Multiple routes of phosphatidylethanolamine biogenesis ensure membrane integrity of *Toxoplasma gondii*

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I. Zusammenfassung

Toxoplasma gondii ist ein weit verbreiteter, obligat-intrazellulärer, einzelliger Parasit, der die lebensbedrohliche Krankheit Toxoplasmose in Menschen und Tieren hervorrufen kann.

Der schnell replizierende Parasit benötigt erhebliche Mengen an Phospholipiden zur Biogenese intra- und extrazellulärer Membranen. Phosphatidylethanolamin (PtdEtn) ist ein wichtiges und ubiquitäres Phospholipid in Pro- und Eukaryoten und das zweithäufigste Lipid in *T. gondii*. Dieses kann *de novo* über den CDP-Ethanolamin Stoffwechselweg oder durch Decarboxylierung von Phosphatidylserin synthetisiert werden. Aufgrund seiner konischen Struktur, scheint PtdEtn vor allem eine wichtige Funktion bei der Krümmung, Fusion und Abschnürung von Membranen, sowie der Stabilisierung von Membranproteinen einzunehmen. Zudem ist PtdEtn essentiell für die Integrität und Funktion von Mitochondrien in Eukaryoten.

Im Rahmen dieser Arbeit konnte die Expression von zwei distinkten Phosphatidylserin Decarboxylasen (PSDs) in *T. gondii* nachgewiesen werden: *Tg*PSD1pv ist partiell löslich und wird über Dichte Granula in die Parasitophore Vakuole sekretiert, während sich *Tg*PSD1mt im Mitochondrium von Tachyzoiten befindet.

*Tg*PSD1mt ist in der Lage einen Ethanolamin-auxotrophen *S. cerevisiae* Stamm zu komplementieren, wohingegen *Tg*PSD1pv in Hefe nicht funktionell ist. Ein Knock-down von *Tg*PSD1mt verursacht eine verlangsamte Parasitenreplikation, welche zu einem verminderten *in vitro* Wachstum führt. Der PtdEtn-Gehalt in der Mutante bleibt unverändert, was auf eine stringente Homöostase des zellulären PtdEtn Reservoirs durch alternative Lipidbiogenesewege hindeutet. Tatsächlich verfügt *T. gondii* zusätzlich über einen aktiven CDP-Ethanolamin Stoffwechselweg im Endoplasmatischen Retikulum, welchen die *Δtgpsd1mt*-Mutante für einen erhöhten Einbau von ^{14}C-Ethanolamin in PtdEtn nutzt. Dies deutet darauf hin, dass der Parasit versucht den Verlust von *Tg*PSD1mt über eine Hochregulierung des *de novo* PtdEtn Stoffwechselweges auszugleichen. Das zweite und *Coccidia*-spezifische *Tg*PSD1pv-Enzym hingegen scheint zwar für das Parasitenwachstum *in vitro* entbehrlich zu sein, verfügt jedoch über einzigartige enzymatische Eigenschaften, die sich nicht in anderen PSD Enzymen finden.

Infektionsversuche mit [^{14}C]-Ethanolamin markierten Wirtszellen zeigten zudem eine Aufnahme von PtdEtn oder PtdEtn-Derivaten in *T. gondii* als weiteren Mechanismus der PtdEtn-Homöostase in intrazellulär replizierenden Tachyzoiten.

Diese Ergebnisse demonstrieren eine außergewöhnliche Kompartmentalisierung und Plastizität der PtdEtn-Synthese in *T. gondii*, welche dem Parasiten sehr wahrscheinlich eine flexible Membranbiogenese bei unterschiedlichem Nährstoffangebot ermöglicht.

II. Abstract

Toxoplasma gondii is a remarkably successful and widespread obligate intracellular protozoan parasite, which can cause the potentially life-threatening disease Toxoplasmosis in humans and animals.

This fast proliferating parasite requires a significant amount of phospholipids for biogenesis of organelles and enclosing vacuolar membranes. Phosphatidylethanolamine (PtdEtn) is one of the most ubiquitous phospholipids and the second most abundant lipid in *T. gondii*. It can be produced *de novo* by the CDP-ethanolamine pathway or by decarboxylation of phosphatidylserine. Due to its conical structure, the lipid plays important roles in membrane curvature as well as fusion and fission events. Furthermore, PtdEtn is critical for the mitochondrial function and integrity in eukaryotes.

This work revealed the expression of two distinct PtdSer decarboxylase (PSD) enzymes in *T. gondii*: One of which is *Coccidia*-specific and partially soluble and secreted into the parasitophorous vacuole via dense granules (*Tg*PSD1pv), and a second enzyme that localizes in the mitochondrion (*Tg*PSD1mt) of tachyzoites.

The mitochondrial PSD can complement a *S. cerevisiae* mutant auxotrophic for ethanolamine, whereas *Tg*PSD1pv is not functional in yeast. A conditional knockdown of the *Tg*PSD1mt gene impairs the parasite growth *in vitro*. Surprisingly, the mutant displayed an unaltered total PtdEtn content, which suggests a stringent homeostasis of the cellular PtdEtn pool by alternative routes of lipid biogenesis. Consistently, the parasite encodes an active CDP-ethanolamine pathway in the endoplasmic reticulum. Metabolic labeling of the *Tg*PSD1mt mutant displayed an increased utilization of ethanolamine into PtdEtn, indicating an upregulation of the *de novo* CDP-ethanolamine pathway. Likewise, exogenous ethanolamine partially restored the growth phenotype of the mutant. In contrast, the *Tg*PSD1pv enzyme is dispensable for the parasite growth. However, the enzyme displays unique enzymatic characteristics, not shared with other PSD enzymes. Host cell labeling with [^{14}C]-ethanolamine prior to infection indicated a potential uptake of host-derived PtdEtn or PtdEtn-derivates, which could ensure PtdEtn homeostasis in intracellular parasites.

Taken together, these results demonstrate an exceptional compartmentalization and plasticity of the PtdEtn synthesis in *T. gondii*, which likely ensures a flexible membrane biogenesis in dissimilar nutritional milieus.

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V. List of abbreviations

ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
bp	base pairs
CAT	Chloramphenicol acetyltransferase
CD	catalytic domain
CDP	Cytidine diphosphate
cDNA	complementary DNA
CL	Cardiolipin
CMV	Cytomegaolovirus
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DER1	Degradation in the endoplasmatic reticulum protein 1
DHFR-TS	Dihydrofolate reductase thymidylate synthase
DIC	Differential interference contrast
DMEM	Dulbeccos's modified Eagle medium
DNA	Deoxyribonucleic acid
ECT	CTP:phosphoethanolamine cytidylyltransferase
EDTA	Ethylendiamine tetraacetate
EK	Ethanolamine kinase
ECT	Ethanolaminephosphate cytidyltransferase
EPT	CDP-ethanolamine:diacylglycerol ethanolamine-phosphotransferase
ER	Endoplasmatic reticulum
EtOH	Ethanol
F(wd)	forward
F1B	ATPase subunit F1-β
FACS	Fluorescence-associated cell sorting
FASI/II	Fatty acid synthase type I/II
FCS	Fetal calf serum
F-dUMP	5´-Fluorodeoxyuridine monophosphate
FUDR	5´-Fluorodeoxyuridine
GAL	Galactose
gDNA	genomic deoxyribonucleic acid
Gap45	gliding-associated protein (45 kDa)
GFP	Green fluorescent protein
GRA	dense granule protein
GSH	Glutathione
h(r)	hour
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human foreskin fibroblast
Hsp90	Heat-shock protein (90 kDa)
HXGPRT	Hypoxanthine-xanthine-guanine phosphoribosyltransferase
ICM	Intracellular-type medium
IFA	Indirect immunofluorescence assay
IPTG	Isopropyl-ß-D-1-thiogalactopyranoside
IT	Insertional tagging
IVN	Intravacuolar network
kb	kilo bases
KO	Knockout
KOV	Knockout verification
ME	Mammalian expression
MEM	Modified Eagle´s medium
MeOH	Methanol
MOI	Multiplicity of infection
MOPS	3-(N-morpholino) propanesulfonic acid
MPA	Mycophenolic acid
mt	mitochondrion/mitochondrial
mTP	mitochondrial targeting peptide
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
dNTP	desoxyribo nucleotide triphosphates
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEMT	Phosphatidylethanolamine methyltransferase
PEtn-Cer	Phosphoethanolamine ceramide
PMSF	Phenylmethylsulfonyl fluoride
PIC	Protease inhibitor cocktail
PSD	Phosphatidylserine decarboxylase
PSR	Phosphatidylserine receptor
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
PYR	Pyrimethamine
R(ev)	reverse
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
s(ec)	second
Sag1	Surface antigen 1
Sc	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
SP	Signal peptide
TaTi	Trans-activator trap identified
Tet	Tetracycline
TEM	Transmission electron microscopy
Tg	*Toxoplasma gondii*
TGD	Targeted gene disruption
TLC	thin-layer chromatography
OD	Optical density
U	Unit(s)
UMP	Uridine-5´-monophosphate
UPRT	Uracilphosphoribosyltransferase
UTR	Untranslated region

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1. Introduction

1.1. *Apicomplexa*

Apicomplaxa [lat. *apex* = top/tip, lat. *complexus* = infolds] represent a large group of non-flagellated protists within the superphylum of *Alveolata* (1). The phylum comprises thousands of versatile species, which are exclusively obligate (endo)parasites in a variety of host organisms (2). Several members of this phylum have the capacity to cause severe diseases in humans and domestic animals, and are therefore of high medical, veterinary and (socio-) economic importance (3). Most *Apicomplexa* display a strict host-specificity and seven genera among them are known to infect humans. For example *Plasmodium* spp., the causative agent of malaria, inflicting approximately 0.5 million annual deaths particularly in children (2,4). Other widely prevalent cyst-forming apicomplexan species, such as *Eimeria* and *Toxoplasma,* can cause coccidiosis and toxoplasmosis [see 1.2.1.] in animals and humans, respectively (5,6).

The main characteristic of apicomplexan parasites is the presence of unique ultrastructural features, including the *apical complex* at the anterior end, which consists of specialized secretory organelles and longitudinal cytoskeletal structures. The apical complex facilitates attachment, penetration and invasion of the host-cell [see 1.2.2.]. The presence of a laterally located micropore for nutritional uptake via pinocytosis, as well as cortical alveolar structures, are other common features in this phylum (7). Most *Apicomplexa* harbour a non-photosynthetic but metabolically important plastid-like organelle surrounded by four membranes, termed as *apicoplast* (= apicomplexan plastid), which was likely derived by secondary endocytobiosis from an algal ancestor (8,9). With exception of the male gametes, all developmental stages lack flagella. In lieu thereof *Apicomplexa* show a unique form of active movement, described as *gliding motility* (10) [see 1.2.3.].

The general life cycle of *Apicomplexa* comprises alternating sexual and asexual generations produced via three distinct processes: sporogony, merogony/schizogeny and gamogony [schematized in Fig. 1]. Different characteristic forms of daughter cell formation are found during schizogeny for the generation of various numbers of progeny (2). All developmental forms, with exception of the zygote, harbour a haploid genome and vary in their organelle equipment and morphological characteristics. Whereas in monoxenous parasite species, like *Eimeria* sp., all developmental processes take place in the same host, heteroxenous *Apicomplexa*, such as *Toxoplasma* and *Plasmodium*, use different host species to complete their life cycle.

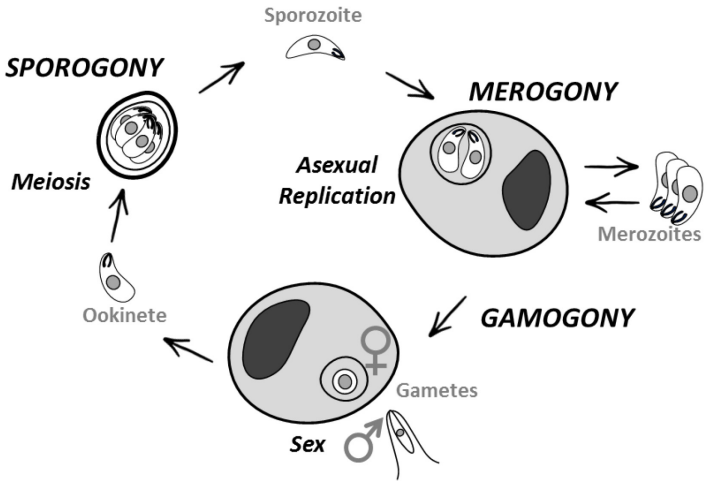

Figure 1: Developmental stages of Apicomplexa
Female and male gametocytes and gametes are formed via gamogony, which fuse to form a zygote/ookinete. During sporogony, sporozoites develop within a resistant thick-walled oocyst. Sporozoites in turn can develop into merozoites by a process called merogony or schizogeny giving rise to a large number of infective merozoites. [*adapted from Striepen et al. 2007* (11)]

1.2. *Toxoplasma gondii*

T. gondii is the most prevalent and cosmopolite species of the phylum *Apicomplexa,* which can be isolated from an enormous range of terrestrial and even marine hosts worldwide (12). The parasite was discovered and described first in the tissue of a small rodent named *Ctenodactylus gundi* in 1908 by Nicolle and Manceaux (13). Based on its cellular morphology and the first described host organism, the crescent-shaped parasite was named *Toxoplasma gondii* (lat. *toxo* = arc, bow; *plasma* = life; *gondii* = from *C. gundi*). To date, three dominant clonal lineages (referred to as type I-III) and a large amount of atypical genotypes have been identified in *T. gondii* isolates, which differ in their global distribution, prevalence and clinical severeness of the disease (14-17).
Approximately one third of the human population is estimated to be sero-positive for *T. gondii*. Seroepidemiology however strongly varies between countries and depends on the hygienic and nutritional conditions and habits in the respective area as well as the considered age cohort (18).

1.2.1. Toxoplasmosis in humans

The first indications for *T. gondii* as the inducing agent of a disease, was drawn in 1910 in an infected puppy. About 30 years later, *T. gondii* was also detected in an infant, which lead to an subsequent increased discovery of the parasite and its clinical outcome in a variety of hosts in the following years (19).
In immune-competent humans the primary infection with *T. gondii* is seldom recognized, because it causes mild flu-like symptoms and is usually controlled by the humoral and adaptive immune system involving a strong TH1-cell/IFN-γ mediated immune-response (20). Immune-incompetent patients, however, can develop a serious or even fatal clinical outcome. In the acute phase of the infection, fast

replicating tachyzoites [greek. *tachy* = fast] are distributed throughout the body and are able to cross blood-tissue barriers. This can cause severe ocular and cerebral toxoplasmosis via tissue lesions, eventually (18). Congenital Toxoplasmosis can occur through dia-placental transmission of parasites to the developing, immune-immature foetus during primary infection of a pregnant woman. It can result in serious neurological disabilities or the death of the unborn child. The probability of fetal transmission and the fatality of the disease also coincide with the trimester of infection. In many cases *in utero* infections remain unrecognized until the child develops clinical symptoms in the first lifespan (2).

Upon immune response, tachyzoites can convert into slow-dividing/quiescent bradyzoite [greek. *brady* = slow] stages, surrounded by a protective cyst wall, with a tissue tropism for neural and muscular tissue, leading to a chronic but mostly asymptomatic infection of the host (21). Upon a decline in immune-response of the chronically infected host, dormant tissue cysts can be reactivated. Hence, *T. gondii* poses a high-risk, especially for AIDS-patients and individuals that receive immune-suppressive treatments, e.g. transplant recepients (18). Furthermore, chronic *T. gondii* infections may also be associated with neuropsychiatric disorders (22,23) and behavioural changes in animals and humans (24).

Whereas acute toxoplasmosis can be treated with a combination therapy of pyrimethamine and sulfadiazine with folic acid supplementation (18), there is no effective treatment to prevent or eliminate chronic infections. Although vaccination strategies are successfully applied in sheep and goats (25) and experimentally tested in other animals, no vaccination protecting humans against *T. gondii* infection is available to date (26).

1.2.2. The life cycle of *T. gondii*

The definite host of *T. gondii* are members of the genus *felidae* (27,28), whereas asexual reproduction can take place in all nucleated cell types of warm-blooded animals including humans (29).

Merogony and gamogony [see 1.1.] take place exclusively in the feline intestine tissue where upon the fusion of male and female gametes resistant oocysts are formed. These are shed with the cat feces into the environment. The oocysts develop into two sporoblasts each containing four sporozoites. Sporulated oocysts can be infectious for several months or years (30) and upon oral ingestion, the released sporozoites can invade the gut epithelium of the intermediate hosts. Here, sporozoites differentiate into fast dividing tachyzoites. In immunocompetent organisms, tachyzoites will convert into quiescent bradyzoites, which are surrounded by a thick cyst wall and can persist in the neural or muscular tissue causing a lifelong chronic infection. By ingestion of contaminated food

(31), water (32) or soil, the life cycle can proceed in the infinite host closing the heteroxenous life cycle of *T. gondii* (33) [Fig. 2]. The parasite can also transmit from intermediate to intermediate host by predation, which is a major cause of its high prevalence.

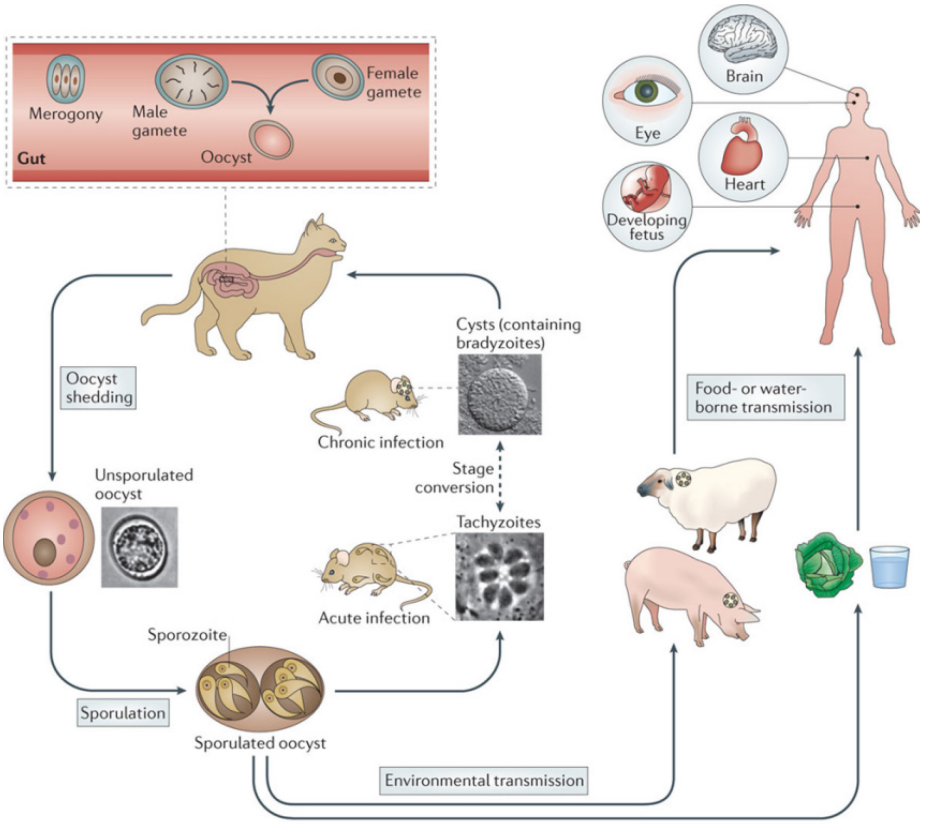

Figure 2: Life cycle of T. gondii
The sexual reproduction of *T. gondii* takes place in the gut epithelium of feline species, where oocysts are formed and shed with the feces into the environment. Sporulated oocysts can be ingested by other vertebrates, e.g. rodents, where they can initiate an acute and subsequent chronic infection leading to the formation of tissue cysts. Predation of an infected animal by a cat, will initiate another round of the life cycle. If sporulated oocysts are taken up by an intermediate host via contaminated water, soil or food, they can generate acute and chronic infections, especially affecting neural and muscular tissues and are particularly harmful to immune incompetent hosts. [*from Hunter and Sibley, 2012* (34)]

In terms of *T. gondii* evolution and propagation, special attention is drawn to the house cat and house and wild mouse infection cycle (35). Wild type or knockout mice are widely used as infection model to explore the immune response that is triggered by the parasite infection. One drawback, however, is the difficulty of investigating the development of sexual stages of the parasite, because these processes take place exclusively in feline species, which are less amenable to experimentation and less suitable as laboratory animals.

1.2.3. Morphology and lytic cycle of *T. gondii*

The most extensively studied developmental form of *T. gondii* is the tachyzoite stage, which causes the acute form of the parasite infection [see 1.2.1.]. Tachyzoites are highly-polarized, displaying several characteristic morphological features at the apical end of the cell. Besides common eukaryotic organelles, like the endoplasmic reticulum, Golgi apparatus, a single mitochondrion and nucleus, the parasite harbours unique secretory organelles, described as *dense granules*, *micronemes* and *rhoptries* [Fig. 3A].

Whereas dense granules are present throughout the parasite body, elongated micronemes and club-shaped rhoptries are located at the apical end, which is highly specialized to enable active host cell invasion, mediated by its enzymatic and cytoskeletal equipment. At the apical tip, a thimble-shaped cytoskeletal structure is found, called *conoid* (= cone shaped), which consists of two annular structures and spirally wound microtubules (36) [Fig. 3B]. The parasite is surrounded by a multi-layered pellicle, built of the outermost plasma membrane, two tightly adjacent membranes (*inner membrane complex* = IMC) and longitudinal cytoskeletal structures to confer rigidity and flexibility to the highly dynamic parasite cell (37) [Fig. 3C].

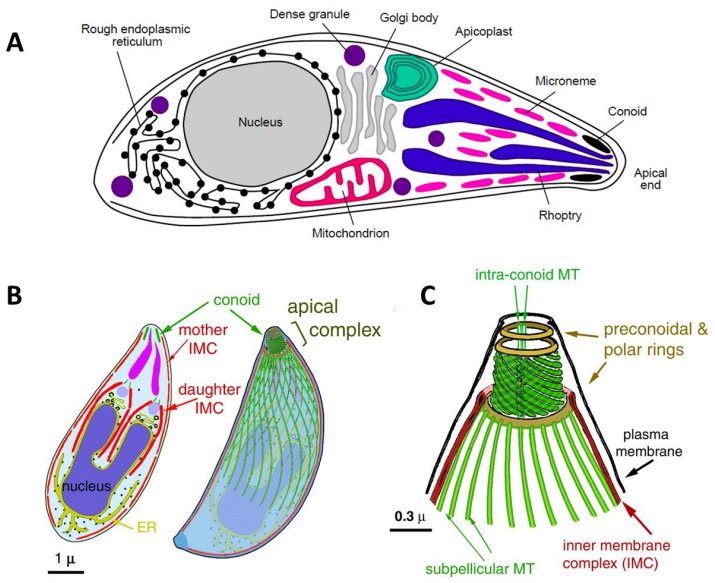

Figure 3: Ultrastructure of T. gondii tachyzoites
(A) The tachyzoite stage of *T. gondii* displays a highly polarized structure and is marked by the presence of parasite-specific secretory organelles (dense granules, micronemes, and rhoptries) and unusual cytoskeletal structures, like the conoid, a cone-shaped structure at the apical tip. A plastid-like organelle with four membranes - the apicoplast - is also present in the parasite body. Besides that, the tachyzoite cell contains a complete set of conventional eukaryotic organelles. **(B)** The conoid consists of a preconoidal and two polar ring structures containing two intra-conoidal microtubules (MTs). The preconoidal ring is anchored to the inner membrane complex. Spirally wound and longitudinal MTs stabilize the conoid and the parasite body. **(C)** Longitudinal section of a dividing tachyzoites showing the organellar and IMC (red) organisation during intracellular daughter cell formation. Besides, the organisation of the subpellicular microtubuli is shown (in green).[*Tachyzoite morphology* (38)*, inner membrane complex* (37) *and cytoskeletal conoid organisation* (37)]

The asexual reproductive cycle is hallmarked by certain critical steps: Invasion of the host cell, intracellular replication, and egress from the infected cell to subsequently infect another cell [Fig. 4]. These processes are found to be highly complex and orchestrated. The non-flagellated parasites developed a specialized form of active movement, termed as *gliding motility*, which is driven by the secretion of adhesion molecules via an actin/myosin motor-complex, that is elementary for the invasion and egression process (39). After apical orientation and attachment to the host cell, the parasite forms a *moving junction* complex with the host cell membrane (40) and enters the cell while creating its own surrounding membranous replication niche, the so called *parasitophorous vacuole* (PV)(41). The vacuole is secluded from host endocytosis and exocytosis, but and is permeable to small molecules (42). Furthermore, it presumably functions as a protective barrier and nutritional interface with the host cell. Within the PV and coherent with secretion of dense granular content, an intramembranous filamentous network is formed, described as intravacuolar, nanotubular or tubovesicular network (43,44). The IVN (intravacuolar network) presumambly acts as a mechanical stabilisator (45) and is also discussed to be involved in nutrient acquisition (46).

Tachyzoites replicate by an extraordinary process called *endodyogeny*, where two daughter zoites are formed synchronously within a mother cell following mitosis and organelle duplication [see Fig. 3C]. The doubling time varies from 6 to 8 hours generating approximately 64 - 128 progeny. Eventually the parasite replication leads to the physical rupture of the host cell and infectious tachyzoites can initiate another round of the lytic cyle [Fig. 4].

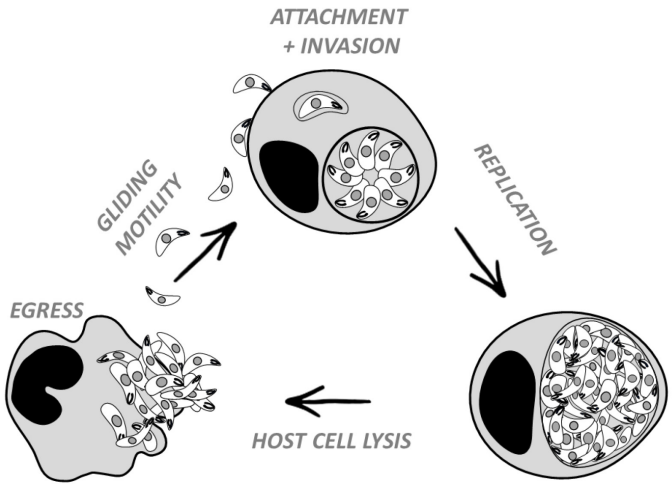

Figure 4: Lytic cycle of T. gondii
During its acute infection phase *in viv*o and during *in vitro* cultivation, *T. gondii* undergoes a fast lytic cycle, where infective tachyzoites replicate in a parasitophorous vacuole to generate large amounts of progeny and eventually lyse the infected cell. New infective and motile tachyzoites egress, and initiate another round of the lytic cycle.

Infection and intracellular replication of *T. gondii* is accompanied by active remodelling and reprogramming of the host cell. Soon after invasion, host mitochondria and ER membranes are recruited to the PV membrane (47), which presumably provides nutrients to the parasite (48).
Additionally, the parasite is known to manipulate the infected host cell via secretion of immune and apoptosis modulators to circumvent the detection and subsequent defense by the host cell, both of which enable unhindered intracellular multiplication of the parasite (21,34,49). Most extensively studied is the interaction of parasite secreted rhoptry (pseudo)kinases with downstream signalling pathways like the JAK/STAT pathway and INF-γ production of the host cell (50,51). Furthermore, the interaction of strain-specific rhropty kinases with immunity-related GTPases was shown as a critical determinant for virulence of *T. gondii* strains in mice (52-54). To ensure its fast intracellular replication, the parasite has to satisfy his extensive needs for nucleic acids, proteins, and membrane lipids, which must be either generated *de novo* by the parasite itself and/or scavenged from the host cell [see 1.3.3. and 1.3.4.].

1.2.4. Genetic manipulation of *T. gondii*

Its capability to infect nearly all animal cells and the accessibility of an annotated genome (55,56) along with the relative ease of genetic manipulation and *in vitro* cultivation make *T. gondii* an excellent model to study parasite-host interactions. A variety of selection markers have been identified that enable stable expression and deletion of parasite genes: The hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) (57) and the uracil-phosphoribosyltransferase (UPRT) (58) targeting the nucletoide biosynthesis and salvage pathways can be used for positive and negative selection of drug-resistant parasites, respectively. Furthermore, the dihydrofolate reductase thymidilate synthase (DHFR-TS) conferring resistance to pyrimethamine (59) as well as the chloramphenicol-acetyltransferase (60) are other commonly used as selection markers in *T. gondii*.
Since the haploid genome of *T. gondii* does not favour homologous recombination, long DNA sequences (2-3 kb) are needed to facilitate this event (61). The efficiency of gene replacement and tagging was significantly enhanced by the use of *T. gondii* strains lacking the non-homologous end joining (KU80) repair system (62,63). Meanwhile, a variety of single- or multi-stepped strategies have been developed to permit the conditional ablation of essential genes. The generation of a stable *T. gondii* TATi-1 line, carrying tetracycline trans-activator elements, can be used to repress the transcription of a gene of interest by anhydrotetracycline (64). While the original two-step strategy is relatively laborious, a variety of improved techniques have been established, such as the single-stepped promotor-displacement/replacement directly targeting the genomic locus of interest (65).

The application of the DD/Shield-1 system in *Toxoplasma* allows the destabilisation of cytosolic proteins (66). Transgenic strains, particularly knockout mutants, can be analysed for their phenotype *in vitro* as well as for their virulence *in vivo*. The continuous development, improvement and refinement of methods to genetically manipulate and phenotype *T. gondii* opens the door for characterizing the molecular basis of the tachyzoite lytic cycle (67). Another important but understudied branch in *Toxoplasma* research is the analysis of intrinsic and extrinsic factors, which regulate tachyzoite to bradyzoite interconversion. This phenomenon is more complex to study, since genetic manipulation and cultivation of bradyzoite-forming strains is more challenging.

1.3. Lipids and membrane biology

Lipids [greek *lipos* = fat] are classically defined as amphipathic or hydrophobic molecules that are readily soluble in organic solvents. But lipids can be also described as fatty acids and their naturally occurring derivatives and functionally related molecules. These molecules display a wide diversity in molecular structure and biological function, like energy storage and signalling events, but most importantly serving as structural matrix of cellular membranes (68). Fatty acids build the fundamental structure of biological lipids, consisting of a hydrocarbon chain, most commonly with 14 – 20 carbons terminating with a carboxylic acid group. Neutral lipids, such as triglycerides (triacylglyceroles) mainly act as energy storage molecules [Fig. 5].

Polar lipids can be broadly categorized into (glycero)phospholipids and glycolipids. Furthermore, sphingolipids can be either glycosphingolipids or phosphospingolipids. Glycerophospholipids, commonly referred to as phospholipids, are the predominant molecule species in biological membrane bilayers [Fig. 5].

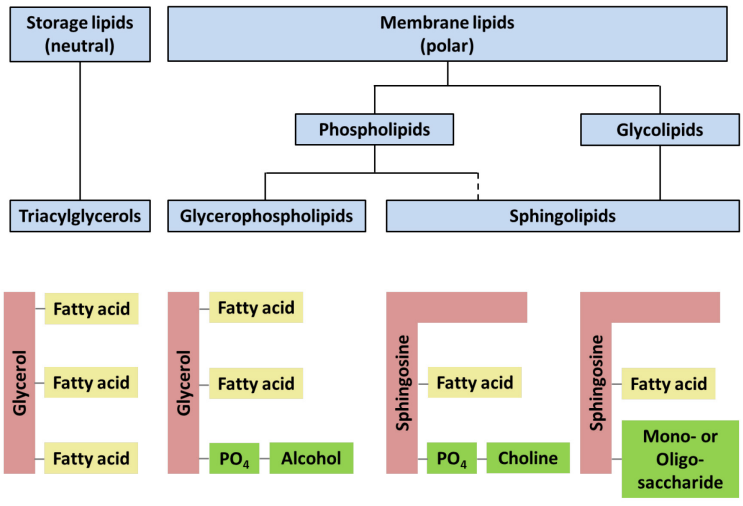

Figure 5: Structure and classification of neutral and polar lipids
[*adapted from Lehninger* (69)]

In eubacteria and eukaryotes, the diacylglycerol backbone of phospholipids is formed by *sn*-3-glycerol esterified with long chain fatty acids at positions 1 and 2 (68). In addition, the chain length and degree of acyl chain saturation as well as the polar head group extends the phospholipid diversity. Most commonly choline, ethanolamine, serine, glycerol and inositol are found as substituents of the diacylglycerophosphate [Fig. 6A].

Due to their amphipathic nature, phospholipids self-assemble in aqueous solutions into micelles or bilayers [Fig. 6C]; the latter comprise a hydrophobic core and two hydrophilic interfaces constituting a robust but dynamic barrier. Phospholipid bilayers surround all living organisms and are a prerequisite for intracellular compartmentalization in eukaryotic cells, enabling a cross protection of diverse biochemical sub-milieus. The inherent capacity for membrane fission and fusion also enables cell division and vesicular trafficking of cellular metabolites (70).

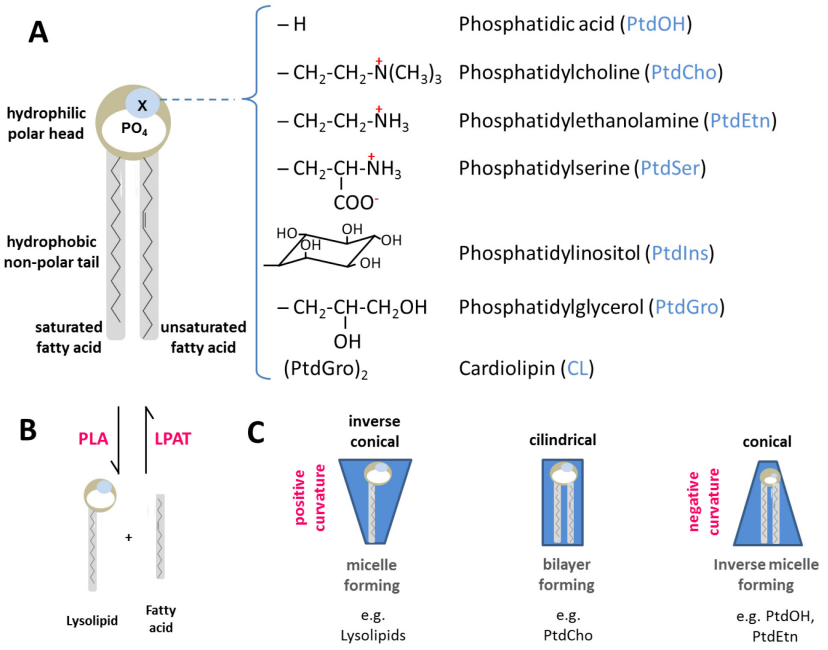

Figure 6: Structure and remodelling of major phospholipids
(A) Phospholipids are generally composed of two fatty acid chains with different degree of saturation esterified to a glycerophosphate backbone carrying a polar amino alcohol head group (choline, ethanolamine, serine or inositol). Cardiolipin is a dimeric structure, which consists of two phosphatidylglycerol molecules. The charge of the aminoalcohol group is indicated in red colour. **(B)** Phospholipid remodelling and recycling occurs via the *Lands´ cycle*, a deacylation and reacylation reaction conferred by phospholipase A (PLA), and lysophospholipid acyltranserase (LPAT) enzymes, respectively. **(C)** Phospholipids show a differential geometry and behaviour based on the size and charge of the head group and the fatty acid composition.

1.3.1. Membrane composition of eukaryotes

The major structural lipids in eukaryotic cells are glycerophospholipids, which reconstitute up to 65% of membrane lipids. Phosphatidylcholine (PtdCho) accounts for the vast majority (>50%) of lipids in most eukaryotic cells, followed by phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and phosphatidic acid (PtdOH) (70). Another class of structural lipids, the sphingolipids, with ceramide as hydrophobic backbone, account for ~10% of polar lipids. Sphingomyelin and glycosphingolipids are the most abundant sphingolipid species in mammals (68). Non-polar sterol molecules are another major lipid class in eukaryotic membranes (~25%). Cholesterol, as a predominant species in mammalian cells, serves as an important precursor for fat-soluble vitamins and steroid hormones (70).

