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OBSERVATIONS ON BRADYZOITE BIOLOGY

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Abstract

Tachyzoites of the Apicomplexan *Toxoplasma gondii* cause acute infection, disseminate widely in their host, and eventually differentiates into a latent encysted form called bradyzoites that are found within tissue cysts. During latent infection, whenever transformation to tachyzoites occurs, any tachyzoites that develop are removed by the immune system. In contrast, cysts containing bradyzoites are sequestered from the immune system. In the absence of an effective immune response released organisms that differentiate into tachyzoites cause acute infection. Tissue cysts, therefore, serve as a reservoir for the reactivation of toxoplasmosis when the host becomes immunocompromised by conditions such as HIV infection, organ transplantation, or due to the impaired immune response that occurs when pathogens are acquired *in utero*. While tachyzoites and bradyzoites are well defined morphologically, there is no clear consensus on how interconversion occurs or what exact signal(s) mediate this transformation. Advances in research methods have facilitated studies on *T. gondii* bradyzoites providing important new insights into the biology of latent infection.

Keywords

Toxoplasma gondii; Bradyzoite; Cyst; Differentiation; Gene regulation; Stage Specific Genes; Cyst Wall; Latent Infection; Metabolism

INTRODUCTION

Toxoplasma gondii, an Apicomplexan parasite, can cause significant morbidity in both immune competent and immune compromised humans. Transmission of *T. gondii* is due to either tissue cysts containing bradyzoites, the latent asexual stage found in animal tissues, or oocysts containing sporozoites, the sexual stage found in the cat intestinal tract and feces. Dissemination of the infection following ingestion of either of these life stages is due to their transition to tachyzoites which rapidly disseminate in the host, causing disease. *T. gondii* is unusual, among the coccidia, in that it can transition from bradyzoite to tachyzoite and vice-versa, thereby bypassing its definitive host enabling this parasite to propagate clonally in its intermediate hosts. *T. gondii* infects a wide range of hosts and can be transmitted through carnivory, propagating this protozoan through many mammals and birds when infected meat is consumed. Within the human population, it is conservatively estimated that more than one

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billion people are infected. For humans, common routes of infection include ingestion of encysted bradyzoites in infected meat or consuming vegetables contaminated with oocysts from the environment [1]. Ingested parasites can convert to the quickly growing tachyzoites which are able to disseminate throughout the body and with the development of an immune response the infection transitions into a latent (chronic) stage manifested by bradyzoites within tissue cysts (a modified parasitophorous vacuole) [2–4]. Cysts are not static structures, but probably regularly rupture and reinvade new host cells. In individuals who are immunosuppressed or immunocompromised, reactivation of *Toxoplasma gondii* can occur when bradyzoites erupt from cysts and transition back to rapidly proliferating tachyzoites, causing Toxoplasmosis presenting as chorioretinitis or encephalitis [5].

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The success of this intracellular parasite can be attributed its ability to infect and replicate within virtually any warm blooded host while also having the ability to differentiate into a form that can evade the host's defenses. Current drugs such as pyrimethamine and sulfadiazine target tachyzoites, the rapidly replicating growth stage; however, no clinical treatment exists for eliminating *T. gondii* cysts and curing latent infection [6,7]. Discovering drug targets against bradyzoites or the cyst wall may lead to novel compounds for curing latent *Toxoplasma gondii* infection, thereby preventing reactivation. One of the limitations in finding efficient drugs to eliminate chronic infection is that, to date, the majority of research on this organism has been done on tachyzoites; however, advances in technology have allowed investigators to advance our understanding of bradyzoites and the biology of latent infection. In this review, we will highlight research on bradyzoites conducted within the past few years that have provided insights into bradyzoite differentiation and the biology of this important life cycle stage of *T. gondii*.

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BRADYZOITE DEVELOPMENT

Defining the molecular mechanisms that coordinate bradyzoite development has not been a straightforward task. As with most biological systems, there are many components at play when the parasite converts from tachyzoites to bradyzoites and vice versa. In this section, we will discuss the transcription factors and post translational modifications that regulate bradyzoite development and the markers used to identify bradyzoites.

