Phosphatidylinositol synthesis, its selective salvage, and inter-regulation of anionic phospholipids in *Toxoplasma gondii*

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Phosphatidylinositol (PtdIns) serves as an integral component of eukaryotic membranes; however, its biosynthesis in apicomplexan parasites remains poorly understood. Here we show that *Toxoplasma gondii*—a common intracellular pathogen of humans and animals—can import and co-utilize myo-inositol with the endogenous CDP-diacylglycerol to synthesize PtdIns. Equally, the parasite harbors a functional PtdIns synthase (PIS) containing a catalytically-vital CDP-diacylglycerol phosphotransferase motif in the Golgi apparatus. Auxin-induced depletion of PIS abrogated the lytic cycle of *T. gondii* in human cells due to defects in cell division, gliding motility, invasion, and egress. Isotope labeling of the PIS mutant in conjunction with lipidomics demonstrated de novo synthesis of specific PtdIns species, while revealing the salvage of other lipid species from the host cell. Not least, the mutant showed decline in phosphatidylthreonine, and elevation of selected phosphatidylserine and phosphatidylglycerol species, indicating a rerouting of CDP-diacylglycerol and homeostatic inter-regulation of anionic phospholipids upon knockdown of PIS. In conclusion, strategic allocation of own and host-derived PtdIns species to gratify its metabolic demand features as a notable adaptive trait of *T. gondii*. Conceivably, the dependence of *T. gondii* on de novo lipid synthesis and scavenging can be exploited to develop new anti-infectives.

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A sexual reproduction of apicomplexan parasites in respective host cells involves successive rounds of lytic cycle leading to acute infection. A corresponding biogenesis of organellar membranes is imperative to sustain intracellular proliferation of these parasites. Previous research on Toxoplasma, Plasmodium, and Eimeria has revealed the occurrence of lipid biosynthesis networks in these parasites, occasionally involving a balance of endogenous synthesis and salvage of lipids from sheltering host cells\(^{1-3}\). Our own work focusing on phospholipids has demonstrated the expression and synthesis of generic, as well as exclusive lipids in \(T. gondii\) and \(E. falciformis\)^{3-9}. In context of this report, we showed that the acute stage of \(T. gondii\) can synthesize several major classes of phospholipids, namely phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylthreonine (PtdThr), and phosphatidylycerine (PtdSer). Among these, PtdThr is an exclusive ethanolamine (PtdEtn), phosphatidylthreonine (PtdThr), and phospholipid species, namely phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), and CDP-DAG.

The diagnosis\(^{12}\) of PtdIns-derived second messengers (phosphoinositides) has been performed in apicoplast homeostasis (\(T. gondii\))^{16,11}. PtdThr, PtdCho, and PtdSer are made in the endoplasmic reticulum (ER), whereas PtdEtn is produced at multiple locations including the ER, mitochondrion, and parasitophorous vacuole (PV)^{3-9}\). PtdIns is yet another primary class/family of membrane phospholipids in \(T. gondii\); however, its biosynthesis and importance in the parasite have not yet been examined.

In parasitic protists, PtdIns itself and its derivative lipids are known to facilitate a repertoire of functions. Especially, metabolism of PtdIns-derived second messengers (phosphoinositides) has drawn notable attention due to their crucial roles in parasite pathogenesis and potential as the target for drugs, vaccines or diagnosis\(^{12-20}\). They are considered essential cellular mediators involved in apicoplast homeostasis (Toxoplasma)^{21,22}\), protein export (Plasmodium)^{23,24}\), motility and egress (Plasmodium)^{25,26}\), endocytosis (Trypanosoma)^{27,28}\), autophagy (Plasmodium, Trypanosoma)^{29,30}\), and gametogenesis (Plasmodium)^{31}\). Another vital class of metabolites is glycosylphosphatidylinositol (GPI), which serves as a membrane anchor to glycoproteins. Several GPI-anchored surface proteins have been implicated in modulation of the host’s immune response\(^{32-35}\) and parasite survival\(^{36,37}\). A multifarious requirement of PtdIns species and metabolites derived thereof has necessitated understanding the mechanism and regulation of PtdIns synthesis in protozoan parasites.

Synthesis of PtdIns is catalyzed by PtdIns synthase (PIS), which co-utilizes CDP-diacylglycerol (CDP-DAG) and myo-inositol as precursors\(^{38}\). Thus far only one protozoan PIS, isolated from the kinetoplastid parasite, \(T. brucei\), has been studied in substantial details. \(TbPIS\) localizes in the ER and Golgi network of the blood stages of \(T. brucei\)^{39}\), generating two distinct PtdIns pools. In the ER, de novo-synthesized myo-inositol is utilized to produce PtdIns, which subsequently supports GPI synthesis\(^{40,41}\). On the other hand, PtdIns production in the Golgi complex employs exogenous myo-inositol, and this lipid pool is used to synthesize inositol phosphorylceramides\(^{42,43}\). Among intracellular parasites, the presence of PIS enzymes has been reported in \(Plasmodium\), Toxoplasma, and Eimeria\(^{3,44-46}\), although their contribution to the making of different PtdIns species and their physiological relevance have not been investigated. In addition, it is unclear how these parasites meet a balance between the synthesis of PtdIns and its import from host cells. In this work, we characterized PtdIns synthase of \(T. gondii\) and demonstrated a vital role of de novo PtdIns synthesis along with the salvage of designated host-derived lipid species by the parasite.