The phospholipid composition, fatty acid chain length and degree of saturation, as well as the insertion of sphingolipid and sterol molecules, together determine the membrane fluidity and rigidity (71). A tremendous diversity of phospholipid species can be generated via the *Lands´ cycle* (72), which involves phospholipase A_2 activity cleaving fatty acids at the sn-2 position to produce lysolipid species. The reverse reaction is accomplished by lysophospholipid acyltransferases reacylating lysophospholipids to constitute new phospholipid species for lipid remodelling [see Fig. 6B]. As a consequence of their head group charge and size as well as their fatty acid composition, phospholipids can show a conical, inverted conical or cylindrical structure [Fig. 6C] that influences their behaviour in aquous solutions. Inverse conical lysolipids form micellar structures and confer membrane curvature in bilayers, whereas cylindrical species will naturally form bilayers (70,73).

Another fundamental constituent of biological membranes are integral and peripheral membrane proteins, which are either tightly intercalated into the hydrophobic membrane matrix or loosely bound to the hydrophilic membrane surface by electrostatic or non-hydrophobic interactions. Many of these proteins fulfil essential cellular functions, e.g. acting as transporters, channels or receptors (68). The composition and dynamics of cell membranes was postulated in 1972 in the *fluid-mosaic model* (74), which has been further refined, e.g. by the inclusion of membrane-associated cytoskeletal structures (75). Inter- and intramembranous as well as lateral and transversal diffusion and transport of lipids maintains a selective lipid distribution in different organelles and even between the leaflets of the bilayer (76,77). In addition to their structural role, phospholipids can fulfill a number of other important cellular functions, such as PtdIns and its phosphorylated derivates as important keyplayers in cellular signalling and membrane-trafficking (78,79). Furthermore, not only the molecule itself but also its membrane orientation can be crucial for the cellular function, like PtdSer exposed at the outer leaflet of the cell membrane, which acts as an apoptosis signal for macrophage clearance (80).

1.3.2. *De novo* phospholipid synthesis in the mammalian (host) cell

The basic building blocks for the mammalian phospholipid biosynthesis are AcylCoA derivates and glycerol 3-phosphate (G-3-P) as a glycolytic by-product as well as head group precursors, such as choline, ethanolamine, serine or inositol [Fig. 7].

The main sites of phospholipid synthesis in the mammalian cell are the endoplasmic reticulum and the mitochondrion, where the major phospholipids, PtdCho, PtdEtn, PtdSer and PtdIns, are produced by multiple interconnected routes [Fig. 7]. The ER and Golgi apparatus produce the bulk of structural lipids, like PtdCho, sphingomyelin and cholesterol, which are distributed throughout the cell via the endomembrane system. The mitochondria produce and inherit high amounts of bacterial-derived lipids, like PtdEtn, phosphatidylglycerol (PtdGro), and cardiolipin (CL) (76).

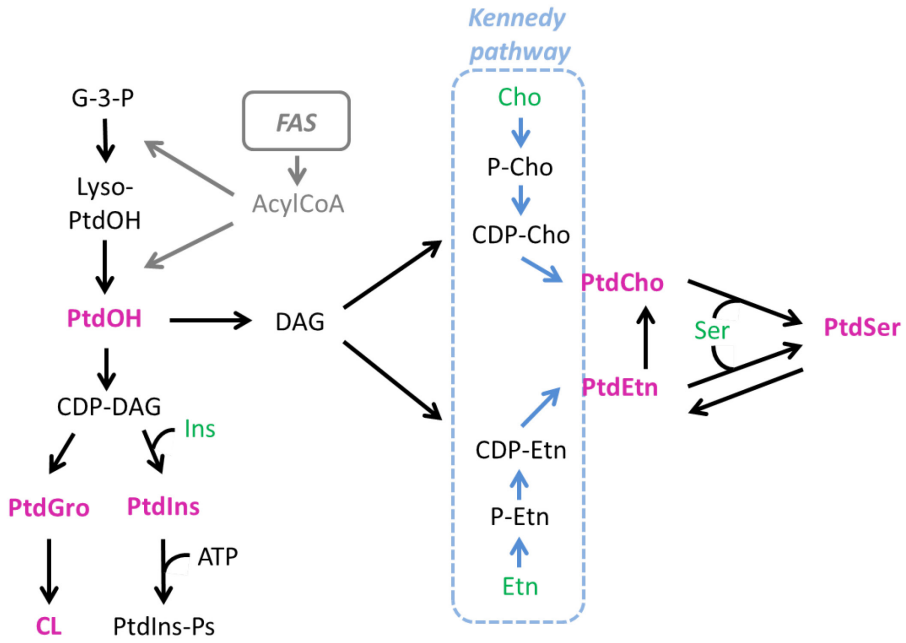

Figure 7: Major phospholipid synthesis pathways in the mammalian cell
Phospholipids are generated via multiple interconnected routes. The synthesis starts with condensation of fatty acids and glycerol-3-phosphate (G-3-P) to produce phospatidic acid (PtdOH). Cytidine diphosphate diacylglycerol (CDP-DAG) is further metabolized into PtdGro, CL and PtdIns. DAG and CDP-choline or CDP-ethanolamine are used as substrates in the *Kennedy pathway* to form PtdCho and PtdEtn, both of which can be converted into PtdSer by a base-exchange reaction using serine as a substrate. Decarboxylation of PtdSer produces PtdEtn, which can be further methylated to PtdCho. Headgroup precursors are shown in green, phospholipid species are marked pink. *Cho*: choline; *Etn*: ethanolamine; *Ins*: Inositol; *Ser*: Serine

The *de novo* synthesis of PtdCho and PtdEtn is mediated by the three-stepped *Kennedy pathway*. The final step is an aminoalcohol-phosphotransferase reaction in the ER, which uses sn-1,2-diacylglycerol and either CDP-choline or CDP-ethanolamine as substrates (81) [Fig. 7]. Both branches are interconnected by the PtdEtn-N-methyltransferase (PEMT) enzyme, converting PtdEtn into PtdCho by sequential trans-methylation in the ER (82). An alternative route of PtdEtn production is the decarboxylation of PtdSer, mediated by PtdSer decarboxylase (PSD) enzymes located in the inner mitochondrial membrane (83). PtdSer is synthesized by two PtdSer synthases (PSS) exchanging the choline or ethanolamine moiety of PtdCho or PtdEtn by serine (84) [Fig. 7]. The mitochondrial cardiolipin is synthesized by the fusion of CDP-DAG and PtdGro via a reaction catalyzed by cardiolipin synthase (85). PtdIns, PtdGro and CL are all synthesized from cytidine diphosphate diacylglycerol (CDP-DAG), which in turn is derived from condensation of phosphatidic acid (PtdOH) and cytidine triphosphate (CTP) (76,86).

1.3.3. Membrane synthesis in *T. gondii*

The membrane synthesis in *T. gondii* seems to reflect a balance of retained pathways for autonomous lipid synthesis and scavenging of host cell lipids. The lipid signature of the parasite shows a mixture of neutral and polar lipids as well as a few unusual species derived from bacterial- and plant-like enzymes. The fatty acid biogenesis in *T. gondii* is mediated by three different concerted intrinsic pathways and salvage of host fatty acids (87), which provide the essential building blocks for the synthesis of phospholipids [see 1.3.4].

Triacylglycerols (TAGs) are formed in the ER via the glycerol-3-phosphate-pathway and stored as lipid bodies (88). Upon nutritional stress, TAGs can be hydrolyzed into diacylglycerols (DAGs) that are another main constituent of membrane building phospholipids. Additionally, galactosylglycerolipids are synthesized in the apicoplast from fatty acids and glycerol-3-phosphate (89). The major sterol molecule in *T. gondii*, cholesterol, is incorporated from LDL-particles derived from host lysosomes (90). Sphingolipids and their precursors can be synthesized *de novo* or retrieved from the extracellular environment. General indications for the import of host lipids were drawn indirectly by the use of fluorescently labelled lipid-analogs (46,91).

1.3.4. Phospholipid biogenesis in *T. gondii*

Phospholipids constitute for a majority of the parasite´s membrane mass and were shown to be derived from intra- and extracellular sources (46,91,92).

Compared with human host cells, *T. gondii* has an unusually high content of PtdCho, which is also the most prevalent parasite lipid accounting for 60-75% of total phospholipid species (92-94). The parasite also harbours an unusual plant-related lipid, phosphoethanolamine-ceramide (PEtn-Cer) (95). Recently, an exclusive and naturally occuring phospholipid was identified in *T. gondii*, which harbours threonine at the headgroup (PtdThr) (96). The relative percentage of the different phospholipid species varies between the respective sample preparation and analysis methods used. But the general sequence of phospholipids ranked by quantity can be described as follows: PtdCho (60-75%) > PtdEtn (12-25%) > PtdIns (8-12%) > PtdThr (2-8%) > CL (~6-8%) > PtdSer (1-6%) > PtdOH (~1,5%) (92,95-97). Futhermore, the parasite was shown to be enriched in shorter and unsaturated fatty acid chains compared to the mammalian host cell (human foreskin fibroblasts) (95,98).

To date, different enzymatic activities in *T. gondii* have been characterized, which catalyze the synthesis of major phospholipids in tachyzoites [Fig. 8]. The presence of a functional phosphatidlyinositol synthase (PIS), expressed exclusively in the tachyzoite stage, was shown by heterologous expression in yeast (99). The two functionally-related (base-exchange) phosphatidylserine synthase (PSS) and Phosphatidylthreonine synthase (PTS) enzymes have also been characterized for their activity and biological relevance in tachyzoites (96).

Catalytic activity of the *Kennedy pathway*, leading to *de novo* PtdCho and PtdEtn formation, was proven by metabolic labelling and characterization of the choline and ethanolamine kinase enzymes. An active Phosphatidylethanolamine N-methyltransferase (PEMT) enzyme seems to be missing in the parasite, making The CDP-choline branch of the *Kennedy pathway* vulnerable to a lipid analogue, Dimethylethanolamine (DME) (92,100).

An unusual high PSD activity had been described in *T. gondii* cell extracts compared to yeast and mammals (92) but the identity of the respective enzyme(s) remained elusive.

Cardiolipin synthesis in *T. gondii* has also been demonstated by acetate-labelling experiments (101). However, the functional characterization of a cardiolipin synthase enzyme (CLS) as well as the identity of other key enzymes, such as cytidyldiphosphate-1,2-diacylglycerol-synthase (CDS) and phosphatidate phosphatase (PAP), remain undefined in *Toxoplasma* to date.

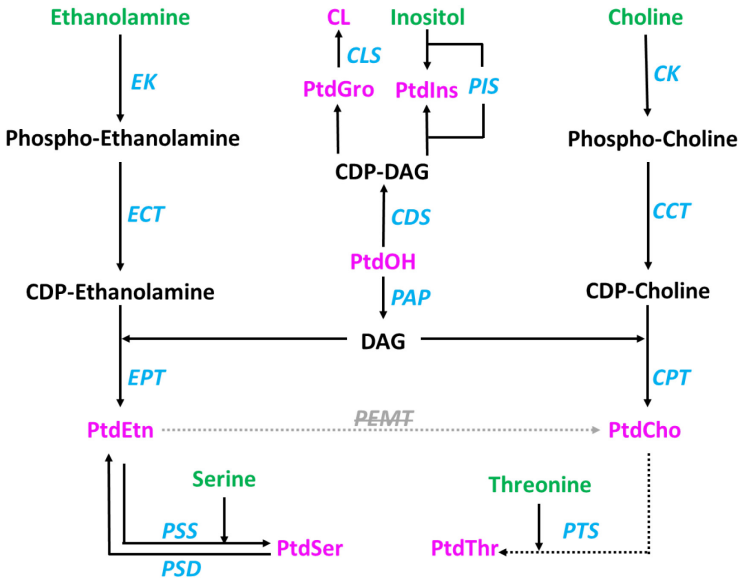

Figure 8: Model of phospholipid synthesis pathways in T. gondii
Current model of the phospholipid biosynthesis pathways in *Toxoplasma* comprising several interconnected routes. A phosphatidate phosphatase (PAP) confers DAG formation from phosphatidic acid, which is an essential component for the synthesis of many lipid species. The synthesis of PtdEtn can be achieved via the CDP-entanolamine pathway, which is comprised of ethanolamine kinase (EK) CTP: phosphoethanolamine cytidylyltransferase (ECT) and CDP-ethanolamine:1,2-diacyglycerol ethanolamine-phosphotransferase (EPT), or by decarboxylation of PtdSer by a phosphatidylserine decarboxylase (PSD) enzyme. A phosphatidylserine synthase (PSS) can use PtdEtn to form PtdSer. There is no evidence for an active PEMT (phosphatidylethanolamine methyltransferase) enzyme in *T. gondii* which indicates an essential CDP-choline pathway mediated by choline kinase (CK). CTP:phospho-choline cytidylyltransferase (CCT) and CDP-choline:1,2-diacyglycerol cholinephosphotransferase (CPT) enzymes. PtdThr is proposed to be formed by a base-exchange reaction of the phosphatidylthreonine synthase (PTS) from PtdCho. The biogenesis of PtdGro, CL and PtdIns rely on CDP-DAG formation via CDS (Cytidyldiphosphate-1,2-diacylglycerol-synthase). A cardiolipin synthase (CLS) is likely involved in CL formation from PtdGro. Phosphatidylinositol synthase (PIS) activity is needed for PtdIns synthesis using CDP-DAG and inositol. Headgroup precursors are depicted in *green*, phospholipids are marked *pink* and the respective enzymes appear in *blue* colour.

Besides the capability of *de novo* phospholipid biosynthesis pathways as shown in figure 8, the possibility of phospholipid salvage of host-derived lipids has also been postulated. Labelling experiments with fluorescent BODIPY-tagged PtdCho and PtdOH have shown accumulation of the phospholipid analogs into the parasite plasma membrane, intracellular compartments as well as the intravacuolar network (46,91). The biological significance of this process for membrane biogenesis is still unknown.

1.4. Objective of this study

T. gondii, as an obligate intracellular parasite, has to sustain and expand its organellar and surrounding membranes during its fast proliferation inside the host cell. The parasite genome encodes a nearly complete enzymatic machinery to synthesize the major phospholipids by the utilization of glycolytic precursors and polar head groups (92). Phosphatidylethanolamine is the second most abundant phospholipid in *T. gondii* and is known to be a critical component for membrane stability and curvature as well as vesicular fusion and fission and mitochondrial function (102).

The aim of this study was the identification of the concrete enzymes responsible for PtdEtn synthesis in the tachyzoite stage. Special emphasis was given to the parasite-encoded phosphatidylserine decarboxylase (PSD) enzymes, since previous work showed an exceptional high PSD activity in tachyzoites compared to yeast and mammalian cell extracts (92), and indicated the presence of two distinct PSD pools with differential enzymatic behaviour (103).

In addition, the relative contribution of PtdSer decarboxylation for PtdEtn homeostasis and its connection to other PtdEtn routes should be determined within this study.

2. Materials and methods

2.1. Materials

2.1.1. Biological resources

COS-7 cells	Isabelle Coppens, Johns Hopkins University, USA
Human Foreskin Fibroblasts (HFF)	Carsten Lüder, University Göttingen, Germany *or* Cell Line Service, Germany
Jurkat T-cells c6.1	Carsten Lüder, University of Göttingen, Germany
T. gondii (RH *Δhxgprt* and RH TaTi strains)	D. Soldati-Favre, University of Geneva, Switzerland
T. gondii (TaTi-*Δku80*)	Boris Striepen, University of Georgia, USA
T. gondi (*Δku80*)	Vern Carruthers, University of Michigan, USA
E. coli XL-1blue	Stratagene, Germany
E. coli M15	Qiagen, Germany
E. coli JA-200 (PSS overexpression strain)	Dennis Voelker, National Jewish Health, USA
S. cerevisiae BY23480 (*Δpsd1Δpsd2*)	Akio Toh-e, Chiba University, Japan
S. cerevisiae PTY44 (*Δpsd1Δpsd2*)	Dennis Voelker, National Jewish Health, USA
S. cerevisiae JSY9750 (*Δpsd2*)	Dennis Voelker, National Jewish Health, USA

2.1.2. Chemical reagents

Agarose	Biozym, Germany
Anhydro tetracycline (ATc)	IBA, Germany
Bromphenol blue	Merck, Germany
DNA marker (1kb ladder)	Fermentas, Germany
Delipidated fetal calf serum	Biowest or PAA, Germany
Dialysed fetal calf serum	PAA, Germany
dNTP-Mix (100mM)	Fermentas, Germany
Dulbecco`s Modified Eagle Media (DMEM)	Biowest or PAA, Germany
Fetal calf serum	PAN Biotech, Germany

Fluoromount-G + 4,6-diamidino-2-phenylindole (DAPI)	SouthernBiotech, USA
GeneRuler DNA-Ladder (1kb)	Fermentas, Germany
L-Glutamine (100x stock)	Biowest, Germany
MEM essential amino acids (50x stock)	Biowest, Germany
MEM non-essential amino acids (100x stock)	Biowest, Germany
MEM vitamins (100x stock)	PAA, Germany
Natural and synthetic lipids	Avanti Polar Lipids, USA
NBD-Lipids	Avanti Polar Lipids, USA
Paraformaldehyde	Merck, Germany
Penicillin/ Streptomycin (100x stock)	Biowest, Germany
Phosphat Buffered Saline (PBS)	Biowest or PAA, Germany
Potassium hydroxide	Merck, Germany
Protein marker (prestained)	Fermentas, Germany
Pyrimethamine	AK Scientific, USA
RPMI-1640 medium	PAA, Germany
Sodium pyruvate (100x stock)	Biowest, Germany
Tris-Hydrochloride	Promega, USA
Trypsin/EDTA	Biowest, Germany

Other standard chemicals were purchased from AppliChem (Germany), Carl Roth (Germany) or Sigma Aldrich (Germany), if not stated otherwise above.

2.1.3. Materials for radioactive work

L-[1,2-^{14}C]-Ethanolamine	American Radiolabled Chemicals *or* Hartmann Analytic, Germany
L-[1-^{14}C]-Serine	ICN Radiochemicals, USA
L-[1-^{3}H]-Serine	American Radiolabled Chemicals
Liquid scintillation cocktail	Perkin-Elmer, USA
Scintillation vials	Perkin-Elmer, USA
24-well scintillation plate	Perkin- Elmer, USA

2.1.4. Vectors

Vector description	Source
pcDNA3.1(+)	Isabelle Coppens, Johns Hopkins Univ. , USA
pESC-Ura	Agilent Technologies, USA
pNTP3	Isabelle Coppens, Johns Hopkins Univ., USA
pNTP3-TetO7-Sag1	modified *pNTP3*
pQE-60	Qiagen, Germany
pTgS9$_{(33-159)}$-GFP	Frank Seeber, RKI Berlin, Germany (DeRocher et al., 2000 (104))
pTet-UPKO	modified *pNTP3*
pTKO-HXGPRT	John Boothroyd, Standford Univ., USA
pTKO-DHFR	modified *pTKO-HXGPRT*
pTub8-TgDer1-GFP	Boris Striepen, Univ. of Georgia, USA
pYES2.1 TOPO	Life technologies, USA

2.1.5. Antibodies and working dilutions

Antigen (produced in)	Dilution	Source
α-*Tg*Actin (mouse)	1:1000	D. Soldati-Favre, Univ. of Geneva, Switzerland
α-*Tg*CK (mouse)	1:200	Sampels et al., 2012 (100)
α-*Tg*F1B (mouse)	1:1000	P. Bradley, Univ. California, USA (105)
α-*Tg*Gap45 (rabbit)	1:3000	Plattner et al., 2008 (106)
α-GFP (rabbit)	1:10000	Life technologies, Germany
α-*Tg*Gra1 (mouse)	1:500	M. Cesbron-Delauw, CNRS Grenoble, France (43)
α-*Tg*Gra3 (rabbit)	1:500	Bermudes et al., 1994 (107)
α-*Tg*Gra5 (mouse)	1:500	M. Cesbron-Delauw, CNRS Grenoble, France (108)
α-HA (mouse)	1:10000	Sigma Aldrich, Germany
α-HA (rabbit)	1:1000	Sigma Aldrich, Germany
α-His (mouse)	1:2000	Dianova, Germany
α-*Tg*Hsp90 (rabbit)	1:1000	Echeverria et al., 2005 (109)
α-KDEL (mouse)	1:1000	Kaufusi et al., 2014 (110)
α-*Tg*Sag1 (mouse)	1:1000	Dubremetz et al., 1985 (111)
α-V5 (mouse)	1:1000	Abcam, United Kingdom

Secondary antibodies (produced in)	Dilution	Source
Alexa Fluor 488 anti-mouse IgG (goat)	1:3000	Life technologies, Germany
Alexa Fluor 594 anti-rabbit IgG (goat)	1:3000	Life technologies, Germany

2.1.6. Enzymes

Antarctic phosphatase	New England Biolabs, Germany
Dream Taq polymerase	Fermentas, Germany
Pfu Ultra II Fusion HS DNA polymerase	Stratagene, Germany
Proteinase K	Sigma Aldrich, Germany
Restriction endonucleases, Klenow enzyme	New England Biolabs, Germany
T4 ligase	Life technologies, Germany

2.1.7. Instruments

Amaxa Nucleofector	Lonza, Switzerland
Analytical balance FA-30100-2	FAUST, Germnay
BioPhotometer	Eppendorf, Germany
BTX Square Porator ECM 830	BTX, USA
Centrifuges	Eppendorf, Germany
Counting chamber (Neubauer improved)	Fuchs Rosenthal, Germany
ELISA microplate reader	Biotek, Germany
Fluorecence microscope Axio Image.Z2	Zeiss, Germany
Gel documentation & EASY Enhanced Analysis	Herolab, Germany
Gel electrophoresis system EasyPhor	Biozym, Germany
Heraeus Function Line Incubator	Hereaus, Germany
Incubation shaker „innova 4000“	New Brunswick, USA
Invertical microscpe LABOVERT	Leitz, Germany
Light optical microscope DM750	Leica, Germany
Micropipettes	Eppendorf, Germany
Multichannel-Pipettes	Brand, Germany
NanoDrop ND-1000 Spectrophotometer	Peqlab, Germany
PCR Thermocycler (FlexCycler)	JenaAnalytic, Germany
Safety work benches HeraSafe	Heracell, Germany
Scintillation counter (1450 MicroBeta TriLux)	PerkinElmer, USA

Thermomixer comfort	Eppendorf, Germany
TLC developing tank	Sigma Aldrich, Germany
Waterbath julabo U3	Julabo, Germany
Ultra-Centrifuge Avanti J-26S XP	Beckmann Coulter, USA
UV-screen UVT-20M/W	Herolab, Germany

2.1.8. Plasticware and other disposables

Cell culture well plates (6, 24, 96 well)	Costar, USA
Cell culture dishes (60x15 mm)	Greiner Bio One, Germany
Cryo preservation tubes (1.8 ml)	Sarstedt, Germany
Borosilicate glass tubes (16 ml)	Sigma Aldrich, Germany
DuraSeal Laboratory sealing film	Diversified Biotech, USA
Electroporation cuvettes	Eppendorf, Germany
Falcon-Tubes (15 ml; 50 ml)	Greiner Bio One, Germany
Filter (5 µm)	Merck Millipore, Germany
Filter sterilizer (0.22 µm)	Schleicher Schuell, Germany
Glass bottom dishes (MatTek)	MatTek Corporation, USA
Glass cover slips and microscopic slides	Carl Roth, Germany
Hamilton Syringes	Hamilton, USA
High performance chemiluminescence film	GE Healthcare, Germany
Hypodermic needles	BD Bioscience, Germany
Nitrocellulose transfer membrane	AppliChem, Germany
Parafilm	Bemis Company, USA
Pasteur pipettes	A. Hartenstein, Germany
PCR-tube-stripes (0.2 ml)	Biozym, Germany
PCR-tubes (0.2 ml)	Sarstedt, Germany
Petri dishes (94x16 mm)	Greiner Bio One, Germany
Pipette tips (10 – 1000 µl)	Greiner Bio One, Germany
Polypropylene culture tubes (12 ml)	Greiner Bio-One, Germany
Reaction tubes (1.5 ml; 2 ml)	Sarstedt, Germany
RNAase-free barrier tips	Biozym, Germany
SDS Electrophoresis Unit SE250 Mighty Small II	Hoefer Inc, USA
Semi-dry Electro blotter SEDECM	Peqlab, Germany

Serological pipettes (10 ml; 25 ml)	Greiner Bio One, Germany
Size exclusion columns (30 kDa)	Merck Millipore, Germany
Syringes	BD Bioscience, Germany
TLC silica gel 60 plates	Merck Millipore, Germany
Whatman paper (3 MM)	A. Hartenstein, Germany

2.1.9. Commercial kits

Annexin-V-FLUOS staining kit	Roche, Germany
cAMP Biotrak EIA kit	GE healthcare, USA
cloneEZ PCR cloning kit	GenScript, Germany
innuPREP DOUBLEpure kit	Analytik Jena, Germany
innuPREP Plasmid Mini kit	Analytik Jena, Germany
Lipofectamine 2000	Life Technologies, Germany
Membrane recycling kit	Alpha Diagnostics Intl., USA
pDrive PCR cloning kit	Qiagen, Germany
Protein Assay kit (BCA)	Thermo Scientific, Germany
Pure Link RNA Mini kit	Life Technologies, Germany
PureLink HiPure Plasmid Midiprep kit	Life Technologies, Germany
QIAGEN PCR Cloning kit	Qiagen, Germany
Reverse transcription PCR (SuperScript III)	Life Technologies, Germany
Trizol reagent	Life Technologies, Germany
WesternBright Quantum WB Detection kit	Advansta Inc., USA

2.1.10. Reagent preparations

Media and buffers for *E. coli*

Standard media (Lysogeny Broth, Super Optimal Broth or Super Optimal Broth for Catabolite repression) for the cultivation of *E. coli* were made according to Sambrock et al. (112) or other following standard protocols. 15 g agar-agar (Carl Roth, Germany) was added to 1 liter of liquid medium before autoclaving for the preparation of solid media.

Media and buffers for *T. gondii*

Cytomix:	120 mM	KCl
	25 mM	HEPES (pH 7.6)
	5 mM	$MgCl_2$
	2 mM	EDTA
	0.15 mM	$CaCl_2$
	10 mM	K_2HPO_4/KH_2PO_4 (pH 7.6)
D10-medium:	500 ml	DMEM (4500 mg/l glucose)
	50 ml	iFCS (heat-inactivated)
	5.5 ml	Penicillin/Streptomycin (100x)
	5.5 ml	Non-essential amino acids (100x)
	5.5 ml	L-Glutamine (200 mM)
	5.5 ml	Sodium pyruvate (100 mM)
Intracellular-type medium (ICM):		
2x Salts for ICM:	280 mM	KCl
	20 mM	NaCl
	5 mM	$MgCl_2$
	40 mM	HEPES/NaOH (pH 7.4)
	10 mM	Glucose
	0.2µM	$CaCl_2$
100 ml ICM contain:	50 ml	2x Salts
	1 ml	100x MEM Vitamins
	2 ml	50x MEM Amino Acids
	1 ml	100x Sodium pyruvate
	1 ml	MEM non-essential amino acids
	45 ml	dH_20
	1 mM	MgATP (freshly added)
Lysis buffer (gDNA-extraction):	10 mM	Tris-HCl (pH 8)
	5 mM	EDTA
	0.5%	SDS
	200 mM	NaCl
	0.1 g/ml	Proteinase K
Parasite extract buffer	50 mM	Potassium phosphate buffer (pH 6.8)
	0.25 M	Sucrose
	0.5 mM	PMSF
	3 mM	EDTA
PBS	2.7 mM	KCl
	1.5 mM	KH_2PO_4
	136.9 mM	NaCl
	8.9 mM	Na_2HPO_4 $7H_2O$
Toxo-freezing medium:	10% DMSO in iFCS	

Media and buffers for *S. cerevisiae*

YPD-media	20 g	Peptone
	10 g	Yeast extract
	20 g	Agar-agar (for solid media)
	ad 950 ml dH_2O	
	2% filter-sterile glucose was added after autoclaving.	
10x amino acid mix	400 mg adenine hemisulfate, 200 mg L-Arg, 1000 mg L-Asp, 1000 mg L-Gln, 200 mg L-His, 600 mg L-Leu, 300 mg L-Lys, 200 mg L-Met, 500 mg L-Phe, 3750 mg, L-Ser, 2000 mg L-Thr, 400 mg L-Try, 300 mg L-Tyr, 1500 mg L-Val, 200 mg Uracil ad 500 ml ddH_2O (Uracil was omitted for preparation of selective media.)	
Synthetic complete (SC) medium	1.7 g	Yeast nitrogen base (w/o $(NH_4)_2SO_4$)
	5 g	$(NH_4)_2SO_4$
	ad 500 ml dH_2O.	
	Sterile amino acids and glucose, galactose or lactate as carbon source (filter sterile 40% stock solution) were added after autoclaving to a final concentration of 2%.	

Standard buffers and reagents

SDS loading buffer (5x)	0.25%	Bromophenol blue
	0.5 M	Dithiothreitol (DTT)
	50%	Glycerol
	10%	Sodium dodecyl sulfate (SDS)
	0.25 M	Tris-HCl (pH 6.8)
SDS-running buffer (10x)	30 g	Tris base (pH 8.3)
	144 g	Glycine
	10 g	SDS
	ad 1000 ml dH_2O	
SEM buffer	250 mM	Sucrose
	1 mM	EDTA
	10 mM	MOPS/KOH (pH 7.2)
Semi-dry transfer buffer	5.8 g	Tris base
	2.9 g	Glycine
	0.37 g	SDS
	200 ml	Methanol
	Ad 1000 ml dH_2O	
TBS-buffer (10x)	60.6 g	Tris-HCl (pH 7.6)
	87.6 g	NaCl
	ad 1000 ml dH_2O	

2.1.11. Oligonucleotide primers

All oligonucleotides listed below were synthesized by Life Technologies, Germany.

Table 1: Oligonucleotide primers used in this study

Primer Name (restriction site)	Nucleotide Sequence (restriction site underlined)	Cloning Vector (research objective)
Expression of *Tg*PSD1mt-HA, *Tg*PSD1mt$_{(91-427)}$-HA und *Tg*PSD1mt $_{(113-427)}$ in *T. gondii* (Δ*ku80-hxgprt*$^-$ and Δ*ku80-TaTi strains*)		
*Tg*PSD1mt-F (*Nco*I)	CTCATCCCATGGGCAGTTACTTGCGGTTTTCG	*pUPKO* (Ectopic expression of *Tg*PSD1mt-HA)
*Tg*PSD1mt-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGGT AGTAAAATGCAAACAGGCGTTC	
*Tg*PSD1-IT-F (*Xcm*I)	CTCATCCCACCGGTCACCTGGGAGCCATGTGGCAGATATTC	*pTKO-HXGPRT* (3'HA-tagging of the *TgPSD1mt* gene locus)
*Tg*PSD1-IT-HA-R (*Hpa*I)	CTCATCCAATTGTCAAGCGTAATCTGGAACATCGTATGGGT AGTAAAATGCAAACAGGCGTTC	
*Tg*PSD1mt$_{(91-427)}$-F (*Nco*I)	CTCATCCCATGGTTGGCATGACCGCG	*pTETO7SAG1-UPKO* (Ectopic expression of *Tg*PSD1mt$_{(91-427)}$ at the *TgUPRT* gene locus)
*Tg*PSD1mt$_{(91-427)}$-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGTAAAATGCAAACAGGCGTTCATCTT	
*Tg*PSD1mt$_{(113-427)}$-F (*Nco*I)	CTCATCCCATGGCGACAG ACAATGTTGCAGA	*pTETO7SAG1-UPKO* (Ectopic expression of *Tg*PSD1mt$_{(113-427)}$ at the *TgUPRT* gene locus)
*Tg*PSD1mt$_{(113-427)}$-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGTAAAATGCAAACAGGCGTTC	
Expression of *Tg*PSD1pv-HA in *T. gondii* (Δ*ku80-TaTi and RH HX*$^-$ *strain*)		
*Tg*PSD1pv-5'UTR-F (*Eco*RV)	CTCATCGATATCTGAAGGGAAGAAGCGAAGG	*pUPKO* (Cloning of the *TgPSD1pv* gene promoter)
*Tg*PSD1pv-5'UTR-R (*Msc*I)	CTCATCTGGCCACCAGGGCCACGCACAC	
*Tg*PSD1pv-F (*Msc*I)	CTCATCTGGCCATTTCGGTACCAGCAGCGT	*pUPKO* (Expression of the *Tg*PSD1pv-HA ORF under its native promotor)
*Tg*PSD1pv-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGTAAAATGCAAACAGGCGTTCA	
*Tg*NTP3-3'UTR-F (*Pac*I)	CTCATCTTAATTAAAATGTCGATTGATGGTGTCG	*pUPKO* (Cloning of the *Tg*NTP3-3´UTR)
*Tg*NTP3-3'UTR-R (*Not*I)	CTCATCGCGGCCGCACTAGTGTGGCGCCACGG	
*Tg*PSD1pv-F2 (*Eco*RV)	CTCATCGATATCATGGCTAGGGTTATGAGGCTTATC	*pNTP3-DHFR* (Ectopic over-expression of *Tg*PSD1pv)
*Tg*PSD1pv-HA-R2 (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGAGATCCCCATTGGTAAGCA	

Expression of *Tg*PSD1mt, *Tg*PSD1mt$_{(113-427)}$ and *Sc*PSD1 in *S. cerevisiae* (BY23480 or PTY44 strain)		
*Tg*PSD1mt-F (*Not*I)	CTCATCGCGGCCGCATGCGCAGTTACTTGCGGT	*pESC-Ura* (Ectopic expression of *Tg*PSD1mt ORF)
*Tg*PSD1mt-R (*Not*I)	CTCATCGCGGCCGCTCAGTAAAATGCAAACAGGCGT	
*Tg*PSD1mt$_{(113-427)}$-F (*Bgl*II)	CTCATCAGATCTATGGCGACAGACAATGTTGCAGAGAT	*pYES2.1* (Ectopic expression of *Tg*PSD1mt$_{(113-427)}$)
*Tg*PSD1$_{(113-427)}$-R (*Eco*RI)	CTCATCGAATTCTCAGTAAAATGCAAACAGGC	
*Sc*PSD1-F (*Not*I)	CTCATCGCGGCCGCATGTCAATTATGCCAGTTAAGAACG	*pESC-Ura* (Ectopic expression of the *Sc*PSD1 ORF)
*Sc*PSD1-R (*Not*I)	CTCATCGCGGCCGCTCATTTTAAATCATTCTTTCCAATT	
Expression of *Tg*PSD1pv, *Sc*mTP-*Tg*PSD1pv, *Tg*PSD1pv-CD and *Sc*mTP-*Tg*PSR in *S. cerevisiae* (BY23480 or PTY44 strain)		
*Tg*PSD1pv-SC-R (*Not*I)	CTCATCGCGGCCGCATGGCTAAGGTTATGAGGCTTATC	*pESC-Ura* (Ectopic expression of *Tg*PSD1pv ORF in yeast)
*Tg*PSD1pv-SC-R (*Not*I)	CTCATCGCGGCCGCTCAGAGATCCCCATTGGTAAG	
*Sc*PSD1mtp-F (*Eco*RI)	CTCGAATTCATGTCAATTATGCCAGTTAAGAACG	*pESC-Ura* (Targeting of *Tg*PSDs to yeast mitochondria)
*Sc*PSD1mTP-R (*Not*I)	CTCGCGGCCGCATCGAGACATCGCATTCAGC	
*Tg*PSD1pv-woSP-Y-F (*Not*I)	CTCGCGGCCGCATGCTCACGTTGAATCGACG	*pESC-Ura* (for tagging with *Sc*PSD1mTP)
*Tg*PSD1pv-CD1-F (*Not*I)	CTCGCGGCCGCATGAGTGACAAGGACAGACGC	*pESC-Ura* (Ectopic expression of *Tg*PSD1pv catalytic domain in yeast)
*Tg*PSD1pv-CD1-R (*Not*I)	CTCGCGGCCGCTCAGAGATCCCCATTGGTAAG	
*Tg*PSD1pv-CD2-F (*Not*I)	CTCGCGGCCGCATGTGGGAATTTCCTGACAC	*pESC-Ura* (Ectopic expression of *Tg*PSD1pv catalytic domain in yeast)
*Tg*PSD1pv-CD2-R (*Not*I)	CTCGCGGCCGCCTAAAATGCGTTGCATCTCTCT	
Generation of the Δ*tgpsd1mt/Tg*PSD1mt-HA$_r$ mutant in *T. gondii* (Δ*ku80-TaTi* strain)		
*Tg*PSD1mt-5´UTR-F1 (*Xcm*I)	CTCATCCCACCGGTCACCTGGACTTCTTCAGCACATCGTGTGT	*pTKO-DHFR-TS* (Cloning of the *Tg*PSD1mt-5´UTR)
*Tg*PSD1mt-5´UTR-R1 (*Spe*I)	CTCATCACTAGTGCAAACATCTCAAGAGAAGCAC	
*Tg*PSD1mt-3´UTR-F1 (*Hpa*I)	CTCATCGTTAACTTTGACTGAATCGCTTTGTTG	*pTKO-DHFR-TS* (Cloning of the *Tg*PSD1mt-3´UTR)
*Tg*PSD1mt-3´UTR-R1 (*Apa*I)	CTCATCGGGCCCACAGCGAAACCCCTTCAG	

