Vitamin and cofactor acquisition in apicomplexans: Synthesis *versus* salvage

Published, Papers in Press, November 25, 2019, DOI 10.1074/jbc.AW119.008150

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Edited by Chris Whitfield

The Apicomplexa phylum comprises diverse parasitic organisms that have evolved from a free-living ancestor. These obligate intracellular parasites exhibit versatile metabolic capabilities reflecting their capacity to survive and grow in different hosts and varying niches. Determined by nutrient availability, they either use their biosynthesis machineries or largely depend on their host for metabolite acquisition. Because vitamins cannot be synthesized by the mammalian host, the enzymes required for their synthesis in apicomplexan parasites represent a large repertoire of potential therapeutic targets. Here, we review recent advances in metabolic reconstruction and functional studies coupled to metabolomics that unravel the interplay between biosynthesis and salvage of vitamins and cofactors in apicomplexans. A particular emphasis is placed on *Toxoplasma gondii*, during both its acute and latent stages of infection.

Members of the Apicomplexa encompass a large number of parasites exhibiting a great level of diversity in their life cycles, with morphologically distinct stages in one or more hosts. The phylum includes coccidians, hemosporidians, piroplasms, *Cryptosporidia*, and gregarines that occupy divergent niches (1). *Toxoplasma gondii* is the most successful zoonotic parasite of the cyst-forming subclass of coccidians. The proliferative tachyzoites infect and replicate in most cell types and are responsible for an acute infection, whereas the dormant cyst-forming bradyzoites are responsible for chronic infection, predominantly in the brain and striated muscles (2, 3). *Plasmodium falciparum* is the deadliest form of the human malaria parasites that proliferate in erythrocytes and hepatocytes. *T. gondii* and malaria parasites replicate intracellularly within a parasitophorous vacuole membrane that is permeable to small metabolites (4–8). In contrast, *Theileria* and *Babesia* species that belong to the genera of piroplasms rapidly escape the vacuole and proliferate freely in the cytoplasm of lymphocytes and red blood cells, respectively, with a more direct access to host nutrients (9, 10). *Cryptosporidium*, an enteric pathogen that relies only on a single host for both its sexual and asexual reproduction, develops in an extracytoplasmic compartment confined to the apical surfaces of epithelial cells and in a vacuole connected to the host cell via an extensively folded membrane structure called the feeder organelle (11). In humans, the causative agents of malaria, toxoplasmosis, and cryptosporidiosis are responsible for over a million deaths each year. From an evolutionary point of view, it is useful to compare the needs and capabilities between the closely related alveolates from the Apicomplexa and Chromerida phylum that group species capable of photosynthesis (12).

Our knowledge of apicomplexan metabolism has greatly benefited from the assembly of parasite genomes and has advanced through functional studies, in particular of *T. gondii* and *Plasmodium* spp. A necessary step toward a global understanding of the central carbon metabolism as well as the synthesis and uptake of amino acids, lipids, vitamins, and cofactors involves the use of *in silico* methods capable of predicting essential reactions, genes, and synthetic lethal pairs (13–16).[3] Currently available genome-scale computational models for *T. gondii* and the malaria parasites (14–17)[3] have recently been challenged by an impressive series of genome-wide gene fitness screens (17–19) and stage-specific transcriptomics data (20–22). These global approaches have turned out to be instrumental for the curation and validation of computational networks. Ultimately, incorporating functional analyses of metabolic pathways with molecular biology and metabolomic techniques will improve the accuracy of computational predictions.

In the recent past, several studies have illustrated the power of combining genetic and metabolomics approaches to understand metabolic functions in *T. gondii*. To summarize, it was shown that glucose and glutamine are the major carbon sources utilized by *T. gondii* tachyzoites (23, 24) and that glycolysis is essential for bradyzoites (25). The gluconeogenic enzyme fructose bisphosphatase was essential to regulate glycolytic flux in a futile cycle with phosphofructokinase (26). Uniquely, acetyl-CoA in the mitochondrion was shown to be produced via the branched-chain α-ketoacid dehydrogenase complex and not the canonical pyruvate dehydrogenase (PDH)[4] complex (27).

This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program under Grant Agreement 695596 (to J. K. and M. L.).

This article contains Table S1.

[1] Supported by Grant 51PHP0_157303, an interdisciplinary Ph.D. program of SystemsX.ch awarded by the Swiss National Science Foundation (SNSF). The authors declare that they have no conflicts of interest with the contents of this article.

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[3] Krishnan, *et al.*, Functional and computational genomics reveal unprecedented flexibility in stage-specific Toxoplasma metabolism. *Cell Host & Microbe., in press*.

[4] The abbreviations used are: PDH, pyruvate dehydrogenase; TPK, thiamine diphosphokinase; PBAL, pantoate-β-alanine ligase or PAN synthase; PAN,

PDH is required for a functional fatty acid (FA) synthase complex, also known as the FASII, in the apicoplast that produces medium-chain FAs, further elongated at the endoplasmic reticulum to form long monounsaturated FAs (28, 29).

Given the availability of large-scale data sets, systems-wide analysis of parasite metabolism offers a great opportunity to identify essential metabolic functions for targeted drug intervention. In a recent study,[3] a well-curated computational genome-scale model, iTgo (*in silico T. gondii*), was generated. iTgo contains 556 metabolic genes and integrates all available data sets to serve as a valuable platform for model-guided investigations. To harmonize the model with the genome-wide fitness scores for metabolic genes, additional constraints on substrate availabilities from the host as well as reaction utilization based on transcriptomics data were applied (16, 30). The workflow led to a model, 80% consistent with experimentally observed phenotypes,[3] allowing for reliable hypothesis generation for experimental validation. The two previous metabolic reconstructions (13, 15) identified several essential metabolic functions and differences within the clonal strains of *T. gondii* that display distinct virulence profiles. Within the apicomplexans, the most studied and comprehensive metabolic reconstructions were generated for *P. falciparum* and the rodent malaria parasite, *Plasmodium berghei* (14, 16, 31). Constant modeling efforts with the incorporation of physiological parameters, such as metabolomics and fluxomics, continue to expand our knowledge of the metabolic versatility of the apicomplexans. Although challenging, future models should consider the kinetic properties of reactions, allowing the simulation of altered enzymatic activities in both the host and parasite (31). Ideally, as complementary constituents of an iterative process, both computational and experimental efforts will ultimately lead to the identification of potential drug targets, mechanisms of drug action and complex host-pathogen interactions.

Among the indispensable pathways for parasite proliferation and persistence, the biosynthesis of vitamins and cofactors offers potential targets for intervention. Vitamins are essential precursors for the production of cofactors and, in humans, can be acquired solely through the diet (32). To date, 13 metabolites are classified as vitamins, required for the functioning of a mammalian cell, facilitating numerous enzymatic reactions. Nine of the 13 vitamins are known to be utilized by the apicomplexans, with three of them (vitamins B_5, B_6, and B_9) being *de novo*–synthesized by some parasites (33). The vitamins that can be synthesized *de novo* are probably low in abundance in one or more niches and cannot be sufficiently salvaged. Comparison across the phylum can reveal interesting insights into the origins and subsequent loss of several pathways in certain genera, such as the *Cryptosporidia* and piroplasms (34–36) (Fig. 1). Both genera possess limited biosynthesis capabilities, reflecting their lifestyle in a nutrient-rich environment and adaptation to

