretation of this observation is that cell mechanisms such as exocytosis in Apicomplexans evolved early, and so accumulated more critical functions with greater evolutionary conservation – e.g., PtdIns4P synthesis at the Golgi. A recently proposed model for the origin of the proto-eukaryotic cell, called the inside-out theory, suggested that secretion and exocytosis evolved before endocytosis (Baum and Baum, 2014). This argument was supported by cell biology and phylogenetic data analysis of the Ras superfamily (Rojas et al., 2012; Jékely, 2003; Boureux et al., 2007; Langford et al., 2002). The Ras superfamily is formed by three major clades: Ran, Sar1/Arf, and Rab/Ras/Rho, which can be interpreted as specializing in nuclear transport, secretion, and endocytosis, respectively (Rojas et al., 2012). Strikingly, Ran and Sar1 functions predated Rab and Rho functions (Jékely, 2003). Following these findings, it is reasonable to assume that exocytosis evolved before endocytosis in *T. gondii*, and most

likely in the proto-eukaryotic cell, and that the trafficking of synthesized proteins from the ER was regulated early in these cells. Early PtdIns4P-modulated cell secretion, in particular, may have resulted in the emergence of the conserved Sec14-like PITP family, allowing the connection of PtdIns4P signaling with acquired metabolic pathways.

4.4. The Sec14-like PITP family and diversified PIP signaling regulation in eukaryotes

How PIP pools are formed is a major question in contemporary cell biology. How are these maintained to establish cell internal communication? How is cell signaling diversified by the usage of a limited number of PIPs? While much effort is invested in understanding the enzymes that produce and consume PIP, fundamental aspects of how PIP production is regulated and physically organized define unappreciated gaps in our understanding. A potential answer to all these questions involves the role of PITPs. Sec14-like PITPs mediate instructive regulation of PtdIns 4-OH kinase activities, and channel PIP production to specific biological outcomes in yeast (Bankaitis et al., 2010). These proteins exert a novel and apparently well-conserved regulatory mechanism, the physiology and dynamics of which are not yet well understood in eukaryotic cells.

PITPs are evolutionarily conserved proteins that fall into two structurally distinct families: the Sec14-like PITPs, of which Sec14 is the founding member, and StAR-related lipid transfer (StART) PITPs (Schaaf et al., 2008; Yoder et al., 2001). These PITP families are structurally unrelated but include members that regulate PtdIns 4-OH

kinase activities through the heterotypic lipid exchange reaction between PtdIns and a secondary ligand (Schaaf et al., 2008; Bankaitis et al., 2010; Xie et al., 2018). Initial identification and characterization of ScSec14p, the founder of the Sec14-like PITP family, resulted in the description of the lipid transfer model for PITPs. In this model, the activity of PITPs is limited to the delivery of PtdIns from the ER to membranes engaged in PIP signaling (Cockcroft et al., 2007). Yeast biochemistry revealed that Sec14-like proteins instead function as nanoreactors that use the lipid-exchange cycle to expose PtdIns from the membrane bilayer to insufficient PtdIns kinases, making these kinases stronger enzymes on demand. This is exemplified by the Sec14 lipid heterotypic exchange cycle of PtdCho for PtdIns that stimulates the yeast PI4K (Pik1) to produce sufficient PtdIns4P to potentiate membrane trafficking (Schaaf et al., 2008; Bankaitis et al., 2010). Yeast genetics revealed that SEC14 is an essential gene. Further studies on "bypass Sec14" mutants demonstrated that secondary ligand-binding – PtdCho for ScSec14p – serves as signaling input into the PITP exchange lipid cycle to regulate PtdIns phosphorylation. Bypass Sec14 mutants are those in which specific gene deficiencies allow for cell survival in the absence of ScSec14. Several key bypass Sec14 mutations are in the CDP-choline pathway for PtdCho biosynthesis. This pathway consumes DAG, and adequate levels of DAG in TGN/endosomal membranes are necessary to initiate vesicle budding and exocytosis. When sufficient DAG levels are available for vesicle biogenesis at the TGN/endosomes and to be used as substrate for PtdCho production, Sec14 senses PtdCho production levels as metabolic information to drive pro-secretory PtdIns4P synthesis (Li et al., 2000). Importantly, phospholipid

binding analyses of the Sec14-like PITP protein family have shown a critical and conserved PtdIns barcode composed of residues that stabilize the PtdIns headgroup. These findings revealed a novel and direct regulatory cell signaling mechanism based on PITPs sensing and channeling lipid metabolic information to tightly regulate PIP signaling pathways.