Screening for 5' and 3' recombination in the Δ*tgpsd1mt/Tg*PSD1mt-HA$_r$ mutant of *T. gondii*		
*Tg*PSD1mt- 5´Scr-F1	GCGAGCAGGGACTAAGTGG	*pDrive* (TA-cloning of 5' PCR product for sequencing)
*Tg*PSD1mt-5´Scr-R1	CACAGTCTCACCTCGCCTTG	
*Tg*PSD1mt-3´Scr-F1	CGGAAAGTGCTTACATCGAAC	*pDrive* (TA-cloning of 3' PCR product for sequencing)
*Tg*PSD1mt-3´Scr-R1	GACCGACGGCAGTATGTTG	
Generation of Δ*tgpsd1mt* mutant in *T. gondii* (Δ*ku80-hxgprt*$^-$ strain)		
*Tg*PSD1mt-5´UTR-F2 (*Kpn*I)	CTCATCGGTACCTCTGAAACCGGTTACAGACCA	*pTKO-HXGPRT* (Cloning of the *Tg*PSD1mt-5´UTR)
*Tg*PSD1mt-5´UTR-R2 (*Xho*I)	CTCATCCTCGAGCTCTGGAAGCCATAACTAGAGAAACA	
*Tg*PSD1mt-3´UTR-F2 (*Hpa*I)	CTCATCGTTAACTCATGCACATGGTTGCTGTG	*pTKO-HXGPRT* (Cloning of the *Tg*PSD1mt-3´UTR)
*Tg*PSD1mt-3´UTR-R2 (*Apa*I)	CTCATCGGGCCCAACCAATGGTCGACGAAGC	
Screening for 5' and 3' recombination in the Δ*tgpsd1mt* mutant of *T. gondii*		
*Tg*PSD1mt-5´Scr-F2	CGGTTTCTTTGTCGTATTCCC	*pDrive* (TA-cloning of 5' PCR product for sequencing)
*Tg*PSD1mt-5´Scr-R2	GACGCAGATGTGCGTGTATC	
*Tg*PSD1mt-3´Scr-F2	ACTGCCGTGTGGTAAAATGAA	*pDrive* (TA-cloning of 3' PCR product for sequencing)
*Tg*PSD1mt-3´Scr-R2	GAAAGGAGTGAAGGAGCCTATCA	
Expression of *Tg*EK-HA in *T. gondii* (Δ*ku80-TaTi strain*)		
*Tg*EK-F (*Xho*I)	CTCATCCCTGCAGGATGGCTCTCCACACTGCA	*pTgGRA1-UPKO* (Ectopic expression of *Tg*EK-HA at the *TgUPRT* gene locus)
*Tg*EK-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGAACGACAAATGCGGGAC	
Expression of putative *Tg*ECT-HA in *T. gondii* (Δ*ku80-TaTi strain*)		
*Tg*ECT-F (*Nsi*I)	CTCATCATGCATATGACGGCGGTAGCGTCG	*pTgGRA1-UPKO* (Ectopic expression of *Tg*ECT-HA at the *TgUPRT* gene locus)
*Tg*ECT-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGAGTTCTGTCAGAGACACCATCTGT	
Expression of putative *Tg*EPT1-HA and *Tg*EPT2-HA in *T. gondii* (Δ*ku80-TaTi strain*)		
*Tg*EPT1-F (*Nsi*I)	CTCATCATGCATATGATGGTCGGTGGCGT	*pTgGRA1-UPKO* (Ectopic expression of *Tg*EPT1-HA at the *TgUPRT* gene locus)
*Tg*EPT1-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAGGAGCTCTTTTTGAGAGCATTAA	
*Tg*EPT2-F (*Nsi*I)	CTCATCATGCATATGGTGTTTGGACACTACATTCCCCC	*pTgGRA1-UPKO* (Ectopic expression of

*Tg*EPT2-HA-R (*Pac*I)	CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGG GTAAGCCCCGCGCCGTCTG	*Tg*EPT2-HA at the *TgUPRT* gene locus)
Expression of *Tg*PSD1mt-V5 and *Tg*PSD1pv-V5 in COS-7 cells		
*Tg*PSD1mt-ME-F (*Nhe*I)	CTCATCGCTAGCATGCGCAGTTACTTGCGG	*pCDNA3.1* (Ectopic expression in mammalian cells)
*Tg*PSD1mt-ME-R (*Xba*I)	CTCATCTCTAGAGTAAAATGCAAACAGGCGTTC	
*Tg*PSD1pv-ME-F (*Hind*III)	CTCATCAAGCTTATGGCTAAGGTTATGAGGCTTATC	*pCDNA3.1* (Ectopic expression in mammalian cells)
*Tg*PSD1pv-ME-R (*Xba*I)	CTCATCTCTAGAGAGATCCCCATTGGTAAGCA	
Cloning of the *pTKO-TgPSD1pv-5´TGD-HXGPRT-3´TGD construct*		
*Tg*PSD1pv-5´TGD-F (*Not*I)	CTCATCGCGGCCGCTAGGTGTCTTGCCACTATGTGGT	*pTKO-HXGPRT* (Cloning of the *Tg*PSD1pv-5´TGD fragment)
*Tg*PSD1pv-5´TGD-R (*Eco*RI)	CTCATCGAATTCTCGCATAGAGGCACATCTAT	
*Tg*PSD1pv-3´TGD-F (*Hind*III)	CTCATCAAGCTTGGTGGGCATACACCAATGC	*pTKO-HXGPRT* (Cloning of the *Tg*PSD1pv-3´-TGD fragment)
*Tg*PSD1pv-3´TGD-R (*Hpa*I)	CTCATCGTTAACTAGGTCGTCGTCTGGTG	
Screening for 5' and 3' recombination in the Δ*tgpsd1pv-TGD* mutant of *T. gondii*		
*Tg*PSD1pv-5´TGD-Scr-F3	CAGACACACAAACTGACTCAAACAG	*pDrive* (TA-cloning of 5' PCR product for sequencing)
*Tg*PSD1-5´TGD-Scr-R3	GACGCAGATGTGCGTGTATC	
*Tg*PSD1-3´TGD-Scr-F3	ACTGCCGTGTGGTAAAATGAA	*pDrive* (TA-cloning of 3' PCR product for sequencing)
*Tg*PSD1pv-3´TGD-Scr-R3	CAATTTTCGTCGTCGCCA	
RT-PCR to analyse transcript abundance in *T. gondii* cDNA		
*Tg*ELf1α-F	AGTCGACCACTACCGGACAC	Control housekeeping gene for RT-PCR
*Tg*ELf1α-R	CTCGGCCTTCAGTTTATCCA	
Expression of *Tg*PSD1pv-CD1 and *Tg*PSD1pv-CD2 in *E. coli*		
*Tg*PSD1pv-EC1-F1 (*Bgl*II)	CTCATCAGATCTATGTTCCGACGCCGTGG	*pQE-60* (Expression of *Tg*PSD1pv without signal peptide in *E.coli* M15 strain)
*Tg*PSD1pv-EC1-R1 (*Bgl*II)	CTCATCAGATCTGAGATCCCCATTGGTAAGCA	
*Tg*PSD1pv-EC2-F1 (*Bgl*II)	CTCATCAGATCTATGTTCCGACGCCGTGG	*pQE-60* (Expression of *Tg*PSD1pv w/o SP and C-term. extention in *E.coli* M15)
*Tg*PSD1pv-EC2-R1 (*Bgl*II)	CTCATCAGATCTAAATGCGTTGCATCTCTCTTG	

2.1.12. Software

AxioVision	Carl Zeiss, Germany
CLC sequence viewer	CLC bio, Netherlands
FlowJo	FlowJo LCC, USA
FigTree (v4.1)	http://tree.bio.ed.ac.uk/software/figtree/
GraphPad Prism 5.0	GraphPad software Inc., USA
ImageJ (v1.45s)	http://imagej.nih.gov/ij/index.html
Microsoft Office 2010	Microsoft Corporation, USA
Protein Molecular Weight	http://www.bioinformatics.org/sms/prot_mw.html
SnapGene	GSL Biotech LLC, USA

2.2. Methods – Molecular Cloning and Protein analysis

2.2.1. PCR reactions

To amplify DNA fragments for molecular cloning Pfu-Ultra Fusion II high-fidelity polymerase (Stratagene) was used, whereas Fermantas Dream Taq Polymerase was used for analytical PCR reactions including colony PCRs. 10-200 ng of DNA was used as template in standard PCR reactions. The reaction was performed in a Thermocycler (FlexCycler, Analytik Jena) according to the manufacturer's protocol. For the screening of bacterial colonies, cells were suspended in 20 µl of sterile dH_2O, and 5 µl of the solution was used as PCR template. The DNA fragments mixed with DNA-loading dye were separated on 0.8-1.2% agarose gels (stained with RedSafe DNA staining solution) at 90-120 V in 1 x TAE buffer followed by UV-visualization.

2.2.2. DNA restriction and ligation

PCR products were analysed for their purity and expected fragment size. They were either column purified or cut from agarose gel and extracted using the innuPREP DOUBLEpure Kit (Analytik Jena). Plasmid DNA was isolated from *E. coli* overnight cultures using the innuPREP Plasmid Mini Kit (Analytik Jena). For DNA digestion, 0.5-50 µg DNA was used as substrate for the restriction endonucleases (New England Biolabs). 0.4 U/µg DNA was used for the linearization of plasmids, whereas 2.5 U/µg DNA was used for the restriction of PCR-fragments. The reaction was performed according to the manufacterers protocol. For non-directional cloning, plasmids were dephosphorylated using Antarctic phosphatase. Insert and plasmid DNA were used in a molar ratio of

3:1 or 5:1 (fmol insert : fmol vector) for sticky and blunt end ligation reactions, respectively. The T4-DNA-ligase reaction was performed over night at RT or at 4 °C prior to transformation into chemical competent *E. coli* Xl1-blue or M15 cells.

2.2.3. Transformation of *E. coli*

90 µl of competent *E. coli* cells were mixed with the ligation reaction and incubated on ice for 30 min. The bacterial cells were heat-shocked for 45 sec at 42 °C in a waterbath and then immediately chilled on ice for 1-2 min prior to the addition of 700 µl liquid SOC-medium and incubation at 37 °C and 250 rpm for 1 hr. Cells were pelleted by centrifugation and suspended in 200 µl of fresh SOC-medium for plating on selective LB-agar containing ampicillin (0.1 mg/ml) or kanamycin (0.05 mg/ml). Plates were incubated over night at 37 °C and appearing colonies were screened for plasmid expression by PCR. Positive clones were used for plasmid preparations and/or protein expression in *E. coli* M15 cells. Overnight cultures were cryo-preserved in 25% glycerol at -80 °C.

2.2.4. Nucleic acid preparation

For the preparation of plasmid DNA, 5 ml of *E. coli* liquid cultures were processed using the innuPREP Plasmid Mini Kit. The DNA was eluted in 2 x 20 µl dH_2O. For large-scale plasmid preparation, 200 ml of bacterial overnight cultures were processed with the PureLink HiPure Plasmid Midiprep kit. The DNA was precipitated by addition of 2 volumes 96% ethanol and sodium acetate (0.3 M) for 1 h at 4 °C and subsequently pelleted at 16.000 x g, washed with 1 volume 70% Ethanol, air dried and resuspended in an adequate volume of dH_2O. Genomic *T. gondii* tachyzoite DNA was extracted by resuspending the parasite pellet in 200 µl of Toxo lysis buffer [2.1.10.] supplemented with 1 µl fresh Proteinase K (50 mg/ml) and incubation at 55 °C for 30 min. The gDNA was precipitated with 1 volume of pure isopropanol and centrifugation at 16.000 x g for 45 mins. The final pellet was dissolved in 10-50 µl ddH_2O. To extract RNA, RNase-free plasticware and DEPC-treated water were used throughout the procedure. Trizol reagent and PureLink RNA MiniKit were used for RNA preparation and purification, which was eluted with 10-30 µl RNase-free water. cDNA synthesis was performed using the SuperScript III First-strand synthesis kit (Life Technologies) with either oligo-dT- or random hexamer primers. To determine the nucleic acid concentrations, the NanoDrop Spektralphotometer 1000 was used, and samples were stored at -20 °C (plasmid DNA), -80 °C (RNA) or 4 °C (genomic DNA).

2.2.5. Expression of recombinant proteins in *E. coli*

For heterologous expression of *T. gondii* proteins in *E. coli*, parasite cDNA was used to amplify truncated versions of *Tg*PSD1pv. The DNA fragments were cloned into the *pQE-60* expression vector at *Bgl*II restriction site, which resulted in a C-terminal 6xHis-tag fusion. The *E. coli* M15 strain was used for protein expression. 20 ml of *E. coli* overnight culture were diluted to an OD_{600} of 0.1 in 500 ml medium and grown to an OD_{600} of 0.4 before induction with 1 mM IPTG over night at 30 °C. Cells were pelleted by centrifugation (3000 x g, 20 min) and stored at -80 °C until use. The pellets were resuspended in 6 ml of lysis buffer containing 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 10 mM imidazol, 10% glycerol and 60 µl protease inhibitor cocktail (containing 2 mM AEBSF, 0.3 µM Aprotinin, 130 µM Bestatin, 1 mM EDTA, 14 µM E-64 and 1 µM Leupeptin). Cells were disrupted by 6 x 30 sec probe sonication intervals on ice followed by centrifugation at 12.000 x g for 20 min to remove intact cells. The supernatant containing the cell lysate was loaded on a NiNTA column, washed with 8 ml washing buffer (20 mM NaH_2PO_4 pH 7.8, 500 mM NaCl, 20 mM imidazol) and eluted with 100 mM and 200 mM imidazole in 20 mM NaH_2PO_4 buffer (pH 7.8) containing 500 mM NaCl. The eluate was stored on ice and PIC was added before concentrating the sample using an 30 kDa size exclusion column (Merck Millipore, Germany). Samples were stored at -80 °C until use. The protein concentration was determined by BCA assay and 5-10 µg of purified protein was used for SDS-PAGE and Western Blot analysis using an anti-His antibody.

2.2.6. Protein extraction, preparation and Immuno-blot analysis

Fresh extracellular parasites (~3-5 x 10^7) were washed with PBS and pelleted (400 x g, 10 min, 4 °C) before snap-freezing in liquid nitrogen. The pellets were either directly thawed in SDS-sample buffer or in 10 mM MOPS/KOH buffer (pH 7.2) containing 250 mM sucrose, and 1 mM EDTA supplemented with fresh PIC for the preparation of membrane proteins. The samples were probe sonicated on ice (3 x 30 s burst) followed by centrifugation (2000 x g, 5 min, 4 °C) to remove intact cells. The cell free extract was centrifuged at 30.000 x g for 1 hr. The resulting membrane pellet was suspended in 1% Triton X-100 and 2x SDS sample loading buffer. SDS-PAGE and Western Blot Analysis were performed using standard protocols (112). Briefly, proteins were separated on 10-12% SDS-polyacrylamid gels at 100 V in SDS-running buffer followed by transfer to a nitrocellulose membrane at 0.8mA/cm^2. The membrane was blocked in 5% skim milk (in TBS-0.1% Tween) over night at 4 °C. Primary antibodies (α-HA 1:500 – 1:1000, α-His 1:2000, α-*Tg*Actin 1:1000, α-*Tg*Hsp90 1:1000 in blocking solution) were used for epitope binding for 2-4 hrs at RT or over night at 4 °C. HRP-conjugated secondary antibodies were used for primary antibody binding (1:20.000) and detection on X-ray film (WesternBright

Quantum WB Detection kit Advansta Inc., USA.). The membrane was washed 3 x 10 min with TBS-0.1% Tween after primary and secondary antibody incubation. Where necessary, the membrane was stripped using the Membrane recycling kit (Alpha Diagnostics Intl., USA) and probed again as described above.

2.3. Methods – Cell culture and transfection

2.3.1. Host cell cultivation

Human foreskin fibroblasts were cultured in D10 medium [2.1.10.] at 37 °C and 5% CO_2 in a humidified incubator. Cells were harvested by trypsin/EDTA treatment and seeded into multi-well plates, dishes or cell culture flasks as required.

2.3.2. *T. gondii* cultivation and genetic manipulation

T. gondii tachyzoites were routinely propagated by serial passage in 60 mm cell culture dishes with confluent HFF monolayers at a multiplicity of infection (MOI) of 3, if not mentioned otherwise. Cultures were incubated in D10 medium [2.1.10.] at 37 °C and 5% CO_2. Parasites were diluted appropriately and counted using a Neubauer counting chamber. To generate transgenic parasite lines, freshly egressed or syringe-released tachyzoites (1-2 x 10^7) were washed with PBS and resuspended in 700 µl cytomix solution supplemented with fresh ATP (30 µl of a 100 mM stock solution) and glutathione (2 µl of a 250 mM stock solution). 50 µg of circular or linearized plasmid was used for the transfection using a BTX630 instrument (2 kV, 50 ohm, 25 microfarads, 250 µs). Alternatively, 10 µg plasmid and 100 µl cytomix plus additives were used for parasite transfection using the Amaxa nucleofector (programm T-16). Transfected parasites were used to infect HFF cells immediately. The culture medium was replaced 4-12 hrs post transfection to remove dead parasites and residual transfection reagent. Drug selection was performed as listed below. Stable transgenic strains were used to generate clonal lines by limiting dilutions in 96-well plates and used for follow up assays.

Selection drug	Concentration used	Drug target (Reference)	Added post transfection
Pyrimethamine	1 µM	DHFR-TS (59)	8 - 24 h
Mycophenolic acid/ Xanthine	25 µg/ml 50 µg/ml	HXGPRT (57)	8 - 24 h
FUDR	5 µM	UPRT (58)	after 2 passages (~96 h)
Chloramphenicol	20 µM	CAT (60)	8 - 24 h

2.3.3. Cultivation and transfection of COS-7 cells

COS-7 cells were cultivated in D10 medium, and monolayers were harvested using trypsin/EDTA regularly to avoid overgrowing and reduced cell viability. For heterologous expression, the parasite ORFs were amplified from tachyzoite cDNA and cloned into the *pCDNA3.1(+)* vector at *Hind*III or *Nhe*I and *Xba*I sites, which resulted in a C-terminal fusion with a V5-tag and stable expression under the *pCMV* promotor. Prior to transfection, 800 ng of *Bgl*II-linearized plasmid was resuspended in 50 µl Opti-MEM (reduced serum) medium containing 0.5 µl Lipofectamine 2000. The solution was incubated at RT for 20 mins and then added to the COS-7 cells grown in a 24-well plate. After 24 h incubation (37 °C, 5% CO_2), cells were harvested with trypsine/EDTA and seeded in T-75 flasks in a 1:10 dilution. Geneticin (800 µg/ml) was added 2 days post-transfection for the selection of stable transgenic cells. Fresh medium was provided every second or third day. The cells were analysed for transgene expression 24-48 hrs post-transfection and after 4 weeks of drug selection by immuno-fluorescence assay.

2.3.4. *S. cerevisiae* cultivation and transformation

The ethanolamine-auxotrophic yeast strains BY23480 (*Δpsd1Δpsd2*) and PTY44 (*Δpsd1Δpsd2*) were maintained at 30 °C on synthetic complete (SC) solid media containing 2 mM ethanolamine and 2 % glucose or 2 % lactate as carbon source, respectively. The strains were used for transformation with the *pESC-Ura* or *pYES2.1-TOPO* vector carrying ORFs amplified from *T. gondii* cDNA to test for complementation in ethanolamine-free media and the URA3 gene for selecting yeast transformants in uracil-deficient medium. Both plasmids allowed a galactose-inducible expression of a protein of interest under the control of the GAL promoter of *S. cerevisiae*. Empty plasmids and *Sc*PSD1-expressing plasmids were used as negative and positive controls, respectively. Transformation was performed using standard protocols (113,114). Briefly, yeast overnight cultures grown in 5 ml of synthetic complete media with 2% glucose or lactate at 30 °C on an incubation shaker, were used to inoculate a 50 ml culture at initial OD_{600} = 0.1 and grown to an OD_{600} of 0.4-0.6. Cells were collected by centrifugation (5 min, 2500 x g, RT) and washed with 25 ml of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5) for 5 min at 2500 x g and RT. The pellet was then washed in 10 ml of 100 mM lithium acetate (LiAc) buffered in TE (5 min, 2500 x g, RT) and suspended in 500 µl TE/LiAc and incubate at RT for 30 min. 100 µl of the competent yeast cells were mixed with 100 – 200 ng of plasmid DNA and 100 µg of Salmon sperm carrier DNA. 0.6 ml of PEG3350/LiAc/TE solution was added to the transformation reaction and vortexed vigorously for 10 sec followed by horizontal incubation at 30 °C for 30 min at 200 rpm in a shaker. 70 µl of DMSO (100%) was added to each tube and inverted. Cells

were heat-shocked at 42 °C for 15 min in a waterbath and chilled on ice for 1-2 min. Cells were then pelleted and washed in 200 µl of TE-buffer (5 sec, 14.000 x g, RT). The final pellet was suspended in 100 µl fresh TE-buffer and plated on in synthetic uracil-dropout minimal medium supplemented with 2 mM ethanolamine and 2% glucose or 2% lactate at 30 °C. Positive clones were identified by PCR and sequencing. To test for complementation, yeast cells were grown in liquid medium to an OD_{600} of 0.1, and serial dilutions (1:5) were stamped, spotted or streaked on synthetic uracil-dropout plates with or without ethanolamine (2 mM) containing either glucose, galactose, or lactate (2%) as carbon source. Plates were incubated for 2-4 days at 30 °C. To perform plasmid loss assays, yeast cells were serially propagated for 5 days under non-selective conditions (2% yeast extract, 1% peptone, and 2% glucose) and then evaluated by replica plating on selective (uracil-free) and nonselective (uracil-replete) plates in synthetic minimal medium. They were also simultaneously examined for their ability to grow with or without ethanolamine to examine phenotypic reversion.

2.4. Methods – Biological and Biochemical Assays

2.4.1. Indirect immuno fluorescence assay

Parasite-infected HFF monolayers cultured on glass coverslips were washed with PBS at 24-30 hrs post-infection, fixed with 2-4% paraformaldehyde (10 min), and then neutralized with 0.1 M glycine/PBS (5 min). Cells were permeabilized with 0.2% Triton X-100/PBS for 20 min, and nonspecific binding was blocked with 2% bovine serum albumin in 0.2% Triton X-100/PBS (30 min). Samples were stained using primary antibodies produced in mouse or rabbit for 1 hr [for dilution see 2.1.5.]. Cells were washed 3x with 0.2% Triton X-100/PBS, and stained with Alexa488- or 594-conjugated antibodies (anti-rabbit or anti-mouse, 1:3000) for 45 min. Following three additional washing steps, samples were mounted in fluoromount-G/DAPI (Southern Biotech, USA) and stored at 4 °C. Samples were analyzed and imaged using a Zeiss fluorescence microscope with the filter sets 49 (DAPI), 38 (GFP) and 43 (DsRed).

2.4.2. Transmission electron microscopy

Parasite-infected HFF cells grown on glass cover slips were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 30 min at room temperature. The reaction was neutralized with 0.1 M glycine/PBS or cacodylate buffer followed by three subsequent washings for 5 min in cacodylate buffer. Cells were then fixed with 2% OsO_4 in 0.1 M cacodylate-buffer for 1 hr on

ice, washed 3 x 5 min with ddH_2O and contrasted with 0.5 % uranylacetate for 1 hr in the dark. After another 3 x 5 min washing steps with ddH_2O, cells were dehydrated with an ascending alcohol series as follows: 40% EtOH, 50% EtOH, 60% EtOH, 70% EtOH, 80% EtOH for 5 min each and 90% EtOH, 95% EtOH, 100% EtOH and a second 100% EtOH dehydration for 30 min each. Embedding of the cells was performed in 100% EtOH/EMBed 812 (3:1; v/v), 100% EtOH/EMBed 812 (1:1; v/v), 100% EtOH/EMBed 812 (1:3; v/v) and pure EMBed 812 for 1 hr each and subsequently in fresh pure EMBed 812 over night with open lid. The glass cover slips were attached to an object plate and an EmBed 812 filled capsule was imposed to the cell monolayer. The sample was hardned at 70 °C for 48 hrs, heated for 12 sec on a heating plate and ripped off the glass slide followed by trimming into 70 nm ultra thin sections with an ultra microtome (Reichert Ultracut S, Leica). Imaging was done using a Zeiss EM 900 transmission electron microscope.

2.4.3. Correlative microscopy

Correlative microscopy combines immunofluorescence and transmission electron microscopy (TEM) to superimpose antibody-stained epitopes with the cellular ultrastructures. Host cells were grown to a density of 50% in 35 mm cell culture dishes with gridded glass coverslip bottom (MatTek, USA). Infected cells were fixed 24 hrs post infection and immunofluorescently labled as described in 2.4.1.. Fluorescent signals were imaged with an inverse fluorescence microscope and located based on their position on the grid. Samples were subsequently subjected to transmission electron microscopy as described in 2.4.2.. Both microscopic images were combined to correlate the fluorescence signal with the respective cellular structures detected by TEM.

2.4.4. *T. gondii* plaque assay

Plaque assays, recapitulating successive rounds of lytic cycles [see 1.2.3], were used to examine parasite fitness. Confluent HFF cells in 6-well plates were infected with 200-400 parasites/well. Parasitized host cells were incubated in standard D10 medium with 10% FCS. For nutrient-depletion assays, normal FCS was replaced by dialyzed (PAA) or by lipid-depleted FCS (Biowest) with additives as indicated, at the time of infection. After 7 days of unperturbed incubation at 37 °C and 5% CO_2, cells were washed twice with PBS, fixed with ice-cold methanol for 2 mins, and stained with crystal violet for 10 mins. The plaques were imaged and measured using the ImageJ suite.

2.4.5. *T. gondii* replication assay

Confluent HFF cells cultured on glass coverslips in 24-well plates were infected with tachyzoites (MOI = 1), incubated for 12–60 hrs, fixed with 4% PFA, and then immunostained using an anti-*Tg*Gap45 antibody (1:3000; rabbit). The mean numbers of parasites per vacuole were scored to compare the replication rates of different strains.

2.4.6. Annexin binding on Jurkat T-cells

Clonal Jurkat T-cells (E.61) were grown in RPMI medium supplemented with 10% iFCS, penicillin (10 U/ml) and streptomycin (100 µg/ml) at 37 °C and 5% CO_2 in a humidified incubator. The lymphoblast suspensions were grown to a maximum cell density of 6-8 x 10^5 cells and splitted 1:4 in T-75 cell culture flasks. For induction of apoptosis, 1x 10^6 Jurkat T-cells were resuspended in 1 ml of RPMI medium containing 1 µM staurosporine in DMSO or DMSO only for 2 hrs. Cells were harvested by centrifugation (400 x g, RT), washed twice with fresh medium and co-incubated with respective *T. gondii* tachyzoites at an MOI = 30 or plain medium as a control. Cells were then resuspended in 100 µl of annexin V-FLUOS labelling reagent, stained for 15 min in the dark and fixed for 15 min with 2% paraformaldehyde. Cells were washed according to protocol and resuspended in 200 µl HEPES buffer for FACS analysis or seeded on poly-L-lysine coated cover slips for microscopic analysis. FACS analysis was performed using LSRFortessa cell analyser (BD Biosciences). Parasites and T-cells were fractionated based on their cell size prior to analysis of annexin V-FLUOS staining.

2.4.7. Subcellular fractionation of *S. cerevisiae*

Yeast cells were grown to an OD_{600} of 0.4-0.8 in synthetic uracil-dropout medium supplemented with 2 mM ethanolamine and 2% galactose. All subsequent steps were performed at 4 °C. Cells were washed once with ddH_2O and suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 3 mM EDTA, 0.5 mM PMSF, and 0.25 M sucrose. Cell extract was prepared by disrupting yeast cells with glass beads (0.5-mm diameter) using a bead beater (Biospec Products) and removing the beads and cell debris by centrifugation (2000 x g, 5 min). Crude mitochondrial preparation was obtained by centrifugation of the cell extract at 10.000 x g for 10 mins and two washings with buffer. The resulting supernatant was used to obtain the microsomes and cytosolic fractions (100.000 x g, 1 h). Yeast cells expressing only mitochondrial *Sc*PSD1 (*Δpsd2*) were included when indicated, to ascertain the efficiency of fractionation.

2.4.8. Radioactive phosphatidylserine decarboxylation assay

PSD activity was measured by trapping $^{14}CO_2$ (released from Ptd[U-^{14}C]Ser or Ptd[1´-^{14}C]Ser) on filter paper impregnated with 2 M KOH. Reaction samples were prepared in 50 mM potassium phosphate (pH 6.8) buffer containing 10 mM β-mercaptoethanol, 0.25 M sucrose, 0.5 mM PMSF, 1 mM EDTA, and standard protease inhibitors. Ptd[1-^{14}C]Ser was purchased from ICN radiolabelled chemicals (USA) or made from L-[1-^{14}C]serine and dioleoyl CDP-diacylglycerol using PtdSer synthase. PtdSer synthase was purified from *E. coli* strain JA-200 harboring the plasmid pPS3155 as described previously (115). Briefly, L-[1-^{14}C]-serine was vacuum-dried, suspended in dH_20 and mixed with a 4-fold access of Triton-X solubilized CDP-DAG in 0.1 M KPO_4 at pH 7.4, 1 mM DTT, BSA (1 mg/ml) and *E. coli* JA-200 membrane fraction. The reaction was incubated at 37 °C for 1 h in a shaking incubator followed by lipid extraction and Ptd[1-^{14}C]Ser-recovery as described in 2.4.11. and liquid scintillation counting.

The PSD assay was performed with either yeast or *T. gondii* cell extracts at 30 °C or 37 °C in 16x100-mm borosilicate tubes sealed with an airtight rubber septum holding the KOH-saturated filter paper. The 0.8-ml assay mixture contained 60 mM potassium phosphate (pH 6.8), 0.17 M sucrose, 0.35 mM PMSF, 2 mM EDTA, 0.5 mM β-mercaptoethanol, 0.5 mM dioleoyl Ptd[U-^{14}C]Ser (0.1µCi/µmol), and 0.1% (w/v) Triton X-100. The reaction was started by addition of 0.2 ml of the protein fractions and terminated after 1 h by addition of 0.5 ml of 0.25 M H_2SO_4, introduced through the rubber septum using a hypodermic needle. The emitted $^{14}CO_2$ was trapped for 1 h prior to recovering the filter paper for liquid scintillation spectrometry.

2.4.9. Metabolic labelling of *T. gondii* and host cells

For metabolic labelling of host-free parasites, HFF cells were infected with tachyzoites (MOI = 3), and parasites were collected 42 h post infection. Samples were maintained on ice throughout the parasite isolation procedure. Infected cells were passed twice through 27-gauge needles to release the parasites, followed by removal of host cell debris by centrifugation (30 x g, 5 min). Parasites in the supernatant were pelleted (2000 x g, 10 min) and washed twice with intracellular type medium. Fresh extracellular tachyzoites (5 x 10^7) were incubated with [1,2-^{14}C]ethanolamine (20-40 nCi/nmol) or [1-^{3}H]serine (1 µCi/nmol) in 1 ml of intracellular type medium in glass tubes (2 h, 37 °C) followed by lipid extraction and quantification of radiolabeled phospholipids. To examine the scavenging of host-derived lipids by intracellular parasites, HFF cells cultured in T-25 flasks were labeled with [1,2-^{14}C]-ethanolamine (5 µCi, 25 µM) in DMEM containing aforementioned additives and lipid-depleted FCS (24 h, 37 °C, 5% CO_2). Cells were then infected with tachyzoites (MOI = 2). At the time of infection,

cultures were supplemented with a 200-fold excess of unlabeled ethanolamine (5 mM) to dilute the residual pool of radioactive ethanolamine in the host cell cytosol, if any. Parasitized cells were incubated (48 h, 37 °C, 5% CO_2), and the parasites were released using 27-gauge needles. Tachyzoites were washed twice with PBS, counted, and subjected to lipid extraction. The total radioactivity accumulated in parasite lipids was quantified to deduce the import of host phospholipids by the replicating tachyzoites.

2.4.10. Lipid extraction, separation and analysis

Lipid extraction from *T. gondii* was performed using the Bligh and Dyer method (116). Briefly, cells were suspended in 1.8 ml water or buffer in a round bottom glass tube. The following chemicals were added with subsequent vortexing after each addition: 4 ml methanol, 2 ml chloroform, 1.8 ml 0.2 M KCl, 2 ml chloroform. The lipid-containing chloroform-phase was backwashed twice with practical upper (CH_3OH:KCl (0.2 M):$CHCl_3$ (1:0.9:0.1, v/v)). The lipid-containing chloroform phase was either used for liquid scintillation spectrometry or dried under nitrogen gas stream and suspended in chloroform and methanol (9:1). Lipids were resolved by one-dimensional TLC on silica gel 60 plates in chloroform, methanol, and acetate (65:25:10, v/v/v). To achieve a higher resolution, they were either separated twice in chloroform, ethanol, water, and triethylamine (30:35:7:35, v/v/v/v) (117), or by 2D-TLC using chloroform, methanol and ammonium hydroxyide (30%) (84.5:45.5:6.5, v/v/v) in the first dimension and chloroform, glacial acetic acid, methanol and H_2O (80:12:9:2, v/v/v/v) in the second dimension. Lipids were visualized by iodine or 8-anilino-1-naphthalenesulfonic acid staining and/or by autoradiography.

The lipids were identified based on their co-migration with authentic standards and quantified by liquid scintillation counting or by lipid phosphorus assay (118). Briefly, 180 µl of perchloric acid (70%) was added to the lipid samples and heated for 30 min at 160-180 °C with a marble as lid. A silica-blank was added when samples were scraped from TLC-plates. A phosphorous standard series was performed using a 1 mM Na_2HPO_4 stock solution. 1 ml of dH_2O was added to samples and standards and vortexed followed by the addition of 200 µl ammonium molybdate (2.5 %) and freshly prepared ascorbic acid (10%). The reaction was accompanied by yellow colour change. Samples were heated in a waterbath at 50 °C for 15 min and developed a blue colour, which was measured at 820 nm using a spectral photometer.

2.4.11. Preparation of *T. gondii* secretome and NBD-PSD assay

As an alternative to the radioactive PSD assay [2.4.8.] fluorescently-labled lipids were used to detect the enzymatic activity. For preparation of tachyzoite secretome, T-150 flasks containing HFF cells were infected with respective *T. gondii* strains at MOI = 3. Parasites were syringe released at 40-42 hrs p.i., pelleted at 400 x g for 15 min at 4 °C and washed with ICM. The parasite pellet was subsequently resuspended in 1 ml ICM containing 1 mM fresh ATP and stored on ice. Samples were counted and adjusted to similar parasite numbers (1-1.5 x 10^7) in 1.2 ml ICM containing 1 mM ATP in a 1.5-ml tube. Tachyzoites were allowed to secrete for 2 hrs at 37 °C in a waterbath or with slightly open lid in the incubator. Afterwards, parasites were pelleted for 10 min at 2000 x g and 4 °C, and then 1.1 ml of the supernatant containing the secreted proteins was transferred to a fresh tube whilst the pellet was stored on ice and resuspended in 900 µl ICM. The secretome was centrifuged again for 10 min at 2000 x g at 4 °C, and subsequently 1 ml of supernatant was transfered to a fresh tube followed by another centrifugation step (10 min, 2000 x g, 4 °C) and transfer of 0.9 ml supernatant to a new tube to ensure gradual removal of parasites from the final secretome sample. Protease inhibitor cocktail was added to the samples, which were then snap-frozen in liquid nitrogen and stored at -80 °C until further use. Prior to the NBD-PSD-assay, the samples were thawed on ice and the cell extract was generated by repeated freezing and thawing of the pellet in liquid nitrogen/37 °C waterbath and vortexing.