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AP2 transcription factors

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Toxoplasma gondii expresses transcription factors that contain the plant-related DNA binding domain Apetala2 (AP2). Apicomplexan AP2 proteins were originally discovered in Plasmodium and have been found to be involved in malaria development as transcription factors [8]. In the *Toxoplasma gondii* genome, approximately 68 genes have been annotated to contain the AP2 domain [9], and several of these AP2 transcription factors have been found to be regulated in tachyzoites in a cell-cycle manner [10]. Research on these proteins has begun to uncover roles for several AP2s in bradyzoite differentiation. The binding motifs of some of these AP2 transcription factors have been demonstrated to bind to the promoter regions of bradyzoite markers BAG1 and B-NTPase, providing evidence that these AP2 proteins are trans regulators of bradyzoite genes [11–15]. Radke et al. identified the

upregulation of 15 AP2 mRNAs during bradyzoite induction, of which some transcriptionally oscillate in cell cycle manner [11].

AP2 proteins AP2XI-4 and AP2IV-3 have been characterized as bradyzoite activators. Deletion of AP2XI-4 results in the downregulation of bradyzoite markers and impairment of cyst formation. AP2XI-4 binds to a 'CACACAC' cis motif that appears on the promoter of many bradyzoite upregulated genes [15]. Likewise, deletion of AP2IV-3 also results in a downregulation of bradyzoite genes and a modest decrease in cyst numbers during pH 7.8 *in vitro* induction compared to the parental strain. Overexpression of AP2IV-3 upregulated bradyzoite markers such as BAG1 and LHD2 and increased the frequency of cyst formation [12].

Other AP2 proteins, such as AP2IX-9, AP2IX-4, and AP2IV-4, have been characterized as bradyzoite repressors [11–14]. Overexpression of AP2IX-9 suppressed the rate of cyst formation, whereas deletion of this protein increased this rate [11]. Interestingly, AP2IX-4 deletion affected cyst formation negatively even though there was upregulation of bradyzoite markers BAG1, LDH2, and MCP4 [13]. Lastly, AP2IV-4 has been shown as another repressor of bradyzoite differentiation where deletion of this AP2 transcription factor resulted in the upregulation of bradyzoite markers such as BAG1, B-NTPase, ENO1, LDH2, and cyst wall proteins BPK1 and MCP4 and an increase in cyst formation within the RH strain [14]. It is possible, that bradyzoite formation is a default pathway in differentiation and that these types of AP2 repressors are critical for allowing continued tachyzoite gene expression, facilitating acute infection and dissemination of this pathogen in its host.

Research on *Toxoplasma gondii* AP2 has unraveled parts of the genetic programming of bradyzoite differentiation. Uncovering the upstream regulators of these AP2 transcription factors would further elucidate the genetic pathway that regulates bradyzoite differentiation. In addition, discovering additional genes regulated by these AP2 proteins will increase our understanding of the molecular pathways involved in bradyzoite differentiation. These studies on AP transcription factors provide evidence for an important role for AP2 factors in bradyzoite development most likely through interactions with epigenetic markers and binding *cis* elements of diverse sequence motifs.

Post-transcriptional regulation

In addition to transcriptional regulation of bradyzoite differentiation, *Toxoplasma gondii* utilizes post-translational modifications of proteins to exert control over its differentiation process. One aspect of this post-translational control involves eukaryotic Initiation Factor 2 (eIF2) kinases. In other eukaryotes, these kinases have been well characterized to phosphorylate the alpha subunit of eIF2, an initiation factor involved in the start of protein translation in times of stress. Generally, eIF2 α phosphorylation leads to a decrease in global protein synthesis in favor of translating certain transcription factor mRNAs related to the stress response [16]. Within *Toxoplasma gondii*, it has been demonstrated that stress factors, such as pH 8 media, that cause bradyzoite differentiation also led to TgIF2 α phosphorylation by stress-related eIF2 kinases and subsequently, a decrease in protein translation [17–19]. In addition, there is an association of TgIF2 α phosphorylation with preferential translation of AP2 factors in response to endoplasmic reticulum stress with

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tunicamycin treatment [20]. Interestingly, TgIF2 α remains phosphorylated after the parasite has differentiated into bradyzoites, which help explain the slower growing nature of bradyzoites compared to tachyzoites [18]. Konrad et al. has shown that inhibiting TgIF2 α dephosphorylation *in vitro* leads to decreased tachyzoite replication, increased cyst formation, and lower reactivation rates [19]. They also show that these inhibitors of eIF2 α phosphatases inhibited TgIF2 α dephosphorylation and that their results were not from host eIF2 α effects.