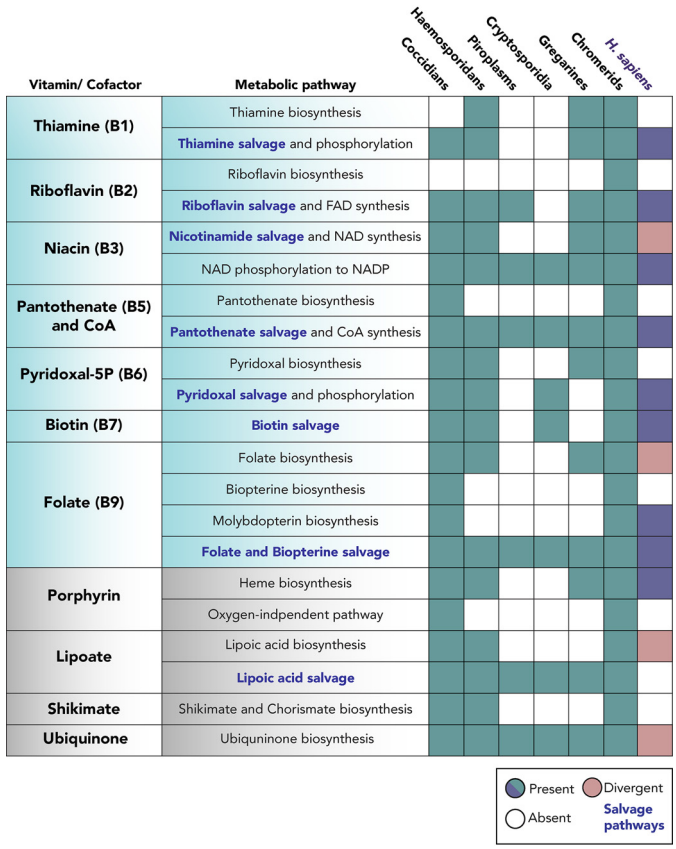

Figure 1. Conservation of vitamin and cofactor biosynthesis or scavenge pathways within the apicomplexans and the human host. The presence or absence of metabolic pathways within the Apicomplexan and Chromerida phylum and the human host, *Homo sapiens*, is summarized. The gene identifiers and enzyme names in each pathway can be found in Table S1. For each genus, representative organisms were chosen: coccidians (*T. gondii*), hemosporidians (*P. falciparum*), piroplasms (*Babesia bovis* and *Theileria annulata*), *Cryptosporidia* (*Cryptosporidium muris*), gregarines (*Gregarina niphandrodes*), and chromerida (*C. velia* and *V. brassicaformis*).

mechanisms for metabolite acquisition from the host. Concordantly, the genome of *Cryptosporidium hominis* was shown to encode more than 80 genes with strong similarity to known transporters and several hundred genes with transporter-like properties (37). *Cryptosporidia* are also in close contact with the microbiome in the intestinal gut, thus expanding their capacity for nutrient acquisition (38).

In the next sections, we review the progress made in *T. gondii* and apicomplexans in general, to better understand the interrelationship of *de novo* synthesis and scavenge routes for vitamins and cofactors and their utilization in different life cycle stages. An overview of the pathways in both *T. gondii* and its mammalian host is presented in Fig. 2. Further, the latest observations are discussed in the context of long-standing questions on the roles of the metabolic pathways for latency and their potential as drug targets.

Vitamin B_1

Vitamin B_1, or thiamine, is an important precursor for its metabolically active form, thiamine pyrophosphate (TPP). TPP acts as a cofactor for enzymes implicated in carbohydrate and amino acid metabolism, such as the PDH complex, 2-oxoglutarate dehydrogenase, pyruvate decarboxylase, and dihydrolipo-

pantothenate; PLP, pyridoxal 5′-phosphate; PLK, pyridoxal kinase or PdxK; CPO, coproporphyrinogen oxidase; CPDH, coproporphyrinogen dehydrogenase; FC, ferrochelatase; FA, fatty acid; TPP, thiamine pyrophosphate; FS, fitness score(s); ETC, electron transport chain; KPHMT, Ketopantoate hydroxymethyltransferase; KPR, α-ketopantoate reductase; PanK, pantothenate kinase; DOXP, 1-deoxy-D-xylulose 5-phosphate; ACCase, acetyl-CoA carboxylase; ALA, δ-aminolevulinic acid; ProtoIX, protoporphyrin IX.

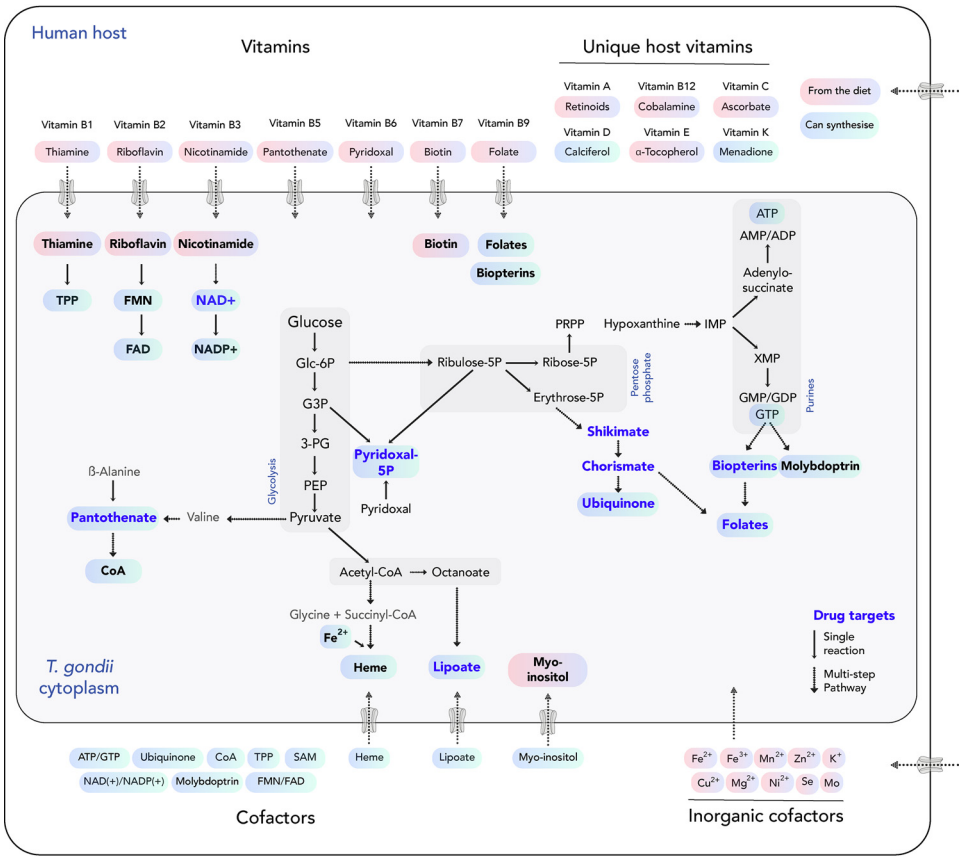

Figure 2. Vitamins and cofactors biosynthesis *versus* scavenge pathways in *T. gondii* and its mammalian host. Metabolites that can either be *de novo* produced (*blue*) or must be salvaged (*pink*) from an external source are depicted. Enzymes for the production of metabolites (*boldface blue type*) are potential drug targets, given the unique synthesis capability of the parasite, but not the host.

amide dehydrogenase. In *T. gondii*, these enzymes are either residents of the secondary endosymbiotic organelle, called the apicoplast, or the mitochondrion, suggesting a need for the cofactor within these subcellular compartments. Like their mammalian host, the parasites do not possess the pathway for thiamine biosynthesis and must therefore acquire it. Hemosporidians (in particular *P. falciparum*) are the only apicomplexans that possess the enzymes to synthesize thiamine, like bacteria, plants, and fungi (39–41). The genes implicated in the synthesis of TPP are, however, expressed only in the mosquito vector (salivary gland sporozoites) stage (42). Despite the ability to synthesize thiamine, *Plasmodium* spp., like other apicomplexans, harbor the key enzyme thiamine diphosphokinase (TPK) to convert the scavenged thiamine into TPP. TPK is expressed in all stages of the *Plasmodium* life cycle, and several studies have shown that parasite replication is inhibited by thiamine analogues that generate toxic anti-metabolites (43, 44). Deduced from the genome-wide CRISPR-Cas9 screen for *T. gondii* performed *in vitro*, TPK is critical for *in vitro* tachyzoite survival with a high negative fitness score (FS) (−3.28) (Fig. 3). FS are experimentally observed measures (ranging from −7 to +3) and assess the fitness cost of a given gene for parasite survival (17).