The Sec14-like PITP mechanism of signaling regulation has also been described in human Sec14-like proteins, such as α-tocopherol transfer protein (α-TTP) and cellular retinaldehyde-binding protein (CRALBP), which prime other lipophilic ligands (α-tocopherol and 11-cis-retinal, respectively). Mutations within the PtdIns binding barcode of these proteins are associated with specific pathologies (Nile et al., 2010; Kono et al., 2013).

The yeast Sec14-like PITP system has been exploited to comprehend this unique and critical mechanism of PIP and lipid signaling regulation. Six proteins have been characterized in yeast. The collected data demonstrated that the PtdIns and PtdCho binding/exchange activities and the Sec14-like PITP-dependent PtdIns4P synthesis stimulation are not fixed rules in this family. The proteins that do not follow this described classical mechanisms are called non-canonical Sec14-like PITPs and these feature different ligands, metabolic regulation of other lipidic species, and lipid regulatory functions that uncouple from PtdIns binding activities. For instance, ScSfh3 is a yeast PITP that binds PtdIns to stimulate PtdIns4P production and to negatively regulate neutral lipid mobilization through inhibition of TAG lipolysis rate (Ren et al., 2014). In this case, the proposed Sfh3-regulated PtdIns4P pool regulates lipid droplet

metabolism. Another example is the non-canonical Sec14-like protein ScSfh4, which directly modulates Psd2-dependent PtdSer decarboxylation through physical association with Psd2. In this role, the PtdIns-binding/exchange activities of Sfh4 (and therefore its ability to stimulate PtdIns 4-OH kinase activity) are dispensable for Psd2 activation. In this second case, the biological function of a Sec14-like PITP is cleanly uncoupled from its canonical in vitro PtdIns-transfer activity (Wang et al., 2020). Overall, these data uncovered unusual Sec14-like PITP-dependent regulatory mechanisms and indicated that this family of proteins modulates a variety of lipid metabolic pathways, and that regulation is not restricted to the PtdIns-exchange activity. Moreover, the identity of lipid ligands among the Sec14-like PITP family is diverse. This is the result of hydrophobic lipid-binding cavities of Sec14-like PITPs presenting chemically distinct landscapes that translate to functional diversities for “second-ligand” binding (Tripathi et al., 2019). This feature allows Sec14-like PITPs to chaperone a tightly channeled "on-demand" synthesis of privileged, spatially discrete phosphoinositide pools – or other membrane lipids – dedicated to controlling specific biological outputs.

4.5. Apicomplexa as a model to study Sec14-like-dependent PIP regulation

Is stimulation of PI4P synthesis the original role of Sec14 proteins? Is the PtdIns-binding barcode invariably conserved? How has evolution shaped the ligand binding specificity of each Sec14-like PITP type? These questions are of great interest in the field of lipid signaling in eukaryotic cells. The protein architecture and sizes of *T. gondii* Sec14-like candidates described in this work are more diverse than those described in the

yeast *S. cerevisiae*. Curiously, multidomain PITP proteins are usually encoded in higher eukaryotes, while unicellular eukaryotic cells produce single domain PITPs. Multidomain PITPs serve to integrate more complex developmental and metabolic signaling networks with lipid signaling-dependent regulatory processes that emerged in higher eukaryotes (Montag et al., 2020). The finding of a Sec14-like PITP cohort with complex protein architectures in *T. gondii* is an indication that this parasite has overcome several adaptations to new environments – e.g., infection of multiple emerging host species – but still maintained basic and primitive physiological eukaryotic cell features. Thus *T. gondii* is a proper model to address evolutionary questions about Sec14-like PITPs as it would provide an understanding of how these proteins undergo diversification and versatility in an evolutionarily tractable organism.