For the preparation of substrate, C6-labled NBD-PtdSer was mixed with L-α-PtdSer to a final concentration of 0.5 mM. The substrate was dried in a 15 ml falcon tube under nitrogen gas and disolved in 100 µl ICM (+0.1 mM ATP) per reaction and vortexed vigorously for 1 min and sonicated twice for 30 s on ice. 900 µl of secretome or cell extract were mixed with 100 µl of substrate. The 5 mM substrate working stock solution was diluted 1:10 in ICM to a 0.5 mM final concentration in the reaction mixture. The reaction was performed for 1-2 hrs at 37 °C in a waterbath and the samples were subsequently subjected to lipid extraction and 1D-TLC analysis [2.4.11.]. NBD-PtdSer and NBD-PtdEtn were used as migration standards and the fluorescent lipids were visualized under UV light.

2.5. Bioinformatics and data analyses

Initial identification of the parasite gene sequences was performed using *T. gondii* genome database (ToxoDB) (55) and NCBI BLAST (119). The *TargetP* algorithm was used to predict subcellular targeting (120). *SignalP* was used to identify signal peptide sequences and their cleavage sites (121). The *MitoProt* tool is trained to identify mitochondrial proteins (122). *PlasmoAP* is designed to predict apicoplast-targeting sequences in *Plasmodium* species (123). And the *TMHMM* algorithm gives a general prediction of transmembrane domains (124). CLC sequence viewer was used for protein alignments. For phylogenetic analysis, protein sequences were aligned based on the PFAM Hidden Markov Modes (PF02666) using hmmer3 (125) and sequences were trimmed using trimAl (126). The phylogenetic tree was built using PhyML (127) and the LG model for amino acid substitution. Data plotting and statistical analyses were performed using the GraphPad Prism suite. All assays presented in this study were performed at least three independent times unless specified otherwise. The error bars in the figures represent the standard error of the mean. Statistics were done using the student's *t*-test, ANOVA, or one-way variance tests (*, $p<0.05$, **, $p<0.01$; ***, $p<0.001$) using Graphpad Prism, as indicated in respective figure legends.

3. Results

3.1. *T. gondii* encodes multiple pathways to synthesize phosphatidylethanolamine

Previous studies have shown that tachyzoites are capable of producing PtdEtn from ethanolamine and serine as precursors, the latter of which involves phosphatidylserine decarboxylation (92). However, the identity of the underlying enzymes remained undefined.

To investigate whether the *T. gondii* genome encodes the enzymes that are responsible for the *de novo* synthesis of PtdEtn, as described in other eukaryotic organisms [see 1.3.2.], database mining was performed using the *Toxoplasma* genome database (ToxoDB.org) (55). *S. cerevisiae* orthologs (128) were used to screen for the respective candidate genes in the *Toxoplasma* genome applying BLAST algorithms [Tab. 2]. It was found that the parasite encodes a complete set of coding genes to putatively perform an autonomous PtdEtn synthesis via PtdSer-decarboxylation and the CDP-ethanolamine-pathway [Tab. 2].

Table 2: Genes encoding putative enzymes for the synthesis of PtdEtn in T. gondii

ToxoDB accession no.	**Gene description**	**Protein length** [amino acids]	**Genomic location**	**Protein identities/positives** [compared to Yeast via BlastX]	**Abbreviations used in this study**
TGGT1_225550	phosphatidylserine decarboxylase	427	Chr. X	37%/55% [ScPsd1p; GI:584373840]	***Tg*PSD1mt**
TGGT1_269920	phosphatidylserine decarboxylase	968	Chr. VIII	37%/51% [ScPsd1p; GI:584373840]	***Tg*PSD1pv**
TGGT1_306540	phosphotransferase enzyme family protein	547	Chr. X	24%/40% [ScEki1p; GI:628157091]	***Tg*EK**
TGGT1_310280	phosphoethanolamine-cytidyltransferase	1128	Chr.XI	37%/52% [ScEct1p; GI:766989628]	***Tg*ECT**
TGGT1_261760	CDP-alcohol phosphatidyltransferase superfamily protein	449	Chr. VIIb	30%/47% [ScCpt1p; GI:767249243]	***Tg*EPT1**
TGGT1_257510	CDP-alcohol phosphatidyltransferase superfamily protein	467	Chr. VIIb	33%/53% [ScEpt1p; GI:768505096]	***Tg*EPT2**

The candidate proteins for PtdEtn-synthesis in *T. gondii* showed sequence similarities of approximately 50% to the queried yeast sequences that were used as a basis for further analyses. The parasite encodes two phosphatidylserine decarboxylases located on different chromosomes, which differ significantly in their sequence parameters. Consistent with the presence of an active ethanolamine kinase (*Tg*EK) and a bifunctional choline kinase, which can also use ethanolamine as substrate (100), the parasites encodes a putative phosphoethanolamine-cytidyltransferase (*Tg*ECT), a phospocholine-cytidyltransferase (*Tg*CCT) (94) and two CDP-alcohol phosphatidyltransferase proteins

(*Tg*EPT), which could perform the second and last reaction of the three-stepped Kennedy pathway [see 1.3.4.].

It should be noted that the annotated gene sequences were partially incomplete especially at their 5´ end at the beginning of this study. Therefore the start-codons had to be tested, sequenced and annotated by PCR and sequencing. Genome annotations have been significantly improved in the meantime, confirming the acquired cDNA sequences. Initial sequence screens were based on the genome of the *T. gondii* ME49 strain, which was best described and annotated in the ToxoDB. PCR primers for cDNA cloning were generated based on the *T. gondii* GT1 strain, however. This strain is more closely related to the *T. gondii* RH strain routinely used in the laboratory (129). The cDNA sequences used in this study and the ToxoDB accession numbers are attached in appendix 1.

3.1.1. Predicted subcellular localizations of PtdEtn-synthesis proteins

To predict the subcellular localization of the candidate proteins, a diverse set of *in silico* tools was applied. The information was subsequently used to compare the localizations with the corresponded targeting in other organisms and to design an appropriate tagging-strategy for microscopic detection.

Table 3: Predicted subcellular localization of putative PtdEtn synthesis proteins
mTP = mitochondrial targeting peptide; *SP* = Signal Peptide. [see also materials and methods section 2.5.].

Protein	TargetP	SignalP	MitoProt	PlasmoAP	TMHMM
***Tg*PSD1mt**	**0.859 mTP** 0.023 SP 0.244 other	No SP	**0.9850** (Cleavage site: A 97^ S 98)	No	0
***Tg*PSD1pv**	0.122 mTP **0.960 SP** 0.012 other	**Yes** (Cleavage site: S25^V26)	**0.9292**	No	1 (= SP)
***Tg*EK**	0.662 mTP 0.059 SP 0.333 other	No SP	0.2578	No	0
***Tg*ECT**	0.688 mTP 0.189 SP 0.346 other	No SP	0.3397	No	0
***Tg*EPT1**	0.227 mTP 0.226 SP 0.490 other	No SP	0.0686	No	**7**
***Tg*EPT2**	0.208 mTP 0.427 SP 0.397 other	No SP	0.1617	No	**9**

Using different algorithms, *Tg*PSD1mt was predicted to localize in the parasite mitochondrion (98,5%), which is in agreement with the localization of most PSD proteins in other eukaryotes (83,130). *Tg*PSD1pv has contradictory predictions of being potentially localized in the mitochondrion (~93%) but also carrying a signal peptide with a very high probability (96%) [Tab. 3]. In fungi and plants

however, additional non-mitochondrial PSD proteins have been identified localizing to the Golgi and vacuolar/endomambrane compartments (131-134). The expression of a single, but non-mitochondrial PSD enzyme was also reported in *Plasmodium falciparum* (135). The proteins of the CDP-Ethanolamine pathway could not be clearly allocated to a certain subcellular compartment. But ethanolamine kinases are known to be cytosolic in other organisms (136,137) including *T. gondii* (94). ECT enzymes are considered to be mainly soluble cytosolic but can also interact with nuclear and ER membranes (138). Consistently, *Tg*CCT showed nuclear localization in tachyzoites as well (94). The final reaction of the CDP-ethanolamine pathway is known to take place in the ER in other eukaryotes (81,137). No signal peptide or ER-retention signals could be identified by the used prediction tools in the two *T. gondii* EPT candidate proteins however [Tab. 3]. Unlike the other proteins, both EPTs harbour 7 and 9 predicted transmembrane domains, respectively [Tab. 3].

To gain insight into the biological importance of the candidate proteins, they were analyzed for functional domains and post-translational modifications as well as for transcript and protein abundance in different *T. gondii* life cycle stages, including tachyzoites, bradyzoites and oocysts.

Table 4: Catalytic domains, expression and modification of enzymes for PtdEtn synthesis
Tz = tachyzoites; *Bz* = bradyzoites; *Ooc* = Oocysts

Protein	Interpro domains	Transcript detection	MS-based expr. evidence in Tz	Post-transl. modifications
***Tg*PSD1mt**	PF02666 Phosphatidylserine Decarboxylase	Tz Bz Ooc	Yes	---
***Tg*PSD1pv**	PF02666 Phosphatidylserine Decarboxylase	Tz Bz Ooc	Yes	---
***Tg*EK**	PF01633 Choline/ethanolamine kinase	Tz Bz Ooc	Yes	**14** phosphoryl. sites
***Tg*ECT**	PF01467 Cytidylyltransferase	Tz Bz Ooc	Yes	**25** phosphoryl. sites
***Tg*EPT1**	PF01066 CDP-alcohol phosphatidyltransferase	Tz Bz Ooc	Yes	**6** phosphoryl. sites
***Tg*EPT2**	PF01066 CDP-alcohol phosphatidyltransferase	Tz Bz Ooc	Yes	**3** phosphoryl. sites

As shown in table 4, all enzymes were expressed during the three considered stages of *T. gondii*, indicating a requirement of PtdEtn synthesis throughout the parasite life cycle. The presence of functional domains and the expression evidence in tachyzoites was a good prerequisite for the experimental characterizations in terms of their subcellular localization, enzymatic function and biological relevance.

3.1.2. Phylogenetic origin of phosphatidylserine decarboxylases in *T. gondii*

To analyse the phylogenetic context of the two PSD homologs in *Toxoplasma,* a phylogenetic analysis of various PSD enzyme sequences was performed. Therefore, a maximum likelihood tree (127) of various pro- and eukaryotic, pathogenic- and non-pathogenic organisms was generated [Fig. 9]. The PS decarboxylase PFAM domain PF02666 was used as a basis for the protein alignments.

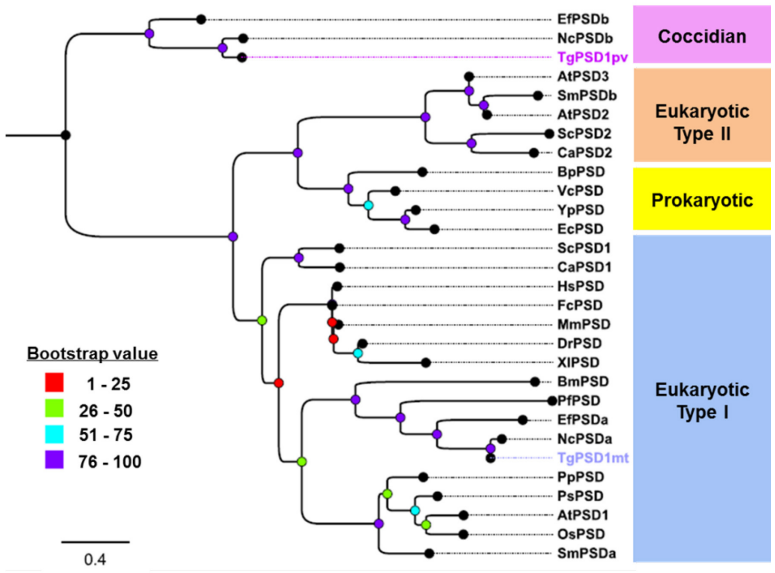

Figure 9: TgPSD1mt and TgPSD1pv are of different phylogenetic origin.
Phylogenetic clades of *Tg*PSD1mt, *Tg*PSD1pv, and orthologs. Branch support was estimated by 100 bootstrap replicates. AtPSD1, *Arabidopsis thaliana* (GI 42566885); AtPSD2, *A. thaliana* (GI 240256448); AtPSD3, *A. thaliana* (GI 186513660); BmPSD, *Babesia microti* (GI 399218717); BpPSD, *Bordetella pertussis* (GI 33592417); CaPSD1, *Candida albicans* (GI 68473808); CaPSD2, *C. albicans* (GI 68468048); DrPSD, *Danio rerio* (GI 63102372); EcPSD, *E. coli* (GI 15804752); EfPSDa, *Eimeria falciformis* (unpublished); EfPSDb, *E. falciformis* (unpublished); FcPSD, *Felis cattus* (GI 410976945); HsPSD, *Homo sapiens* (Q9UG56); MmPSD, *Mus musculus* (GI 74195621); NcPSDa, *Neospora caninum* (GI 401409734); NcPSDb, *N. caninum* (GI 401408937); OsPSD, *Oryza sativa* (GI 115450115); PfPSD, *Plasmodium falciparum* (XP_001352149), PpPSD, *Physcomitrella patens* (GI 168030155); PsPSD, *Picea sitchensis* (GI 116788855); ScPSD1, *S. cerevisiae* (GI 6324160); ScPSD2, *S. cerevisiae* (GI 841244); SmPSDa, *Selaginella moellendorffii* (GI 302802812); SmPSDb, *S. moellendorffii* (GI 302818837); VcPSD, *Vibrio cholera* (GI 446280068); XlPSD, *Xenopus laevis* (GI 148236972); YpPSD, *Yersinia pestis* (GI 22124534).

The indicated PSD orthologs from different organisms show a distinct clustering pattern and were therefore categorized into prokaryotic, eukaryotic type I (mitochondrial) and eukaryotic type II PSDs. The phylogenetic clustering showed that *Tg*PSD1mt belongs to the eukaryotic type I PSD enzymes, and is distinct from the *Tg*PSD1pv protein. Database search identified only one PSD in most protozoan parasites, except for a small subgroup of coccidians (*Eimeria*, *Hammondia*, *Neospora*), which express two discrete PSD enzymes related to *Tg*PSD1mt and *Tg*PSD1pv. *Tg*PSD1pv can therefore be considered as a coccidian-specific subtype of PSD enzymes [Fig. 9].

Alignment of *Tg*PSD1mt with *P. falciparum* and human orthologs revealed a fairly high conservation, particularly in the PSD domain [Fig. 10]. *Tg*PSD1mt is 40% identical (62% similar) to *Pf*PSD and 35% identical (51% similar) to *Hs*PSD. The PSD proenzymes in prokaryotes as well as in eukaryotes are proteolytically processed between the G and S residues into a membrane-anchored β-subunit and a smaller α-subunit harboring the pyruvoyl-moiety at its N-terminus (139,140). The pyruvoyl prosthetic group is part of the active site and needed for the catalytic function of PSD enzymes (83). A similar processing of *Tg*PSD1mt is predicted to yield a mature protein with ~32-kDa β-subunit and ~6-kDa α-subunit, when cleaved at the predicted LG^ST motif [Fig. 10].

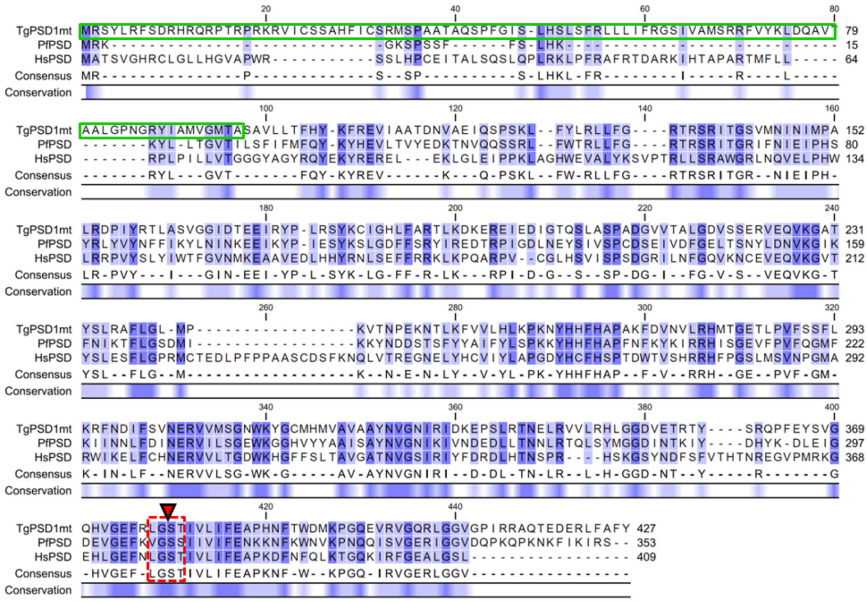

Figure 10: TgPSD1mt shares sequence homologies with type I PSDs.
Alignment of PSD sequences from *T. gondii* (TgPSD1mt), *P. falciparum* (PfPSD) and *H. sapiens* (HsPSD). Entirely and partially conserved residues are shown in dark and light blue, respectively. The green and red boxes represent the predicted mitochondrial targeting peptide and conserved cleavage motif at the catalytic site, respectively. The PSD domain in TgPSD1mt spans from 184-408 (PFAM domain PF02666). TgPSD1mt and PfPSD have extended C-termini. NCBI accession numbers: TgPSD1mt, DQ450198; PfPSD, XP_001352149; HsPSD, Q9UG56.

The second PSD, *Tg*PSDpv, cannot be adequately aligned to PSD orthologs, other than from the coccidian PSD subgroup, which are all relatively big proteins showing a conserved FG^ST motif at the active site [Appendix 2B].

3.2. Phosphatidylserine decarboxylases localize to distinct organelles

To assess the subcellular localization of the two PSD enzymes of *T. gondii*, we performed different tagging approaches using a C-terminal fused haemagglutinin-tag under different promotors, and co-localized their expression-patterns with established organelle markers.

3.2.1. *Tg*PSD1mt is localized in the mitochondrion of *T. gondii* tachyzoites

To determine *Tg*PSD1mt localization, the ORF was amplified from *T. gondii* cDNA and cloned downstream of the *TET07SAG1* promotor flanked by the 5´and 3´UTR elements of the *Tg*UPRT gene. The sequence of *Tg*PSD1mt was verified by PCR and sequencing (NCBI accession number DQ450198). Parasites expressing a single copy of the C-terminally tagged *Tg*PSD1mt-HA protein show a clear mitochondrial localization in intracellular as well as in extracellular parasites as verified by co-staining with *Tg*S9$_{33\text{-}159}$-GFP, an established mitochondrial marker (104) [Fig. 11].

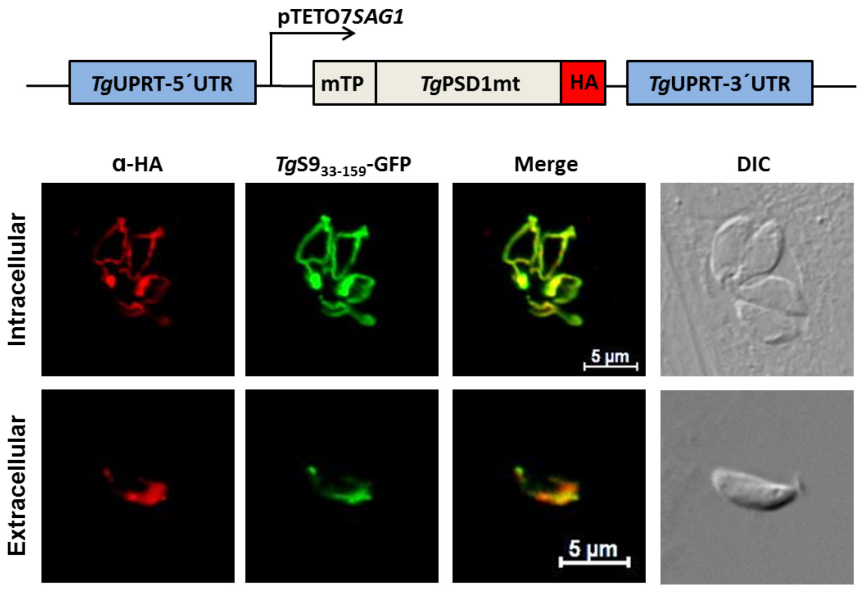

Figure 11: Ectopic expression of TgPSD1mt-HA in intracellular and extracellular tachyzoites.
Immunofluorescence imaging of intracellular parasites 24 hrs post infection, and fresh extracellular parasites of the Δ*ku80*/TaTi strain. The complete *Tg*PSD1mt ORF with the predicted mTP and a C-terminally fused HA epitope-tag was expressed under the control of the pTETO7*SAG1* promoter at the UPRT locus of *T. gondii*. Stable transgenic parasites expressing *Tg*PSD1mt-HA were co-transfected with a plasmid encoding for the mitochondrial marker *Tg*S9$_{33\text{-}159}$-GFP (green) and stained with anti-HA/Alexa594 (red) antibodies as described in materials and methods. *DIC* = differential interference contrast, *HA* = haemagglutinin, *mTP* = mitochondrial targeting peptide

Because ectopic expression of a protein is prone to localization artefacts, we generated stable transgenic parasites by 3'-HA tagging of the *Tg*PSD1mt gene, which subsequently expressed *Tg*PSD1mt-HA under the control of its native promoter [Fig. 12]. Immunostaining of the intracellular parasites showed a clear mitochondrial localization of *Tg*PSD1mt-HA, which co-localized with a known organelle marker, the mitochondrial ATPase subunit *Tg*F1B (105).

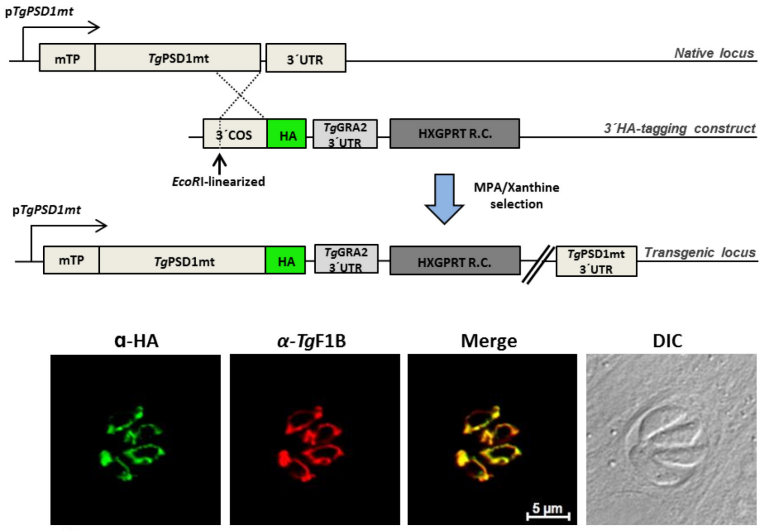

Figure 12: 3´-insertional-tagging of TgPSD1mt confirms its mitochondrial localization.
Genomic tagging of the *Tg*PSD1mt gene and immunofluorescence imaging of intracellular parasites expressing *Tg*PSD1mt-HA under the control of its endogenous promoter and *Tg*Gra2-5´UTR. The construct for 3´-HA tagging of *Tg*PSD1mt (*pTKO-HXGPRT-TgPSD1mt-3´IT*) was transfected and selected in the *Δku80-Δhxgprt* strain. Parasitized cells were stained with anti-HA and anti-*Tg*F1B antibodies after 24 h of infection. *COS* = crossover sequence; *mTP* = mitochondrial targeting peptide; *R.C.* = resistance cassette

We next tested the importance of the predicted mitochondrial targeting peptide for localization and enzymatic activity [see 3.4.2.], by expressing two HA-tagged deletion variants of *Tg*PSD1mt lacking the N-terminal peptide (*Tg*PSD1mt$_{(91–427)}$-HA and *Tg*PSD1mt$_{(113–427)}$-HA). None of the two truncated forms localized in the parasite mitochondrion, and displayed a diffuse fluorescence throughout the parasite body instead [Fig. 13].

Taken together, these localization studies demonstrate that *Tg*PSD1mt is indeed a mitochondrial protein in *T. gondii* tachyzoites, as suggested from in *silico* analyses [see 3.1.] and that the predicted mitochondrial targeting peptide is crucial for correct subcellular localization.

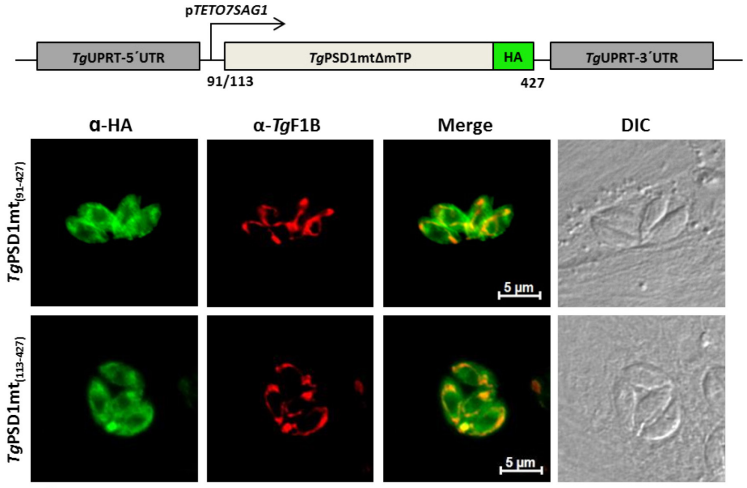

Figure 13: The mTP-deletion variants of TgPSD1mt-HA fail to localize in the mitochondrion.
Immunofluorescence imaging of the two truncated HA-tagged *Tg*PSD1mt forms (*Tg*PSD1mt$_{(91–\ 427)}$-HA and *Tg*PSD1mt$_{(113–\ 427)}$-HA) expressed in *T. gondii* tachyzoites of the *Δku80-Δhxgprt* strain under the control of the *TETO7SAG1* promoter. Intracellular parasites were stained using anti-HA (green) and anti-*Tg*F1B (red) antibodies after 24 h of infection.

3.2.2. *Tg*PSD1pv is secreted into the parasitophorous vacuole *via* dense granules

Since the bioinformatical prediction did not reveal a clear indication for the subcellular localization of *Tg*PSD1pv, and due to the observation that this protein seems to be highly divergent from other PSD enzymes, the determination of its localization was particularly crucial. The full length ORF of *Tg*PSD1pv was amplified from tachyzoite cDNA and sequenced (annotated at NCBI as accession number JN003619). For localization studies, the protein was expressed in *T. gondii* tachyzoites, C-terminally fused with a HA-tag (*Tg*PSD1pv-HA) under the control of the *Tg*NTP3 promoter [Fig. 14]. Immuno-staining of the transgenic parasites present within the host cell revealed that *Tg*PSD1pv was secreted into the parasitophorous vacuole. The secreted PSD co-localizes with *Tg*Gra1, *Tg*Gra3 and *Tg*Gra5, which are *bona fide* markers of the dense granules and secreted into the parasitophorous vacuole (141). *Tg*Gra1 is completely soluble within the vacuolar space (43), and *Tg*Gra3 and *Tg*Gra5 are associated with the vacuolar membranes (108,142). Depending on the focal plane used for microscopic imaging, *Tg*PSD1pv was detectable within the dense granules of the intracellular parasites as well as in the vacuolar space and at the vacuolar membrane [Fig. 14A]. Fluorescence images of parasites after their release from host cells [Fig. 14B] reveal co-localization of *Tg*PSD1 with *Tg*Gra1 and *Tg*Gra3. These results confirm that *Tg*PSD1pv resides in the dense granules of *T. gondii*, prior to its secretion into the parasitophorous vacuole.

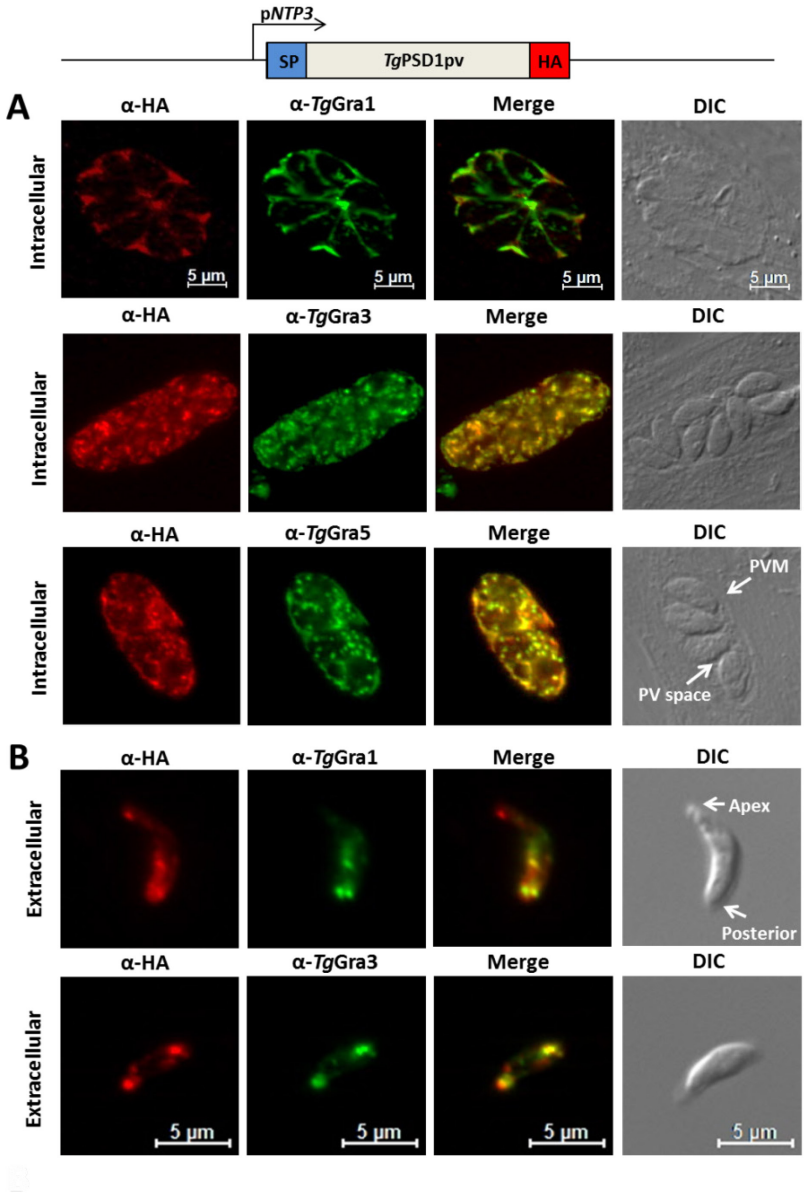

Figure 14: TgPSD1pv is stored in the dense granules and secreted into the PV.
Immunofluorescent co-localization of *Tg*PSD1pv with dense granule proteins in intracellular (*panel A*) and extracellular parasites (*panel B*). Purified tachyzoites of the TaTi strain ($1x10^6$) were transfected with the *Tg*PSD1pv-HA expression construct under the control of p*NTP*3 elements. Stable pyrimethamine-resistant transgenic lines were generated prior to immuno-fluorescence detection using the indicated antibodies. A schematic version of the *Tg*PSD1pv construct is shown with the signal peptide (SP), core enzyme (*Tg*PSD1pv) and epitope tag (HA) domains highlighted.

Since the overexpression of proteins under a strong promotor, like *p*NTP3, could have misdirected *Tg*PSD1pv into the secretory pathway, as shown for micronemal proteins (143), we additionaly expressed the *Tg*PSD1pv-HA protein under its native promotor. Stable integration of *Tg*PSD1pv was achieved at the *Tg*UPRT-locus. The respective immunofluorescence analysis confirmed its secreted nature and co-localization with the dense granule protein *Tg*Gra5 [Fig. 15].

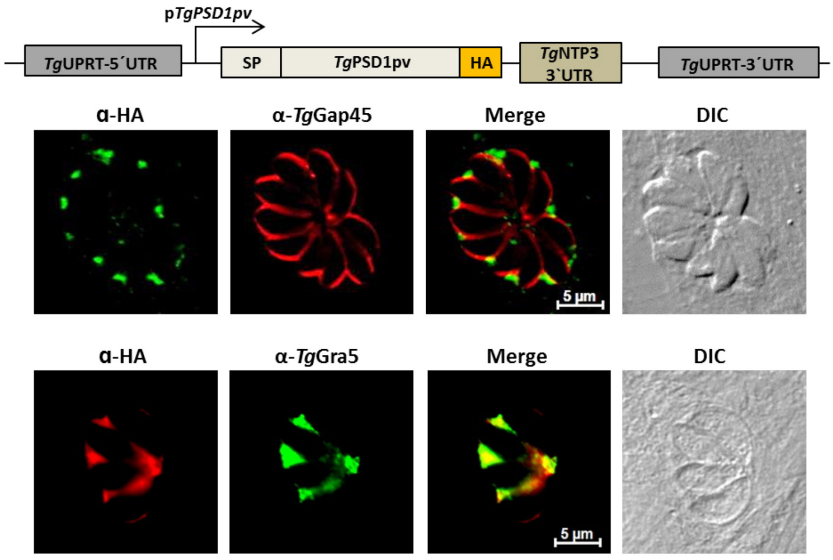

Figure 15: Localization of TgPSD1pv in the parasitophorous vacuole under its endogenous promotor element. Intracellular parasites stained for *Tg*PSD1pv-HA expression under the control of its native promoter and *Tg*NTP3-3´UTR at the *UPRT* locus. The parasite periphery is stained by anti-*Tg*Gap45 antibody (*red*, upper panel), whereas the *Tg*Gra5 antibody stains the PV lumen (*green*, lower panel). *SP* = signal peptide.

Electron microscopic analyses were performed to examine whether *Tg*PSD1pv binds to intravacuolar membranes and/or the PV membrane after its secretion. Unfortunately, the detection of the HA-tag using immuno-gold-labelling was not successful. Therefore, correlative microscopy was used, combining immune-fluorescence and transmission electron microscopy [Fig. 16].

The correlation of the fluorescence signal with the electron micrograph showed that *Tg*PSD1pv is located in the PV lumen [Fig. 16D], as well as at the PV membrane. No definite conclusion could be drawn whether the protein binds to the intra-vacuolar or PV membrane structures. In comparison to *Tg*Gra5 (108), which accumulated at the parasite and PV surface, *Tg*PSD1pv showed a more diffused localization pattern within the PV space [Fig. 16B]. No fluorescence signal within the parasite interior (dense granules) was detected with both antibodies in this experiment.

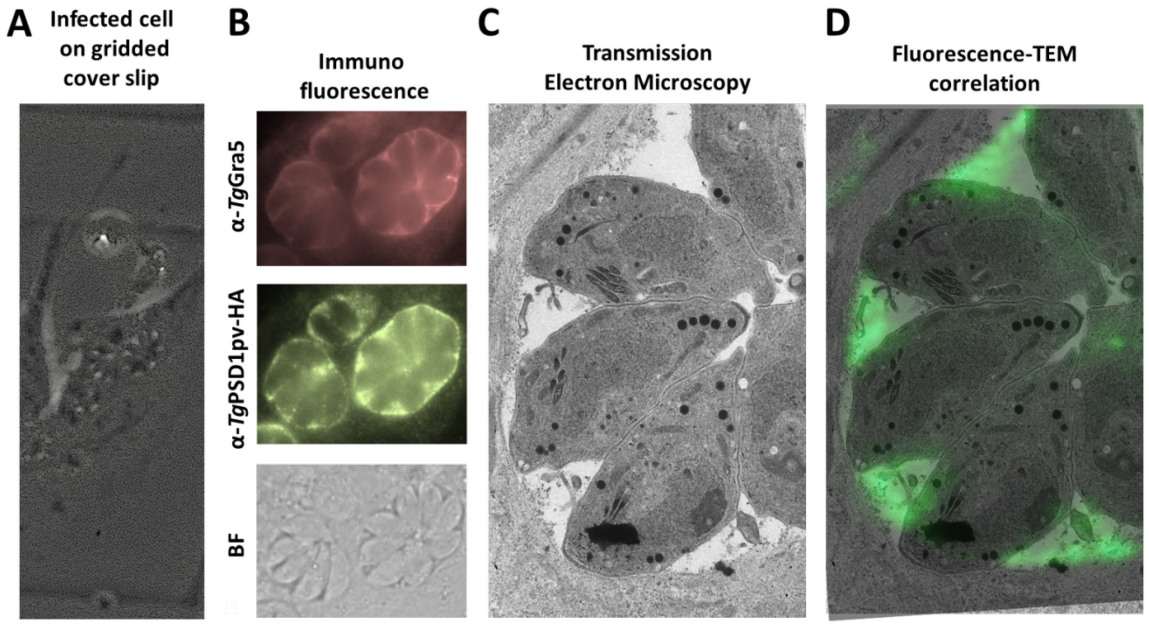

Figure 16: Localization of TgPSD1pv-HA in the parasitophorous vacuole by correlative imaging.
(A) HFF cells infected *T. gondii* tachyzoites stabily expressing *pUPKO-pTgPSD1pv-TgPSD1pv-HA* grown on a gridded glass cover slip were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer 24 h post infection. **(B)** Samples were used for immuno-fluorescence analysis with anti-HA (green) and anti-*Tg*Gra5 antibodies (red). **(C)** The same vacuole was subsequently analysed by transmission electron microscopy (TEM). **(D)** Merge of *Tg*PSD1pv-HA fluorescence and TEM-image.