The mechanism by which thiamine is taken up and translocated across organelles where it is needed is yet to be determined. In humans the thiamine transporters, hThTr1 and hThTr2 have been well-characterized (45, 46), but no obvious orthologs within the parasite's genome could be identified. Interestingly, in certain apicomplexans, such as *Cryptosporidia* and piroplasms, salvage of the phosphorylated form (TPP) must occur.

Vitamin B_2

Vitamin B_2, or riboflavin, is crucial for flavin-dependent processes occurring in all subcellular compartments. FMN and FAD participate in redox reactions and play an essential role for the proper functioning of the electron transport chain (ETC), tricarboxylic acid cycle, and fatty acid biosynthesis. Like their mammalian hosts, most apicomplexans are unable to synthesize riboflavin but possess the capacity to convert riboflavin into FMN and FAD. The two genes coding for their synthesis, riboflavin kinase and FAD synthase, are present in *T. gondii* and are fitness-conferring with an FS of −3.97 and −4.87, respectively (Fig. 3). Exceptionally, *Cryptosporidia* appear to lack these enzymes and therefore must take up both FMN and FAD, suggesting an exquisite adaptability to scavenge phosphorylated cofactors. Outside the Apicomplexa phylum, the absence of an FMN/FAD synthase can be seen in obligate intracellular α-proteobacteria, *Rickettsiae* (47).

Vitamin B_3

Vitamin B_3, or nicotinic acid, also known as niacin, is essential for generation of coenzymes NAD^+ and $NADP^+$, which act as key electron carriers in a cell. The apicomplexans are unable to synthesize nicotinate or nicotinamide *de novo*, indicating

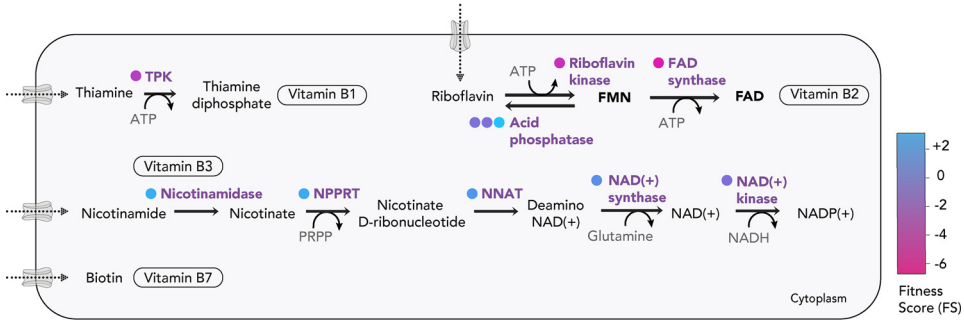

Figure 3. The scavenge pathways and bioconversion of vitamins (B_1, B_2, B_3, and B_7). *T. gondii* must uptake vitamins B_1, B_2, B_3, and B_7 via unknown transport mechanisms and subsequently convert them into the cofactors for utilization within the parasite. FS for the enzymes for the bioconversion are *color-coded* (in *circles*). *NPPRT*, nicotinate phosphoribosyltransferase; *NNAT*, nicotinate-nucleotide adenylyl transferase.

that these metabolites are salvaged from the host. All apicomplexans possess the enzymes for the subsequent conversion into NAD^+ and $NADP^+$, although the corresponding genes appear dispensable for *T. gondii* tachyzoites, based on their FS (NAD^+ synthase, +0.03; NAD^+ kinase, −1.33) (Fig. 3). Of relevance, the CRISPR-Cas9 screen was performed with cultured human foreskin fibroblasts grown in rich media containing an abundance of amino acids, vitamins, salts, and sugars. This may allow certain genes to seem dispensable than they actually would be in a physiological environment more restricted in nutrients. Fitness scores might also vary, depending on the metabolic rates and capabilities of different host cell types (*in vitro* or *in vivo*).

In *P. falciparum*, infected erythrocytes showed a 10-fold increase in NAD^+ content compared with uninfected cells, suggesting an efficient and functional biosynthesis pathway in the parasite (48). Due to the substantial release of NAD^+ from *Plasmodium*-infected erythrocytes, NAD^+ has been proposed as a potential clinical biomarker for malaria (49). The impact of blocking the parasite nicotinate mononucleotide adenylyl transferase, which synthesizes NAD^+ from nicotinate, validates the biosynthesis pathway as an antimalarial target (50).

Vitamin B_5

Vitamin B_5, or pantothenate (PAN), is the precursor for the biosynthesis of the essential cofactor, CoA. PAN synthesis takes place in most bacteria plants and fungi, but not in animals. The biosynthesis of CoA from PAN, on the other hand, is present in almost all organisms. The *de novo* synthesis of PAN requires three enzymatic activities: hydroxymethyl transfer to ketoisovalerate (KPHMT), α-ketopantoate reduction (KPR) to pantoate, and pantoate-β-alanine ligation (PBAL). Interestingly, *T. gondii* encodes the pathway in two sequences conserved within the coccidians, which include *Hammondia*, *Neospora*, *Besnoitia*, *Cyclospora*, and *Eimeria* genera. The PAN synthesis pathway has been partially characterized in *T. gondii*, and its essentiality has been proposed based on the use of chemical inhibitors (51). However, the tested drugs had been developed for *Mycobacterium tuberculosis* homologue (panC), and off-target effects cannot be excluded.

In *T. gondii*, the first enzyme in the PAN synthesis pathway is bifunctional, encoding the first two enzymatic steps ketopantoate hydroxymethyl transferase and ketopantoate reductase (KPHMT-KPR). The KPHMT and KPR domains of the protein present conserved key catalytic residues (52, 53) when compared with *Escherichia coli* panB and panE, respectively. The fusion of the two catalytic domains into one ORF can also be found outside the Apicomplexa phylum in Dinoflagellates (*Perkinsus marinus*) and free-living photosynthetic Chromerida (*Vitrella brassicaformis* and *Chromera velia*), where, interestingly, a single ORF comprises all three enzymes for the synthesis of PAN (Table S1).

The final step of PAN synthesis is catalyzed by PBAL, which ligates pantoate with β-alanine. Sequence comparison with *E. coli* panC indicates that >30% of the catalytic domain and all catalytic residues (54) are conserved in *T. gondii* PBAL, pointing to a possible conservation of function. In all members of the phylum, the protein presents an extended N and C terminus (the latter conserved >45% within *Neospora*, *Hammondia*, and *Besnoitia* genera), although no known molecular function has been associated to date. The respective FS of KPHMT-KPR (+0.09) and PBAL (+0.72) indicate *in vitro* dispensability for PAN synthesis, suggesting that *T. gondii*, as demonstrated for *P. falciparum* (55), utilizes host derived PAN for CoA synthesis. Except for the coccidians, the apicomplexans lack PAN synthesis enzymes, and attempts to identify a PAN transporter by orthology have proven difficult (56–58).