4.6. PI4P signaling and Sec14-like proteins as therapeutic targets for intervention in Apicomplexan parasitic infections

Apicomplexa are an exceedingly effective group of obligate intracellular parasites that have a global impact on human health and disease. As a result, there is a strong incentive to research these parasites to lessen the morbidity and mortality they cause. The severe manifestations of *T. gondii* most often occur when tissue cysts (Bradyzoites) reactivate in the setting of immunocompromised diseases (Grant et al., 1990; Wang et al., 2017). The first-line therapy for this acute affection consists in highly toxic drug combinations, such as pyrimethamine and sulfadiazine with leucovorin, the side effects of which usually lead to discontinuation of the therapy as these impair

important host cellular pathways – e.g., synthesis of folate, DNA synthesis (Alday and Doggett, 2017; Pelphrey et al., 2007; Gwilt and Tracewell et al., 1998). Moreover, treatment of malaria – caused by *Plasmodium* – also consists in combination therapies that require primaquine, a drug capable of eliminating the hypnozoite reservoir in the liver, in combination with artemisinin and chloroquine. Although this is a potent therapy against malaria, it causes a high risk of hemolysis in patients, thus requiring blood transfusions (John, 2016). Besides the significant toxicity of these therapies, the emergence of resistant parasites to these treatments is on the rise (Montazeri et al., 2018; Nsanzabana, 2019)

The evolutionary analysis of PIP signaling in *T. gondii* indicated that synthesis of PtdIns4P in secretory compartments is an essential and conserved trait in the Apicomplexa phylum. Furthermore, PtdIns4P in these organisms carries out critical functions as regulator of membrane trafficking. The development of imidazopyrazine compounds resulted in the successful inhibition of Plasmodium PI4KIIIβ activity – the isoform that catalyzes PtdIns4P synthesis at the TGN – in both in vitro and in vivo conditions. This may therefore be an efficient and highly selective drug with potential to treat malaria in multiple stages of the parasite life cycle (McNamara et al., 2013). These data argue for the importance of characterization of *T. gondii* PI4KIIIβ and for the development of compounds that target this enzyme. Most of the anti-*T. gondii* therapeutics are ineffective against bradyzoites, and the cyst form persists in the tissues of healthy individuals. Thus *T. gondii* PI4KIIIβ is a prospective therapeutic target to help eliminate the tissue-cyst stage in compromised patients.

Finally, several lines of evidence point to Sec14-like PITPs as highly selective gateways for targeting PIP signaling and as underutilized routes for pharmacological suppression of specific PIP signaling pathways in cells. Studies in yeast provided evidence that NPPM-dependent inhibition of Sec14-PITPs offer significant advantages for chemically modifying PIP signaling, and that the selectivity attained is superior to those obtained by methods that target specific PtdIns-kinase isoforms or specific PIP species (Nile et al., 2014; Khan et al., 2016). Therefore, the pharmacological targeting of Sec14-like PITPs that are associated with PIP metabolism in *T. gondii* and other parasitic Apicomplexans may offer more effective therapeutics.

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5. CONCLUSIONS

This thesis summarized current knowledge of PIP signaling and regulatory processes in the Apicomplexa phylum, particularly in *T. gondii*. This work also presented the first report to address the location of PtdIns4P pools and to identify the functional role of PtdIns4P signaling, which was to promote TGN/post-TGN membrane trafficking and maturation of DGs in *T. gondii*. Moreover, the study of PtdIns4P in this ancient model organism indicated that the subcellular localization and functional role of this PIP isoform are well preserved. And secretion is a primordial function among eukaryotic systems.

The present work also reported the first description of the Sec14-like PITP cohort in an Apicomplexa organism. The in silico analysis of *T. gondii* Sec14-like PITPs revealed that although this organism conserves markedly ancient physiological features, it has overcome important adaptation processes. Thus, this makes *T. gondii* a proper model to study how ancient eukaryotic systems developed and conserved the Sec14-like PITP-dependent regulatory mechanisms. And how evolution shaped the lipid ligand binding capabilities of multiple PITPs to allow proper lipid signaling diversification in eukaryotic cells.