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Other means of translational control in *Toxoplasma gondii* include *cis*-acting regulatory RNA hairpins [22], silencing by microRNA [23], tRNA cleavage [24], and modulatory RNA-binding proteins [25,26]. The tachyzoite-associated lactate dehydrogenase 1 (LDH1) mRNA contains a *cis*-acting regulatory element in the form of an RNA hairpin structure [22]. This hairpin has the ability to repress translation, most likely through binding of the 5'UTR to an unidentified *trans*-element for sequestration away from the translation pool. These *cis*-acting elements are a mechanism that *Toxoplasma gondii* may use to repress translation of tachyzoite genes after differentiation. In *Plasmodium*, Puf RNA-binding proteins, which have been characterized to bind to the 3' UTR regions of select mRNAs to control their translation, modulate the differentiation of sporozoites [27,28]. In *Toxoplasma gondii*, a Puf homologue that has RNA-binding capacity has been characterized to be upregulated in bradyzoites although the function of this protein in the differentiation process of the parasite remains unknown [25]. TgAlba proteins have been shown to bind to RNA and interact with proteins that regulate translation. In addition, knockout of TgAlba shows reduced cyst formations *in vitro* and *in vivo* [26]. Other RNA-binding proteins that may play a role in controlling translation during bradyzoite development include Argonautes, DEAD-box helicases, and KH-type splicing regulatory proteins [23,29]. It is possible that upon differentiation, tachyzoite transcripts are translationally repressed within bradyzoites and sequestered by ribonucleoproteins into granules awaiting translation upon reversion back to tachyzoites. Additional mechanisms of translational control in Apicomplexans are reviewed by Holmes et al. [30].

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Sugi et al. characterized *T. gondii* Protein Kinase A catalytic subunit 3 (TgPKAc3) and its effects on bradyzoite differentiation [31]. PKAc isoforms are typically involved in modulating the different cellular processes in other organisms such as response to nutrition starvation and respiratory functions through phosphorylation of its target substrates, modulating their activity. In *Toxoplasma gondii*, they have been characterized to play a role in regulating cell division [32] and bradyzoite differentiation in response to elevated cAMP levels [33]. Sugi et al. showed that deletion of TgPKAc3 resulted in parasites that differentiated at higher rates within human foreskin fibroblast cells, both in normal culture conditions and with pH 8 induction [31]. However, cyst numbers within the mice brain were attenuated when infected with TgPKAc3-KO parasites. Further analysis on the substrates of TgPKAc3 will help elucidate its role in the pathway of bradyzoite differentiation. Other kinases proposed to play a role in parasite differentiation include calcium-dependent protein kinase 1 (TgCDPK1) and mitogen-activated protein kinase like 1 (TgMAPKL1). Inhibitors of bump kinases upregulates bradyzoite markers and affects parasite replication through interactions with TgCDPK1 and TgMAPKL1 [34,35]. While transcription of TgMAPKL1 is increased during early bradyzoite differentiation, deletion of TgMAPKL1 leads to

downregulation of known bradyzoite markers and affects parasite attachment and replication [36].

Transcriptional expression of several rhoptry kinases (ROPK) have been found to be increased during chronic infection compared to acute infection [37,38]. While rhoptry kinases have been characterized for their roles in parasite virulence and protection of the parasite against host immunity-related GTPases [39], the rhoptry kinome has also been shown to play a role in chronic infection [37,40]. Of 32 deleted ROPKs in the Pru Type II strain, 11 ROPK-KOs showed a modest decrease in cyst burden while 5 ROPK-KOs showed a significant reduction in cyst numbers. Further characterization on the substrate and functional activities of these rhoptry kinases during bradyzoite differentiation are required.