**Results**

\(T. gondii\) harbors a functional PtdIns synthase in the Golgi complex. We next focused on establishing the genetic basis of PtdIns synthesis in \(T. gondii\). Previous work has reported the occurrence of a “tachyzoite-specific” isoform that can functionally complement a PIS mutant of \(S. cerevisiae\)^{46}. This protein (ToxoDB, TGGT1_007710; NCBI GenBank, KX017549) comprises 258 residues with four transmembrane helices and a CDP-alcohol phosphotransferase domain with DXDGDYXARX,GX,D motif (Fig. 2a). Besides several residues conserved in CDP-alcohol phosphotransferase-type enzymes (Supplementary Table 1), we identified glutamine (Q103) and arginine (R117), present only in the PIS sequences (Supplementary Figure 1a, Supplementary Table 1). \(TgPIS-6xHis\) was expressed in \(E. coli\), which lacks the PtdIns synthase activity, and thus well suited for functional analysis of the PIS proteins. Successful IPTG-inducible expression of the full-length recombinant protein was confirmed by Western blot analysis (28-KDa band, Fig. 2b).

The catalytic activity was assessed by thin-layer chromatography in conjunction with the quantification of phosphorus in TLC-resolved lipid bands (Fig. 2c–d). As expected, the \(E. coli\) strain harboring the empty vector (negative control, N.C.) did not show any PtdIns synthesis, whereas the expression of \(TgPIS-6xHis\) produced PtdIns in a myo-inositol-dependent manner. The site-mutations or deletion of most conserved residues abolished the
catalysis except for Q103G mutation, which could still produce PtdIns, albeit with a notably reduced efficiency (Supplementary Figure 1a–c), which endorsed the functional importance of the predicted signature residues.

In our preceding work, we have reported localization of ectopically overexpressed TgPIS in the Golgi network. Herein, we expressed TgPIS-HA under the control of its endogenous promoter and found it exclusively in the Golgi body, as determined by co-localization with a known organelle marker TgERD247 (Fig. 2e). In further work, we tested the relevance of a prolonged N-terminal extension in TgPIS (Fig. 2a) by ectopically expressing a mutant lacking the specified extension (TgPIS258-HA). A single copy of the PIS expression cassette directed by the pTETO7SAG1 regulatory elements was inserted at the uracil phosphoribosyltransferase (UPRT) locus by double homologous recombination (Fig. 2f). TgPIS258-HA mutant localized in the Golgi complex, as judged by its co-staining with TgERD2-Ty1. We also performed expression of TgPIS258-HA in E. coli (23-kDa band, Fig. 2b), but observed only a minor reduction in PtdIns synthesis by the truncated mutant (Fig. 2c–d). Based on all these results, we conclude that TgPIS encodes a functionally-active enzyme, which localizes in the parasite’s Golgi apparatus, and its extended N-terminus is dispensable for the subcellular localization and catalytic activity.

PtdIns synthase is essential for the lytic cycle of T. gondii. We attempted to delete the TgPIS gene by double homologous recombination in tachyzoites of T. gondii. However, the locus was refractory to deletion, suggesting a vital requisite of this enzyme during the lytic cycle. To confirm our premise, we implemented the Cre-mediated gene-swap strategy48. In this regard, the TgPIS locus was first replaced by a cassette comprising the loxP-flanked (floxed) open reading frame of TgPIS-HA, followed by yellow fluorescence protein (YFP), TgDHFR-TS-3’UTR, and TgHXGPRT selection cassette (Fig. 3a). The method enabled us to get a viable strain (TgPIS-HAfloxed), as confirmed by genomic PCR of clonal transgenic parasites (Fig. 3b). Transfection of the TgPIS-HAfloxed strain with the pSAG1-Cre construct expressing Cre recombinase caused the excision of floxed TgPIS-HA, and repositioned YFP in proximity of the TgPIS promoter that resulted in fluorescing Δtgpis-HAexcised mutant (Fig. 3c). The mutant could not be drug-selected because it was not feasible to...
generate a clonal knockout strain. Nevertheless, it allowed us to investigate the effect of genetic ablation on the parasite growth by scoring the progression of the YFP-positive Δtgpis-HAExcised strain up to 10 days of transfection (Fig. 3d–e). Indeed, parasites without TgPIS-HA signal but expressing YFP had much smaller vacuoles, and vice versa, indicating a replication defect. The YFP-positive vacuoles disappeared from mixed cultures within 7–10 days (Fig. 3e), disclosing an indispensable role of TgPIS during the lytic cycle.

Auxin-induced degradation of TgPIS function is detrimental to tachyzoites. A viable TgPIS deletion mutant could not be generated, hence we implemented the conditional mutagenesis by tagging the enzyme with an auxin-inducible degradation (AID) domain for translational control of the protein stability49. In this regard, a CRISPR/Cas9 construct expressing a single guide RNA targeted to the 3′UTR of the TgPIS gene (pU6-Cas9-TgPISsgRNA) was co-transfected with a PCR-amplicon containing the 5′- and 3′-homology arms, minimal AID (mAID) motif, 3xHA tag, GRA1–3′UTR and HXGPRT selection marker to permit crossover-mediated epitope-tagging of the native locus by positive selection (Fig. 4a). Successful mAID-3xHA-tagging of TgPIS was confirmed by recombination-specific PCR (Fig. 4b), its subcellular localization in the Golgi apparatus (Fig. 4c), and immunoblot analysis revealing the expected 35-kDa band (Fig. 4d). As reckoned, the HA signal was not detectable after auxin exposure (Fig. 4c–d).