These results also coincide with previous biochemical experiments showing two pools of PSD activity in *T. gondii* tachyzoites, of which one is soluble and the other membrane-bound. They also confirm previous data for the presence of a secreted PSD activity in axenic parasites (103).

3.3. Expression and subcellular localization of *Tg*PSD enzymes in COS-7 cells

To assess the localization of the two parasite enzymes in mammalian cells, the cDNAs of *Tg*PSD1mt and *Tg*PSD1pv were cloned into the *pCDNA3.1* vector and transfected into COS-7 cells, derived from monkey kidney tissue. Transgenic COS-7 cells expressed the C-terminally V5-tagged parasite proteins. *Tg*PSD1mt-V5 revealed a mitochondrial localization, as verified by co-localization with mitotracker reagent. Additionally, the protein showed a nuclear (membrane) localization [Fig. 17A]. *Tg*PSD1pv showed a diffuse expression in the parasite cytosol or endomembrane system and partial co-localization with the anti-KDEL antibody designating ER and Golgi compartments [Fig. 17B].
A stable expression of both proteins in the COS-7 cell line by drug-selection with geneticine was not successful, however. Prolonged cultivation under drug-selecton led to impaired cell viability and the eventual loss of the V5-signal, which impeded further analysis of the catalytic activity in this cell line.

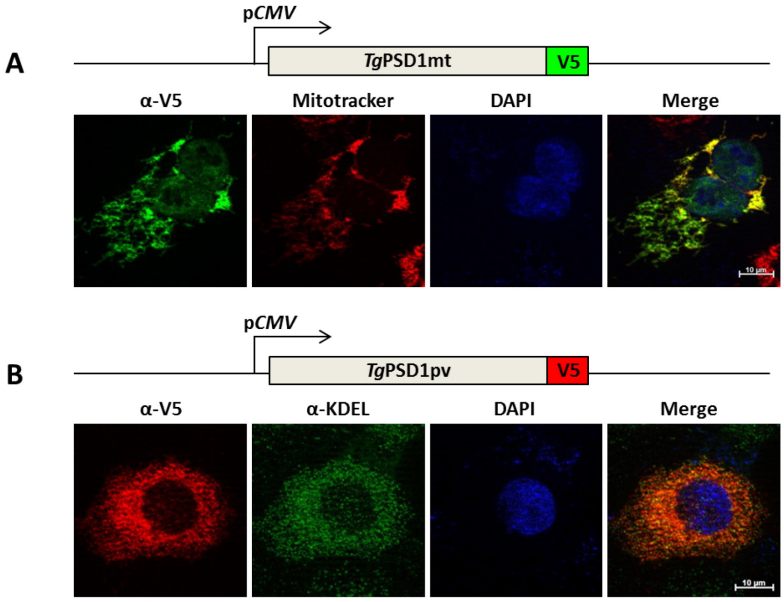

Figure 17: TgPSD1mt localizes to the mitochondria and nucleus and TgPSD1pv to the endomembrane system in monkey kidney fibroblasts.
*Tg*PSD1mt and *Tg*PSD1pv cDNAs fused to a C-terminal V5-tag were expressed in COS-7 cells under the control of the CMV promotor. Cells were fixed 48 hrs post-transfection for immunofluorescence analysis with an anti-V5 antibody. **(A)** For mitochondrial visualization cells were stained for 20 min with 10 nM Mitotracker reagent in serum-free DMEM prior fixation. **(B)** For co-localization of *Tg*PSD1pv-V5, an anti-KDEL antibody was used to stain endomembrane compartments.

These localization studies demonstrate that the mitochondrial targeting peptide of *Tg*PSD1mt allows mitochondrial expression of *Tg*PSD1mt in a mammalian system. *Tg*PSD1pv is targeted to the cytosol or endomembrane compartments in COS-7 cells.

However, the use of a different heterologous expression system was needed for stable expression of these parasite proteins for their functional analysis.

3.4. *Tg*PSD1mt can functionally complement a *S. cerevisiae* PSD mutant

To verify the catalytic function of the predicted PSD enzymes expressed in *T. gondii*, *Tg*PSD1mt and *Tg*PSD1pv were expressed in a *S. cerevisiae* mutant strain lacking its endogenous *psd1* and *psd2* genes. The yeast Δ*psd1*Δ*psd2* strain is auxotrophic for ethanolamine, which can be utilized to produce PtdEtn via the Kennedy pathway in the ER [Fig. 18].

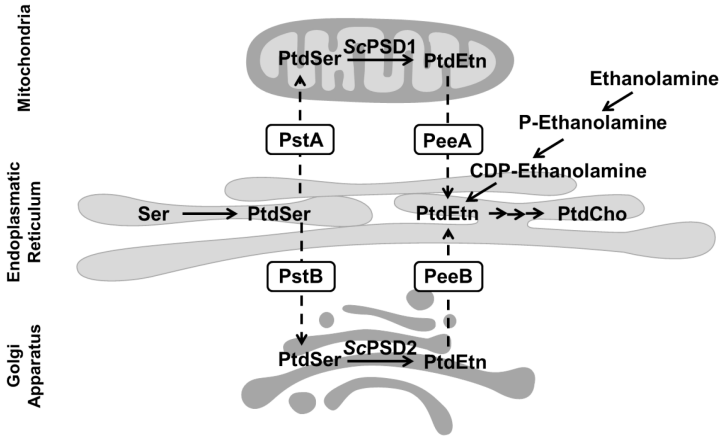

Figure 18: Schematic overview of the phospholipid synthesis in S. cerevisiae
Yeast expresses two PSD enzymes, *Sc*PSD1 and *Sc*PSD2 to produce PtdEtn from PtdSer in its inner mitochondria and the Golgi apparatus, respectively. The last reaction of the three-stepped CDP-Etn pathway is located in the ER generating PtdEtn. Exchange of PtdSer and PtdEtn between the organelles requires membrane contact sites and lipid transfer proteins (PstA/B and PeeA/B routes). [*Adapted from Voelker* (144)]

Different variants of the parasite PSDs were amplified from cDNA and cloned into the *pESC-Ura* vector, which allowed expression under the control of the galactose-inducible *Sc*GAL10 promotor. The transformed yeast strains were then examined for their growth under ethanolamine starvation.

3.4.1. *Tg*PSD1mt is expressed in its active form in yeast mitochondria

*Tg*PSD1mt cDNA was expressed in the Δ*psd1*Δ*psd2* yeast mutant strains BY23480 and PTY44. Empty vector and *Sc*PSD1-expression plasmid served as negative and positive controls, respectively [Fig. 19]. As expected, all transgenic yeast strains were able to grow in ethanolamine-replete conditions. The empty-vector negative control did not show any detectable growth in ethanolamine-free medium, whereas *Tg*PSD1mt and *Sc*PSD1 rescued the growth of the mutant in a galactose-inducible manner [Fig. 19A].

Next, subcellular fractions of the transgenic yeast strains were produced to determine the distribution of *Tg*PSD1mt in the mitochondrial, microsomal and cytosolic fractions. A Δ*psd2* yeast strain (JSY9750), expressing only the native *Sc*PSD1 in the mitochondria, was included to ascertain a successful fractionation [Fig. 19B]. Each fraction was used to perform PSD assays by trapping $^{14}CO_2$ that was released by decarboxylation of radioactive PtdSer. As anticipated, the Δ*psd1*Δ*psd2* mutant harbouring the empty vector did not show any measurable activity, and overexpression of *Tg*PSD1mt via the *Sc*GAL10 promoter was quite evident when compared to the positive control. Similar to *Sc*PSD1 (131,145), a majority of the recovered *Tg*PSD1mt activity (>80%) was expressed in the mitochondria-enriched fraction.

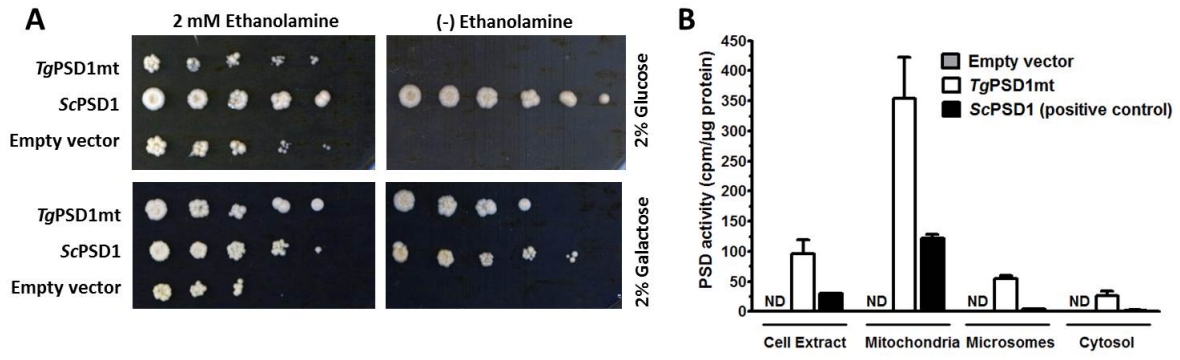

Figure 19: TgPSD1mt can rescue the growth of a S. cerevisiae mutant lacking its endogenous PSD activity.
(A) Yeast complementation by heterologous expression of *Tg*PSD1mt in a *S. cerevisiae* Δ*psd1*Δ*psd2* mutant (BY23480). *Tg*PSD1mt was expressed under the control of the *Sc*GAL10 promotor. Empty *pESC-Ura* and *Sc*PSD1-expression vectors were included as negative and positive controls. **(B)** PSD activity in subcellular fractions of the yeast strains expressing *Tg*PSD1mt, or endogenous *Sc*PSD1, or harbouring empty vector. Indicated organelle-enriched fractions were subjected to PSD enzyme assays. *ND*, not detectable

To validate the specificity of the complementation, we allowed yeast cells to lose the plasmid by growing for several generations without selection pressure in full growth medium and subsequent analysis under different selective conditions via replica plating. As a result, cells that lost the vector also lost their ability to grow on uracil-dropout plates, as well as in ethanolamine-deficient non-selective medium [Fig. 20].

These results verify the predicted specific enzymatic function of the mitochondrial PSD enzyme of *T. gondii* in baker's yeast.

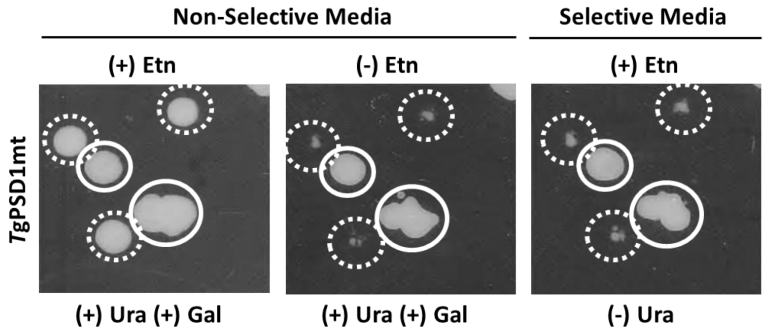

Figure 20: Loss of TgPSD1mt results in a phenotypic reversion of the Δpsd1Δpsd2 S. cerevisiae mutant.
The transgenic yeast strain BY23480 (*Δpsd1Δpsd2*) expressing *Tg*PSD1mt was grown in non-selective medium for multiple generations, and plated on the indicated media to screen for the colonies that had lost or retained the plasmid. Colonies in dotted circles show a plasmid loss and subsequent reversion to ethanolamine auxotrophy, whereas colonies fully circled retained the plasmid and grow in the absence of ethanolamine.

3.4.2. *Tg*PSD1mt is active in yeast irrespective of its mitochondrial localization

We also expressed *Tg*PSD1mt$_{(113-427)}$ in the Δ*psd1*Δ*psd2* strain of yeast to test the role of the N-terminal sequence for its catalytic activity and subcellular distribution. Notably, the truncated enzyme was catalytically active and functionally complemented the yeast growth in ethanolamine-free cultures [Fig. 21A], even though most of the *Tg*PSD1mt$_{(113-427)}$ activity (>70%) was present in the microsomes and ~13% was cytosolic [Fig. 21B]. Only ~17% of the total enzyme activity was distributed in the mitochondria-enriched fraction.

These assays demonstrate that the N-terminal mitochondrial targeting peptide of *Tg*PSD1mt is required for its mitochondrial localization but not for the catalytic function in yeast.

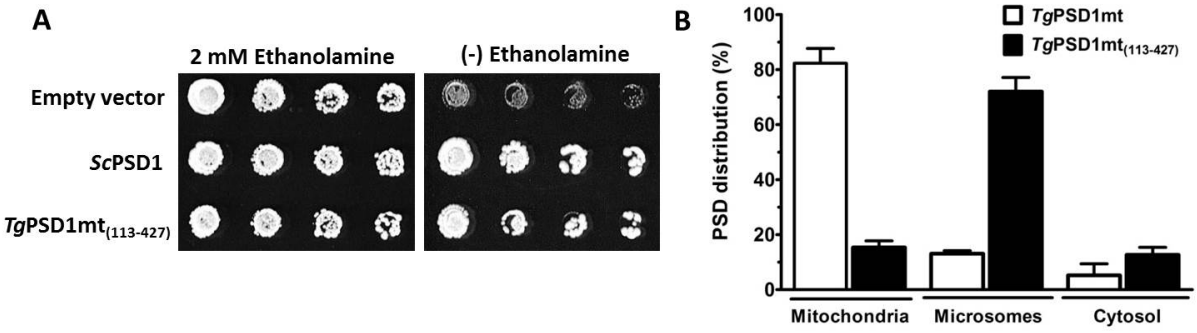

Figure 21: The mitochondrial targeting peptide in TgPSD1mt is required for an efficient localization but not for enzyme activity in S. cerevisiae.
(A) Complementation of *S. cerevisiae* Δ*psd1*Δ*psd2* mutant (PTY44) by *Tg*PSD1mt$_{(113-427)}$ in medium containing 2% lactate / 0.5% galactose. Empty *pYES2.1* and *Sc*PSD1-expression vectors were used as negative and positive controls. **(B)** Enrichment of the PSD activity in subcellular fractions of yeast cells expressing *Tg*PSD1mt or *Tg*PSD1mt$_{(113-427)}$. The PSD assay was performed by radioactive $^{14}CO_2$-trapping.

3.4.3. *Tg*PSD1pv is not able to complement for PSD function in *S. cerevisiae*

We also tested *Tg*PSD1pv for its ability to rescue the growth of the aforementioned yeast *Δpsd1Δpsd2* mutant strains. Our initial attempts to complement the *S. cerevisiae* BY23480 strain by the expression of full lengh *Tg*PSD1pv cDNA were futile, unlike *Sc*PSD1 and *Tg*PSD1mt, that were used as positive controls [Fig. 22]. Based on the assumption that the predicted signal peptide of *Tg*PSD1pv could mistarget the protein in yeast, we replaced the N-terminal targeting sequence by a yeast mitochondrial targeting peptide (*Sc*mtp). Additionally, we expressed truncated forms of *Tg*PSD1pv carrying only the catalytic domain (CD, 392 – 730 AA) fused with or without *Sc*mtp [Fig. 22A]. Targeting of the *T. gondii* proteins to yeast mitochondria inhibited growth of the respective mutants even under uninduced culture conditions [Fig. 22A, 4-6]. We also expressed full length *Tg*PSD1pv and

*Tg*PSD1pv-CD in a different Δ*psd1*Δ*psd2* yeast strain (PTY44), growing on a non-fermentable carbon source [Fig. 22B]. However, none of the *Tg*PSD1pv variants were able to restore the growth of the mutant strains on ethanolamine-free media [Fig. 22].

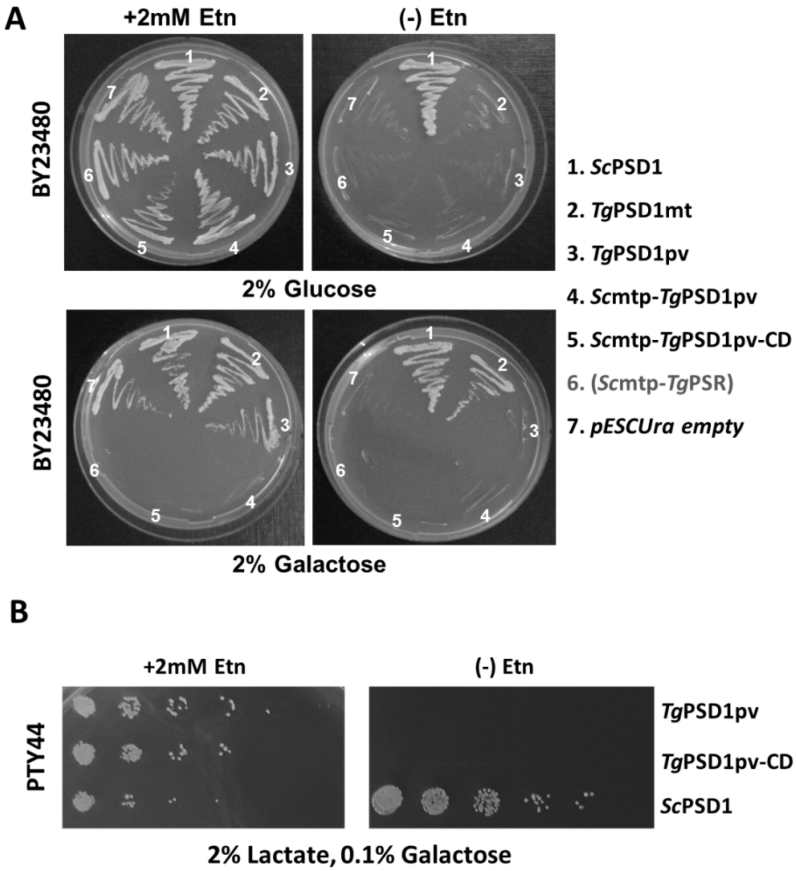

Figure 22: TgPSD1pv fails to rescue Δpsd1Δpsd2 yeast mutants.
(A) Different versions of *Tg*PSD1pv were cloned in the *pESC-Ura* vector and expressed in the yeast BY23480 strain. *Sc*PSD1 and *Tg*PSD1mt served as positive and the empty vector as negative control. Galactose-supplemented media was used for induction of protein expression. **(B)** *Tg*PSD1pv and *Tg*PSD1pv-CD were expressed in the PTY44 strain on the *pYES2.1* vector and lactate as a carbon source. Galactose was used to induce expression via the *Sc*Gal10 promotor. *Scmtp = S. cerevisiae* mitochondria targeting peptide, *CD* = catalytic domain (392 – 730 AA).

3.5. Overexpression of *Tg*PSD1pv in *T. gondii*

Due to the fact that heterologous expression of *Tg*PSD1pv in yeast had been futile, a parasite strain overexpressing the protein under control of a strong promotor (p*NTP3*) [see 3.2.2.] was further analysed for its PSD activity to verify the enzymatic identity and behaviour of *Tg*PSD1pv.

3.5.1. The *Tg*PSD1pv overexpression strain displays a many-fold higher PSD activity

To examine whether the p*NTP3*-*Tg*PSD1pv-HA strain indeed displays an increased PtdSer decarboxylation, we incubated extracellular parasites or secretome of the transgenic strain with fluorescent NBD-PtdSer and performed lipid extraction and thin-layer chromatography [Fig. 23].

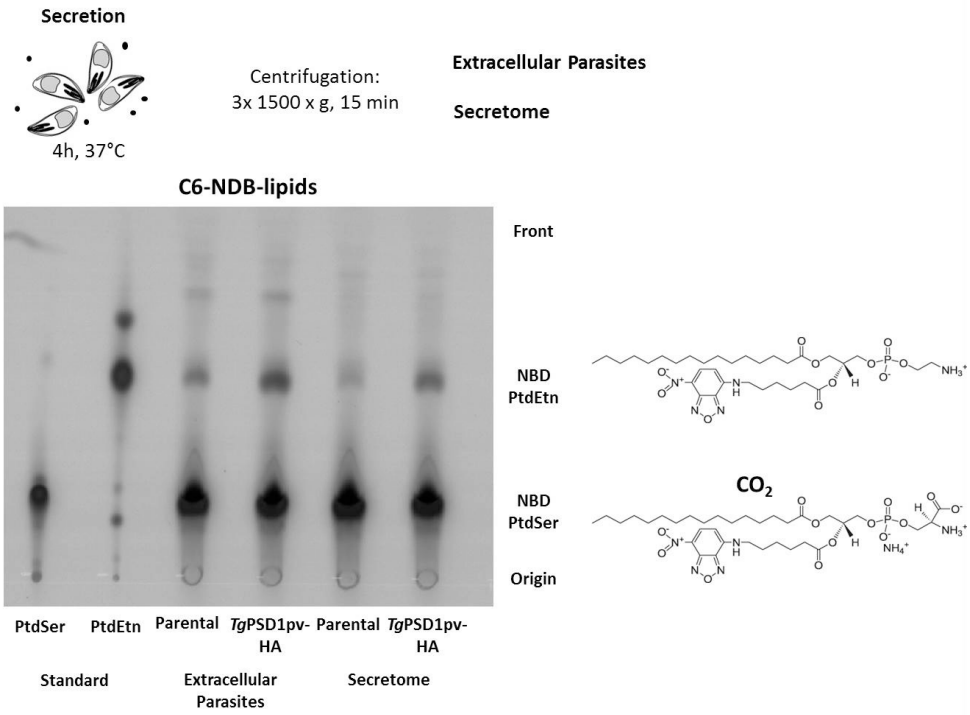

Figure 23: The pNTP3-TgPSD1pv-HA strain shows increased decarboxylation of NBD-PtdSer
Fresh syringe-released tachyzoites of the pNTP3-*Tg*PSD1pv-HA and parental strain were incubated in ICM medium for the collection of secretome as described in the scheme. After centrifugal separation, extracellular parasites and secretome were used for NBD-PSD assays. Fluorescent lipids were resolved by TLC and detected with UV light.

The observation that the *Tg*PSD1pv-HA mutant converted a higher amout of PtdSer substrate into PtdEtn, indicated the overexpression of a functional and secretory PSD enzyme [Fig. 23].

To quantify the overexpression, the transgenic tachyzoites were fractionated into soluble and membrane-bound proteins by ultra-centrifugation [Fig. 24A]. Uninfected host cells and the parental strain were used alongside as controls.

Immunoblotting of the subcellular fractions revealed that the majority of *Tg*PSD1pv-HA is found in the high speed pellet (HSP), containing membranes and dense granules. The enzyme was detectable almost excusively in its processed form (~33 kDa α-subunit), which indicated that nearly all of the proenzyme was efficiently processed to the mature enzyme [Fig. 24B,C]. A schematized protein structure and its predicted processing compared to the yeast PSD2 enzyme, which is composed of a large β- and a very small α-subunit, is shown in appendix 3.

The overexpression of *Tg*PSD1pv-HA was quantified by measuring the enzyme activity in the subcellular fractions isolated of infected HFF cells. The transgenic strain displayed a ~10-fold higher PSD activity in homogenates compared with the parental strain and ~20% of the enzymatic activity was detectable in the soluble protein fraction represented in the HSS [Fig. 24D].

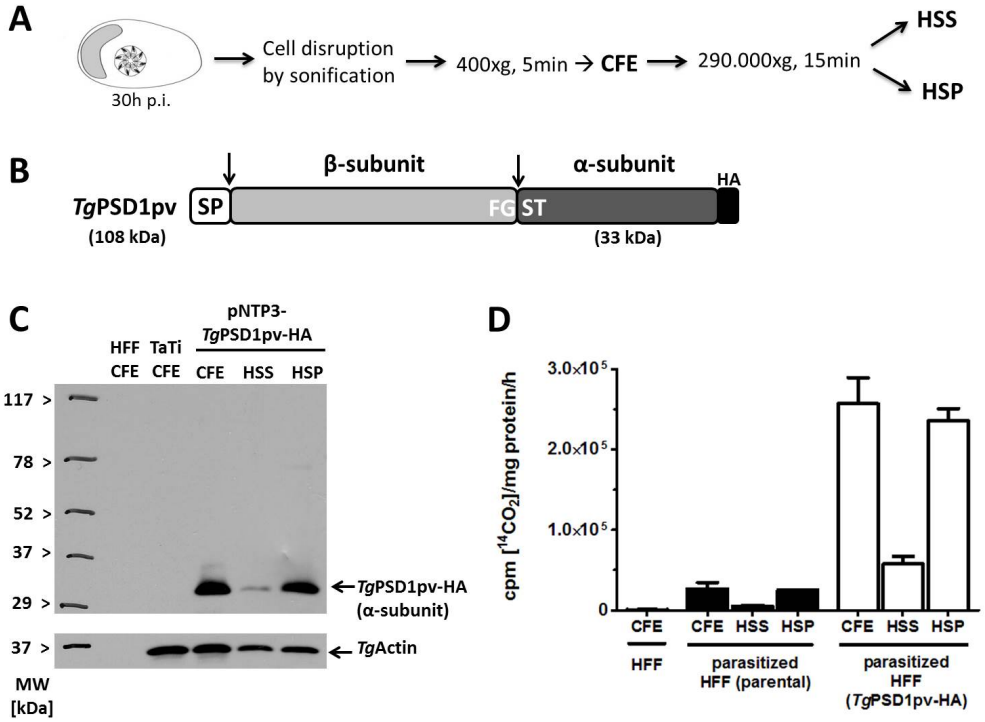

Figure 24: Overexpression of TgPSD1pv causes a 10-fold higher PSD activity in T. gondii.
(A) Experimental setup of the cell fractionation procedure. HFF cells infected with the parental (TaTi) strain or the transgenic *Tg*PSD1pv-HA overexpression strain were used to generate cell free extract (CFE) that was further separated into a high speed supernatant (HSS) and a high speed pellet (HSP) by ultra centrifugation. Uninfected HFF cells were used as a control. **(B)** Scheme of the *Tg*PSD1pv protein and its predicted processing. **(C)** Cell fractions were analyzed by Western Blot using anti-HA (1:500) and anti-*Tg*Actin (1:1000) antibodies. **(D)** The PSD activity of the homogenates and cell fractions were used to measure PSD activity with [^{14}C]dioleoyl-PtdSer (2 nCi/nmol, solubilized in 0,1% Triton X-100).

The respective parasite strains were further analysed for PSD activity in the soluble secreted protein fractions [Fig. 25]. The transgenic *Tg*PSD1pv-HA strain secreted ~10-times more soluble PSD activity than the parental strain, and neither strain showed a significant amount of active PSD in the secretome, when active secretion was inhibited by low temperature.

Collectively, the data confirm the activity of *Tg*PSD1pv as well as its soluble and secreted nature in *T. gondii*.

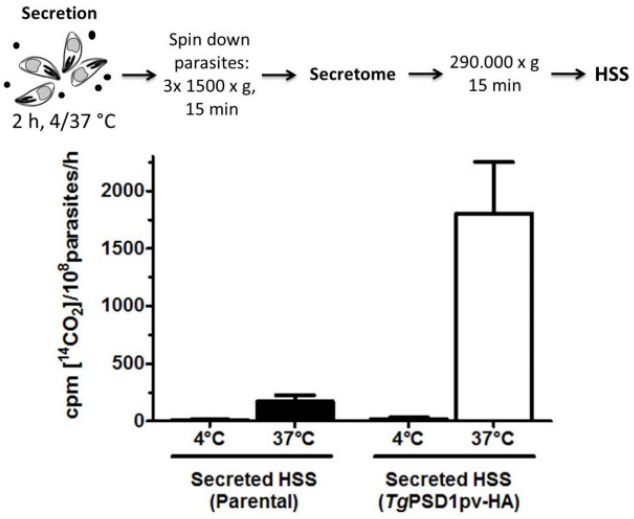

Figure 25: Overexpression of TgPSD1pv leads to an increased secretion of soluble PSD activity.
Freshly released extracellular parasites were used to prepare the high speed supernatant (HSS) from the parasite secretome collected after secretion at 4 °C and 37 °C respectively. The PSD activity in the soluble secreted fraction was assayed for 2 hrs at 37 °C in intracellular type medium (ICM) using [^{14}C]dioleoyl-PtdSer (2 nCi/nmol, 0.2 mM solubilized in 0.1% Triton X-100).

3.5.2. *Tg*PSD1pv can perform interfacial catalysis at mammalian plasma membranes

In an effort to understand the biological role of *Tg*PSD1pv, we examined the ability of the secreted enzyme to act upon PtdSer exposed on cell surfaces. Therefore, we compared the amounts of externalized PtdSer present on Jurkat T-cells treated with staurosporine, either in the absence or presence of axenic parasites secreting *Tg*PSD1pv. Staurosporine elicts an apoptotic host cell response that induces PtdSer externalization at the plasma membrane (146). The exposed PtdSer was detected by measuring fluorescent annexin binding (147) [Fig. 26].

Cultures of DMSO-treated control Jurkat cells contained a minor population of cells that stained with fluorescent annexin V, detected by microscopy and cell sorting. Treatment of cells with staurosporine increased the annexin staining in almost the whole cell population. When drug-treated cells were exposed to wild type parasites for 2 hrs, the annexin binding remained unchanged. In contrast, a co-incubation with transgenic *T. gondii* overexpressing *Tg*PSD1pv caused a significant 34% reduction of fluorescence intensity of staurosporine-treated cells [Fig. 26B].

These experiments reveal that under the given experimental conditions, *Tg*PSD1pv can reduce the amount of surface PtdSer present on host cells. From a biochemical perspective, the data show that the interfacial catalysis performed by *Tg*PSD1pv with a liposomal substrate (103) is recapitulated with PtdSer present in the exofacial leaflet of plasma membranes.

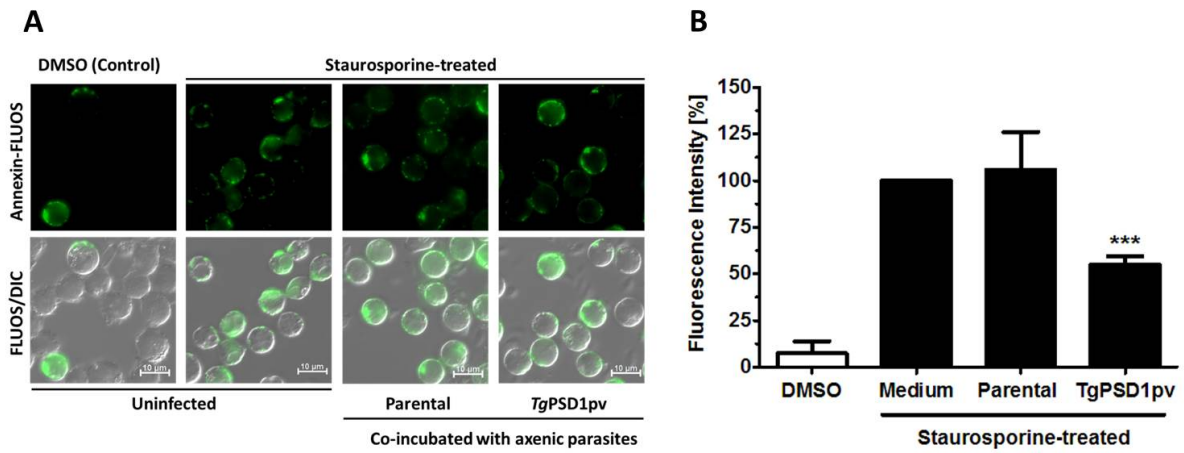

Figure 26: Secreted TgPSD1pv can decarboxylate PtdSer on the surface of Jurkat T-cells.
(A) Microscopic imaging of Jurkat cells following induction of apoptosis with 1 µM staurosporine for 2 h at 37 °C. DMSO was included as a control. PtdSer exposed on the cell surface was visualized by staining with annexin V-FLUOS. Cells were fixed with 2% PFA and immobilized on poly-L-lysine-coated coverslips. **(B)** Quantification of the annexin binding by FACS analysis. Jurkat cells were treated with staurosporine or DMSO and co-incubated with axenic *T. gondii* tachyzoites as indicated, followed by staining with annexin V-FLUOS. Cells were subjected to FACS analysis and fluorescence intensity of the fractionated Jurkat cells was determined using FlowJo software. Statistical analysis was determined by one-way analysis of variance test. Values are the ± S.E. for three experiments. Statistics was done using one-way ANOVA *** $p<0.005$.

3.5.3. Overexpression of *Tg*PSD1pv does not affect tachyzoite growth

Plaque assays were performed to assess whether the 10-fold higher expression of the active *Tg*PSD1pv enzyme has an influence on the parasite *in vitro* growth. The assay allows quantification of the parasites ability to complete their lytic cycle in a host cell monolayer. The transgenic *Tg*PSD1pv-HA strain showed no significant difference in plaque number and area compared to its parental strain [Fig. 27].

Furthermore, the overexpression strain was analysed for its phospholipid composition by 1D and 2D thin layer chromatography. Surprisingly, no obvious changes in the relative amount of PtdEtn could be detected (Appendix 4), suggesting a strict homeostasis of the PtdEtn content. If, however, the protein is only active after its secretion into the PV, potential changes in intravacuolar or PV membranes in the strain overexpressing *Tg*PSD1pv could not be determined using extracellular tachyzoites.

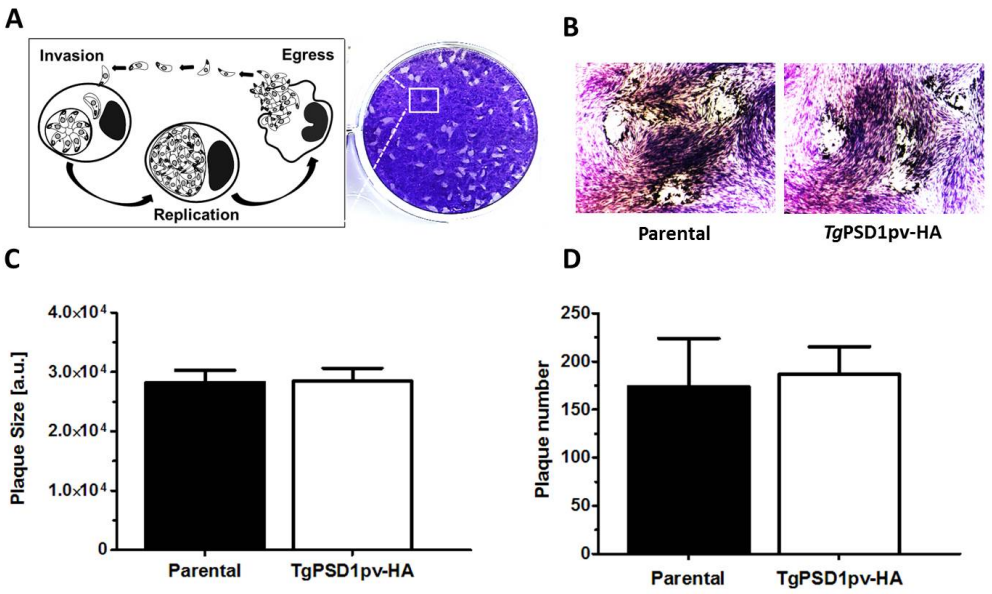

Figure 27: Overexpression of TgPSD1pv does not affect tachyzoite growth.
The transgenic *Tg*PSD1pv-HA strain and its parental were used to infect a monolayer of HFF cells. 400 parasites were inoculated into a 6-well plate, and the plate was incubated for one week without disturbance (37 °C, 5% CO_2) prior to methanol fixation and staining with crystal violet. **(A)** Schematized lytic cycle of *T. gondii* tachyzoites depicting the events of invasion, replication, and egress. Successive rounds of parasite cycles in confluent human fibroblasts over a 1-week period are leading to plaque formation. **(B)** Representative plaques formed by the parental and *Tg*PSD1pv-HA strains. **(C)** Plaque area of parental and *Tg*PSD1pv-HA strain. **(D)** Number of plaques per well were scored from three independent assays.