CoA, the end product of the pathway, is essential for a broad range of metabolic functions. It provides activated acyl groups for various metabolic pathways, such as the tricarboxylic acid cycle, fatty acid synthesis, and heme synthesis, as well as for gene regulation and post-translational modification of proteins (59). Pantothenate kinase (PanK), which catalyzes the first step in CoA synthesis, has been extensively characterized in *P. falciparum* (60), allowing pantothenamides (pantothenate mimetic compounds) to be catabolized into CoA antimetabolites (61) with deleterious effects for the parasite (62). Interestingly, of the five enzymes required for CoA synthesis, phosphopantetheine-cysteine ligase and phosphopantothenoylcysteine decarboxylase, which catalyze the second and third step, respectively, are dispensable in both the rodent malaria parasites *Plasmodium yoelii and P. berghei* (19, 63). This observation could be explained by the promiscuous activity of PanK (64), allowing usage of pantetheine (an intermediate) scavenged from the host cell (65). In *T. gondii*, the FS of all of the enzymes of the CoA synthesis pathway indicate essentiality (including the two different genes encoding for PanK) (Fig. 4). We have recently identified the gene coding for the final step, dephos-

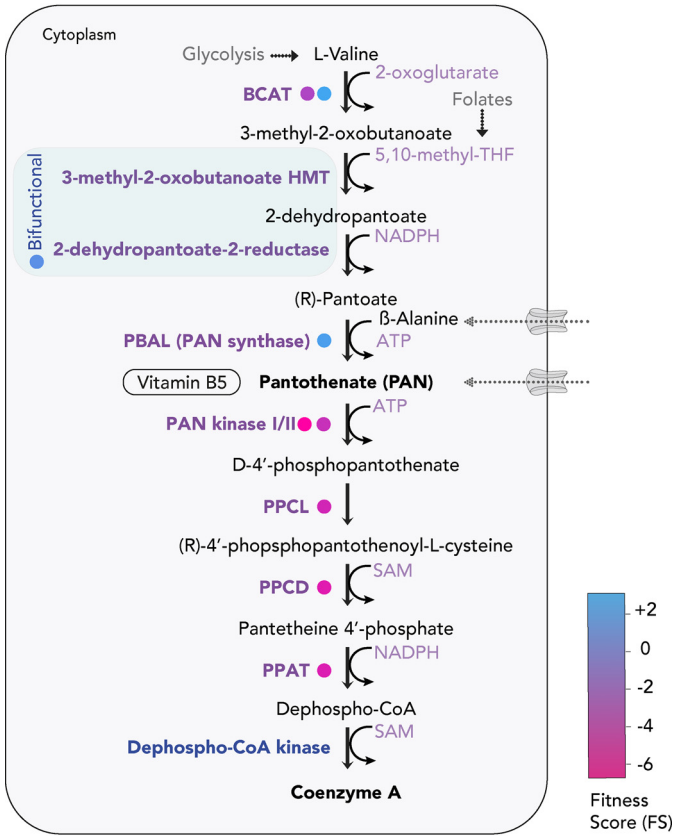

Figure 4. PAN (vitamin B_5) and CoA biosynthesis pathway. *T. gondii* can *de novo*–synthesize or uptake PAN and subsequently convert it into CoA within the parasite. The bifunctional enzyme for PAN synthesis is shown in *blue*. FS for the enzymes are *color-coded* (in *circles*). *BCAT*, branched-chain amino acid transaminase; *HMT*, hydroxymethyltransferase; *PPCL*, phosphopantetheine-cysteine ligase; *PPCD*, phosphopantothenoylcysteine decarboxylase; *PPAT*, pantetheine-phosphate adenylyl transferase.

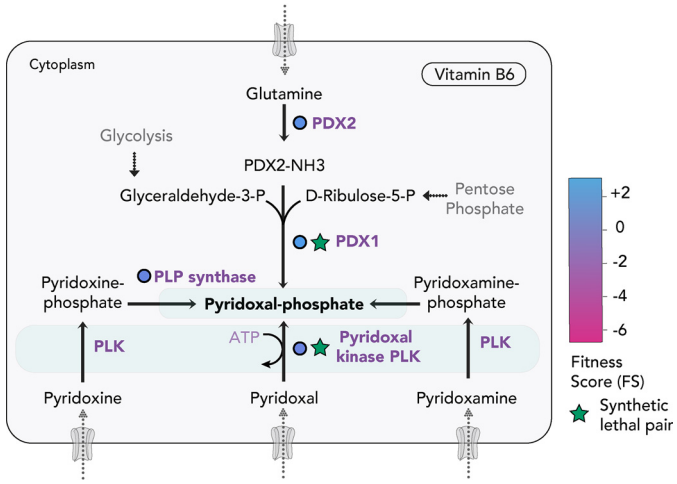

Figure 5. Pyridoxal-5P (vitamin B_6) biosynthesis and scavenge pathways. *T. gondii* can *de novo* synthesize PLP or uptake the vitamers to subsequently convert them into PLP within the parasite. PLK (in *blue*) can phosphorylate any of the vitamers—pyridoxal, pyridoxamine, or pyridoxine—and is synthetically lethal with the synthesis enzyme, PDX1. FS for the enzymes for the bioconversion are *color-coded* (in *circles*). Experimentally validated enzymes are *circled* in *black*.[3]

pho-CoA kinase, previously thought to be missing from the genome, and have shown that the activity is essential for parasite survival by conditional disruption.[5]

Taken together, it appears that most apicomplexans share the capability to scavenge PAN from their host. Hence, the retention of the PAN synthesis pathway among the coccidians is intriguing. It is likely that PAN synthesis is required in life cycle stages where exogeneous PAN levels are limiting, such as in sporozoites or in the cyst-enclosed bradyzoites of *T. gondii*. Importantly, PAN synthesis requires β-alanine, for which no synthesis pathway has been clearly identified in the genome of *T. gondii*. Thus, the parasite would have to acquire this metabolite from its environment. Further research is necessary to delineate the relevance of PAN synthesis in coccidians.

Vitamin B_6

Vitamin B_6 is part of the essential vitamin B group of molecules, consisting of pyridoxal, pyridoxamine, and pyridoxine. The metabolically active form is pyridoxal 5′-phosphate (PLP). PLP is a crucial cofactor for the activity of over 140 enzymes, several of them involved in amino acid metabolism (66, 67). Two different routes for the *de novo* synthesis of PLP exist in organisms: 1-deoxy-D-xylulose 5-phosphate (DOXP)-dependent and DOXP-independent (68). The DOXP-dependent route occurs in proteobacteria and most other bacteria, whereas eukaryotes, including the apicomplexans, utilize the DOXP-independent route. In this route, PLP is synthesized via the activity of two enzymes, PDX1 (PLP synthase subunit) and PDX2 (class I glutamine amidotransferase). Free vitamin B_6 forms can also be phosphorylated via the action of pyridoxal kinase (PLK or PDXK). The subsequent conversion of pyridoxamine-5P and pyridoxine-5P to PLP can be performed via a different enzyme, pyridoxal 5′-phosphate synthase (PLP synthase).

Both coccidians and hemosporidians possess all of the enzymes for *de novo* synthesis as well as scavenge of the vitamin (69, 70). The FS for the genes coding for PDX1 (+0.59), PDX2 (+0.08), PLP synthase (−0.33), and PLK (−0.41) indicate dispensability *in vitro* (Fig. 5), indicating redundancy between synthesis and salvage for PLP production. In a recent study, disrupting *de novo* biosynthesis of PLP via conditional knockdown of PDX1 was detrimental in parasites lacking the PLK gene.[3] The synthetic lethality showed that blocking both routes for cofactor generation is deleterious, and several PLP-dependent enzymes must become inactive. One such enzyme is glycogen phosphorylase, which breaks down the storage polysaccharide amylopectin (71). In *T. gondii*, loss of glycogen phosphorylase is associated with amylopectin accumulation and lethal for both tachyzoites and bradyzoites (72). Indeed, amylopectin accumulation was observed in mutants depleted of PLP.[3] Although PLP requirement for several enzymes is fulfilled with either the biosynthesis or scavenge pathway *in vitro*, contrastingly, the deletion of PDX1 alone was sufficient to abolish *T. gondii* virulence in mice.[3] This points to limited or insufficient amounts of pyridoxal in the organs or tissues infected with *T. gondii in vivo* (73). The sole reliance on the *de novo* pathway for PLP production *in vivo* makes PDX1 an attractive drug target or candidate for an attenuated live vaccine.