The AID method resulted in a strain that allowed us to undertake biochemical and phenotypic analyses. [13C]-myo-inositol labeling
of the auxin-treated extracellular tachyzoites caused a significant reduction in the synthesis of most detectable PtdIns species, confirming a functional ablation of PIS and a role of enzyme in de novo synthesis of several lipid species (Fig. 4e). Although a depletion of enzyme activity was evident within 6 h of auxin treatment, it was more pronounced (nearly complete) after 96 h. As anticipated, none other [13C]-labeled phospholipid was detected in the mutant irrespective of the auxin exposure. The content of major phospholipids except for PtdIns, PtdGro, and PtdThr were unaltered (Supplementary Figure 2, also see below).

In plaque assays, we observed a severely impaired parasite growth following the hormone-mediated depletion of PIS (Fig. 4f–g). The plaque size was reduced to less than 10% when TgPIS-mAID-3xHA mutant was treated with auxin. Not least, the parasite ceased to grow after 3–4 passages in routine cultures, once more revealing a critical role of PtdIns synthesis for the lytic cycle.

Auxin-mediated depletion of PIS impairs all major events during the lytic cycle. The auxin-regulated TgPIS-mAID-3xHA mutant also enabled phenotyping of the lytic cycle events such as, replication, egress, invasion, and gliding motility (Fig. 5, Supplementary Figure 3). Assuming a standard role of PtdIns in membrane biogenesis, we first set up immunofluorescent assays to monitor the formation of daughter cells (endodyogeny) by staining with the anti-IMC3 antibody before and after auxin exposure (Fig. 5a). Compared to the parental strain, a minor impairment in the cell division of the PIS mutant was observed even under the control condition (Fig. 5b) that can be attributed to the mAID-3xHA tagging and GRA1–3′UTR. More importantly, unlike the parental strain, the proportion of budding cells declined gradually in the PIS mutant upon prolonged exposure to the hormone (24–120 h). In accord with these and aforesaid findings using the Δtgpis-HAExcised mutant (Fig. 3c–d), we...
recorded a significant defect in the intracellular replication of the auxin-treated TgPIS-mAID-3xHA strain (Fig. 5c). Yet again, as shown above, a modest decline in the proliferation of the mutant was apparent in the absence of auxin.

TgPIS-mAID-3xHA mutant also exhibited egress defect in normal cultures (-IAA), which was radically accentuated upon inclusion of auxin (Supplementary Figure 3a). To test whether curtailed egress may be caused by slow replication of the mutant, we examined induced egress in response to activation of cGMP signaling by zaprinast, a phosphodiesterase inhibitor51,52. Indeed, we recorded a marked recovery in egress of the auxin-treated mutant but the impairment was still evident (Supplementary Figure 3b). Likewise, we noted a reduction in invasion efficiency of the mutant in auxin-supplemented cultures, albeit only after 96 h (Supplementary Figure 3c). Last but not least, our appraisal of the parasite motility demonstrated a modest but significant reduction in the motile fraction and trail length of the PIS mutant after auxin exposure (Supplementary Figure 3d–e). Collectively, our results show a multifaceted impact of PtdIns synthase depletion on the lytic cycle of tachyzoites.

Knockdown of PtdIns synthase disrupts homeostasis of major anionic lipids. To gain insight into observed phenotypes, auxin-regulated conditional mutant of TgPIS was subjected to lipidomic analysis. In this regard, we first performed the parasite yield assay, as reported earlier53, to determine a suitable time point for the sample collection (Supplementary Figure 4a). Parasitized cultures of the mutant and parental strains with a defined multiplicity of infection were incubated in the absence or presence of auxin and the parasite yield was calculated after consecutive passages. As expected, the growth rate of the parental strain remained
unaltered irrespective of hormone treatment. In contrast, the yield of auxin-exposed mutant cultures declined gradually during two serial passages. In other assays, we confirmed the viability and infectivity of these parasites, both of which were normal after 96 h treatment when compared to untreated samples. Besides, we immunostained major organelles, namely plasma membrane, IMC, apicoplast, mitochondrion, and ER (Supplementary Figure 4b). No apparent difference was observed in hormone-treated vs. untreated control samples for any of these organelles. All indicated marker proteins including the GPI-anchored SAG1 and SAG2 were targeted and expressed with no marked anomaly.

In accord with other phenotyping assays, we collected lipidomic samples after 96 h of auxin treatment. Lipid analyses of the parental and mutant strains revealed six phospholipid classes, namely PtdCho, PtdEtn, PtdSer, PtdThr, PtdIns, and PtdGro, and two sphingolipids (sphingomyelin and ethanolamine phosphorylceramide), confirming our earlier reports.\(^3\)\(^4\) (Supplementary Figure 4c). Even though the amount of all major lipid classes remained statistically unaltered, we observed a perturbation in the levels of PtdIns, PtdSer, and PtdThr. Given a strong phenotype in the \(Tg\)PIS-mAID-3xHA mutant, we quantified the most abundant species of each lipid class in untreated and auxin-treated samples (Fig. 6, Supplementary Figure 5). As expected, the content of none of the shown lipid species was affected in the parental strain. In contrary, the mutant showed a clear shift in the specific species of PtdIns, PtdSer, and PtdThr after auxin exposure. Especially, PtdIns species with short-to-medium chains (C30, C32, C34) were highly reduced, whereas PtdIns C38 was increased, though not statistically significant. On the other hand, designated species of PtdThr (C36, C38) were significantly declined, while of PtdSer (C34, C36) were increased. Species of PtdCho, PtdEtn, and PtdGro did not change upon auxin treatment in any of the two strains (Supplementary Figure 5). The data, therefore, disclosed a selective modulation of major anionic phospholipid species following knockdown of PIS.