3.6. Overexpression of *Tg*PSD1pv variants in *E. coli*

*Tg*PSD1pv fused to a 6xHis-tag in the *pQE60* expression vector was expressed in *E. coli* M15 cells for subsequent protein purification. Since earlier attempts to express the full-lengh enzyme in *E. coli* were not successful, two isoforms of *Tg*PSD1pv lacking the predicted signal peptide (EC1) and/or the C-terminal extension (EC2) were used in this approach [Fig. 28A].

Both *Tg*PSD1pv variants, EC1 and EC2, could be successfully expressed and purified as confirmed by Western Blot analysis. The purified proteins displayed different protein sizes as predicted, however [Fig. 28B]. To test the purified proteins for their activity, decarboxylation assays using fluorescent NBD-PtdSer were performed. Subsequent lipid assays revealed a moderate PSD activity in the*Tg*PSD1pv-EC1 and -EC2 protein samples [Fig. 28C].

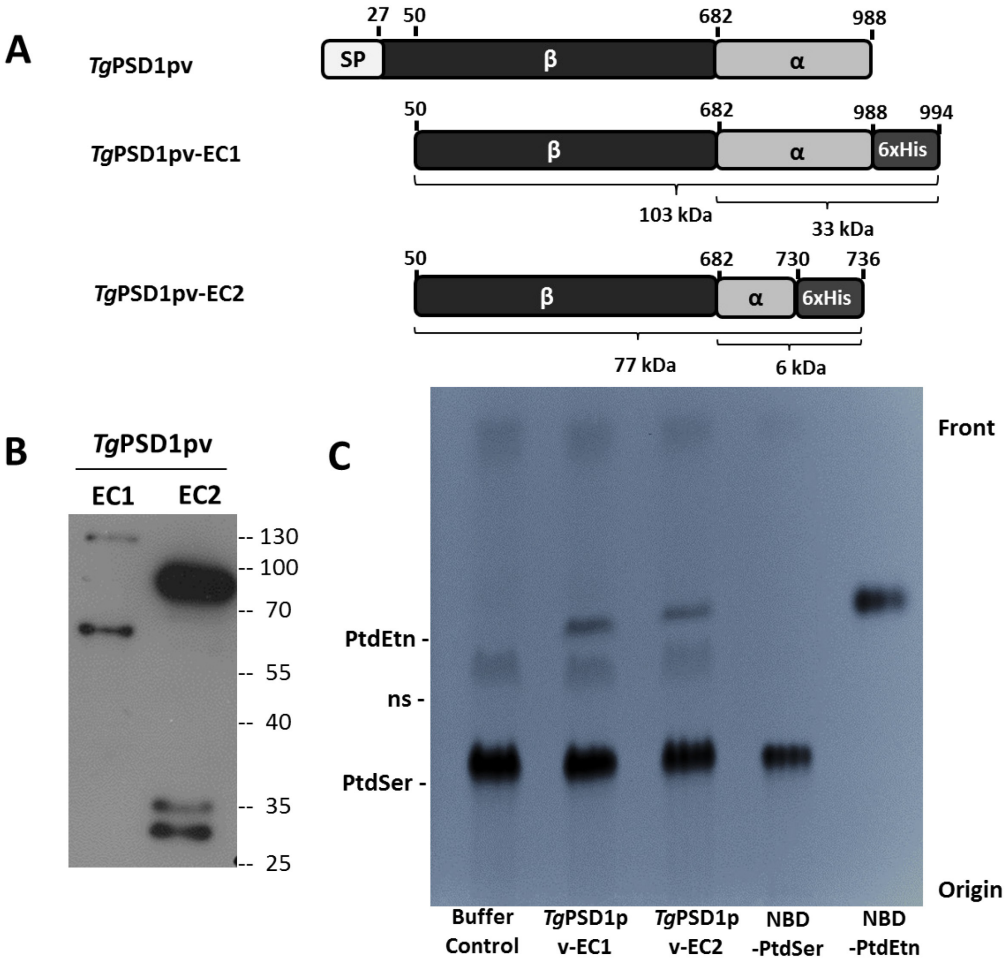

Figure 28: Expression of functional TgPSD1pv-6xHis isoforms in E. coli.
(A) Schematic depiction of the truncated *Tg*PSD1pv forms expressed in *E. coli* M15 cells. PSD enzymes are proteolytically cleaved into a β- and α-subunit. Proteins were C-terminally fused with a 6xHis-tag. **(B)** Western Blot analysis of 5 µg NiNTA-column puried *Tg*PSD1pv-EC1 and -EC2 probed with anti-His antibodies. EC1 showed two protein bands both with higher molecular weight as expected, whereas EC2 showed high expression of a ~80 kDa protein as well as two smaller anti-His signals at approximately 35 and 30 kDa. **(C)** 35 µg of purified *Tg*PSD1pv-variants were used for NBD-PSD assays (1 hr, 37 °C). NBD-PtdSer and NBD-PtdEtn were used as migration standards on TLC. Both *Tg*PSD1pv variants showed a moderate decarboxylation of NBD-PtdSer. A second non-specific (*ns*) fluorescence signal was detected in both samples as well as in the buffer control.

3.7. Conditional and direct knockout of *Tg*PSD1mt

Ablation of mitochondrial PSD is known to cause severe mitochondrial dysfunction in other eukaryotes (148-150) and genetic knockout of PSD causes embryonic lethality in mice (151). Therefore, our further work focused on examining the physiological significance of *Tg*PSD1mt for tachyzoite growth and survival.

3.7.1. *Tg*PSD1mt is dispensable for the parasite survival

First, we generated a tetracycline-regulatable conditional mutant in *Δku80*-TaTi tachyzoites [Fig. 29A]. To this end, we ectopically expressed a tetracycline-repressible *Tg*PSD1mt-HA ORF (*Tg*PSD1mt-HA$_r$) at the *Tg*UPRT locus. Then, the merodiploid strain expressing *Tg*PSD1mt-HAr was subjected to the deletion of the *Tg*PSD1mt locus by double homologous recombination. The mutant strain lacking the *Tg*PSD1mt gene showed PCR bands specific for 5'- and 3'-crossovers [Fig. 29B]. Subsequent DNA sequencing confirmed the successful recombination events. The *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ mutant showed an anhydrotetracycline-dependend regulation of *Tg*PSD1mt mRNA by RT-PCR [Fig. 29C], which confirmed the conditional nature of the strain.

As expected, *Tg*PSD1mt-HA was localized in the mutant's mitochondrion, and displayed a significant expression in the on-state (no tetracycline) [Fig. 29D]. Its expression was markedly downregulated by anhydrotetracycline within 1 day, and disappeared after 3 days (off-state). Immunoblot data confirmed a complete knockdown of *Tg*PSD1mt-HA in the conditional strain treated with the drug for one (2 days) or two (4 days) passages [Fig. 29E]. The presence of three distinct anhydrotetracycline-repressible bands corresponding to pre-proenzyme, proenzyme and α-subunit indicated proteolytic maturation of *Tg*PSD1mt. We were able to culture the mutant in its off-state for a prolonged duration, which implied a nonessential nature of the mitochondrial PSD in *T. gondii*.

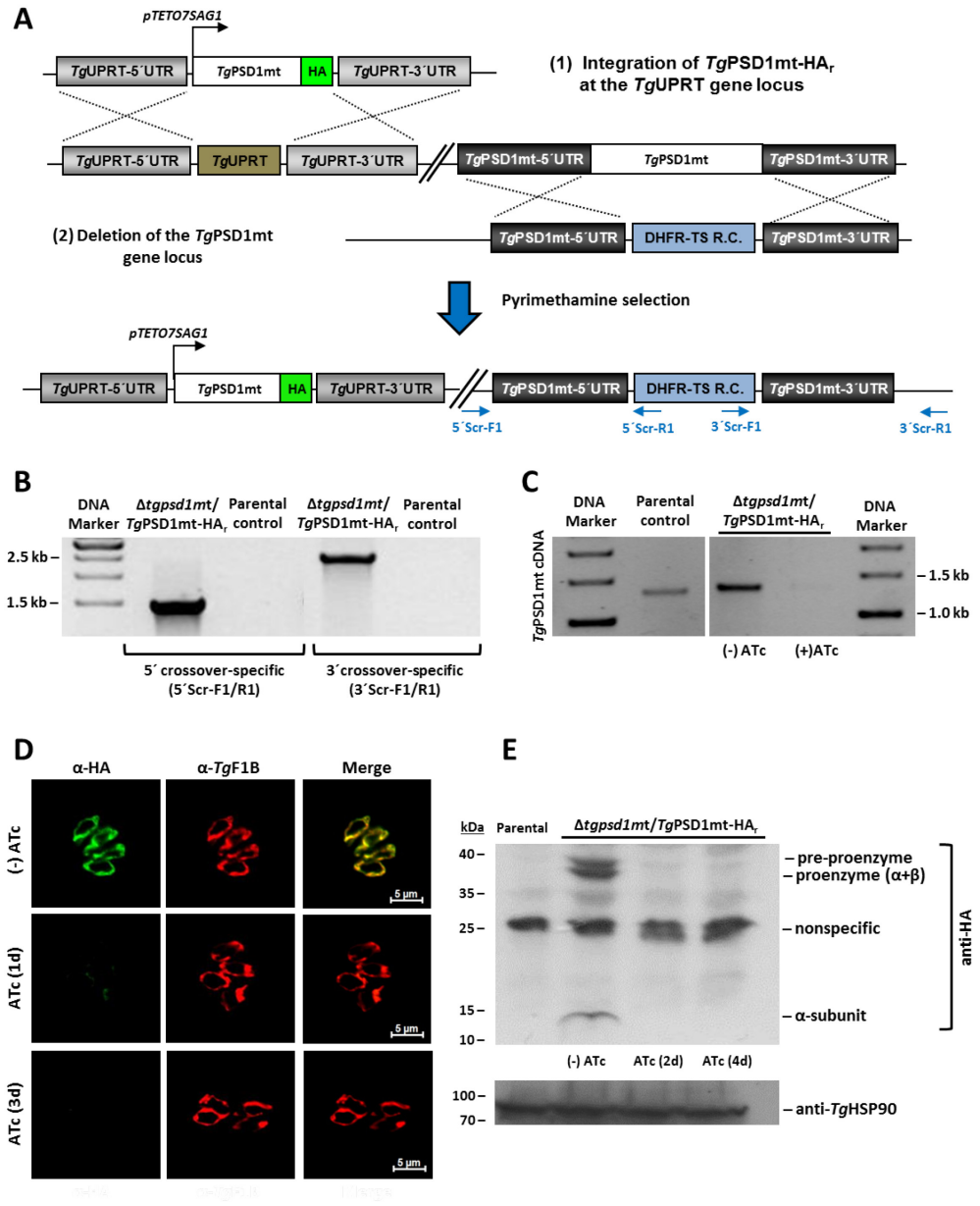

Figure 29: Conditional mutagenesis allows a tetracycline-regulated knockdown of TgPSD1mt in T. gondii.
(A) Scheme for generating the *Δtgpsd1mt/Tg*PSD1mt-HA$_r$ strain. An anhydrotetracycline-regulatable copy of *Tg*PSD1mt-HA was inserted at the *UPRT* locus, and the *TgPSD1mt* gene was deleted using a knockout plasmid (*pTKO-TgPSD1mt-5'UTR-DHFR-TS-TgPSD1mt-3'UTR*). The primer pairs to screen for 5'- and 3'-recombination are depicted in blue. **(B)** PCR verification of the *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ mutant. Pyrimethamine-resistant parasite clones were screened by genomic PCR using 5' and 3' crossover-specific primers (5'Scr-F1/R1, 3'Scr-F1/R1). The parental gDNA was included as a negative control. **(C)** PCR analysis to verify the regulation of *Tg*PSD1mt mRNA by anhydro-tetracycline (ATc). Total RNA isolated from the mutant or parental strain was used to generate cDNA and amplify *Tg*PSD1mt using ORF-specific primers. **(D)** Immunostaining of the mutant showing ATc-regulated expression of *Tg*PSD1mt-HA$_r$. The untreated control and drug-treated (1 d, 3 d) parasites were stained using anti-HA and anti-*Tg*F1B antibodies. **(E)** Immunoblot image confirming the proteolytic processing and the regulation of *Tg*PSD1mt-HA$_r$ protein in the conditional mutant. ATc-treatment was performed for 2 or 4 days in cultures, and fresh host-free parasites were subjected to protein isolation and immunoblot analyses. Note that pre-proenzyme, proenzyme and α-subunit of *Tg*PSD1mt-HA exhibit an aberrant migration in SDS-PAGE. A nonspecific band, which was not regulatable, was also observed in both strains. *Tg*Hsp90 served as the loading controls. [*The final mutant was generated in cooperation with M. Hellmund during her Bachelor´s thesis* (152).]

3.7.2. *Tg*PSD1mt is necessary for an optimal parasite growth and replication

Following a successful genetic mutagenesis of *Tg*PSD1mt, we examined the overall growth of the mutant in human fibroblasts by performing plaque assays. Parasites were allowed to grow in host cell monolayers for multiple rounds of lytic cycles [Fig. 30A,B] and the numbers and areas of plaques were evaluated to assess the relative growth fitness of the mutant. The *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ mutant displayed ~20% reduced plaque growth even in the absence of tetracycline (on-state), which is probably due to a relatively weaker strength of the conditional promoter. A 7-day exposure to the drug accentuated the growth defect to 35% in the conditional mutant, whereas it had no apparent effect on the parental strain. Prolonged drug exposure for 10 days exerted a cumulative 45% growth-impairment in the conditional mutant [Fig. 30B].

Determination of the average numbers of parasites per vacuole during the course of infection confirmed that the observed growth defect is caused by an impaired replication of the mutant tachyzoites [Fig. 30C].

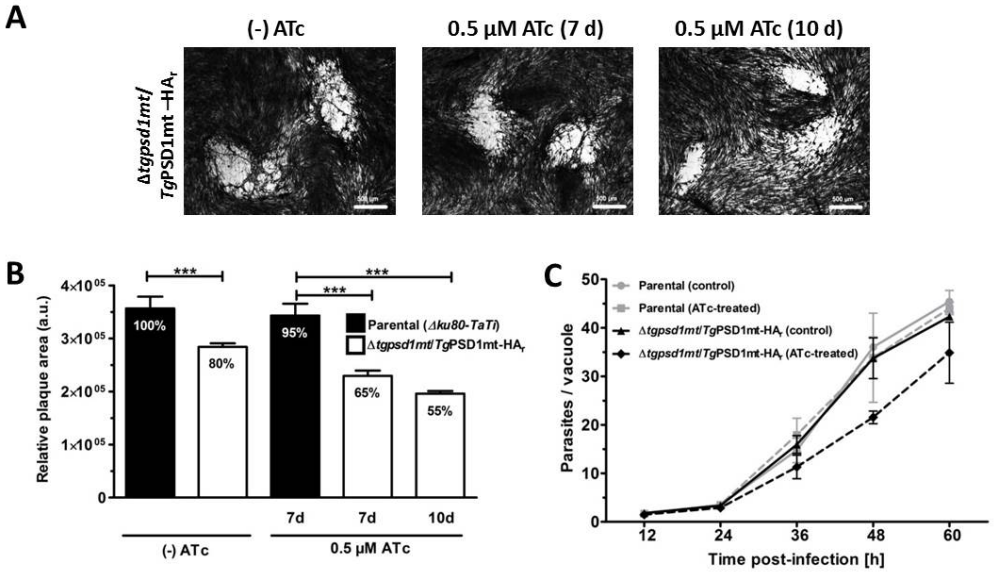

Figure 30: The Δtgpsd1mt/TgPSD1mt-HA$_r$ mutant displays a growth defect due to an impaired replication.
(A) Representative plaques of the parental and *Tg*PSD1mt mutant in the absence or presence of anhydro-tetracycline (ATc, 0.5 µM). For drug treatment exceeding 7 days, parasites were pre-cultured in ATc for an appropriate duration prior to the plaque assays. **(B)** Relative plaque areas of the parental and mutant strains. 200 plaques of each strain from three assays were quantified with the ImageJ software to estimate the parasite growth. Statistics was done using student´s t-test; *** p-value<0.0001. **(C)** Replication assay of the parental and mutant strains. Parasites were cultured for 7 days in ATc, as indicated, and used to infect human fibroblasts. Parasitized cells were fixed at different time points following infection and immuno-stained with anti-*Tg*Gap45/Alexa594 antibodies.

3.7.3. Direct knockout of *Tg*PSD1mt and phenotyping

To further support the data generated with the conditional mutant, suggesting a nonessential function of *Tg*PSD1mt, we generated a direct knockout of *T. gondii* lacking its *tgpsd1* gene by replacing the *Tg*PSD1mt ORF with the HXGPRT resistance cassette by double homologous recombination of *Tg*PSD1mt UTR elements [Fig. 31A]. The *Δtgpsd1mt* strain exhibited about 35% decrease in plaque size compared to its parental strain, which is in agreement with the growth reduction in the conditional mutant [Fig. 30B and 31B].

The *Δtgpsd1* mutant could be maintained in culture for multiple generations, which confirmed a nonessential role of *Tg*PSD1mt for the *in vitro* lytic cycle. Taken together, these results reveal that *Tg*PSD1mt is needed for an optimal growth and replication of *T. gondii*, even though it is dispensable for the parasite survival.

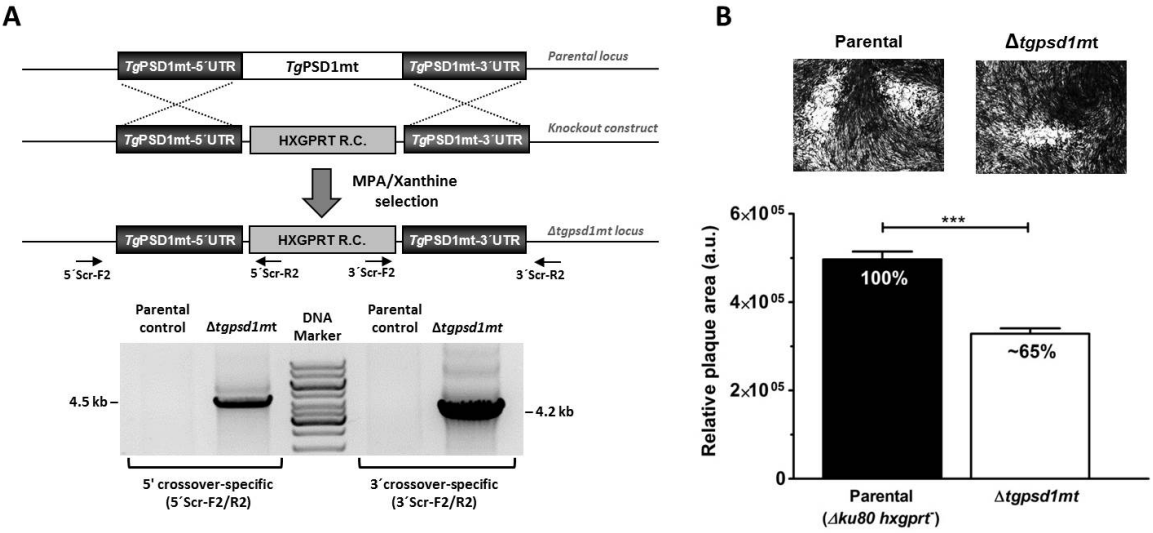

Figure 31: TgPSD1mt is nonessential for the survival but required for an efficient parasite growth.
(A) Scheme for generation of the *Δtgpsd1mt* strain. The *TgPSD1mt* gene was deleted using a knockout construct (*pTKO-TgPSD1mt-5'UTR-HXGPRT-TgPSD1mt-3'UTR*). The deletion of *Tg*PSD1mt was confirmed by 5' and 3' PCR-screening using the indicated primers (5'Scr-F2/R2, 3'Scr-F2/R2). The parental gDNA was included as a negative control. **(B)** Representative plaques formed by the *Δtgpsd1mt* mutant and parental (*Δku80-hxgprt*$^-$) strains and quantification of their sizes. In total, 200 plaques of each strain from 3 assays were measured for their area using the ImageJ software. Statistics was done using student´s t-test; *** p value<0.0001.

3.8. The *Tg*PSD1mt mutant upregulates its CDP-ethanolamine pathway

The parasite is also known to produce PtdEtn from ethanolamine, presumably via the three-stepped CDP-ethanolamine pathway (92) but its subcellular location in *T. gondii* has not been defined, so far. We identified the respective candidate proteins in the parasite genome encoding for a putative ethanolamine kinase (*Tg*EK), a CTP:phosphoethanolamine cytidylyltransferase (*Tg*ECT) and two

putative EPT proteins [3.1.], which could catalyze the first, second and final step of PtdEtn synthesis. The ORFs of all candidate proteins with a C-terminal HA-tag were expressed as a single copy at the *Tg*UPRT-locus under control of the *Tg*Gra1 promotor [Fig. 32]. *Tg*EK-HA was expressed in the cytosol and showed accumulation at the nuclear membrane, whereas *Tg*ECT-HA showed a perinuclear and ER-related expression pattern (138). Both *Tg*EPT-HA proteins co-localized with the known ER-marker *Tg*Der1-GFP [Fig. 32](153).

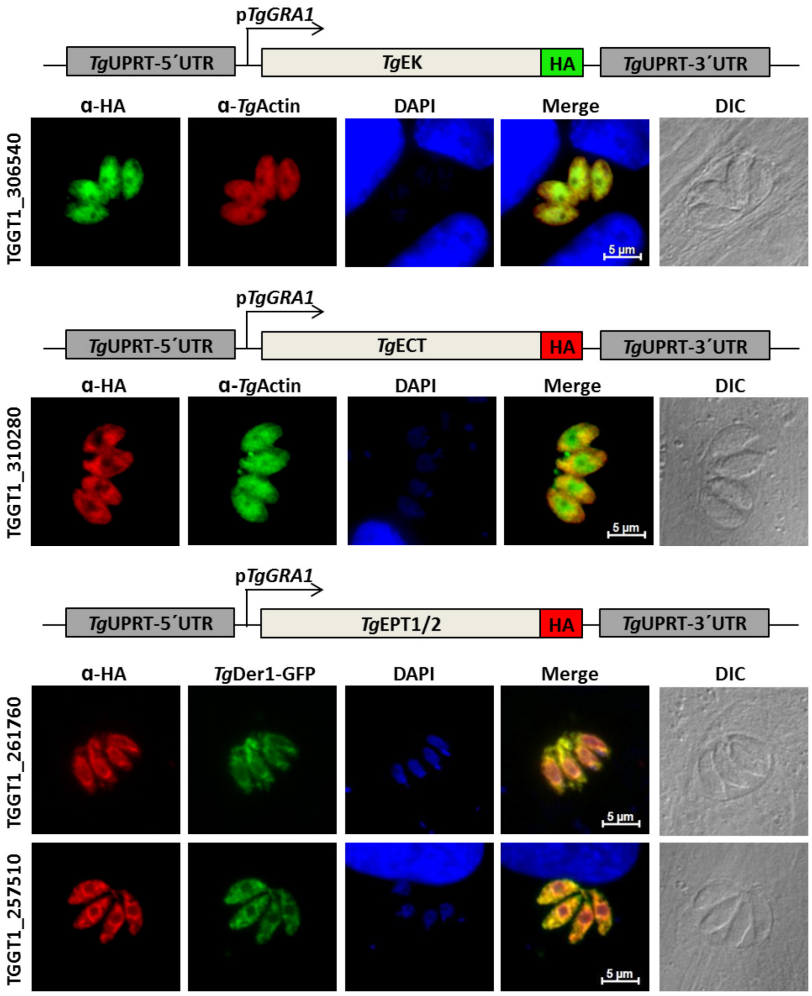

Figure 32: Localization of the CDP-ethanolamine pathway in T. gondii tachyzoites.
Schematic depiction of the constructs used to express the CDP-ethanolamine pathway proteins in *T. gondii* tachyzoites. All proteins were fused to a C-terminal HA-tag and expressed at the *Tg*UPRT locus under control of the *Tg*Gra1 promotor. Cells were fixed 24 h post infection in HFF cells and used for immuno fluorescence assays with anti-HA or anti-*Tg*Actin antibodies or co-transfected with a vector expressing *Tg*Der1-GFP in the ER. Cells were embedded in Fluoromount G/DAPI.

We determined the phospholipid composition of the Δ*tgpsd1mt*/*Tg*PSD1mt-HA$_r$ mutant [Fig. 33A]. And consistent with former results (92), PtdCho was the major lipid in the parental and mutant strains, followed by PtdEtn. Unexpectedly, no significant reduction in PtdEtn was apparent in the

conditional mutant irrespective of ATc-exposure. The relative amounts of other major lipids were also unperturbed [Fig 33A]. These results prompted us to study the alternative routes of PtdEtn synthesis in the mutant. To determine the role of *Tg*PSD1pv, we performed metabolic labeling of parasite lipids with radioactive serine [Fig 33B], which is mainly incorporated in PtdSer, and in PtdEtn following decarboxylation of the nascent PtdSer (92). Compared to the parental strain, only a modest (but statistically not significant) decline in lipid decarboxylation was detected despite a shutdown of *Tg*PSD1mt protein synthesis in the *off-state* mutant. The data indicated a continual PtdEtn synthesis by decarboxylation of PtdSer in the mutant, which is likely due to *Tg*PSD1pv present in the dense granules (103).

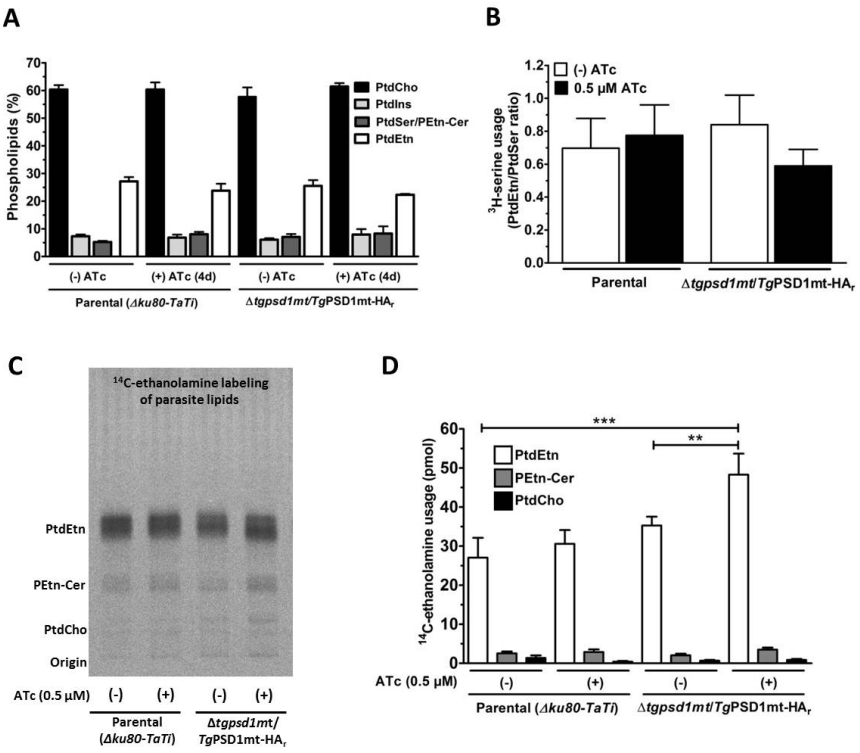

Figure 33: PtdEtn synthesis in the parasite mitochondrion and ER are not mutually exclusive.
(A) Phospholipid fractions of parasite lipids. Lipids isolated from the parental (*Δku80-TaTi*) and mutant (*Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$) strains were resolved by TLC and quantified by phosphorous assay. **(B)** Labeling of parental and *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ strains with radioactive serine. Fresh extracellular parasites (5 x 10^7) of the parent or mutant strain (untreated or treated with ATc for two passages) were incubated with [^{3}H]serine (1 µCi/nmol, 2 h, 37 °C). Lipids were resolved by 1D-TLC (chloroform, methanol, acetate (65:25:10, v/v/v)) and subjected to liquid scintillation counting (n = 3 parental; n = 5 mutant). Differences between the conditions are not significant. **(C)** Metabolic labeling of the parasite lipids with ethanolamine. Axenic parasites (5x10^7) of the parental or mutant strain were incubated with ^{14}C-ethanolamine (20-40 nCi/µM) for 2 hrs prior to lipid extraction and TLC. The radiolabeled lipids were visualized by autoradiography. **(D)** Individual phospholipid bands from *panel C* were visualized by iodine vapor staining and subjected to liquid scintillation counting. Error bars indicate S.E. from three independent assays. Statistics was done using student´s t-test; *** p< 0.001, ** p<0.01.

To test whether the ER-derived lipid can alleviate depletion of the mitochondrial PtdEtn, we performed labeling of parasite lipids with radioactive ethanolamine [Fig. 33C,D]. As expected, ethanolamine was primarily incorporated into PtdEtn, and into phosphoethanolamine-ceramide (PEtn-Cer). Its metabolism was not affected by tetracycline in the parental strain, whereas the mutant in its *on state* showed a modest increase in the nascent PtdEtn. The *off-state* mutant, on the other hand, incorporated about 36% more substrate into PtdEtn than its own *on state*, and 60-77% more with respect to the parental strain [Fig. 33D]. Collectively, these data show a sustained PtdSer decarboxylation as well as an upregulation of the CDP-ethanolamine route in the parasite ER following a knockdown of mitochondrial PtdEtn synthesis.

3.9. The *Tg*PSD1mt mutant can tolerate a depletion of ethanolamine

To dissect the observed metabolic plasticity in *T. gondii*, we assessed whether the conditional mutant can survive simultaneous knockdown of *Tg*PSD1mt and depletion of ethanolamine in culture [Fig. 34A]. To this end, we set up plaque assays using dialyzed FCS. As anticipated, the *Tg*PSD1mt mutant in the *on state* behaved similar to the parental strain, whereas in the *off state* it exhibited an accentuated 56% growth defect that was partially restored by addition of ethanolamine [Fig. 34B]. A further inhibition of the mutant's growth, albeit modest, was observed when lipid-depleted serum was used instead of dialyzed FCS [Fig. 34C].

The survival and durable growth of the *off-state* mutant in both sera types implied yet another route to drive PtdEtn biogenesis, potentially involving import of host-derived lipids. To test this, we first radioactively labeled the lipid pool in human fibroblasts, and then allowed the conditional mutant to replicate and perform lipid biogenesis in pre-labeled host cells [Fig. 34D]. We measured a nearly 2-fold higher accumulation of radiolabeled lipid in the *off-state* mutant. Although these data do not rule out a contribution of the CDP-ethanolamine pathway within the parasite, they are indicative of PtdEtn scavenging from the host cells when *de novo* synthesis by *Tg*PSD1mt is shut down. Taken together, these results reveal a surprising level of metabolic plasticity in the parasite with respect to its growth and lipid biogenesis under varying nutrient conditions.

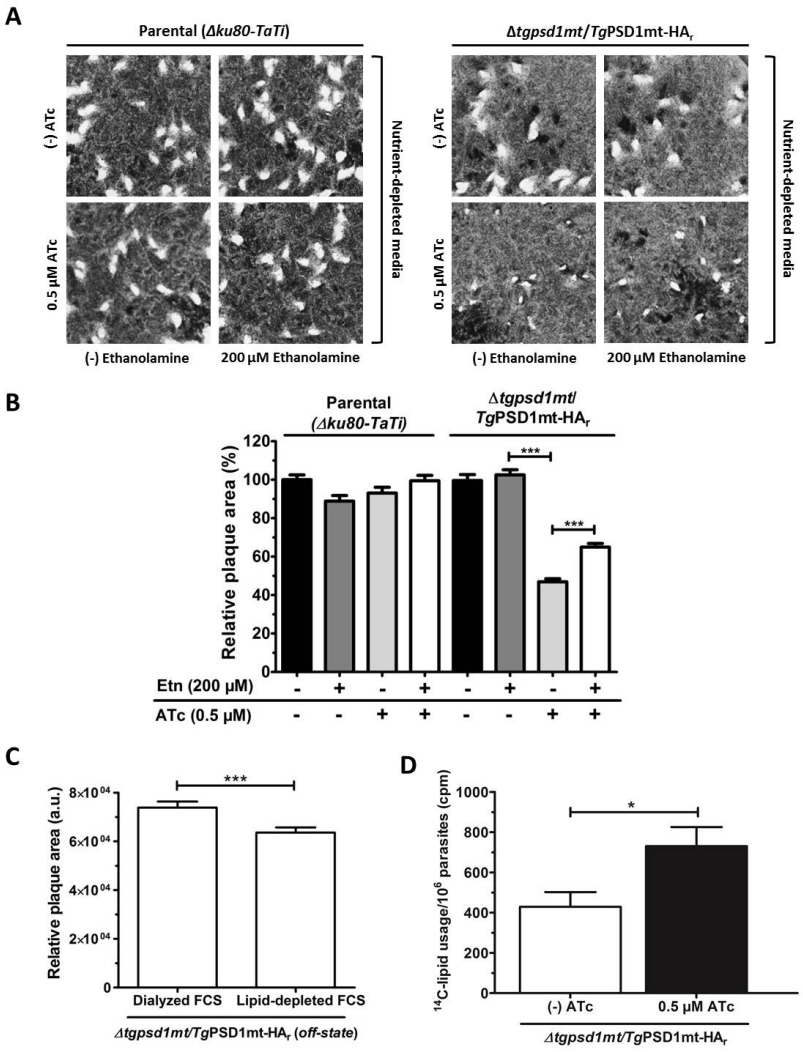

Figure 34: T. gondii can survive a simultaneous knockdown of TgPSD1mt and depletion of ethanolamine.
(A) Representative plaques formed by the parental or *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ strains in media containing dialyzed serum with or without ethanolamine supplementation. The *off-state* parasites were pre-treated for 2 passages with ATc and treatment was maintained during plaque assays. **(B)** Relative plaque areas of the parental and mutant strains from *panel A*. In total, 250 plaques of each strain or condition from three assays were quantified. **(C)** Plaques formed by the conditional mutant in medium supplemented with either dialyzed or lipid-depleted serum. **(D)** Accumulation of radioactive lipids in conditional mutant following its replication in human fibroblasts labeled for PtdEtn. Isotope labeling of host cells was performed with ^{14}C-ethanolamine (40 nCi/nmol) in medium containing lipid-depleted serum (24 hrs, 37 °C, 5% CO_2). Host cells were then infected with parasites (MOI, 2), and incubated for an additional 48 hrs. Lipids isolated from freshly egressed parasites were analyzed by scintillation counting to estimate the import of host-derived lipids (n = 4 assays; * = p<0.05; *** = p<0.001).

3.10. *Tg*PSD1mt depletion does not influence mitochondrial morphology

To test whether the replication defect is caused by an impairement of mitochondrial integrity as reported in other organisms (150,151,154), an ultrastructural analysis of the parasite mitochondrion was performed. Intracellular parasites of the parental and the *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ strain were

examined by transmission electron microscopy. Unexpectedly, the *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ strain did not show apparent alterations in size or internal christae structure [Fig 35].