Given that transcription of parasites genes do not always reflect the protein level [41,42], *Toxoplasma gondii* has been shown to use post-transcriptional processes to temporally regulate translation and post-translational modifications to functionally regulate its proteins during differentiation. Additional post-translational modifications utilized by *T. gondii* such as acetylation and ubiquitination is reviewed by Yakubu et al [43].

Bradyzoite markers

Localizations of the various bradyzoite markers can be found in Figure 1.

Tachyzoites and bradyzoites have different energy demands as bradyzoites primarily utilize glycolysis for their energy source [44]. To this end, this organism has been found to express different stage specific isoforms of metabolic enzymes, which probably play a role in the energy requirements needed for that life cycle stage. During anaerobic growth, lactate dehydrogenase (LDH) is important for supplying energy to the parasite by converting pyruvate to lactate in the glycolytic pathway. *Toxoplasma gondii* LDH occurs in two forms: TgLDH1 (tachyzoite-expressed) and TgLDH2 (bradyzoite-expressed) [45]. TgLDH2 is highly upregulated within bradyzoites [46,47] and is able to function efficiently under acidic and pyruvate-rich conditions [45,48]. Knockout of TgLDH2 (TgLDH2) in the Pru Ku80 strain does not affect bradyzoite differentiation *in vitro*, but does reduce cyst formation *in vivo*. Interestingly, while TgLDH1 expression is higher in tachyzoites, deletion of this protein in the Pru Ku80 strain reduces bradyzoite development *in vitro* and also impairs cyst formation *in vivo*. When both TgLDH1 and TgLDH2 are deleted, a phenotype similar to the TgLDH1 is seen [49]. These results confirmed earlier studies that knock down of TgLDH2 and TgLDH1 reduced cyst numbers *in vivo* [50]. Interestingly, when TgLDH1 and TgLDH2 are overexpressed in Type I RH strain, an increase in *in vitro* cyst formation occurs under alkaline conditions [51].

Enolases (ENO) are enzymes involved in glycolysis that catalyze 2-phosphoglycerate to phosphoenolpyruvate. TgENO1 has been characterized to be highly expressed transcriptionally and translationally in *ex vivo* bradyzoites (tested in the type II 76 K strain), compared to *in vitro* tachyzoites. The tachyzoite isoform of enolase, TgENO2, had the opposite expression, where transcriptional and translational levels were higher in tachyzoites [52]. When TgENO1 is deleted, there is a reduction in cyst burden within mice [53]. As TgENO1 and TgENO2 localize predominately to the nucleus of bradyzoites and tachyzoites,

respectively [54], the stage specific expression and localization of these enolases suggest a role for them in transcriptional stage interconversion. Indeed, these enolases have been shown the ability to bind to chromatin, regulate the expression of many genes, interact with the promoter of MAG1, and bind to 'TTTTCT' DNA motifs [53,55]. In addition, the promoters of TgENO1 and 2 have been characterized to bind of nuclear extracts from tachyzoites or bradyzoites [56]. It has been demonstrated that intron retention of TgENO1/2 and TgLDH1/2 transcripts plays a role in the stage-specific expression of these genes [57]. Lunghi et al. showed that unspliced transcripts of TgENO and TgLDH are produced by the parasite and await a differentiation signal before splicing to mature transcripts.