Changes in anionic lipids reflect perturbed membrane stability in the PIS mutant. To consolidate our results, we plotted the magnitude of change in all detectable species from all shown phospholipids (irrespective of abundance) in volcano plots, which illustrate the fold-change vs. statistical significance in response to auxin treatment (Fig. 7a–b). The majority of lipid species in the parental strain had no perturbation except for some ethanolamine phosphorylceramide and sphingomyelin species that were affected ≥1.5-fold (\(p\) value ≤0.05) (Fig. 7a). The \(Tg\)PIS-mAID-3xHA mutant by contrast exhibited significant modulation of several

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**Fig. 5 Depletion of \(Tg\)PIS impairs the cell division in tachyzoites. a** Immunostaining of the budding daughter cells (endodyogeny) in the \(Tg\)PIS-mAID-3xHA strain precultured without or with auxin. Intracellularly-developing tachyzoites were treated with 500 \(\mu\)M IAA or with 0.1% ethanol (-IAA), as schematized. Samples were stained with \(\alpha\)-HA (green) to monitor the downregulation of \(Tg\)PIS, and with \(\alpha\)-TgIMC3 (red) to visualize the endogeny. Shown are the representative images of \(Tg\)PIS-mAID-3xHA mutant. b Quantification of tachyzoites harboring the progeny. A total of 400-500 vacuoles with IMC3-positive daughter cells were scored for each condition (\(n=3\) assays). c Proliferation rate of the \(Tg\)PIS-mAID-3xHA mutant with respect to its parental strain. Parasites were precultured with 500 \(\mu\)M IAA (+IAA) or 0.1% ethanol (-IAA) for 48 h and subjected to the replication assay (40 h infection). Graphs show the mean percentage of vacuoles containing specified number of parasites. A total of 400-500 vacuoles for each condition were scored (\(n=3\) assays, mean ± S.E; *\(p\) ≤0.05; **\(p\) ≤0.01; ***\(p\) ≤0.001).

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species of PtdIns, PtdSer, and PtdThr above the threshold (Fig. 7b). As depicted in heatmaps (Fig. 7c), we noted evident decline in species of PtdIns and PtdThr, while the level of most PtdSer species was induced. These results encouraged us to deduce the effect of PIS mutation on the physicochemical features of the membrane lipids. In this regard, we generated violin plots based on the calculated equivalent carbon numbers (ECN) for indicated lipid classes (Supplementary Figure 6). The ECN is normally used in reversed-phase chromatography of lipids and proportional to the strength of interaction between a lipid and hydrocarbon tails of the stationary phase.\textsuperscript{54,55} In biological membranes, hydrophobic interactions between lipids with higher ECN are stronger and vice versa, thus it can be regarded as one of the key characteristics determining the membrane stability. Indeed, the ECN density plots displayed altered contour of PtdIns, PtdThr, PtdSer, and PtdGro in auxin-treated PIS mutant but not in the parental strain, resonating with our above-mentioned analysis of individual lipid species.

**Toxoplasma** can salvage specific PtdIns species with long acyl chains from the host cell. One intriguing observation in the lipidomic profiling of the PIS mutant was a somewhat selective increase in an abundant species of PtdIns with long acyl chain (C38), which counterbalanced the decline in most other PtdSer species with shorter chains, and perhaps also offset the expected defects in motility, invasion and egress (see "Discussion"). Further, despite the marked presence of long-chain PtdIns species in parasites (Figs. 6 and 7), the isotope labeling of extracellular tachyzoites resulted in only insignificant inclusion of tracer in such lipid species (Fig. 4e). These unexpected findings led us to investigate the potential salvage of PtdIns from host cells. We first labeled the human fibroblasts with [13C]-myo-inositol and then infected them for propagating parasites (Fig. 8a). Lipidomics of the tachyzoite progeny isolated from prelabeled host cells revealed rather selective accrual of C38:4 PtdIns. A comparison of [13C]-PtdIns in tachyzoites that were labeled extracellularly (host-free) versus those grown in pre-labeled host cells clearly indicated distinct profiles of tracer incorporation (Fig. 8b). While the former displayed a majority of labeling in C34 PtdIns (70%), followed by C36 (20%) and C32 (8%), the latter parasites had primarily C38 (73%), C36 (11%), C40 (8%) and C34 (7%) species. Collectively, our lipidomic and isotope labeling results strongly suggest that *T. gondii* can produce PtdIns species with short-medium acyl chains de novo, while at the same time the parasite can salvage longer-chain lipid species from the host cell.