The development of new therapeutic strategies to treat infections caused by Apicomplexans is highly required. PIP signaling and Sec14-like PITP are recommended pharmacological targets, as these offer potential specificity and treatment of multiple parasitic developmental stages.

APPENDIX A

REGULATION OF PHOSPHOINOSITIDE METABOLISM IN APICOMPLEXAN PARASITES - SUPPLEMENTARY MATERIAL

Supplementary Table S1.1. Summary of PI, PIP kinases, PIP phosphatases and accessory protein homologues in Plasmodium spp. and *T. gondii*. (*) indicates putative orthologues found on the PlasmoDB with e-value < 10-5. (nf): not orthologue was found with e-value < 10-5. (nd): genes which phenotypic score was not determined in *P. berghei* and/or in *T. gondii* in their respective studies of wide genome screen for identification of essential genes (Bushell et al., 2016 and Sidik et al., 2016). (°) In *Plasmodium* and *T. gondii* when is not stated.

PIP	**Protein**	***P. falciparum***	***P. berghei***	**Growth rate phenoty-pes in *P. berghei***	***T. gondii***	**Phenoty-pic score in *T. gondii***	**Function in Apicomplexa (°)**	**Reference**
PtdIns3P	Class III PI3K/ Vps34	PF3D7_0515300 (PfPI3KIII)	PBANKA_111490 (PbPI3KIII)	Essential	TGME49_215700 (TgPI3KIII)	-3.88	Mitophagy and transport of Hb to the FV in *Plasmodium.* Apicoplast biogene-sis in *T. gondii.*	Tawk et al., 2010, 2011; Vaid et al., 2010; Daher et al., 2015

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns3P	Vps15	PF3D7_0823000*	PBANKA_010760*	nd	TGME49_310190*	-2.28		Besteiro et al., 2017
	Beclin-1/ Vps30	nf	nf		TGME49_221360*	0.41		Besteiro et al., 2017
	ATG14L	nf	nf		nf			
	UVRAG/ Vps38	nf	nf		nf			
	Kelch13	PF3D7_1343700 (PfKelch13)	PBANKA_1356700*	nd	nf		PfPI3K proteolysis	Mbengue et al., 2015

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns3P	AKT	PF3D7_1246900 (PfAKT)	PBANKA_1460000*	nd	TGME49_267540*, TGME49_286470*, TGME49_226030*	-0.78, -1.74 -3.04	PtdIns3P synthesis upregulation in *Plasmodium*.	Mbengue et al., 2015
	MTM	nf	nf		nf			
PtdIns(3,5)P_2	PIKfyve/ Fab1	PF3D7_1412400 (PfPIKfyve)	PBANKA_103020 (PbPIKfyve)	Essential	TGME49_256960 & TGME49_256920 (TgPIKfyve)	-3.33 -4.83	Apicoplast biogenesis/mainten ance in *T. gondii*	Daher et al., 2015
	Sac3 /Fig4	PF3D7_0802500*	PBANKA_1227700*	Significa-ntly slow	TGME49_256830*, TGME49_238400*, TGME49_316230*,	-4.73 -3.70 -1.35		

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns(3,5)P_2	ArPIK-fyve/ Vac14	PF3D7_1225700*	PBANKA_1440600*	Essential	TGME49_244040 (TgArPIKfyve)	-4.42	Apicoplast biogenesis/mainten ance in *T. gondii*.	Daher et al., 2015
	Vac7	nf	nf		nf			
	Atg18	PF3D7_1012900 (PfAtg18)	PBANKA_1211300*	Essential	TGME49_220160 (TgAtg18), TGME49_288600*	-1.46 2.23	Apicoplast biogenesis/maint-enance in *T. gondii.*	Bansal et al., 2017
PtdIns(3,4,5)P_3	Class I PI3K	nf	nf		nf			Tawk et al., 2010
	PTEN	PF3D7_1219000 (FRM2)	PBANKA_1434600*	nd	TGME49_206580 (FRM2)	-1.12	Apicoplast maintenance in *T. gondii*	Pathak et al., 2019; Stortz et al., 2018