Bradyzoite antigen 1/Heat shock protein 30 (BAG1/TgHsp30) was one of the first bradyzoite-specific genes to be characterized. As one of the most abundant proteins within the bradyzoite, BAG1 staining is widely used as a marker for bradyzoites with its localization in the bradyzoite cytoplasm [58–60]. Although the expression of BAG1 is high within bradyzoites [46,47], deletion of BAG1 does not prevent bradyzoite development, but it does result in decreased *in vivo* cyst formation (tested in the type II PLK strain) [61]. BAG1 and TgLDH2 promoters have been commonly exploited for upregulating the expression of other markers and proteins within the bradyzoite [11,62–65]. Aside from BAG1, other heat shock proteins have been associated with bradyzoite differentiation. TgHsp60 transcripts from the PLK strain are upregulated within *in vivo* bradyzoites compared to *in vitro* tachyzoites though Hsp60 proteins levels do not seem to differ between these two stages [63]. Likewise, TgHsp70 expression is upregulated in alkaline conditions (tested in the Type II ME49 strain) and localizes to the parasite cytoplasm, similar to BAG1 [64]. Recently, the 3D structures of TgHsp60 and 70 were predicted based on homology modeling, which might aid in the development of drugs against these *Toxoplasma gondii* heat shock proteins [68]. Another cytoplasmic heat shock protein, TgHsp90, has increased expression under alkaline conditions and localizes to the nucleus and cytoplasm in bradyzoites [69]. Deletion of TgHsp90 reduces the expression of BAG1 and MAG1 (tested in a type I strain which typically do not differentiate well to bradyzoites) [70]. DnaK-tetratricopeptide repeat (TgDnaK-TPR), another heat shock protein, has been shown to be upregulated within Type II strains, though its function has yet to be characterized [71].

Lekutis et al. provide an excellent review of *T. gondii* surface proteins [72]. Tachyzoites have specific plasma membrane proteins such as surface antigen 1 (SAG1/p30) [73], 2A (SAG2A/p22), and SAG1-related sequence proteins 1-3 (SRS1-3) [72], which are involved in host cell attachment and invasion, parasite virulence, and regulation of the host immune response to tachyzoites. Bradyzoites also express their own surface antigens including SAG4A [74,75], SAG2C, SAG2D [76], SAG2X, SAG2Y [77], SRS9 [78], and P-type ATPase (TgPMA1) [79]. While P36, an antibody against a bradyzoite surface antigen, was initially reported as recognizing bradyzoite specific recombinant protein 4 (BSR4), a member of the SRS family [80], it is now known that the correct P36 antigen is SRS9 [81]. The crystal structure of BSR4 is significantly distinct from SAG1 with structural and chemical diversity between their ligand binding grooves, which give support that these two proteins interact with different host ligands [82]. A study on SAG2C/D/X/Y that examined the deletion of the locus encoding all of these surface antigen isoforms (in the Type II Pru *T. gondii*) demonstrated a reduction in cyst numbers in SAG2C/D/X/Y *T. gondii* [77].

Another bradyzoite surface antigen SRS9 is highly expressed within bradyzoites [78]. When SRS9 is knocked out (in the Type II Pru strain), these SRS9 parasites persist longer within the brains of Balb/c mice than the parental parasites and also produced fewer cysts in the brains of infected CBA/J mice [83]. These studies suggest that bradyzoite surface antigens may be involved in the persistence of chronic infection. Lastly, TgPMA1 is upregulated in bradyzoites and shows punctate staining on the parasite plasma membrane. *T. gondii*

TgPMA1 parasites have a downregulation of bradyzoite genes and produce fewer bradyzoite-positive vacuoles *in vitro*; however, these parasites are still able to form mature cysts *in vivo* suggesting that TgPMA1 may have a role in early bradyzoite differentiation [79,84].

In addition to dense granule proteins which are found in the intravesicular membrane network (i.e. matrix), tachyzoites have also been shown to secrete Nucleoside Triphosphate Hydrolases (TgNTPases) and rhoptry proteins into the lumen of the parasitophorous vacuole [85,86]. While a bradyzoite-specific NTPase (B-NTPase) has been identified [46,64], its localization within the bradyzoite stage remains unknown. Most rhoptry proteins have been characterized for their roles within tachyzoites. Bradyzoite rhoptry protein 1 (BRP1) is a rhoptry protein that is only secreted from the rhoptries after parasite differentiation and it localizes to the lumen (i.e. the matrix) of the parasitophorous vacuole. Deletion of this protein showed no defect in cyst formation or bradyzoite development and further studies on its function needs to be completed [87].