[5] M. Lunghi, J. Kloehn, and D. Soldati-Favre, unpublished observations.

If the biosynthesis pathway is the major route for PLP production in *T. gondii in vivo*, the presence and role of PLK is puzzling. To test its role during latency, mice infected with parasites lacking PLK were examined for cyst formation.[3] No reduction in cyst number was observed, compared with the WT, suggesting its dispensability for the chronic stage. It is plausible that the enzyme has a role during the sexual or oocyst stages, recycling any free pyridoxal in the cell and preventing toxic accumulation of the vitamers. How the vitamers enter the parasite remains unknown, and the absence of the biosynthesis of PLP in *Cryptosporidia* and piroplasms further indicates an unusual salvage mechanism for the phosphorylated cofactor.

In *P. falciparum*, both PLP biosynthesis and salvage pathways have been shown to be functional. The two genes (encoding for PDX1 and PDX2) are expressed throughout the intraerythrocytic and gametocyte development and have been explored as potential drug targets (74–77). Prodrugs such as pyridoxyl-tryptophan chimeras that interfere with PLP-dependent enzymes and poison the parasite have also been investigated as antimalarials (78, 79). For organisms that lack biosynthesis capabilities, identification of the transporter of pyridoxal and its derivatives would be of significant interest.

Vitamin B_7

Vitamin B_7 or biotin can be synthesized by bacteria, plants, and some fungi, but not by animals. The apicomplexans also lack the biosynthesis capability for biotin. Biotin is an important cofactor for the enzyme acetyl-CoA carboxylase (ACCase), of which ACCase1 was found in the apicoplast of *T. gondii* (80). In bacteria, biotin covalently attaches to the ϵ-amino group of specific lysine residues in the carboxylases via the action of a biotin-ligase (81). A putative biotin-ACC-ligase, with similarity to the *E. coli* biotin operon repressor (BirA) was found in the genome of most apicomplexans. If its role is similar to that of BirA for sensing biotin levels and regulating transcription is unknown (82). How biotin is acquired from the host and transported into the apicoplast, where ACCase1 resides, also remains to be understood. Biotin uptake is mediated by solute transporters in prokaryotes (83) and via a monocarboxylate transporter (MCT1) in mammalian cells (84).

Vitamin B_9

Vitamin B_9 or folate is crucial for DNA replication, cell division, and synthesis of several amino acids. The folate derivative, 5,10-methylenetetrahydrofolate, is essential for the production of dTMP and dUMP nucleotides. In addition to the *de novo* folate biosynthesis pathway from shikimate and chorismate, most apicomplexans can also salvage folate from the host via dedicated BT1 or FT transporters (85, 86) (Fig. 6). The high-affinity folate transporters were shown to take up radiolabeled exogenous folic acid in *T. gondii* (85). If folates are taken up to sustain the acute stage of *T. gondii*, the existence of the biosynthesis pathway is likely relevant for downstream metabolite production or for a different life cycle stage where the parasite encounters limited access to folates or its precursors. Numerous studies have shown the effects of targeting the folate pathway (87, 88). Several anti-parasitic drugs are currently in use, such as sulfonamides targeting dihydropteroate synthase in combination with inhibitors of the dihydrofolate reductase-thymidylate synthase. Although the anti-folates are thought to be safe, recent studies in *P. falciparum* have shown emerging resistance to the once potent drug combination. Future studies would have to unravel the molecular mechanisms of resistance and enable future development of alternative strategies targeting the crucial biosynthesis and scavenge pathways (89). In recent *in vivo* experiments, the contributions of *para*-amino benzoic acid (pABA), a precursor for folate synthesis, were also re-examined (90, 91). pABA is synthesized with the action of two enzymes, aminodeoxychorismate synthase and aminodeoxychorismate lyase. The two genes were knocked out in the rodent malaria parasite *P. berghei*, and the deletions were shown to be dispensable for parasite propagation in mice fed with a conventional diet. However, in mice fed with milk (lacking pABA), the mutants displayed a severe growth phenotype, abolished with the supplementation of pABA (90, 91). In the liver stage, the lack of aminodeoxychorismate synthase was dispensable, suggesting an active salvage, given the folate-rich environment of the liver. The results therefore indicate a combination of salvage and synthesis in *Plasmodium* parasites, to ensure the folate requirements for the fast-growing asexual stages are met.

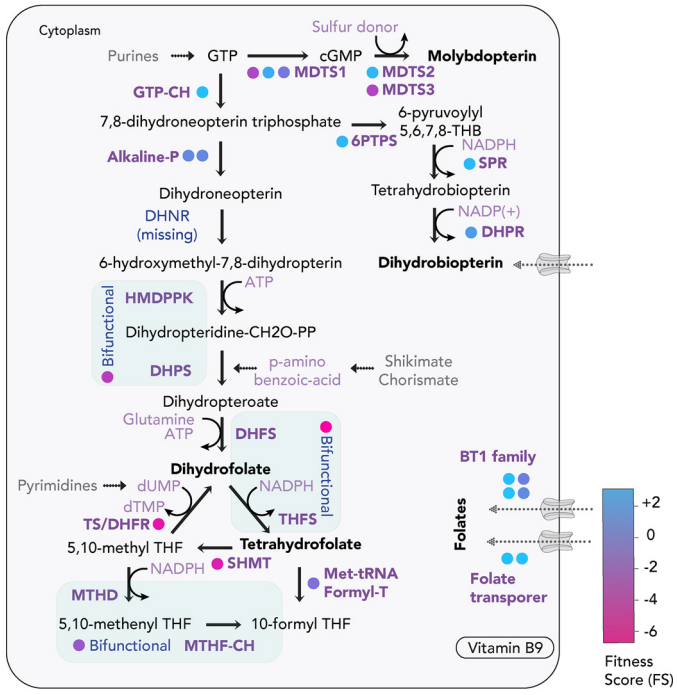

Figure 6. Folate (vitamin B_9) and biopterin biosynthesis and scavenge pathways. *T. gondii* can *de novo*–synthesize or uptake folates and biopterins. FS for the enzymes for the bioconversion are *color-coded* (in *circles*). Enzymes in *blue* are bifunctional, capable of catalyzing two subsequent reaction steps. *TS*, thymidylate synthase; *DHFR*, dihydrofolate reductase; *DHPS*, dihydropteroate synthase; *GTP-CH*, GTP cyclohydrolase; *MDTS1*, molybdopterin cofactor synthesis protein 1 (MOCS1); *MDTS2*, molybdopterin cofactor synthesis protein 2 (MOCS2/MoaE); *MDTS3*, molybdopterin cofactor synthesis protein 3 (MOCS3/MoaB); *6PTPS*, 6-pyruvoyltetrahydropterin synthase; *SPR*, sepiapterin reductase; *DHPR*, 6,7-dihydropteridine reductase; *DHPS*, dihydropteroate synthase; *DHFR*, dihydrofolate reductase; *TS*, thymidylate synthase; *MTHD*, methylenetetrahydrofolate dehydrogenase; *MTHF-CH*, methenyl-tetrahydrofolate cyclohydrolase; *SHMT*, serine hydroxymethyltransferase; *DHFS*, dihydrofolate synthase; *THFS*, tetrahydro-folylpolyglutamate synthase; *Met-tRNA*, methionyl-tRNA formyl-transferase.