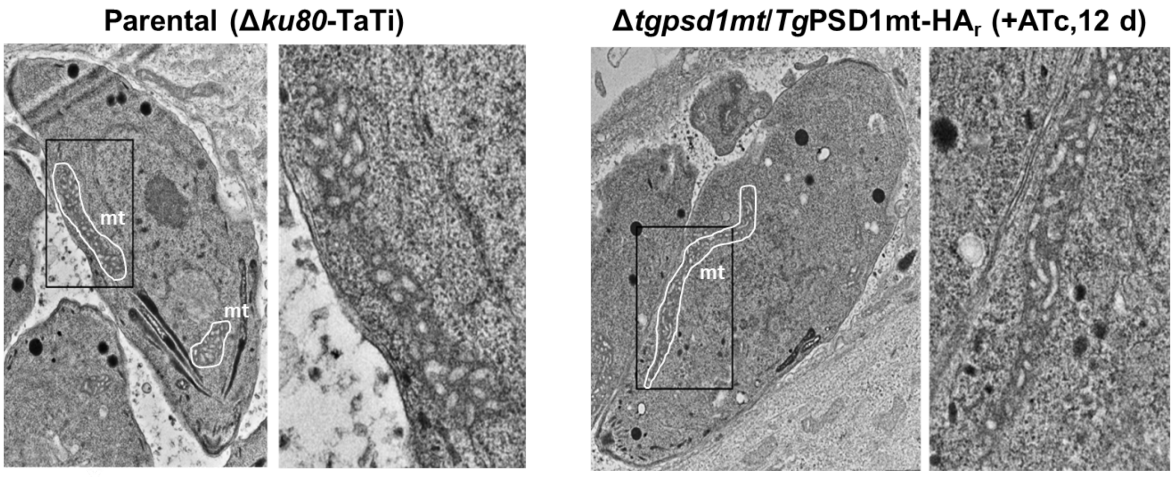

Figure 35: Knockdown of TgPSD1 does not affect the mitochondrial ultrastructure.
Transmission electron micrographs of intracellular tachyzoites of the parental (*Δku80*-TaTi) and *Δtgpsd1mt*/*Tg*PSD1mt-HA$_r$ strain after 12 days of ATc-treatment. *mt* = mitochondrion

In addition, an unperturbed expression pattern of the mitochondrial ATPase subunit *Tg*F1B shown in immunofluoresce analyses in the *Tg*PSD1mt mutant compared to the parental strain indicates an unaltered mitochondrial morphology (and function) in the *Tg*PSD1mt mutant [Fig. 29D]. However, further experiments are needed for clarification.

3.11. Targeted gene disruption of *Tg*PSD1pv

Because the genomic locus of *Tg*PSD1pv encodes upstream and downstream coding genes in close proximity, a targeted gene disruption approach was preferred over a conventional ablation of the gene locus. The experimental design is depicted in figure 36, showing the insertion of the HXGPRT resistance cassette into the catalytic domain of the *Tg*PSD1pv gene. The vector used to generate the gene disruption is attached in appendix 6. Targeted gene disruption was verified by recombination-specific PCR reactions on genomic DNA of the clonal mutant [Fig. 36A]. A copy of the *Tg*PSD1pv ORF under control of its native 5´ promotor region was inserted at the *Tg*UPRT-locus to complement the mutant [Fig. 36B]. Immunofluorescence analysis verified the successful expression of *Tg*PSD1pv-HA in the complemented strain and indicated also an unaltered secretion of *Tg*Gra5 in this mutant [Fig. 36B].

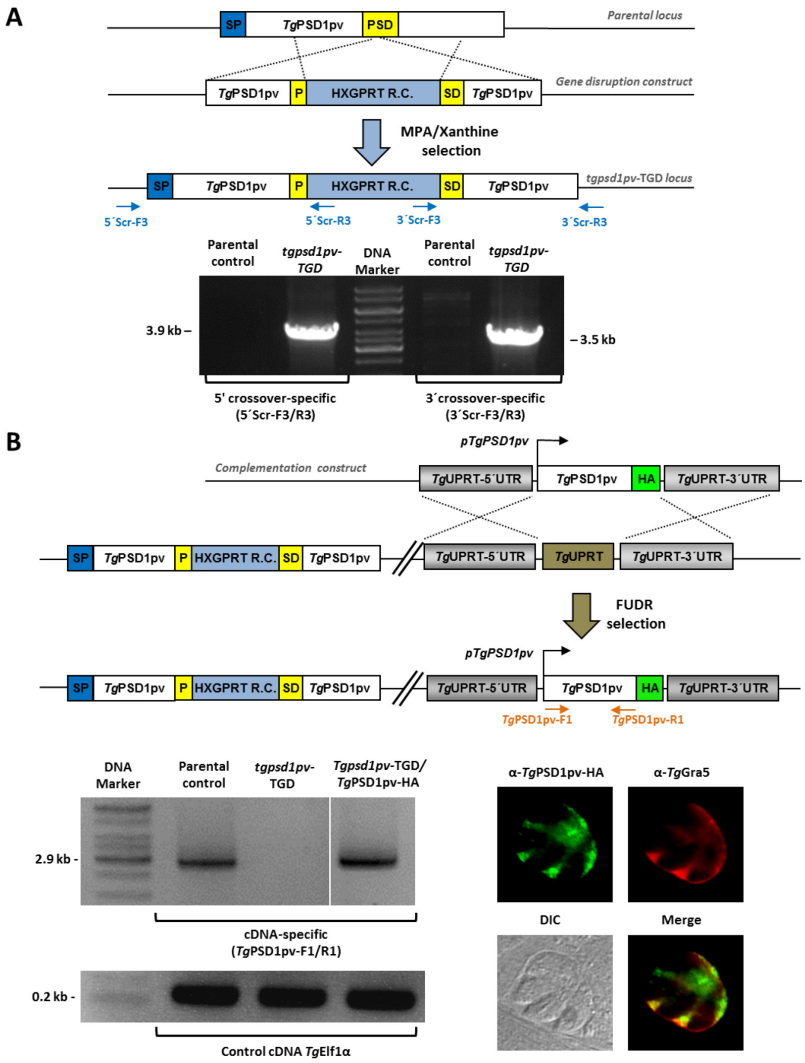

Figure 36: Targeted gene disruption of TgPSD1pv and complementation
(A) Schematics of the *Tg*PSD1pv-TGD mutant and PCR verification of homologous recombination of the 5´ and 3´TGD elements. **(B)** The *Tg*PSD1pv complementation strain expressed the *Tg*PSD1pv-ORF C-termially HA-tagged under control of its native promotor element at the *Tg*UPRT locus. Successful gene disruption in the TGD-mutant and complementation with *Tg*PSD1pv-HA was verified by PCR using *Tg*PSD1pv-specific primers and *Tg*Elf1alpha as a control for successful cDNA synthesis. The expression of the *Tg*PSD1pv-HA protein was verified by immunofluorescence assays using anti-HA (*green*) and anti-*Tg*Gra5 (*red*) antibodies. *PSD* = PSD domain; *R.C.* = resistance cassette; *Scr* = screening; *SP* = signal peptide; *TGD* = targeted gene disruption, *UTR* = untranslated region.

3.11.1. The *Tg*PSD1pv mutant does not secrete PSD activity

To assess whether the *Tg*PSD1pv gene disruption mutant indeed lacks its PSD activity in the secretome and if the same was restored in the complementation stain, secretion assays were performed. Secretome and cell extracts were analyzed for their PSD activity using NBD-PtdSer as a substrate [Fig. 37]. As expected, the parental and complementation strain showed decarboxylation activity in the secretome, whereas no PSD activity could be detected for the *tgpsd1pv*-TGD mutant.

NBD-PtdSer decarboxylation was detectable in all cell extract preparations, due to *Tg*PSD1mt activity. A reduced overall catalytic activity in the *tgpsd1pv*-TGD mutant cell extract also confirmed successful disruption of *Tg*PSD1pv [Fig. 37].

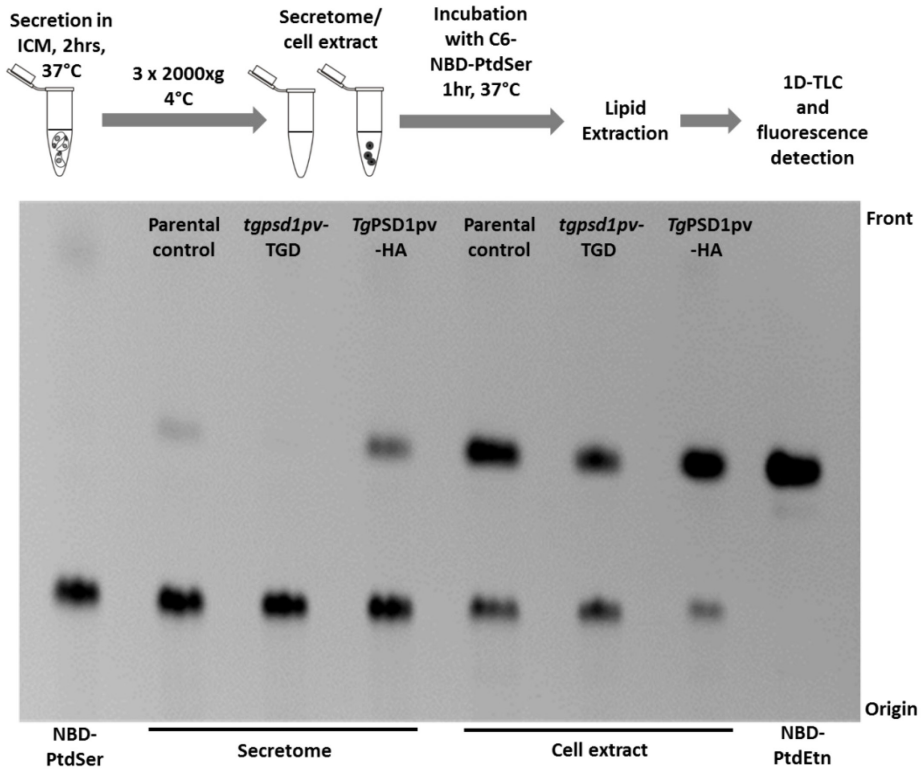

Figure 37: The TgPSD1pv mutant lacks PSD activity in the secreted protein fraction.
Fresh syringe-released tachyzoites were used for the preparation of secretome and cell extracts for PSD assays using fluorescent NBD-PtdSer as substrate. TLC-resolved lipids were visualized by UV light. [*Secretion experiment and picture: Theresa Ring*]

3.11.2. *Tg*PSD1pv is not essential for the lytic cycle of *T. gondii in vitro*

The genetically and enzymatically characterized *Δtgpsd1pv* mutant strains were used for subsequent analysis and phenotying. In routine cultures, the mutant (*tgpsd1pv*-TGD) and complemented (*Tg*PSD1pv-HA) strains did not show any apparent differences in their *in vitro* growth compared to the parental strain, which suggested a nonessential function of *Tg*PSD1pv for the tachyzoite growth. Therefore, we tested the strains for their *in vitro* phenotype in plaque assays under different nutritional depleted and repleted conditions [Fig. 38].

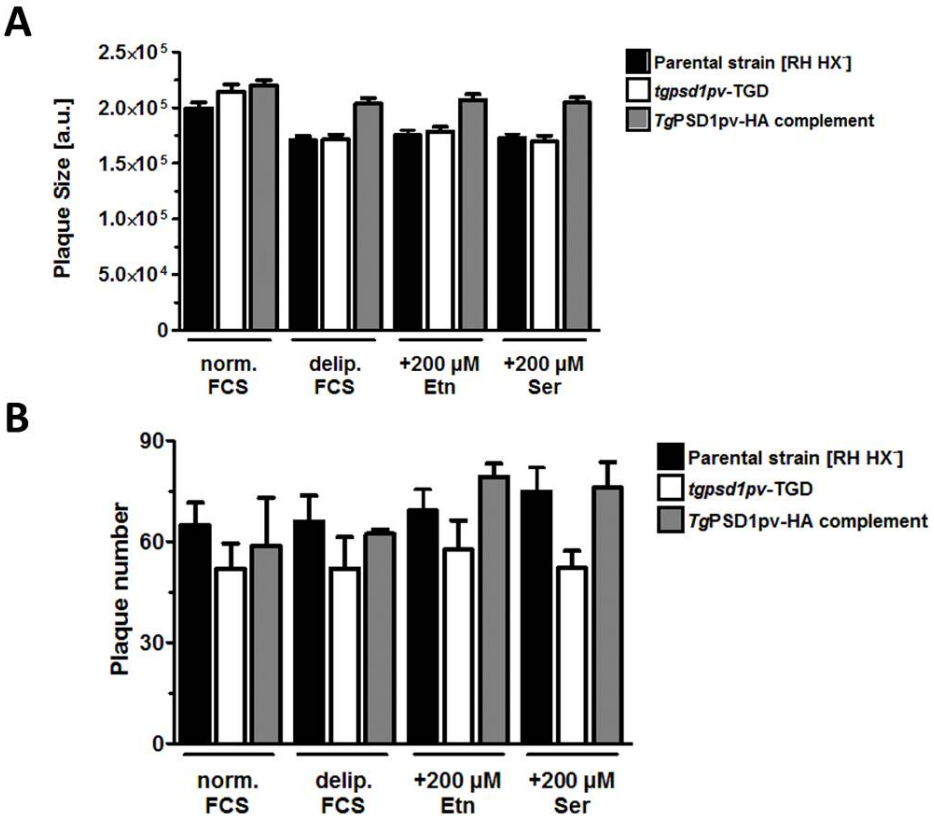

Figure 38: The tgpsd1pv mutant shows a modestly enhanced growth phenotype but lower plaque numbers.
The *tgpsd1pv*-TGD mutant and parental and complemented control strains were used to infect 6 well plates (200 parasites per well). The growth was examined under various nutrient conditions including standard D10 culture medium with normal FCS, as well as in nutrient-depletion medium containing delipidated FCS. For nutrient-repletion experiments, indicated lipid precursors were added in 3 ml of medium during the incubation period of one week. **(A)** The plaque size was measured using microscopic images and the ImageJ software. **(B)** The number of plaques per well was counted and plotted from n= 3-5 independent experiments.

Plaque assays revealed that disruption of the *tgpsd1pv* gene and absence of secreted PSD activity did not negatively affect the fitness of the mutant, which in fact showed slightly larger plaques compared to the parental strain under standard culture conditions [Fig. 38A]. The *Tg*PSD1pv-HA strain showed enhanced growth under all given culture conditions [Fig. 38A]. Interestingly, the *tgpsd1pv*-TGD mutant strain showed a reduced number of plaques under all examined nutritional conditions. The phenotype was specifically restored in the complemented parasite strain [Fig. 38B]. It can be suggested that the absence of secreted PSD activity causes a moderate decrese in tachyzoite infectivity *in vitro*. This observation needs to be further analysed by invasion assays, however.

4. Discussion

4.1. Phosphatidylethanolamine biogenesis in *T. gondii* tachyzoites

Phosphatidylethanolamine (PtdEtn) is the most abundant phospholipid in prokaryotes and the second most prevalent lipid in most eukaryotes, including *T. gondii.* It accounts for ~12-25% of total parasite phospholipids (92,96). Besides its function as a structural component of biological membranes, the conical-shaped PtdEtn plays a critical role for membrane fusion, fission and curvature and the stabilization of membrane proteins (86,137). Unlike the synthesis of PtdCho, which seems to exclusively depend on a functional CDP-Cho-pathway (92,100), this work revealed multiple routes of PtdEtn biogenesis in *T. gondii*. Here, PtdEtn can be produced via the CDP-ethanolamine pathway using ethanolamine (Etn) and diacylglycerol as well as by decarboxylation of phosphatidylserine (PtdSer). The latter reaction is catalized by two distinct PtdSer decarboxylase enzymes (PSDs) in *T. gondii*.

We were able to identify the enzymes which constitute the main routes for PtdEtn biogenesis in the parasite endoplasmatic reticulum and the mitochondrion, as well as in the parasitophorous vacuole of *T. gondii* [Fig. 39]. The presence of a functional *Kennedy pathway* was initially shown by incorporation of Etn and choline into PtdEtn and PtdCho (92). The enzymatic activity of a cytosolic *Tg*EK enzyme and a dual substrate specific *Tg*CK have been shown previously (94,100). These enzymes catalyze the first step of the *Kennedy pathway* in the parasite. However, the enzymatic nature of *Tg*ECT/CCT and *Tg*EPT/CPT and their potential overlapping functions in performing the second and final step of this pathway remain to be investigated.

The localization and characterization of the two PSD enzymes in *T. gondii* was the main focus of this work. Besides the identification of a conserved mitochondrial PSD enzyme (*Tg*PSD1mt), whose enzymatic identity was verified by heterologous expression in yeast, the parasite harbours a unique and partially soluble *Coccidia*-specific PSD enzyme, which could be allocated to be secreted into the parasitophorous vacuole (*Tg*PSD1pv) via dense granule secretory organelles [3.2.2.].

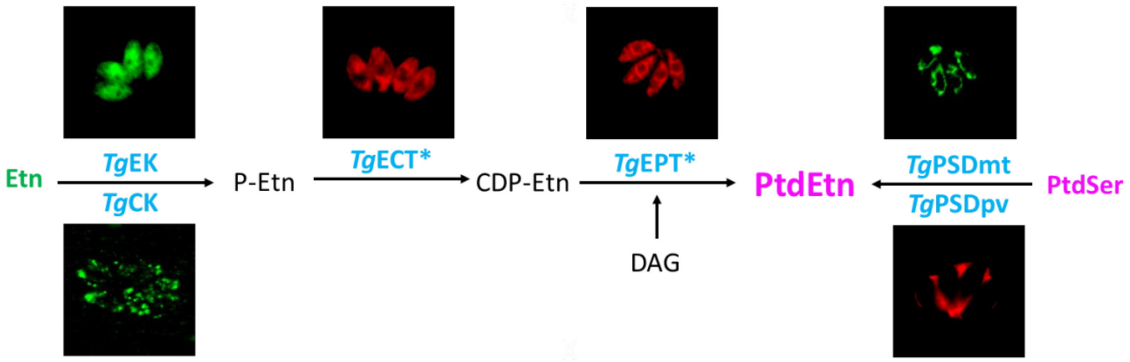

Figure 39: Localization of PtdEtn synthesis in T. gondii
Schematic overview of the PtdEtn-biosynthetic pathways and their subcellular localization in intracellular tachyzoites. *CK*: choline kinase; *EK*: ethanolamine kinase; *ECT*: CTP:phosphoethanolamine cytidylyltransferase; *EPT*: CDP-ethanolamine:1,2-diacyglycerol ethanolamine-phosphotransferase, *P-Etn*: Phospho-ethanolamine *PSD*: phosphatidylserine decarboxylase. *Tg*CK was immunostained using an anti-*Tg*CK serum (1:200) showing subcellular organization in cytosolic clusters, as described by Sampels et al. (100). The enzymatic specifity of the proteins marked with "*" are not experimentally verified.

While PtdSer decarboxlation is the only way to produce PtdEtn in prokaryotes (155), eukaryotic organisms harbour different routes of PtdEtn synthesis (81,86,156). Besides the CDP-ethanolamine and PSD pathways, that are present in most eukaryotes including *T. gondii*, *S. cerevsiae* can also generate PtdEtn via acylation of lyso-PtdEtn. The Acyl-CoA dependent acyltransferase (Ale1) enzyme is located at the mitochondria-associated membranes (MAM) and harbours also broad activity for other lysolipid species (157-159). *The T. gondii* genome encodes one hypothetical protein (TGGT1_259155) with partial sequence similarities to *Sc*Ale1, but for which no functional acyltransferase domain is predicted. In mammals, LPEAT (MBOAT) proteins catalyze the acylaction of lyso-PtdEtn as part of the *Land´s cycle* (160). No similar enzymes could be identified in the *T. gondii* genome so far, indicating that a re-acylation of lysolipids may not contribute to phospholipid synthesis and remodelling of the parasite membranes.

Many differences and exceptions to the conventional PtdEtn pathways can be found in other protozoan parasites (98,161). PtdSer decarboxylation activity was shown to occur in *T. brucei* procyclic and bloodstream forms (81,150). Likewise, PSD genes are encoded in the genome of *Leishmania spp* and *T. cruzi* (98), but the respective enzymes have not been localized or characterized, since The CDP-Etn pathway seems to represent the main PtdEtn source in these species and PSD enzymes are thought to play only a minor role for PtdEtn formation in kinetoplastids (81,150). Interestingly, *Leishmania* can redirect its sphingolipid metabolism to make P-Etn via sphingosine-1-phosphate lyase, which constitutes yet another mechanism to produce PtdEtn. This pathway appears to be crucial for stage differentiation and virulence of the parasite (162). Respective genes are not present in the genomes of *Plasmodium*, *Toxoplasma* and *Theileria* species, however

(98). Additionally, *T. brucei* expresses a bidirectional ceramide ethanolamine-phosphotransferase, which is capable of converting PtdEtn and ceramide to Phosphoethanolamine-ceramide (PEtn-Cer) and DAG and *vice versa* (163). The enzyme producing this unusual sphingolipid PEtn-Cer in *T. gondii* still remains unknown (95). A BLAST search in the *T. gondii* genome identified one conserved hypothetical protein (TGGT1_246780) with minor similarities to the respective *T. brucei* sequence (TritrypDB.org; Tb927.9.9400). *Plasmodium* species express a functional *Kennedy pathway* (98), but in contrast to *Trypanosoma* and *Toxoplasma*, encoding separate EPT and CPT enzymes, *Plasmodium* encodes a single fusion CEPT enzyme used for both, the PtdEtn and PtdCho synthesis (164). The *Kennedy pathway* seems to be essential for blood stage development in *P. berghei* (165). As already indicated, *P. falciparum* encodes a single type I-like PSD enzyme that is likely expressed in the ER (135). Therefore, PtdEtn synthesis is probably confined to the ER in *Plasmodium* species unlike shown for *T. gondii*. The relative importance and contribution of the different pathways used to synthesize PtdEtn appears to be highly divergent among different organisms and tissues (86,150,156). Moreover, different pathways produce functionally-distinct PtdEtn pools with different acyl chain signature (166,167).

The individual phospholipid profile, the loss of the phosphatidylethanolamine methyltransferase reaction and the gain of novel parasite-specific lipid biosynthetic enzymes, such as *Tg*PSD1pv and *Tg*PTS (96), presumambly reflect an adaptation to the intraparasitic lifestyle in *T. gondii*. The resulting phospholipid composition likely enables an interaction with and the exploitation of the host cell whilst achieving unimpaired/undetected growth, movement and propagation within host tissues.

4.2. Evolutionary context and characteristics of *Tg*PSD enzymes

Phosphatidylserine decarboxylases belong to an unique subgroup of decarboxylases harbouring a pyrovoyl at the prothetic group (83). They are maturated post-translationally by an auto-proteolytic cleavage reaction, which splits the pro-protein into an α- and β-subunit at a conserved (L)G^ST motif (83) [Fig. 40]. The unusual non-mitochondrial PSD enzyme of *P. falciparum* (135) carries a VG^SS cleavage motif and *T. brucei* shows a WG^SS at its catalytic site, both representing minor exceptions. All coccidian-specific PSD enzymes display a FG^ST-motif and are predicted to form a relatively large α-subunit upon autoproteolytic cleavage when compared to other PSD enzymes [Fig. 40].

Phylogenetic analysis revealed that both PSD enymes expressed in *T. gondii* are of different phylogenetic origin [3.1.5.]. *Tg*PSD1mt is a conserved mitochondrial protein sharing high similarities to other type I PSD enzymes, whereas the secreted *Tg*PSD1pv forms a novel cluster with other putative PSD enzymes exclusively found in coccidian species [Fig. 9]. The latter are not related to the

type II PSD clade, which includes fungal and plant PSD enzymes, such as PSD2 from *S. cerevisiae* (148) or PSD2 and PSD3 from *A. thaliana* (130,132). The three eukaryotic clades display differential protein features as depicted below [Fig. 40]. The fact that *Tg*PSD1pv seems to be highly different from other characterized PSDs may underlie its inability to functionally complement the yeast PSD double mutant [3.4.3.].

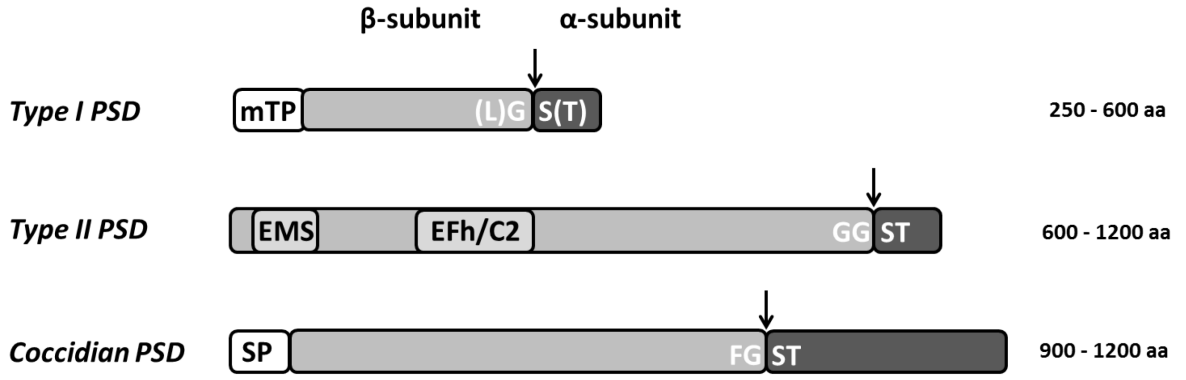

Figure 40: Characteristics of eukaryotic PSD enzyme groups.
Schematic depiction of the proteins features of different eukaryotic PSD enzyme clades. Topological signals, characteristic domains and the conserved cleavage motifs are shown as well as the average protein size. mTP = mitochondrial targeting peptide; *EMS* = endomembrane sorting sequence; *EFh* = EF-hand, calcium binding-motif; *C2* = protein kinase C conserved region 2, calcium binding motif; *SP* = signal peptide.

In line with the observed subcellular localizations observed for various type I and II PSD-proteins, localization sequences are predicted at their N-termini, targeting the enzymes to the mitochondrion or the endomembrane system, respectively. Some PSD enzymes of the type II group carry EFh -Ca^{2+}-binding domains and/or Ca^{2+}-dependend C2-domains, known to be involved in lipid-protein interactions. The C2-domain of *Sc*PSD2 was shown to be nonessential for catalysis but required for sufficient lipid trafficking (168,169). None of these domains are predicted for the PSDs of the coccidian subgroub, but the latter carry a predicted 20 – 26 aa long signal peptide at their N-terminus [Fig. 40], which is consistant with its secretory nature in *T. gondii*.

The successful expression of active recombinant *Tg*PSD1pv variants in *E. coli* [Fig. 28] will enable the study of interfacial catalysis and structural determination, which have not been possible with other membrane-bound PSD enzymes so far.

Occurrence and perpetuation of a coccidian-specific PSD clade suggests a special role of this enzyme in coccidian development, which needs to be further investigated [see 4.6.]. Interestingly, blastx searches using the *Tg*PSD1pv cDNA sequence revealed partial sequence identities to parasitic molds of the *Saprolegina* and *Phytophora* genus and to certain insect species, such as the house fly and the pathogen vector *Aedes aegypti* as well as to the human whipworm *Trichuris trichiura*. This

observation could suggest that coccidian PSD enzymes may be evolutionary related to the PSD enzymes of other (parasitic) pathogens and were probably acquired by lateral gene transfer between different parasitic or symbiontic species and insect vectors (170,171). This hypothesis needs to be analysed in greater detail for further conclusions.

4.3. *Tg*PSD1pv – a common dense granule protein?

The *Tg*PSD1pv protein of *T. gondii* is an exceptional PSD enzyme, displaying certain protein features not shared with other PSDs. It belongs to a novel subgroup of coccidian-specific PSD enzymes [4.2.]. This work revealed that the protein is a functional, partially soluble enzyme, which is secreted by intracellular tachyzoites [3.5.1.]. The secretion of *Tg*PSD1pv was shown to be temperature- and ATP-dependent but remains unimpaired upon Ca^{2+}-depletion with BAPTA-AM (103). Immunofluorescence analysis of C-terminally epitope tagged *Tg*PSD1pv revealed that the protein is secreted into the parasitophorous vacuole via secretory dense granule organelles [Fig. 14].

Whereas the protein is divergent from other PSD enzymes in terms of protein structure and enzymatic properties, it shares many similarities with other dense granule proteins (GRAs) of *T. gondii*, which may provide insights into its biological role. The content of the dense granules is secreted constitutively by subapical exocytosis in a Ca^{2+}-independent manner during as well as after host cell invasion (172,173). Over the last decades an increasing number of canonical GRA-proteins and GRA-like proteins have been described. Their similarities and differences were recently reviewed by Mercier & Cesbron-Delauw (174).

Most of the described GRA-proteins, including *Tg*PSD1pv, harbour a predicted hydrophobic signal peptide at their N-termini, but are in contrast to *Tg*PSD1pv (predicted molecular weight ~ 108 kDa) relatively small proteins of 20 -50 kDa (174). As also observed for *Tg*PSD1pv, GRA-proteins often show aberrant migration behaviour in SDS-PAGE, which is probably due to post-translational modifications, membrane binding domains and a proline rich composition (172,173). Most described GRA-proteins are post-secretory targeted to the PV membrane, the intravacuolar network (IVN) or to the vacuolar space. Interestingly, some GRAs, such as GRA15, 16 and 24 can also be secondary exported to the host cell nucleus (174) [Fig. 41].

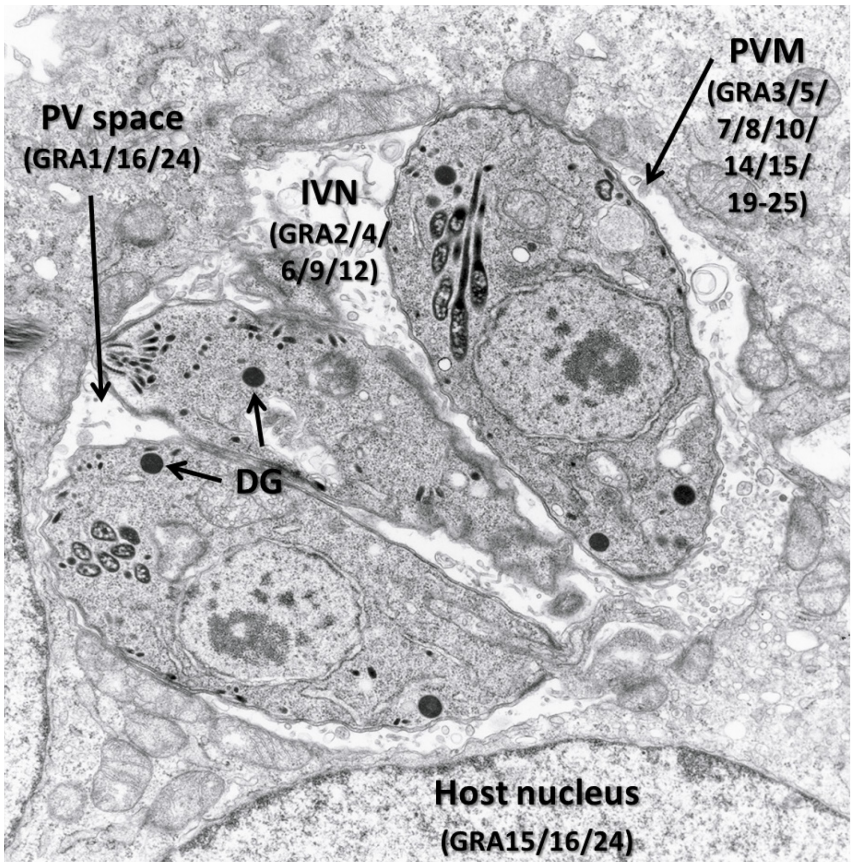

Figure 41:Post-secretory localization of GRA proteins
Transmission electron micrograph of a cell infected with *T. gondii* tachyzoites, and localization of dense granule proteins (GRAs) after their secretion into the parasitophorous vacuole (PV) via dense granules (DG). GRAs are mainly shown to localize to the intravacuolar network (IVN), the PV membrane (PVM) or the PV space, but can also be secondary exported to the host cell nucleus. [*Modified from Mercier and Cesbon-Delauw* (174), *TEM picture from Coppens and Joiner* (175)]

With the exception of GRA1, described as a soluble protein, all GRAs were shown to adopt an intriguing dual behavior, being partly soluble and partly membrane-associated (174), which is also common with *Tg*PSD1pv [Fig. 24]. Whether *Tg*PSD1pv becomes associated with IVN or PV membranes could not be determined due to futile immune-gold labelling of *Tg*PSD1pv-HA in intracellular parasites. However, correlative microscopy indicated that the protein shows a differential localization to *Tg*GRA5, which is known to bind to the PVM (108). In direct comparison to *Tg*GRA5, *Tg*PSD1pv displays a more uniform distribution in the PV-space [Fig. 16].

The biological function of most GRA proteins remains unknown. GRA15, GRA24 and 25 are shown to act as virulence factors by manipulating the host immune system (176-178). Another set of GRA-proteins, GRA2, 6 and 7, are involved in formation, remodelling or stabilization of membranous structures, such as the IVN or host-organelle-sequestering structures (44,179).

Crosslinking-experiments could reveal whether *Tg*PSD1pv interacts with other GRA-proteins, since GRAs proteins usually form high molecular weight complexes by protein-protein interactions (180) and rely on the expression of partner proteins, like GRA2 and GRA6. Here GRA2 was shown to induce IVN-tubulation whilst GRA6 stabilizes the pre-formed tubules (44). A potential role of *Tg*PSD1pv in the remodelling or stabilization of intravacuolar or surrounding PV membranes and proteins is also plausible, because PtdEtn is known to modulate bilayer behaviour. This hypothesis could be addressed by performing electron microscopic imaging with special attention to the morphology of the intravacuolar membranes using the mutant strains generated during this work [Fig. 36].

Like *Tg*PSD1pv [3.11.], most GRA-proteins seem to be non-essential for *in vitro* tachyzoite growth under standard culture conditions (173), but display reduced growth under nutrient starvation (181,182). The *T*gPSD1pv mutant as well as the parental strain showed reduced plaque formation in lipid-depleted medium [Fig. 38]. These experiments should be repeated in medium supplemented with dialysed serum to discern whether the lack of certain nutrients leads to a specific growth reduction or -enhancement of the mutant. The observation that the *Tg*PSD1pv-mutant shows reduced plaque numbers should also be further analysed to reveal if *Tg*PSD1pv could be possibly involved in parasite invasion.

Deletion of GRA2 makes the mutant strain less virulent in mice, disregarding its normal growth behaviour in *in vitro* fibroblast cultures (183). Albeit the secreted *Tg*PSD1pv seems to be not essential for *in vitro* growth [3.11.], this work indicated a reduced virulence of the *Tg*PSD1pv mutant [Fig. 38]. The reduced infectivity observed *in vitro* could also be further tested *in vivo* in mice.

A recent report showed the involvement of GRA17 and GRA23 in passive transport of small molecules through the PV membrane (184). Another indication for a possible role of *Tg*PSD1pv in influencing the PVM permeability by accumulating non-bilayer forming PtdEtn, probably interacting with other GRA proteins, may also enable nutrient/lipid uptake from the host.

Almost all GRAs expressed in *T. gondii* tachyzoites are also found in the bradyzoite stage (185). Based on the expression data extracted from ToxoDB, *Tg*PSD1pv is slightly lower expressed in type II strains (Pru, ME49) compared to non-cyst forming type I strains (RH, GT1), but is expressed also in bradyzoites and oocysts at similar levels as found in the tachyzoite stage. Furthermore, Fox and collegues revealed that GRA4 and GRA6 are important for cyst wall formation (63). Our multiple attempts to disrupt the *Tg*PSD1pv gene in a type II (Pru) background were futile, which could suggest an essential role of *Tg*PSD1pv in bradyzoite/cyst development.

4.4. Other putative functions of *Tg*PSD1pv

Regardless of its allocation as a GRA-protein [4.3.], other possible functions of the *Coccidia*-specific *Tg*PSD1pv can be hypothesized based on its enzymatic features, the preliminary knockout phenotype [3.11.], as well as consideration of potentially related proteins, which are known to interact with PtdEtn in other organisms.

A possible function of *T*gPSD1pv could be to promote the parasite invasion process by introducing large amounts of the non-bilayer forming PtdEtn at the site of cell entry at the host cell surface. In line with that, *Tg*PSD1pv shows perferred catalysis with liposomal substrate and is able to decarboxylate PtdSer at membrane surfaces, as shown by reduced annexin V-binding on apoptotic T-cells when co-incubated whith a *Tg*PSD1pv-overexpression strain secreting significant amounts of active PSD [3.5.]. Additionally, the *Δtgpsd1pv* mutant showed a decreased plaque formation [Fig. 38]. Whether this observation is indeed related to its physiological function *in vivo* requires further investigation.