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns(3,4,5)P_3	SKIP (INPP5K)	PF3D7_0705500*	PBANKA_0803200*	Essential	TGME49_238400*, TGME49_288800*, TGME49_293190*	-3.70 0.51 1.18		
	SHIP1/2 (INPP5D/ INPPL1)	PF3D7_0705500*	PBANKA_0803200*	Essential	TGME49_238400*, TGME49_293190*	-3.70 1.18		
	PIPP (INPP5J)	PF3D7_0705500*	PBANKA_0803200*	Essential	TGME49_238400*	-3.70		
PtdIns(3,4)P_2	Class II PI3K	nf	nf		nf			
	INPP4A	nf	nf		nf			

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns(3,4)P_2	INPP4B	nf	nf		nf			
PtdIns4P	PI4KIIα, PI4KIIβ/ Lsb6	PF3D7_0311300*	PBANKA_0409300*	Significa-ntly slow	TGME49_276170*	-3.91		
	PI4KIIIα/ Stt4	PF3D7_0419900*	PBANKA_0722000*	Significa-ntly slow	TGME49_228690*	-3.01		
	TTC7/ Ypp1	nf	nf		nf			
	EFR3/ Efr3	nf	nf		nf			

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns4P	PI4KIIIβ/ Pik1	PF3D7_0509800 (PfPI4KIIIβ)	PBANKA_1109400 (PbPI4KIIIβ)	Essential	TGME49_296010 (TgPI4KIIIβ)	-3.60	Vesicle trafficking events in *Plasmodium*	McNamara et al., 2013; Stenberg & Roepe, 2020
	Arf1	PF3D7_1020900 (PfArf1)	PBANKA_0505100 (PbArf1)	Essential	TGME49_276140 (TgArf1), TGME49_262860*, TGME49_212950*, TGME49_215060*	-1.44 -0.52 -4.24 -6.29	Trafficking of PEXEL-positive and -negative proteins from ER in *Plasmodium*. Sti-mulate secretion of preformed dense granule in *T. gondii*.	Taku et al., 2021; Liendo et al., 2001; Venupogal et al., 2020; Leber et al., 2009

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenotypes in *P. berghei*	*T. gondii*	Phenotypic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns4P	NCS-1/Frq1	PF3D7_1451700*	PBANKA_1315400*	Significantly slow	TGME49_213800*, TGME49_230490*, TGME49_228750*, TGMAS_242400*, TGMAS_301440*	-3.62 -5.81 -4.13 1.30 -3.30		
	PKD1/2	PF3D7_1123100 (CDPK7)	PBANKA_0925200 (CDPK7), PBANKA_1351500 (CDPK5)	Essential, Essential	TGME49_228750 (CDPK7), TGME49_224950 (CDPK7), TGME49_206590 (CDPK2A), TGME49_233905*	-4.13 1.69 -2.05 -3.54	Intraerythrocytic development and protein trafficking in *Plasmodium*	Brochet et al., 2014; Bansal et al., 2021

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns4P	GGA2	nf	nf		TGME49_313670*	-4.27		
	14-3-3γ	PF3D7_0818200*	PBANKA_0712600*	Essential	TGME49_263090*, TGME49_269582*, TGME49_269960*	-5.79 0.62 -1.12		
	PKG1/2	PF3D7_1436600 (PfPKG)	PBANKA_1008200 (PbPKG)	nd	TGME49_311360 (TgPKG), TGME49_228420*, TGME49_286470*	-2.15 0.14, -1.74	Ookinete gliding and motility. Schizont rupture in *Plasmodium*.	
	Sec14-like PITP	PF3D7_0626400*, PF3D7_0629900*, PF3D7_1127600*	PBANKA_0614800*	nd	TGME49_254390*, TGME49_203390*, TGME49_246330*, TGME49_269390*	-2.16 -0.37 -0.23 0.09		