**Discussion**

The enzyme PIS, utilizing CDP-DAG and myo-inositol to produce PtdIns, is conserved in all eukaryotes including in *T. gondii* (Fig. 9). Many protozoan parasites harbor all three enzymes to produce myo-inositol from glucose besides the sugar transporters, which have been characterized in *Trypanosoma* and *Leishmania*.\textsuperscript{56-58} In *T. brucei*, endogenously-synthesized myo-inositol supports the synthesis of PtdIns and GPI in the ER,\textsuperscript{40,41} while myo-inositol acquired from the milieu drives the bulk production of PtdIns in the Golgi complex.\textsuperscript{42,43} Similarly, *P. falciparum* is dependent on de novo myo-inositol synthesis for GPI assembly.\textsuperscript{59} Intriguingly, one of the genes encoding for inositol-3-phosphate synthase could not

### Fig. 6 Auxin-mediated downregulation of TgPIS perturbs PtdIns, PtdSer, and PtdThr species.

Lipids isolated from the TgPIS-mAID-3xHA mutant and parental strain (RHΔku80Δhxgprt-TIR1) were subjected to lipidomic analysis. For each phospholipid class shown herein, the amount of all major species (accounting for >90% of total lipid) was plotted in pmol/10^6 parasites. Numerical values depict the means with S.E. from 4 independent experiments. Samples without (-) or with (+) IAA treatments are colored differently for the parental and mutant strains. Lipid species are ordered based on their acyl chain length from the top to bottom of the graph. Changes in the contour of violin-like graphs are meant to show the overall variation in given lipids. Statistical significance was scored for each lipid species by comparing the auxin-treated and control samples (Student’s t test; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).
be identified in the genome database of *T. gondii*, and tachyzoites cultivated with [13C]-glucose showed the labeling of glucose-6-phosphate but not of inositol-3-phosphate and myo-inositol60. Equally, we reveal that tachyzoites import and utilize myo-inositol for PtdIns synthesis. Our earlier work identified four sugar transporters in *T. gondii*, of which two reside in the plasma membrane61. One of them, TgGT1, can transport glucose, mannose, fructose, and galactose, while the other (TgST2) may facilitate

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**Fig. 7 Conditional depletion of PtdIns synthase causes modulation of selected lipid species.** a, b Volcano plots illustrating changes in all detectable lipid species upon IAA treatment of the parental and TgPIS-mAID-3xHA strains. Thresholds of false-discovery rate (FDR)-corrected *p*-value (≤0.05) and fold-change (≥1.5) were used to define significantly-altered metabolites. Lipid species (represented as circles) are scaled to abundance; those above the threshold are colored according to phospholipid class, while others are shown in gray/black. The horizontal dashed line corresponds to statistical significance, and the two vertical dashed lines to a decrease or increase by a factor of 1.5 (*n* = 4 assays). c Heatmaps showing the induction or repression of lipid species chosen from a and b.
myo-inositol import. Surprisingly though, TgST2 is dispensable for the lytic cycle of tachyzoites; hence it remains equivocal whether the parasite is indeed strictly dependent on (auxotrophic for) host-derived myo-inositol (Fig. 9).

The other precursor of PtdIns, CDP-DAG, is synthesized by the enzyme CDS using phosphatidic acid and CTP (Fig. 9). The eukaryotic-type CDSs have been identified in the genomes of many protozoan parasites, and proved to be essential for the synthesis of PtdIns in T. gondii, P. falciparum, and T. brucei. Additionally, we found a prokaryotic-type CDS in T. gondii (and other selected parasites) that drives the synthesis of phosphatidylglycerol. This study shows that a knockdown of CDS1 in the ER of T. gondii impaired the incorporation of [3H]-myo-inositol into PtdIns, and caused an equivalent decrease in the lipid content of the CDS1 mutant. Although a dependence of PtdIns synthesis on other CDP-DAG pools cannot be entirely ruled out, it can be concluded that the ER-derived CDP-DAG serves as a key source for PtdIns synthesis in the Golgi network, which would necessitate its transport from the ER to Golgi (Fig. 9). Subsequently, PtdIns should be transported from Golgi to the site of GPI assembly. The mechanism of such inter-organelle exchange of CDP-DAG and PtdIns warrant further investigation.

Autonomous synthesis, trafficking, and physiological relevance of membrane phospholipids are active areas of research in protozoan parasites. In T. gondii, de novo synthesis of PtdCho is critical for normal progression of the lytic cycle, because the parasite seems unable to import sufficient amount of lipid or certain species from the host cells to bypass the ablation of its CDP-choline pathway. Likewise, the endogenous synthesis of PtdThr—a rare coccidian-specific lipid—is critical for the growth and virulence of tachyzoites. In contrast, the parasite shows significant plasticity in synthesis of PtdEtn, which can occur in multiple organelles. This study revealed a vital role of PtdIns synthesis despite the fact that tachyzoites are competent in importing some host-derived lipid species. Using post-translational control of protein stability by auxin, not only were we able to conclude a critical function of PtdIns synthesis for the cell division, gliding motility, invasion, and egress, but also

Fig. 8 Tachyzoites can synthesize as well as salvage selected PtdIns species from host cells. a HFF labeling with [13C]-myo-inositol and intracellular propagation of tachyzoites in prelabeled host cells to test the salvage of [13C]-PtdIns. Briefly, host cells were grown to confluence in the presence of 0.5 mM [13C]-myo-inositol, and then infected with tachyzoites of the PIS mutant (MOI = 1) in the presence of 5 mM myo-inositol to minimize the import and usage of residual intracellular isotope by the parasite’s de novo synthesis. Purified extracellular tachyzoites were examined by lipidomic analysis. Note that this assay was technically (intricate data normalization) not feasible with the auxin-treated TgPIS mutant due to a need of higher MOI for initial infection (and thus more unlabeled PtdIns), and much slower growth in the presence of IAA (and thus prolonged incubation). The data plotted are from 5 independent assays (mean ± S.E.). b Proportion of [13C]-PtdIns species detected after stable isotope labeling of extracellular tachyzoites (left, see Fig. 4e), and in tachyzoites after propagation in prelabelled host cells (right, see a). Only primarily-labeled PtdIns species (≥1%) are named; the minor-labeled species are shown as “Others”.