The vacuolar PtdEtn may also be involved in determing the parasite virulence by influencing the host autophagy system, which is in general responsible for lysosomal degradation of intracellular components (186). Here, PtdEtn functions as an anchor for the autophagy-related protein Atg8 in yeast (187) and its respective mammalian orthologue LC3 (188). It has been reported, that the host autophagy proteins are also required to target IFN-γ-regulated effectors (immunity-related GTPases) to the PVM of *T. gondii* for parasite clearance. An increased PtdEtn content of the PVM through intravacuolar *Tg*PSD1pv could make it more susceptible for LC3- and/or IRG-binding. Furthermore, it was shown that *Tg*Atg3 is essential for the mitochondrial integrity of *T. gondii*, and that *Δtgatg3* parasites fail to conjugate Atg8 to the autophagosomal membrane, indicating the presence of a functional autophagy system in the parasite (105).

In addition, recent studies have shown that a knockout of either PSD1 or PSD2 in yeast and the resulting decrease in cellular PtdEtn promotes autophagy through production of reactive oxygen species, and that the lifespan of the yeast mutants could be extended by providing exogenous Etn or overexpression of *Sc*PSD1 (189). These findings resonate with our results for a sustained and flexible PtdEtn synthesis in *T. gondii* tachyzoites.

PtdEtn was also shown to be involved in additional cellular signalling processes via interaction with a a highly conserved protein family, known as the PtdEtn-binding proteins (PEBPs), which display serine protease inhibitor function and are involved in regulation of several signalling pathways such as the MAP kinase and the NF-κB pathways (190,191). *T. gondii* also encodes a PEBP homologe (TGME49_207930), which may be a component of PtdEtn signalling pathways in *T. gondii*.

4.5. Cooperativity of PtdEtn routes ensures membrane integrity of *T. gondii*

This work revealed that *T. gondii* expresses two functional PSD enzmes in the parasite mitochondrion (*Tg*PSD1mt) and the parasitophorous vacuole (*Tg*PSD1pv) and indicated the expression of a putative *Tg*EPT, catalyzing the last enzyme of the CDP-Etn branch of the *Kennedy pathway*, in the endoplasmatic resticulum of tachyzoites [Fig. 39]. The presence of multiple pathways for the *de novo* synthesis of PtdEtn and the emergence of a novel secreted PSD enzyme suggests the importance of a secure PtdEtn supply and homeostasis for the parasite survival.

PtdEtn is generally enriched in mitochondrial membranes, where it accounts for approximately 25-35% of phospholipids (192,193). Another non-bilayer forming phospholipid, cardiolipin (CL), constituting ~12-25% of mitochondrial lipids (193,194), was shown to have overlapping functions with PtdEtn in stabilizing membrane proteins and conferrering structural organisation of the inner mitochondrial membrane (149,192,195,196). Eukaryotic cells generate CL via a multienzyme cascade in the inner mitochondrial membrane with eventual fusion of CDP-DAG and PtdGro catalyzed by CL synthase (CLS) (192). In contrast, a diphosphatidylglycerol synthase enzyme catalyzes CL formation in prokaryotes (197). There is no experimental evidence for CL synthesis in *T. gondii* and other apicomplexan parasites. But it was shown that *T. brucei* encodes a prokaryotic-type cardiolipin synthase that is essential for mitochondrial function (198). The *T. gondii* genome encodes a protein (TGME49_109940), with high similarities to the *Tb*CLS (Tb927.4.2560), but a relatively low predicted probability of export to mitochondria (~40%), as predicted by MitoProt. Our attempts to localize the respective protein were futile. A compensatory function of CL in maintaining mitochondrial integrity in the absence of *Tg*PSD1mt can not be excluded, however.

Furthermore, metabolic labelling revealed that the *Tg*PSD1mt mutant shows increased utilization of ethanolamine for incorporation into PtdEtn whilst maintaining its cellular PtdEtn levels [Fig. 33]. The mutant strain was able to survive nutrient depletion albeit a ~56% growth defect, which could be partially restored by the addition of exogenous Etn [Fig. 34]. These results indicated that the CDP-Etn pathway can partially compensate for the loss of *Tg*PSD1mt under the given culture conditions and implies an interregulated PtdEtn transport system from ER-derived lipids to the mitochondrion. The highly complex meachnisms of interorganellar lipid transport are still at an early stage of research, but it was shown that phospholipids can be transported between the ER and mitochondria at the so called mitochondria-associated membranes (MAMs) (199-201). MAMs are enriched in certain lipids and lipid-synthetic enzymes (192). It is therefore likely that the parasite also uses this conserved mechanism for interorganellar exchange of PtdEtn.

Extracellular labelling of tachyzoites using [^{3}H]-serine, which is mainly incorporated into PtdSer and PtdEtn (92), revealed only a moderate decline in PSD activity in the *Δtgpsd1mt* mutant [Fig. 33B]. The residual PSD activity is very likely contributed by *Tg*PSD1pv in the dense granules. Nevertheless, it is not yet known how *Tg*PSD1pv is post-translationally transported and maturated, and whether it contributes to the intracellular PtdEtn synthesis or exclusively acts post-secretory in the PV.

The relative contributions of the three distinct PtdEtn *de novo* pathways in *T. gondii* could not be ascertained within this study. Generating a *Δtgpsd1mtΔtgpsd1pv* double mutant could reveal whether PtdSer decarboxylation in general is essential for *T. gondii*, and whether the *Kennedy pathway* can provide sufficient PtdEtn to ensure parasite survival. Likewise, contribution of host-derived lipid would have to be evaluated by generating a triple mutant lacking PSD activity and the CDP-ethanolamine pathway.

To study a possible role of host lipids or lipid intermediates in supporting PtdEtn biogenesis in *T. gondii*, we pre-labeled HFF-cells with [^{14}C]-Etn for 24 hrs prior to infection with tachyzoites of the *Tg*PSD1mt conditional mutant in its *off-state*. After 48 hrs post infection, the *Tg*PSD1mt mutant displayed an increased incorporation of radioactivity into its phospholipid-pool [Fig. 34]. These results imply import of host-derived lipids or lipid intermediates. Former work using radioactive and fluorescently-labled phospholipids already indicated the contribution of host lipids as a source of IVN and parasite lipids (46,91). Labelling of extracellular parasites with NBD-lipids revealed a selective import of PtdEtn and PtdSer into tachyzoites (202) [Fig. 42], which supports the possibility of exteral PtdEtn (or PtdEtn derivates) contributing to the parasite membrane lipid pool.

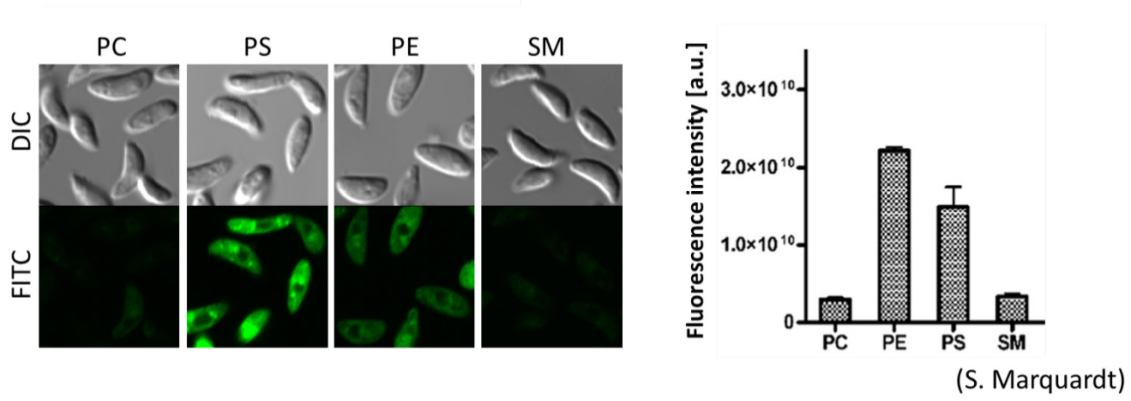

Figure 42: Selective import of NBD-phospholipids by extracellular T. gondii tachyzoites
Labelling of extracellular *T. gondii* tachyzoites with NBD-lipids. NBD-sphingomyelin (SM) served as a negative control. [*from S. Marquardt, diploma thesis* (202)]

Analysis of the parasites labled with NBD-PtdSer revealed that a large fraction of NBD-PtdSer was converted into PtdEtn, which could be inhibited by the use of the PSD inhibitor hydroxylamine [Appendix 7]. These results indicate either external decarboxylation by the secreted *Tg*PSD1pv and

subsequent import of PtdEtn and/or decarboxylation by *Tg*PSD1mt (and *Tg*PSD1pv) after internalization of NBD-PtdSer.

It is known that during *T. gondii* infection, host ER and mitochondrial membranes are recruited to the PVM (47), both of which represent the major sites of lipid biogenesis in the host. These membranes could also serve as contact zones for lipid exchange to the PV. Here, host-derived PtdSer could also be used as a substrate for the vacuolar *Tg*PSD1pv to produce PtdEtn. It was shown that yeast and mammalian cells are capable of transporting (lyso)-phospholipids via P4-type ATPases (203,204), and that the *T. gondii* genome encodes their orthologs (202). Some putative P4-ATPases could be localized in the cytosol and the apical tip of *T. gondii* tachyzoites (205), which rules out their involvement in lipid transport at the parasite surface.

In summary, these results show that *T. gondii* harbours multiple intracellular routes to produce PtdEtn besides a putative mechanism to import host-PtdEtn [Fig. 43].

The remarkable ability of *T. gondii* to perform PtdEtn synthesis via multiple independent routes enables a greater autonomy from the host cell, which may ensure its replication in varying nutritional conditions encountered in different host organisms, tissues and cell types.

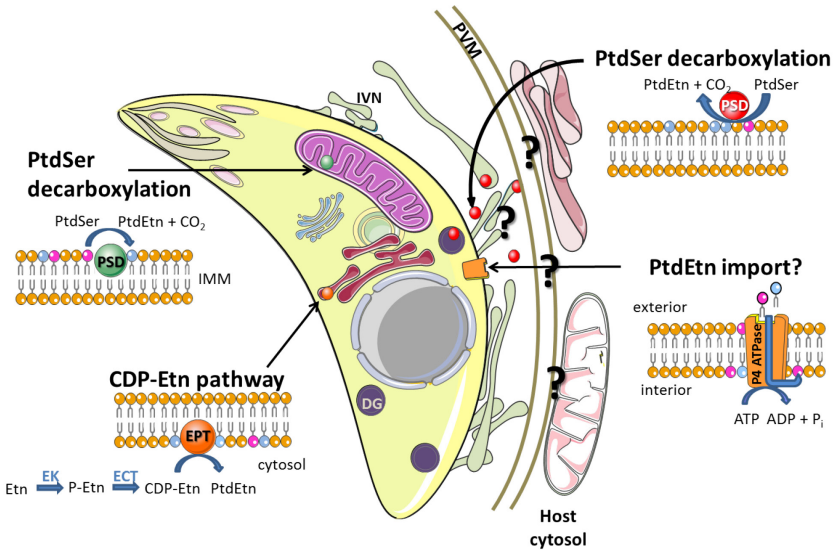

Figure 43: Model of PtdEtn biogenesis in T. gondii

Schematic overview summarizing the PtdEtn routes in *T. gondii* comprising PtdSer decarboxylation in the mitochondrion and the parasitophorous vacuole as well as the CDP-Etn pathway in the ER. Host ER and mitochondria could serve as potential sources of parasite PtdEtn. Additionally, yet undefined lipid-transporters, such as P4-type ATPases, could potentially mediate the import of PtdEtn to the parasite interior.

4.6. Future perspective of PtdEtn research in *T. gondii*

This work established the first model which comprises the major routes for PtdEtn biogenesis in *T. gondii*. Whilst the PSD pathway has been characterized in sufficient detail, the enzymatic identity and importance of the enzymes related to the CDP-ethanolamine pathway remain to be determined.

To reveal the concrete role of the *Coccidia*-specific secreted *Tg*PSD1pv for the parasite biology, further experiments are needed, which involve invasion assays and electron microscopy of the intravacuolar network. *In vitro* tubulation experiments using parasite secretome preparations and/or purified *Tg*PSD1pv could show whether the protein can indeed induce membrane tabulation or curvature. Moreover, the *Tg*PSD1mt and *Tg*PSD1pv mutant strains should be analysed for their lipidome to detect potential minor changes in the membrane lipid composition and to assess substrate-specificity of the PSDs and the CDP-ethanolamine pathway. The generation of PSD double mutants and *Tg*PSD1mt/*Tg*PSD1pv/*Tg*EPT triple mutants could reveal the relative importance of the individual routes.

Analysis of the *Tg*PSD mutants together with other strains deficient in phospholipid metabolism related enzymes could elucidate the interregulation of phospholipid pathways in *T. gondii*. Functional characterization of all putative *T. gondii* P4-type ATPases and other yet undetected lipidtransporters could establish a putative role of host phospholipids for *T. gondii* membrane biogenesis.

An epitope-tagged *Tg*PSD1pv enzyme should be expressed in *T. gondii* type II parasites to follow its expression and subcellular localization in bradyzoites to assess its putative role in cyst formation. Ideally, a genetic knockout in type II parasites should be performed to determine a possible role of *Tg*PSD1pv in bradyzoite development.

The identification of putative interaction-partners of *Tg*PSD1pv, such as GRA- or Atg-proteins, via crosslinking experiments could help to reveal its putative biological function. In addition, immuno-gold labelling of the *Tg*PSD1pv-HA strain should demonstrate whether the protein binds post-secretory to the IVN in the PV space and/or the PVM membrane in infected cells.

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IX. Appendix

Appendix 1

(A) Table of previous and new gene accession numbers of the phosphatidylethanolamine synthesis enzymes in *T. gondii:*

Abbreviations used in this study	ToxoDB accession number	ToxoDB old accession number	NCBI accession number
***Tg*PSD1mt**	TGGT1_225550	TGGT1_080780	EPR62136
***Tg*PSD1pv**	TGGT1_269920	TGGT1_108450	EPR64713
***Tg*EK**	TGGT1_306540	TGGT1_040800	EPR57537
***Tg*ECT**	TGGT1_310280	TGGT1_086510	EPR60847
***Tg*EPT1**	TGGT1_261760	TGGT1_008370	EPR62697
***Tg*EPT2**	TGGT1_257510	TGGT1_013180	EPR63017

(B) List of coding sequences used for *in silico* analysis and primer design:

>TgPSD1mt
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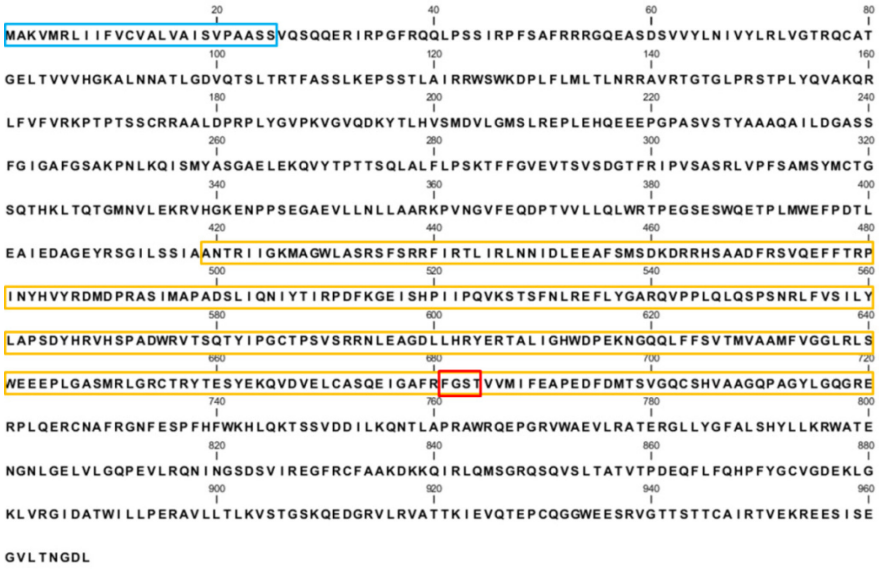
Appendix 2

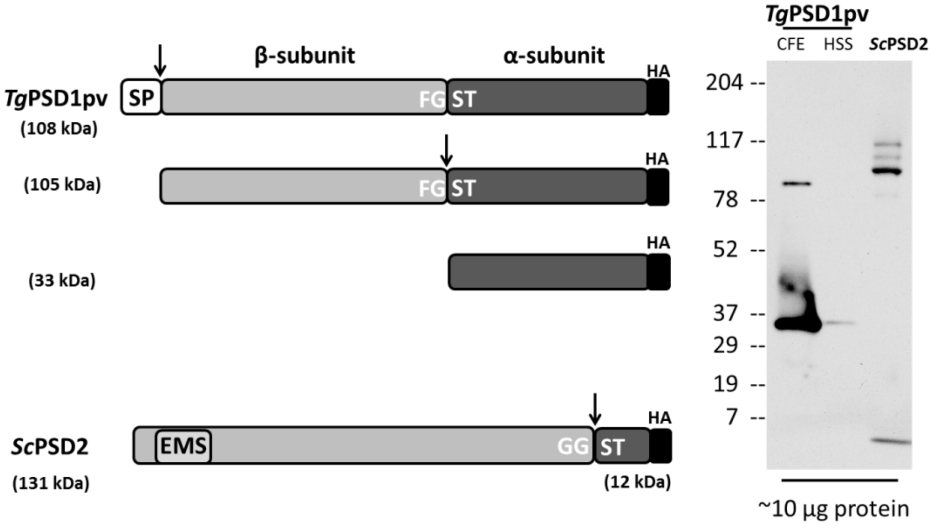
(A) Protein sequence of *Tg*PSD1pv with predicted signal peptide highlighted in *blue*, PSD domain (PF02666) in *orange* and proteolytic cleavage motif in *red* colour:



(B) Protein alignment of coccidian PSD orthologs from *T. gondii* (TgPSD1pv), *Hammondia hammondi* (HhPSDb) and *Eimeria falciformis* (EfPSD2b). Areas of low protein conservation are shown in blue, whereas conserved aminoacids appear in lighter colour.

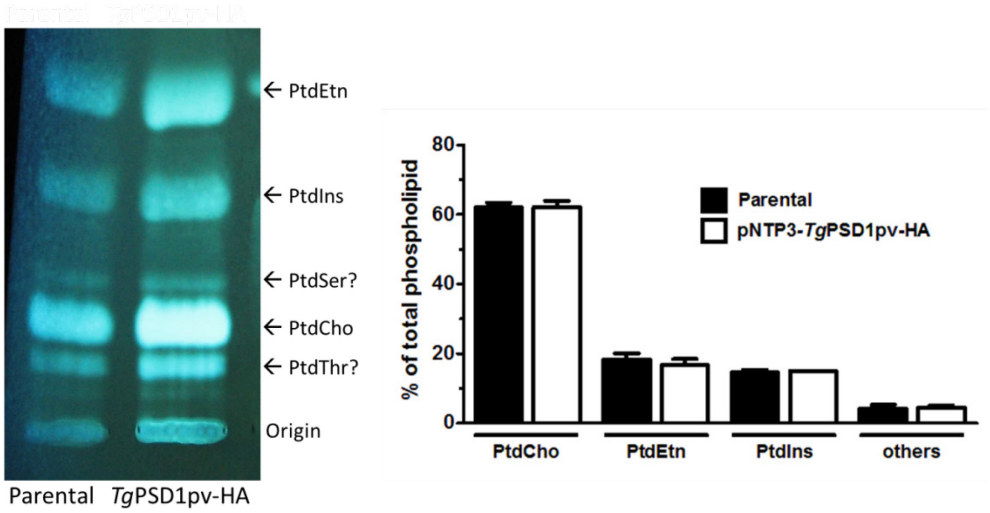
Appendix 3

Processing of *Tg*PSD1pv compared to yeast *Sc*PSD2 as shown by C-terminal HA-tagging and Western blot using anti-HA antibody. PSD enzymes are known to be processed into α- and β- subunits at a conserved XGST cleavage-motif. SP = Signal peptide, EMS = Endomembrane sorting sequence



Appendix 4

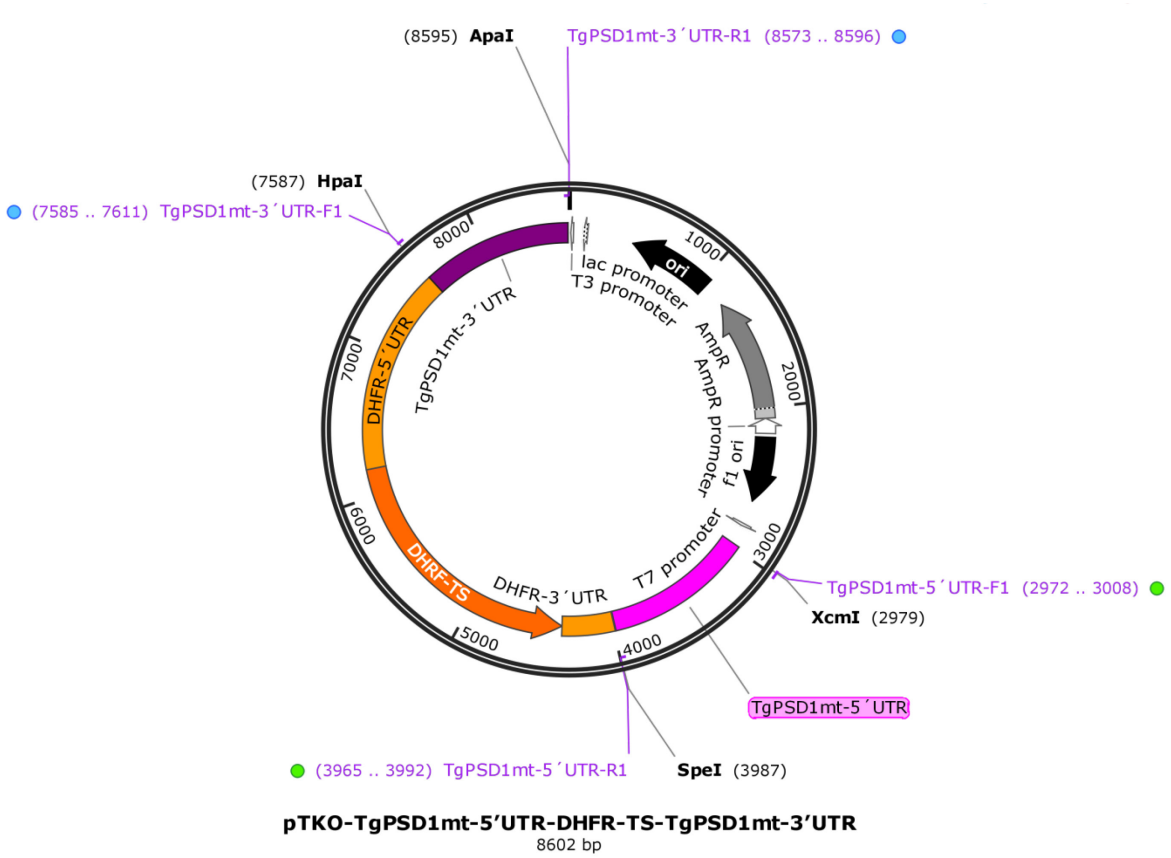
Analysis of the phospholipid composition of the *Tg*PSD1pv overexpression strain via 1D-TLC, and lipid phosphorous assay.



Appendix 5

(A) *pTKO-TgPSD1mt-5'UTR-DHFR-TS-TgPSD1mt-3'UTR*

Plasmid used to generate the *Tg*PSD1mt conditional knockout in the *Δku80-TaTi* strain:



pTKO-TgPSD1mt-5'UTR-DHFR-TS-TgPSD1mt-3'UTR
8602 bp

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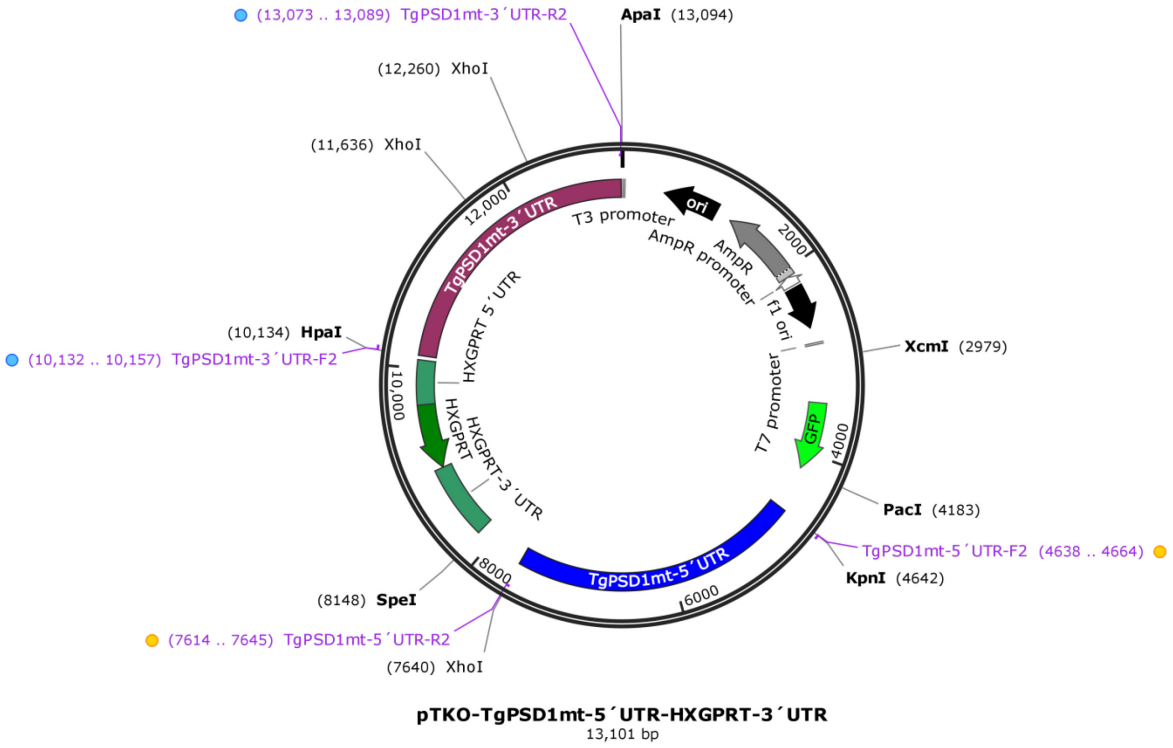
5´UTR fragment of *Tg*PSD1mt
3´UTR-fragment of *Tg*PSD1mt
DHFR-TS resistance cassette coding sequence underlined
*Tg*PSD1mt-5´UTR-KO-F1/R1 primer (Restriction sites underlined *Xcm*I/*Spe*I)
*Tg*PSD1mt-3´UTR-KO-F1/R1 primer (Restriction sites underlined *Hpa*I/*Apa*I)

[The vector was cloned by Maria Hellmund during her Bachelor´s thesis (152)]

(B) *pTKO-TgPSD1mt-5´UTR-HXGPRT-3´UTR*

Plasmid used to generate the *Tg*PSD1mt direct knockout in the *Δku80-hxgprt*$^{-}$ strain:



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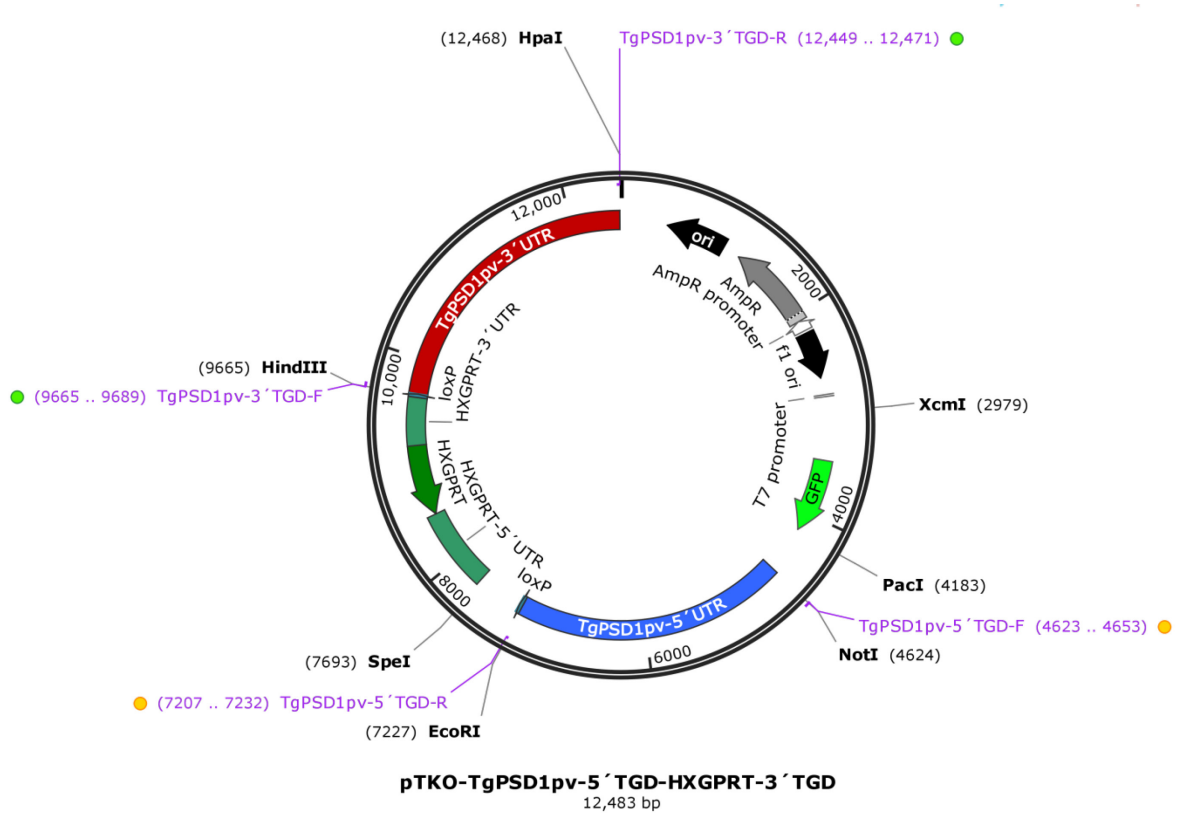
GFP sequence
5´UTR fragment of *Tg*PSD1mt
3´UTR-fragment of *Tg*PSD1mt
*Tg*PSD1mt-5UTR-KO-F2/R2 primer (Restriction sites underlined *Kpn*I/*Xho*I)
*Tg*PSD1mt-3UTR-KO-F2/R2 primer (Restriction sites underlined *Hpa*I/*Apa*I)
HXGPRT-resistance cassette – HXGPRT coding sequence underlined

Appendix 6

pTKO-TgPSD1pv-5´TGD-HXGPRT-3´TGD plasmid used for *Tg*PSD1pv targeted gene disruption in the *RH hxgprt*⁻ strain:



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12,483 bp

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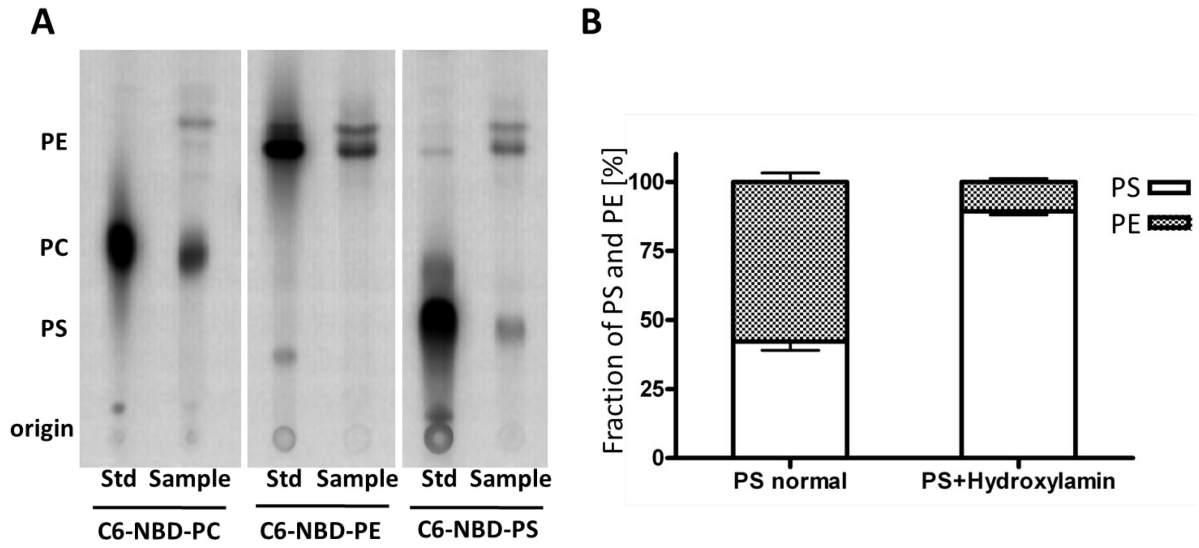
GGGCCCCGTACC

GFP sequence
5´TGD fragment of *Tg*PSD1pv (Introns in lower case)
3´TGD-fragment of *Tg*PSD1pv (Introns shown in lower case)
*Tg*PSD1pv-5´TGD-F1/R2 primer (Restriction sites underlined *Not*I/*Eco*RI)
*Tg*PSD1pv-3´TGD-F1/R1 primer (Restriction sites underlined *Hind*III/*Hpa*I)
HXGPRT-resistance cassette – HXGPRT coding sequence underlined

Appendix 7

(A) Lipid analysis of *T. gondii* tachyzoites after extracellular labelling with NBD-phospholipids by one-dimensional thin layer chromatography. **(B)** Influence of the PSD inhibitor hydroxylamine on NBD-phospholipid turnover.

[S. Marquardt, (202)]



X. List of publications and presentations

The following publications and presentations resulted from this work:

List of publications

Sampels V, **Hartmann A**, Dietrich I, Coppens I, Sheiner L, Striepen B, Herrmann A, Lucius R, Gupta N. "*Conditional mutagenesis of a novel choline kinase demonstrates plasticity of phosphatidylcholine biogenesis and gene expression in Toxoplasma gondii*". J Biol Chem. 2012 May 11;287(20):16289-99.

Gupta N, **Hartmann A,** Lucius R, Voelker DR. "*The obligate intracellular parasite Toxoplasma gondii secretes a soluble phosphatidylserine decarboxylase*." J Biol Chem. 2012 Jun 29;287(27):22938-47

Hartmann A, Arroyo-Olarte RD, Imkeller K, Hegemann P, Lucius R, Gupta N. "*Optogenetic modulation of an adenylate cyclase in Toxoplasma gondii demonstrates a requirement of the parasite cAMP for host-cell invasion and stage differentiation*." J Biol Chem. 2013 May 10;288(19):13705-17.

Hartmann A, Hellmund M, Lucius R, Voelker DR, Gupta N. "*Phosphatidylethanolamine synthesis in the parasite mitochondrion is required for efficient growth but dispensable for survival of Toxoplasma gondii.*" J Biol Chem. 2014 Mar 7;289(10):6809-24.

Presentations at international conferences

Jahrestagung der Deutschen Gesellschaft für Parasitologie, Heidelberg, Germany, 2011: "*Phosphatidylethanolamine Biogenesis in Toxoplasma gondii and its Parasitophorous Vacuole*"

Gordon Research Conference: Biology of Host-Parasite Interaction, Newport, USA, 2012: "*Phosphatidylethanolamine Biosynthesis versus Uptake of exogenous Lipid by Toxoplasma gondii*"

12th International Congress on Toxoplasmosis, Oxford, United Kingdom, 2013:
"*Mitochondrial phosphatidylethanolamine biogenesis is required for an efficient replication of Toxoplasma gondii*"

13th International Congress of Parasitology, Mexico City, Mexico, 2014:
"*Multiple routes of phosphatidylethanolamine biogenesis ensure the membrane integrity in Toxoplasma gondii*"

XI. Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel

"Multiple routes of phosphatidylethanolamine biogenesis
ensure membrane integrity of *Toxoplasma gondii*"

selbständig und nur mit den angegebenen Hilfsmitteln verfasst habe.

Die Abschlussarbeit wurde in gleicher oder ähnlicher Form bisher bei keiner anderen Institution eingereicht.

Berlin, den 15.09.2015 _____