Heme

Heme is an essential cofactor required for the function of various enzymes, including cytochromes, catalases, peroxidases, hemoglobin, and others. Heme alternates between an oxidized and reduced state, enabling heme-containing enzymes to catalyze electron transfer reactions in the ETC and other pathways. Heme can be synthesized *de novo*, via a highly conserved eight-step pathway (92, 93). Alternatively, it can be salvaged via heme-binding proteins and porphyrin transporters, which have been partially identified in protozoan parasites such as trypanosomes but remain elusive in apicomplexans (93–95). Whereas *Trypanosoma cruzi* and *Trypanosoma brucei* are unable to synthesize heme, *Leishmania* spp. have acquired the last three enzymes of the biosynthesis pathway via horizontal gene transfer, possibly acquiring and converting heme precursors from the host (93, 96, 97). Within the Apicomplexa, *Cryptosporidia* have lost all enzymes required for heme synthesis, relying entirely on an uptake mechanism. Coccidians and hemosporidians encode all enzymes necessary for *de novo* synthesis of heme (93). They possess a peculiar synthesis pathway, which spans three subcellular compartments, the mitochondrion, apicoplast, and cytosol, and comprises enzymes with distinct ancestral origins (93, 98) (Fig. 7). The parasites utilize the so-called C4 pathway of α-proteobacterial origin, in which the heme precursor δ-aminolevulinic acid (ALA) is synthesized through condensation of succinyl-CoA and glycine in the mitochondrion. δ-ALA is transported to the apicoplast, where the four-step conversion into coproporphyrinogen III occurs, catalyzed by enzymes originating from the algal endosymbiont (98, 99). Coproporphyrinogen III is exported from the apicoplast to the cytosol, where it is converted to protoporphyrinogen IX by a coproporphyrinogen III oxidase (CPO). Protoporphyrinogen IX is subsequently transported to the mitochondrion and converted to heme through the activity of protoporphyrinogen oxidase and ferrochelatase (FC). The contribution of heme uptake *versus* its *de novo* synthesis has been investigated in depth in *Plasmodium* spp. In its blood stages, *Plasmodium* parasites deal with very high levels of heme, which are released during the digestion of hemoglobin. *P. falciparum* detoxifies heme by depositing it in a large crystalline pigment termed hemozoin. Hemozoin formation is mediated by a multiprotein complex in the food vacuole, which contains several proteases and a heme detoxification protein (100). Whereas protein-driven hemozoin formation has been postulated before (101), lipid-driven mechanisms (102, 103) and an autocatalytic process have also been proposed (104). Unsurprisingly, heme synthesis is not essential for *Plasmodium* during the intraerythrocytic development, but the pathway becomes fitness-conferring during liver stages and is essential for development in the mosquito (105–109). Specifically, the loss of FC impairs male gamete formation and ablates oocyst formation in mosquitoes, indicating that *Plasmodium* can utilize salvaged heme but relies on its synthesis when levels of exogeneous heme become limiting within the insect vector (105, 106).

Heme has also been intensely researched for its role in determining sensitivity of the parasite to the antimalarial drug artemisinin. Heme-bound iron derived from *de novo* synthesis or hemoglobin digestion reacts with artemisinin, forming active cytotoxic artemisinin radicals (110–112). It has been shown that enhancing heme synthesis, by providing excess heme precursors, increases the sensitivity of *Plasmodium* to artemisinin. Conversely, the reduction of heme synthesis by genetic means or through pharmacological inhibition decreases sensitivity of both *T. gondii* and *P. falciparum* to artemisinin (113, 114).

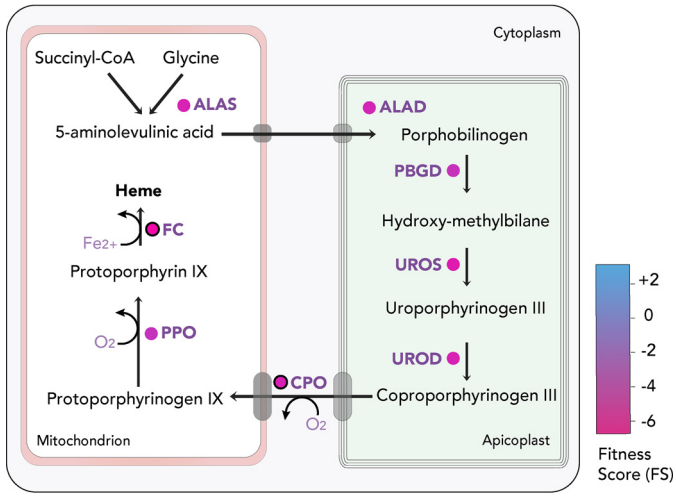

Figure 7. Heme biosynthesis pathway. *T. gondii* can *de novo*–synthesize heme in a complex pathway, compartmentalized between the mitochondrion, cytosol, and apicoplast. FS for the enzymes are *color-coded* (in *circles*). Experimentally validated enzymes are *circled* in *black*.[3] *ALAS*, aminolevulinate synthase; *ALAD*, aminolevulinate dehydratase; *PBGD*, porphobilinogen deaminase; *UROS*, uroporphyrinogen synthase; *UROD*, uroporphyrinogen decarboxylase; *PPO*, protoporphyrinogen oxidase.

Whereas *T. gondii* does not have to deal with copious amounts of heme as in the intra-erythrocytic stage of *P. falciparum*, it is also expected to encounter varying levels of heme during its complex life cycle. Based on their FS, all enzymes implicated in the heme synthesis pathway appear highly fitness-conferring (17) (Fig. 7), indicating that *in vitro* tachyzoites are unable to scavenge sufficient amounts of heme from their host. The enzyme catalyzing the second step of the pathway, ALA dehydratase or porphobilinogen synthase, has been characterized biochemically (115). Its crystal structure revealed that the enzyme functions as an octamer in *T. gondii* and does not contain any metal ions in the active site, although Mg^{2+} ions are present at the intersections between pro-octamer dimers (116). This metal-independent catalysis is unique to apicomplexans and could render the enzyme an attractive target for intervention.

Interestingly, *T. gondii* also encodes two putative and distinct types of coproporphyrinogen oxidases, a CPO and a bacterial-type coproporphyrinogen III dehydrogenase (CPDH) (117, 118). Whereas CPO appears to be highly fitness-conferring based on its FS (−4.64), the oxygen-independent CPDH (+2.29) is the only dispensable enzyme associated with the pathway. Its role in the heme synthesis of *T. gondii* is still unknown, as it may function as the active CPO in a life cycle stage where oxygen levels are limiting. Consistent with this, RNA-Seq data revealed a striking stage specificity, with CPDH being more than 2-fold up-regulated in bradyzoites, oocysts, and sporozoites (20). The role of both enzymes was recently investigated through the generation and characterization of

mutant parasites lacking CPO, CPDH, or both. The results confirmed that in the absence of CPO, parasites are severely impaired in their cell division and the overall lytic cycle is compromised. Contrastingly, parasites lacking CPDH grow normally as tachyzoites and are not affected in stage conversion to bradyzoites or in cyst formation in mice.[3] Furthermore, no aggravation of the phenotype was observed in parasites lacking both enzymes, CPO and CPDH. Overexpression of CPDH in parasites lacking CPO further confirmed a lack of compensation, possibly due to the differential localization of the two enzymes (CPDH in the mitochondrion and CPO in the cytosol).[3] Together, these findings indicate that CPDH is dispensable for both tachyzoites and bradyzoites, highlighting that oxygen levels at these stages are sufficient for the oxygen-dependent CPO to function. Importantly, the activity of CPDH has to date not been formally demonstrated, and misannotations of SAM-dependent enzymes have been reported previously (119). Hence, it remains unclear whether the enzyme truly functions as a CPDH in sporozoites, oocysts, or gametes or whether it functions in a different pathway.