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Phosphate. Successively, Ins3P is dephosphorylated to make inositol by inositol-3P monophosphatase. There is no biochemical and genetic evidence for the synthase; PPM, parasite
myo

and the synthesis of C36 PtdIns species (not shown). Transporters, inter-organelle contact sites, or other lipid traf
PtdThr, PtdSer, PtdGro species). PtdIns species with shorter chains (e.g., C30/C32/C34) are synthesized de novo, while PtdIns species with relatively longer acyl chain (C38/C40) are salvaged by the parasite from its host cell. Based on isoence labeling studies, we speculate that both routes contribute to the synthesis of C36 PtdIns species (not shown). Transports, inter-organell contact sites, or other lipid trafficking pathways, as stated, enable transport of myo-inositol and CDP-DAG. The question-marked reaction specifies yet-unknown enzyme involved in CDP-DAG-dependent PtdSer synthesis. Selected abbreviations: CDS, CDP-DAG synthase; PGPS, PtdGro phosphate synthase; PGPP, PtdGro phosphate phosphate; PSS, PtdSer synthase; PTS, PtdThr synthase; PPM, parasite’s plasma membrane; PVM, parasitophorous vacuole membrane.

Perform comprehensive lipiddomic and isotope-labeling analyses, unveiling additional noteworthy findings. These include de novo synthesis of PtdIns, salvage of specific host-derived lipid species, and integrated regulation of major anionic glycerophospholipids in

T. gondii

(Fig. 9).

A reduction in PtdIns species with short-medium acyl chains (C30, C32, C34) and an increase in lipid species with long-chain (C38) following a knockdown of PIS correlate well with our isotope labeling assays. A few abundant species of PtdIns were either not affected or even slightly increased upon knockdown of

Tg

PIS,

balancing the content of PtdIns. Nonetheless, ablation of de novo synthesis led to a severe phenotype, which can be attributed to a requirement of short-to-medium chain PtdIns species for the membrane biogenesis during endodyogeny.

Besides endogenously made lipid species, the parasite salvages (at least) C38:4 PtdIns from the host cell. Markedly, this particular lipid is found to be enriched in human host cells used herein to propagate tachyzoites.

Moreover, C38:4 is the dominant PtdIns species that drives the synthesis of phosphoinositides in mammalian cells.

Thus, we speculate that scavenging of such lipid species from the host cell may enable the parasite to generate adequate phosphoinositides for operating its own IP3-dependent Ca2+ signaling, which is known to govern the motility, egress, and invasion processes.

Taken together, our phenotypic and lipidomic findings advocate the usage of distinctive PtdIns species for membrane biogenesis and signaling in tachyzoites of

T. gondii

(Lambert et al., 2017).

A decrease in PtdThr on the other hand may be a consequence of its involvement in calcium homeostasis,

and/or due to its inversely-proportional relationship with the PtdSer content, as also observed in other mutants, where PtdThr and PtdSer syntheses were impaired.

A balanced composition of anionic phospholipids, accounting for up to 30% of membrane lipids in eukaryotic cells, is needed for optimal function of many enzymes, e.g., protein kinase C, phospholipase A2, and CTP-phosphocholine cytidylyltransferase. It has been proposed that the negatively charged region of the membrane interacts with the cationic motif of proteins, and the head groups and acyl chains are determinant factors for such interactions.

Anionic lipids are also critical for the functioning of membrane permeases, such as glucose transporters and ATP-sensitive potassium channels in mammalian cells.

Given these and our principal findings on anionic phospholipids, future work should examine their importance and mechanistic roles in

T. gondii

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Methods

Biological reagents and resources. The RHAKaΔhxgprtΔku80 and RHAKaΔhxgprtΔku80Δhxgprt-TIR1 strains of

T. gondii

were kindly offered by Vern Caruthers (University of Michigan, MI) and David Sibley (Washington State University, St. Louis, MO), respectively. The Δg62Ii, mutant was generated in our earlier study. The pG140 plasmid was donated by Markus Meissner (Ludwig-Maximilians University, Munich). Antibodies recognizing the TgHSP90, TgGAP45, TgDH-E1a, TgSAG2, and TgIMC3 proteins were bestowed by Sergio Angelo (IIB-INTECH, Argentina), Dominique Soldati-Favre (University of Geneva, Switzerland), Bang Shen (Huazhong Agricultural University, China), and Marc-Jan Gubbels (Boston College, MA), respectively. Anti-TgSERA and anti-TgPSD1 m antisera were obtained from FriendBio Biscience and Technology (China). Other primary antibodies recognizing the TgSAG1 protein and engineered epitopes (HA and Ty1) were obtained from ThermoFisher Scientific and Sigma-Aldrich (Germany). Corresponding secondary antibodies (Alexa488, Alexa594) were procured from Life Technologies (Germany). [3H]-myo-inositol, and silica plates for TLC were obtained from American Radiolabeled Chemicals (St. Louis, MO) and Merck Millipore (Billerica,
MA), respectively. 