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns4P	START-like PITP	PF3D7_1351000* PF3D7_0104200	PBANKA_1363800* PBANKA_0208900	nd Essential	TGME49_289570*,	-1.70		Van Ooij et al., 2013; Hill et al., 2016
	Sac1	PF3D7_1354200 (PfSac1)	PBANKA_1130800*	nd	TGME49_316230*, TGME49_238400*, TGME49_256830*	-5.27 -3.70 -4.73	Regulation of PtdIns4P levels in ER and Golgi in *Plasmodium*.	Thériault et al., 2017.
	OSBP/ ORP	PF3D7_1131800*	PBANKA_0916600*	Significa-ntly slow	TGME49_264760*, TGME49_294320*, TGME49_289570*	-6.07 0.88 -1.70		

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexa	Reference
PtdIns4P	PI3K/ PI4K-like mTOR	PF3D7_0515300*	nf		TGME49_316430*, TGME49_283702*, TGME49_248540* & TGME49_248530*	0.21 -2.68 -0.88 -2.17		
	Other PI3K/ PI4K-like proteins	nf	PBANKA_1114900*	Essential	TGME49_266010*, TGME49_268370*	-3.21 -3.56		

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexan.	Reference
PtdIns(4,5)P_2	Type I PI4P5K/ Mss4p	PF3D7_0110600 (PfPI4P5K)	PBANKA_0203100 (PbPI4P5K)	Essential	TGME49_230490 (TgPI4P5KA), TGME49_245730 (TgPI4P5KB)	-5.81 -1.93	Modulation of PtdIns(4,5)P_2 production in response to changes in intracellular [Ca^{2+}] in *Plasmodium*.	Leber et al., 2009; Brochet et al., 2014
	Type II PI5P4K	nf	nf		nf			
	PKG1/2	PF3D7_1436600 (PfPKG)	PBANKA_1008200 (PbPKG)	nd	TGME49_311360 (TgPKG), TGME49_228420*, TGME49_286470*	-2.15 0.14 -1.74	Ookinete gliding motility and schizont rupture in *Plasmodium*.	Brochet et al., 2014; Collins et al., 2013

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexan	Reference
PtdIns(4,5)P_2	PLCδ	PF3D7_1013500 (PfPI-PLC)	PBANKA_121190 (PbPI-PLC)	Essential	TGME49_248830 (TgPI-PLC)	-4.54	Exocytosis of microneme proteins	Raabe et al., 2011a, Raabe et al., 2011b, Bullen et al., 2016, Fang et al., 2006
	Synapto-janin1/2	PF3D7_1354200* (1), PF3D7_0705500* (2)	PBANKA_1130800* (1), PBANKA_0803200* (2)	nd Essential	TGME49_238400* (1/2), TGME49_316230* (1/2), TGME49_288800* (2)	-3.70 -1.35 0.51		

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexan	Reference
PtdIns5P	Vps15	PF3D7_1431500*, PF3D7_0623800*	nf		TGME49_310190*, TGME49_233010*, TGME49_233905*, TGME49_267540*	-2.28 -1.53 -3.54 -0.78		
	Class III PI3K	PF3D7_0515300	PBANKA_111490	Essential	TGME49_215700	-3.88		
	aMTM	nf	nf		nf			
	ArPIK-fyve/ Vac14	PF3D7_1225700*	PBANKA_1440600*	Essential	TGME49_244040 (TgArPIKfyve)	-4.42	Apicoplast biogenesis/mainten ance in *T. gondii*	Daher et al., 2015

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexan	Reference
PtdIns5P	Fig4	PF3D7_0802500*	PBANKA_1227700*	Significa-ntly slow	TGME49_256830*, TGME49_238400*, TGME49_316230*	-4.73 -3.70 -1.35		
	Class I PI3K	nf	nf		nf			
	dMTM	nf	nf		nf			
	aMTM	nf	nf		nf			
	Type II PI5P4K	nf	nf		nf			

Supplementary Table S1.1. Continued

PIP	Protein	*P. falciparum*	*P. berghei*	Growth rate phenoty-pes in *P. berghei*	*T. gondii*	Phenoty-pic score in *T. gondii*	Function in Apicomplexan	Reference
PtdIns5P	PtdIns-(4,5)P_2 4-phosphatases	nf	nf		nf			

APPENDIX B

PHOSPHATIDYLINOSITOL-4-PHOSPHATE REGULATES THE FORMATION OF DENSE GRANULES IN *TOXOPLASMA GONDII* - SUPPLEMENTARY MATERIAL

Supplementary Table S2.1 Primers used for subcloning *T. gondii* intracellular organelle marker genes used into the pTub expression vector.