Importantly, although parasites lacking CPO were severely impaired, they remained viable. On the other hand, depletion of the final enzyme, FC, was not tolerated. Mass spectrometry and fluorescence analyses revealed that cells lacking CPO have 10-fold lower heme levels than WT parasites, but 10-fold higher levels of its precursor protoporphyrin IX (ProtoIX).[3] These findings indicate that *T. gondii* likely does not salvage heme itself but rather its precursors ProtoIX or protoporphyrinogen IX from its host. Hence, FC is absolutely essential for the integration of iron into ProtoIX. Conversion of salvaged ProtoIX or protoporphyrinogen IX to heme appears to be inefficient, leading to the described phenotype. This was further supported by the observation that δ-ALA supplementation rescues the growth defect of *T. gondii* lacking CPO. δ-ALA supplementation leads to a drastic increase in host ProtoIX levels, probably boosting its uptake by *T. gondii* and allowing it to restore heme levels.

In parasites lacking CPO, the lack of heme and accumulation of its precursor are expected to cause deleterious impacts on *T. gondii* metabolism and development. Heme is crucial for multiple cellular processes; most notably, it serves as an essential cofactor in several enzymes of the ETC, including cytochrome bc_1 of complex III, soluble cytochrome *c*, and the Cox I subunit of Complex IV (120). It has been proposed that oxidative phosphorylation is the main energy source of tachyzoites and accounts for >90% of the ATP generated in egressed tachyzoites (23). We found that heme depletion in parasites lacking CPO largely disables mitochondrial respiration, although residual low levels of respiration were detected, and parasites devoid of CPO remained sensitive to atovaquone treatment, which inhibits the cytochrome bc_1 complex of the ETC.[3] Strikingly, these parasites appear to survive through markedly increased rates of glycolysis and are unable to survive in the absence of glucose. These observations highlight the importance of *de novo* heme synthesis in *T. gondii* but also demonstrate its astonishing flexibility to adapt and survive solely on an inefficient precursor salvage pathway and rewiring its central carbon metabolism. Given the absence of the heme biosynthesis pathway in *Cryptosporidia* and piroplasms, future research should focus on the identification of heme or hemoprotein transport mechanisms.

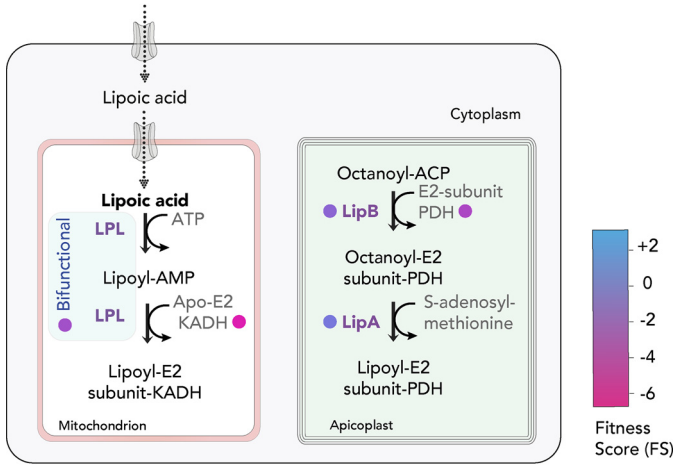

Figure 8. Lipoic acid biosynthesis. *T. gondii* can *de novo*–synthesize lipoic acid in the apicoplast but also scavenge the metabolite from its host for its requirement within the mitochondrion. The bifunctional LPL enzyme (in *blue*) utilizes the scavenged lipoate for the posttranslational modification of branched-chain keto-acid dehydrogenase. LipB and LipA generate lipoate for the modification of the E2 subunit of the apicoplast-resident PDH complex. FS for the enzymes are *color-coded* (in *circles*). *LipB*, lipoyl (octanoyl)-ACP-protein *N*-lipoyl (octanoyl) transferase; *LPL*, lipoate-protein ligase; *LipA*, lipoic acid synthase; *KADH*, branched-chain keto-acid dehydrogenase.

Lipoate

Lipoate, or lipoic acid, is an essential cofactor and, in most eukaryotes, is synthesized in the mitochondrion and transported to other subcellular compartments. In apicomplexans, at least four metabolic complexes use the lipoic acid as a cofactor: PDH, which resides in the apicoplast (121), as well as the α-ketoglutarate dehydrogenase, branched-chain α-ketoacid dehydrogenase, and glycine cleavage complex, which reside in the mitochondrion (27, 122). The coccidians and hemosporidians are able to synthesize and scavenge lipoic acid, whereas the pathways are absent in *Cryptosporidia* and *Piroplasmida*. Unlike plants, which have two isoenzymes, LipA and LipB, for lipoylation in the chloroplast and mitochondria, respectively, apicomplexan genomes encode LipA and LipB. Both enzymes are localized to the apicoplast, and a second enzyme, LplA, is found in the mitochondrion (123). Lipoylation of mitochondrial proteins is dramatically reduced when the parasites are grown in lipoic acid–deficient media without affecting the lipoylation of apicoplast proteins (124). Contrastingly, the reduced lipoylation of mitochondrial proteins could be rescued via exogenous supplementation of lipoate in the media, indicating the salvage pathway primarily supplies lipoate for this organelle (124). As seen by the FS of the LplA gene (−2.60), mitochondrial lipoylation seems essential, whereas LipA (−0.97) and LipB (−1.74) (17) in the apicoplast seem dispensable. In the absence of a lipoylated PDH complex, the parasites likely compensate by taking up fatty acids from the host (Fig. 8). Similar observations were reported during the intraerythrocytic stage of *P. falciparum* (125, 126). The plasma membrane and organellar transporters involved in lipoate salvage have not yet been identified. It is plausible that lipoate is directly scav-

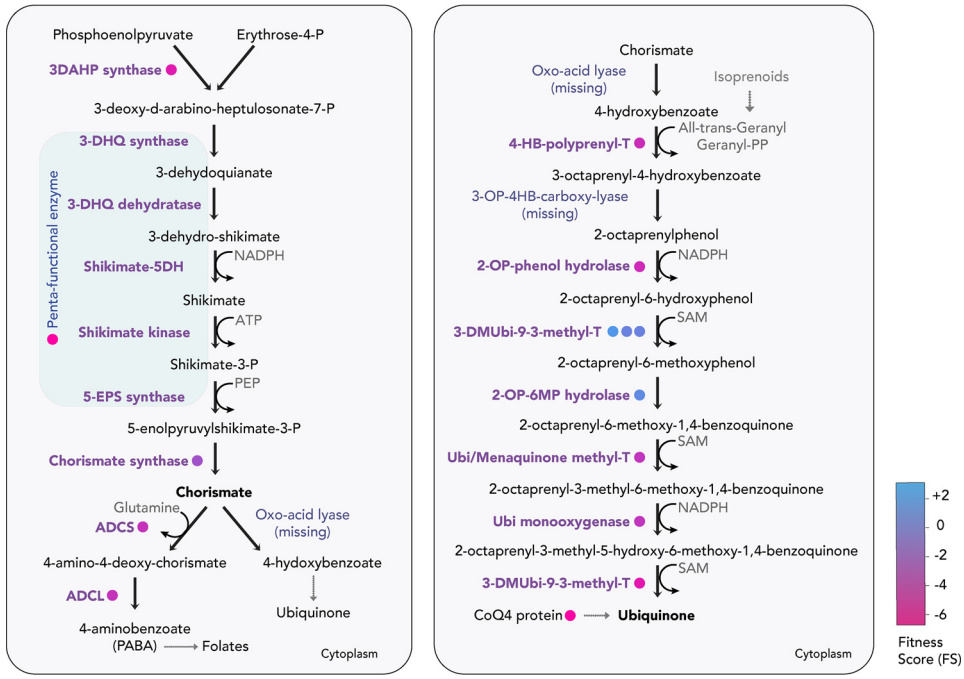

Figure 9. Shikimate, chorismate, and ubiquinone biosynthesis pathway. *T. gondii* can *de novo*–synthesize shikimate and chorismate via a pentafunctional AROM complex, catalyzing the initial five steps (shaded in light blue) and chorismate synthase respectively. Chorismate is a precursor for the biosynthesis of ubiquinone, and the FS for the enzymes are *color-coded* (in *circles*). *3DAHP*, 3-deoxy-D-arabinoheptulosonate 7-phosphate; *5-EPS*, 5-enolpyruvylshikimate-3-phosphate; *3-DHQ*, 3-dehydroquinate; *ADCS*, aminodeoxychorismate synthase; *ADCL*, aminodeoxychorismate lyase; *PEP*, phosphoenolpyruvate; *4-HB*, 4-hydroxybenzoate.

enged from the host mitochondria, which is in close contact with the parasitophorous vacuole (127).