**Radical** expresses an overnight incubation at 25 °C by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures with OD_{600} of 0.4–0.6 (pre-grown at 37 °C), followed by additional incubation with myo-inositol at 37 °C for 4 h. Samples were processed for lipids extraction and TLC.

Lipid extraction, TLC separation, and quantification. Lipids were extracted according to our previously reported protocol based on the original method of Bligh and Dyer. Briefly, cell pellets were suspended in 4 mL methanol/water (2:0.9, v/v), followed by sequential addition of chloroform (2 mL), 0.2 M KCl (1.8 mL), and chloroform (2 mL) each accompanied with vigorous vortex-mixing. Then, the upper chloroform phase was backwashed twice with 2.1 mL of methanol/KCl (0.2 M): chloroform (1:9:90.1, v/v). Lipids obtained from the isotope-labeled parasites or from recombinant bacteria were backwashed 3x each with 2.1 mL of methanol: PBS:chloroform (1:9:90.13, v/v). The chloroform phase containing lipids was filtered and dried under N\textsubscript{2} stream, and resuspended in 50–100 μL of chloroform (methanol: 9:1). Phospholipids were resolved by one-dimensional TLC on silica H plates developed either in a solvent comprising chloroform, methanol, 2-propanol, KCl (0.25%), and triethylamine (20:87.5:13.8:4, v/v), or chloroform, ethanol, water, and triethylamine (90:35:35:5, v/v). Alternatively, two-dimensional TLC on silica 60 plates developed first in chloroform, methanol, and NH\textsubscript{4}OH (65:35:5, v/v), and then in chloroform, acetic acid, methanol, and water (75:25:5.2:2, v/v). They were visualized by incubating the TLC plate in a glass chamber with iodine vapor, and/or by spraying 0.2% (w/v) anilino-1-naphthalene sulfonic acid followed by ultraviolet light exposure, or by using Fuji X-ray films. Phospholipids were identified based on their comigration with authentic standards. TLC-resolved silica scrapings were assayed to quantify individual phospholipids by lipid-phosphorus measurements using defined chemical standards, as described elsewhere.

**Lipidomic analysis.** Pellets of purified parasites (1–2 x 10\textsuperscript{7}) were suspended in 0.8 mL PBS and subjected to lipid extraction according to Bligh and Dyer. Lipid extracts were dried under N\textsubscript{2} stream, dissolved in 100 μL of chloroform and methanol (1:1), and injected (10 μL) into a hydrophilic interaction liquid chromatography column (2.6 μm HILIC 100 Å, 50 x 4.6 mm, Phenomenex, CA). Lipid classes were separated by gradient elution on an Infinity II 1290 UPLC (Agilent, CA) at a flow rate of 1 mL/min. Acetonitrile and acetone (91, v/v) with 0.1% formic acid was used as solvent B. A linear B:C gradient mixture of acetone and acetonitrile (75:25:5, v/v) was used to elute lipids. Gradient elution was done as follows (time in min, % B): (0, 0), (1, 50), (3, 50), (3.1, 100), (4, 100). No re-equilibration of the column was necessary between successive samples. The column effluent was connected to a heated electrospray ionization (hESI) source of either an Orbitrap Fusion or Q-Exactive HF mass spectrometer (Thermo Scientific, MA) operated at ~3600 V in the negative ionization mode. The vaporizer and ion transfer tube were set at a temperature of 275 °C and 380 °C, respectively. Full scan measurements (MS\textsubscript{1}) in the mass range from 450 to 1150 amu were collected at a resolution of 120000. Parallelized data-dependent MS\textsubscript{2} scans were deployed and fragmentation energy was set to 30 eV, using the normalized collision energy of 20% for individual lipid classes using standards, when available. Quantitation was achieved by calculating response factors for individual lipid classes using standards, when available.

**Making of transgenic parasites.** The plasmid constructs (20–50 μg) were transfected into freshly isolated tachyzoites of specific strains (10\textsuperscript{7} cells) suspended in filter-sterile Cytoxim (120 mM KCl, 0.15 mM CaCl\textsubscript{2}, 10 mM K\textsubscript{2}HPO\textsubscript{4}/KH\textsubscript{2}PO\textsubscript{4}, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl\textsubscript{2} supplemented with fresh 5 mM glutathione and 5 mM ATP; pH 7.6) using a BTX electroporation instrument (2 kV, 25 μF, 500 μs). Transfections were performed at the RHΔmu/∅ vector as detailed above. The drug-resistant parasites were cloned by limiting dilution, and individual clones were screened by PCR and/or immunostaining assays. The constructs, primers, and the parasite strains along with other relevant details are described in Supplementary Table 2 and respective Figs. The drug-resistant parasites were cloned by limiting dilution, and individual clones were screened by PCR and/or immunostaining assays. The constructs, primers, and the parasite strains along with other relevant details are described in Supplementary Table 2 and respective Figs.