Construct	**Primer**
Myc-GRA3-RFP	F: 5'-CGC AGATCTATGGACCGTACCATATG-3' R: 5'-AGACCTAGGTTTCTTGGAGGCTTTGTC-3'
Myc-GRA2-RFP	F: 5'-CGCAGATCTATGTTCGCCGTAAAAC-3' R: 5'-ATACCTAGGCTGCGAAAAGTCTGGGAC-3'
Myc-ROP1-RFP	F: 5'-GGAAGATCTAAAATGGAGCAAAGGCTGCCA-3' R: 5'-GTTCCTAGGAAATTGCGATCCATCATCCTG-3
Myc-IMC1-RFP	F: 5'- AAACCTAGGATGGCCTCCTCCGAGGACG-3' R: 5'-AAAGCGGCCGCTTAGGCGCCGGTGGAGTGGCGG-3'
Myc-2XPHPLCδ-EGFP	F: 5'-GCGAGATCTGATATACATATGGACTC-3' R: 5'-AAACCTAGGGCTCACCATGGT-3'
YFP-FAPP1PH-Myc	F: 5'-GAAGATCTAAAATGGAGGGGGTGTTGTACAAG-3' R: 5'- GACCTAGGGTCCTTGTATCAGTCAAACATGC-3'
YFP-SidM-P4M-Myc	F: 5'-AAAAGATC AAGTCCGGACTCAGATC-3' R: 5'-AAACCTAGGTTTTATCTTAATGGTTTG-3'

Supplementary Table S2.1 Continued.

Construct	**Primer**
YFP-GOLPH3-Myc	F: 5'-AAAAGATCTATGACCTCGCTGACCCAG-3' R: 5'- AAACCTAGGTTACTTGGTGAACGC-3'
YFP-FAPP1PH$^{K7A/R18L}$-Myc	R18L: F: 5'-CTCACAGGCTGGCAGCCTCTTTGGTTTGTTTTAGATAATGG-3' R: 5'-CCATTATCTAAAACAAACCAAAGAGGCTGCCAGCCTGTGAG-3' K7A: F: 5'-GGAGGGGGTGTTGTACGCGTGGACCAACTATCTCAC-3' R: 5'- GTGAGATAGTTGGTCCACGCGTACAACACCCCCTCC-3'
YFP-SidM-P4M^{K568A}-Myc	F: 5'-TGATGCTTTAGCTACAGAAATCCTGGCTG-3' R: 5'- CCTCGCATTTGCTGATATTTTTC-3'
YFP-TAPP1PH-Myc	F: 5'-GAAGATCTAAAATGTTTACTCCTAAACCACCTCAAGATAG-3' R: 5'-GACCTAGGATGCTCAGAAGATGCTGATCTGC-3'
Myc-Rab5.1	F: 5'-GGATCCATGAGAGGTTTCGAATCTGC-3' R: 5'-GCGGCCGCTCAACTTTTGCCTCCACATG-3'

Supplementary Table S2.2 List of antibodies in Section 2.