Shikimate

Shikimate is an important metabolite found in bacteria, plants, and fungi but is absent in animals. It is important for several biosynthetic processes, including the biosynthesis of folate, aromatic amino acids, and ubiquinone. Shikimate is primarily synthesized from erythrose 4-phosphate and phosphoenolpyruvate and subsequently converted to chorismate in a seven-step reaction. Steps 2–6 for chorismate biosynthesis are carried out by a pentafunctional protein (Fig. 9). In most apicomplexans, including the coccidians, hemosporidians, and *Cryptosporidia*, a single gene of fungal origin exists, called the AROM complex, encoding for all five activities in a single large polypeptide (128, 129). The presence of all functional domains in *T. gondii* has been verified with bioinformatic analyses (130, 131), although in *P. falciparum* the sequence similarity to the yeast homolog could not be verified for the first two enzymatic activities. However, evidence for the presence of a shikimate pathway was supported in both *T. gondii* tachyzoites and the erythrocytic stage of *P. falciparum*, by treating the parasites with the herbicide glyphosate, inhibitor of the 5-enolpyruvylshikimate-3-phosphate synthase, resulting in a growth defect (132–134). The effect was reversible with the addition of pABA or folate in the media, suggesting an essential role of shikimate in providing precursors for the biosynthesis of folates (133). The role of chorismate in folate biosynthesis has been demonstrated in several studies, but its importance for ubiquinone biosynthesis has not been fully defined. Further, the high negative FS of all enzymes involved in the pathway confirms its essentiality for *in vitro T. gondii* tachyzoites (AROM complex, −5.22; chorismate synthase, −2.84) and could be targeted for intervention against the coccidians and hemosporidians.

Ubiquinone

Ubiquinone, also known as coenzyme Q, is an integral component of the electron transport chain for the transfer of electrons from NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) to cytochrome bc_1 complex (complex III). In most organisms, ubiquinone is synthesized from chorismate in nine enzymatic steps. Most of the pathway is conserved among all apicomplexans, with two enzymes, oxo-acid lyase and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase, missing from the genome, based on bioinformatic approaches. The divergence of these enzymes cannot be ruled out, because a functional synthesis pathway in *P. falciparum* was shown by detecting differences in the ubiquinone side chains when compared with the host (135). The 4-hydroxybenzoate backbone of ubiquinone receives an isoprenoid side chain via the 4-HB-prenyl-transferase, which has been well-characterized in *P. falciparum*, and localized to the apicoplast (136). The production of long-chain isoprenoids, however, occurs in the mitochondrion via farnesyl pyrophosphate synthase (137), which could subsequently be utilized for the synthesis of ubiquinone and other compounds. It was further shown that fosmidomycin, a drug that inhibits the apicoplast-resident isoprenoid biosynthesis pathway, leads to a decline in ubiquinone synthesis (74). In *T. gondii* tachyzoites, the last three steps of the pathway (Fig. 9) display highly negative FS (−3.61, −3.62, and −4.49), highlighting their importance for *in vitro* proliferation.

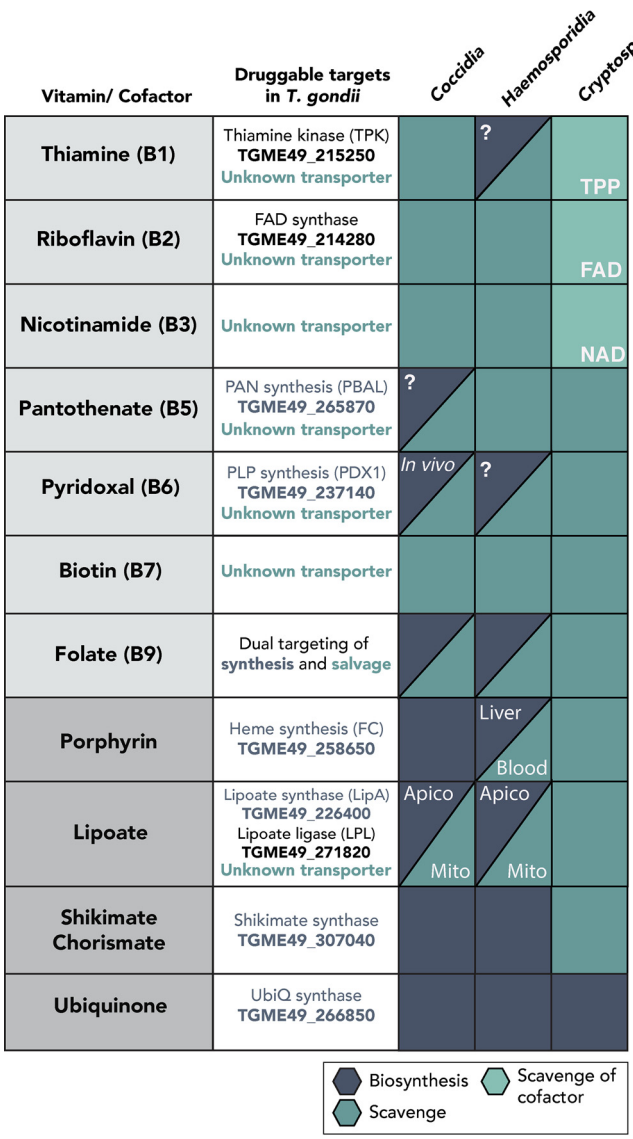

Figure 10. List of potential drug targets in the vitamin and cofactor biosynthesis and salvage pathways within a selected class of apicomplexans. Gene IDs for known genes in *T. gondii* are listed with unknown transporters. Essentialities of the enzymes for known life cycle stages, *in vivo* conditions, or intracellular organelles are marked in *white*. A *question mark* indicates the presence of a biosynthesis enzyme, although its essentiality for a different life cycle stage of the parasite is unknown. *Apico*, apicoplast; *Mito*, mitochondrion.

Conclusion

Apicomplexans possess versatile metabolic capabilities to adapt and adjust to their diverse host environments. Understanding the parasite's requirements for intracellular replication and the contribution of biosynthesis *versus* uptake of essential metabolites is therefore crucial for the identification of new candidate drug targets (Fig. 10). Whereas the genome sequences of the disease-causing pathogens provide us clues on their metabolic capabilities at a global level, an in-depth understanding of the needs at each life cycle stage is vital. Pathways and enzymes that are essential for proliferation during acute infection may be dispensable upon stage conversion to latency and vice versa. Recent studies encompassing computational, molecular, and metabolomic tools have advanced our understanding of metabolic pathways for the production of key vitamins and cofactors, paving the way for targeted drug development. A few commercially available compounds targeting vitamin and cofactor pathways, such as pyrimethamine and sulfonamides, already exist to treat toxoplasmosis or malaria. With the rise in drug resistance, however, identification of new enzymes absent in the mammalian host may be useful for a target-directed intervention against the apicomplexans.

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