**Expression of recombinant proteins in E. coli.** T. gondii whole parasite extracts were subjected to SDS-PAGE and Western blotting using specific antibodies. The percentage of the total protein expression was calculated by densitometric analysis of the Western blots. The positive control was E. coli expressing the recombinant protein. The negative control strain harboring empty vector were cultured in M9 minimal medium containing ampicillin (100 mg/L) and kanamycin (50 mg/L). Protein expression was induced overnight at 25 °C by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures with OD_{600} of 0.4–0.6 (pre-grown at 37 °C), followed by additional incubation with myo-inositol at 37 °C for 4 h. Samples were processed for lipids extraction and TLC.
ΔTgPIS-HA regulated by native promoter and TgDHFR-TS-3’UTR. Parasites were consequently transformed with the pSAG1-Cre plasmid to induce Cre-mediated recombination for deleting TgPIS-HA while concurrently repositioning YFP directly after the TgPIS promoter, which in turn yielded a yellow-fluorescent ΔTgpto-HAExplore mutant. For making the TgPIS-mAID-3xHA construct expressing Cas9 (under TgUB1 promoter), and sgRNA targeting the 3’UTR of TgPIS, Goto et al. was transfected with a donor plasmid comprising 3xHA-TgGRA1-3’UTR and HXGPR marker flanked by short (40 bp) 5’ and 3’ homology arms for crossover at the TgPIS locus, in the RHΔkas803hxgpr-TIR1 strain. Parasites were selected using mycophenolic acid (25 μg/ml) and xanthine (50 μg/ml).59 The method allowed 3’-genomic tagging of the PIS protein with mAID-3xHA and its conditional expression (auxin-regulated) under the control of the native promoter and TgGRA1-3’UTR.

Indirect immunofluorescence assays. The assay was performed essentially as described earlier.80,81 Partitioned HFFs grown on coverslips (24–48 h infection) were washed with PBS, fixed with 4% paraformaldehyde (10 min), and neutralized with 0.1 M glycine in PBS (5 min). Cells were permeabilized by 0.1% Triton X-100/PBS (20 min), followed by staining with primary antibodies (α-HA, mouse or rabbit, 1:1000 or 1:3000; anti-Ty1, mouse, hybridoma, 1:50; α-TgGAP45, rabbit, 1:10000; α-TgDPDH-E1a, rabbit, 1:1000; α-TgMC3, rabbit, 1:2000; α-TgSERCA, rabbit, 1:100; α-TgPSD1, rabbit, 1:100; anti-TgSAG1, mouse, 1:10000; α-TgSAG2, rabbit, 1:1000) for 1 h. Cells were washed three times with PBS, and stained with Alexa488 and Alexa594-conjugated secondary antibodies. The samples were washed three times with PBS, and mounted in Fluoromount G and DAPI mixture (Southern Biotech, Birmingham, AL) and stored at 4 °C. Images were acquired by fluorescence microscopy (Zeiss, Germany).

Immunoblot assays. Tachyzoites (1–2 x 10⁵) were washed twice with PBS, pelleted (400 g, 10 min, 4 °C), resuspended in the Laemmli protein-loading buffer and subjected to denaturing gel electrophoresis. Proteins were resolved by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (85 mA, 90 min). The blot was treated overnight (4 °C) with 5% skimmed milk suspended in Tris-buffered saline (20 mM Tris base and 150 mM NaCl, 0.2% Tween 20, pH 7.4), incubated with α-HA (1:10000), mouse and α-TgHSP90 (1:10000, rabbit) antibodies for 2 h, washed 3x (5 min each), and then incubated with infrared dyes-conjugated secondary antibodies for 45 min. Following additional washing, the samples were visualized using a Li-COR imaging system (Li-COR Biosciences, USA).

Lytic cycle assays. Standard phenotyping methods were used to determine the impact of genetic manipulation on the lytic cycle of tachyzoites in vitro, as described earlier.10,53 Plaque assays were performed by infecting confluent HFF cells in 6-well plates (100–200 parasites/well). Infected cells were incubated unperturbed for 7 days, followed by fixation with ice-cold methanol and staining with cryoprotectant (5%) for 30 min. The plaques were imaged after immobilization using Imaging software (NIH, Bethesda, MD). For invasion assays, confluent HFF cells cultured on coverslips, were infected with tachyzoites (MOI: 10) for 1 h. Cultures were stained with α-TgSAG1 antibody (mouse, 1:10000) prior to detergent permeabilization to visualize the noninvaded or extracellular parasites. Cells were washed 3x with PBS, permeabilized with 0.2% Triton X-100 for 20 min, and then stained with α-TgGAP45 antibody (rabbit, 1:10000) to identify invaded parasites. The fractions of invaded (intracellular) parasites were determined across 10 fields, and trail lengths were quantified by ImageJ software. To test the natural egress, host cell monolayers on coverslips were incubated for 7 days, followed by fixation with ice-cold methanol and staining with antibodies (α-HA, mouse, 1:10000 and α-TgGAP45, rabbit, 1:10000) for 2 h. Images were acquired by using a Li-COR imaging system (Li-COR Biosciences, USA).

Statistics and reproducibility. All values are provided in the main article and associated figure files. The source data for the graphs and charts presented herein can be found in Supplementary Data 1. The lipidomic results are also furnished as Supplementary Data 2 and Supplementary Data 3. All resources are available from the authors upon reasonable request.

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Author contributions

N.G. conceived the study; B.R., P.K., and N.G. designed experiments and wrote the paper; B.R., P.K., F.H., and N.G. performed assays; J.F.B., B.R., and N.G. analyzed lipodomics data. All authors approved the manuscript.

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Competing interests

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