Antibody	Dilution	Source/Brand
Mouse anti-GRA3	1:500	BEI Resources, NIAID, NIH: Monoclonal Anti-Toxoplasma gondii Dense Granule Antigen 3, Clone T6 2H11 (produced in vitro), NR-50269
Mouse anti-IMC	1:500	Dr. Gary Ward
Rat anti-TgSORTLR	1:250	Dr. Stanislas Tomavo
Mouse anti-MIC3	1:500	Dr. Jean François Dubremetz
Rabbit anti-Myc tag	1:400	Cell signaling 2272
Anti-mouse Alexa Fluor 594	1:500	Invitrogen A11032
Anti-rat Alexa Fluor 568	1:500	Invitrogen A11077
Anti-rabbit Alexa Fluor 647	1:500	Invitrogen A21245

APPENDIX C

IDENTIFICATION OF A TOXOPLASMA GONDII STEROL PRECURSOR-EXCHANGER PROTEIN OF THE SEC14-LIKE PITP FAMILY - SUPPLEMENTARY MATERIAL

Supplementary Table S3.1 List of plasmids used in Section 3.

Plasmid	**Observations**
pDR195	Yeast multicopy expression vector. Restriction enzymes used to introduce insert: PspXI/AvrII
pET28a	*E. coli* expression vector. Restriction enzymes used to introduce insert: NcoI/NotI
pTUB	*T. gondii* expression vector. Restriction enzymes used to introduce insert: BglII/AvrII
pSag1-Cas9-NLS-GFP/pU6-gRN	Used to generate Cas9-sgRNA 5' plasmids to obtain clean cuts at the 5' end of the TgPI4KIIIβ gene sequence.
17-V3-5'COR-pT8TATi1-HX-tetO7S1mycNtCOR-Ty	Used as a template to amplify Tet-repressible transactivator cassette. Kindly provided by Dr. Dominique Soldati-Favre.

Supplementary Table S3.2 List of primers used in Section 3.

Construct	Primer
pDR195-TgSfhA	F: 5'-CAGCCTCGAGCATGGCAAGTAGCGCCCCG-3' R: 5'-TTCCCTAGGGCGACCTGCGCCGGTAGACT-3'
pET28b-His8-TgSfhA (E. coli codon optimized)	F: 5'-ATTCCATGGGTCATCATCACCACCACCATATGGCAAGTAGCGCCCCG-3' R: 5'- CATGCGGCCGCTTAGCGACCTGCGCCGGTAG-3'
pTub-YFP-TgSfhA-Myc	F: 5'-AAAAGATCTATGGCTTCGTCAGCGCCG-3' R: 5'-AAACCTAGGCCGCCCCGCCCCCGTAGAC-3'
pTub-Myc-GRA2-RFP	F: 5'-CGCAGATCTATGTTCGCCGTAAAAC-3' R: 5'-ATACCTAGGCTGCGAAAAGTCTGGGAC-3'
pTub-Myc-Rop1-RFP	F: 5'-GGAAGATCTAAAATGGAGCAAAGGCTGCCA-3' R: 5'-GTTCCTAGGAAATTGCGATCCATCATCCTG-3
gRNA-5'-TgSfhA	F: 5'- CACAGAGAAGGTTTTAGAGCTAGAAATAGC-3' R: 5'-GCGGTAGAGACAACTTGACATCCCCATTTAC-3'
Tet-system PCR amplicon	F: 5'-ATTCCTTCCCTCGCATCTCTGCCCTTCTCTCCTCTTCCTGCATGTTTGCGGATCCGGGG-3' R: 5'-CTGGGTCGCTCACAGCAGCGTCGGACGGCGCTGACGAAGCCAGGTCCTCCTCGGAGATGA-3'

Supplementary Table S3.3 List of antibodies used in IFA analysis in Section 3.

Antibody	**Dilution**	**Source/Brand**
Mouse anti-IMC	1:500	Dr. Gary Ward
Rabbit anti-CPL	1:500	Dr. Vernon Carruthers
Mouse anti-MIC3	1:500	Dr. Jean François Dubremetz
Rabbit anti-Myc tag	1:400	Cell signaling 2272
Anti-mouse Alexa Fluor 594	1:500	Invitrogen A11032
Anti-rabbit Texas Red-X	1:500	ThermoFisher T-6390

Supplementary Table S3.4. List of antibodies used in WB assays in Section 3.

Antibody	**Dilution**	**Source/Brand**
Rabbit anti-Myc	1:1000	Cell signaling (2272)
Anti-Rabbit conjugated to horseradish peroxidase (HRP)	1:2000	Millipore (AP307P)