Suppressive subtractive hybridization has been used to find differentially regulated genes and identified a protein with ankyrin domains (TgANK1) and TgDnaK-TPR as bradyzoite-specific genes. Both TgANK1 and TgDnaK-TPR contain tetratricopeptide repeats (TPR) [88]. TPR domains have been reported to play roles in protein-protein interactions, transcription, protein folding, and cell cycle processes [89–91]. Using an ectopic promoter, TgANK1 was shown to localize mainly to the parasite cytoplasm with some staining in the nucleus [88]. TgANK1 was found to be upregulated in the Type I strain RH-ERP over GT1 [92]. TgDnaK-TPR is upregulated during *in vitro* bradyzoite conditions and localizes to the cytoplasm of *in vitro* and *in vivo* bradyzoites. Yeast two-hybrid screens demonstrated that TgDnaK-TPR interacts with co-chaperone p23 [71]. Further experiments looking at these proteins within other *T. gondii* strains will aid in validating these genes as a bradyzoite markers. A *T. gondii* specific deoxyribose phosphate aldolase-like gene (TgDPA) has been described as another protein that is upregulated in bradyzoites (examined in Type II strains) [78,93]. TgDPA antibodies demonstrate that this is a cytosolic protein. Although the catalytic domain of TgDPA is not conserved, TgDPA has been characterized to bind to actin depolymerization factor [94].

Recently, a proteomic study has been performed on *Toxoplasma gondii* on its different developmental stages [95]. This study might aid in identifying other markers specific to each developmental stage. Cyst wall proteins and cyst wall binding lectins have also been used as common markers of bradyzoites, which are discussed in the section on the cyst wall.

BRADYZOITE BIOLOGY

There are many differences between the biology of the tachyzoite and bradyzoite [96]. In this section, we will review recent advances in knowledge on bradyzoite replication, energy storage, and cyst wall structure.

Replication

Tachyzoite replication occurs through synchronous endodyogeny whereby all mother parasites within the vacuole will split into two daughter cells at the same time. Therefore, the number of parasites within a tachyzoite vacuole is usually 2^n , where n is the number of times the parasite has already replicated since invading the host cell [97]. It is known that slowing of the tachyzoite cell cycle is associated with trigger of the differentiation program of the parasite to differentiate into bradyzoites [98,99]. Although blockage of the cell cycle does not lead to bradyzoite differentiation [100], it has been presumed that fully differentiated bradyzoites in tissue cysts reside in a quiescent G_0 state. A recent paper by Watts et al, however, has demonstrated that parasite replication within the cyst is not quiescent. Using inner membrane complex protein 3 (IMC3) as a marker for newly replicated parasites, they have shown that bradyzoites within *ex vivo* cysts can replicate synchronously (cyst shows uniform IMC3 staining amongst all parasites) or non-synchronously (non-uniform staining of IMC3 of parasites within cyst) [101]. Synchronous replication within tachyzoite vacuoles has been attributed to a connective structure between parasites that is lost when certain myosin proteins are deleted [102]. Although this structure seems to be absent within bradyzoite cysts, it does not explain the synchronous division that occurs within certain cysts. Watts et al. demonstrated that *in vivo* bradyzoites are not just dormant entities that reside within cysts and remain metabolically and replicatively inert until transmission.

Amylopectin

One of the obvious morphological differences between tachyzoites and bradyzoites seen using transmission electron microscopy is the abundance of clear vacuolar granules in the bradyzoite cytosol. These structures have been found to contain the plant starch polysaccharide, amylopectin [103,104]. It has been hypothesized that these amylopectin granules serve as energy reserves for bradyzoites for utilization during chronic infection when nutrient acquisition may be limited due to the cyst wall. While it has been known for long that amylopectin catabolic and anabolic genes are upregulated in bradyzoites and tachyzoites, respectively [104], nothing else was known about the regulation of these sugar granules until recently.

Uboldi et al. have demonstrated that a *T. gondii* specific calcium-dependent protein kinase (TgCDPK2) plays a role in the regulation of amylopectin in *Toxoplasma gondii* [105]. Deletion of TgCDPK2 in RH, a type I strain, and Pru, a type II strain, resulted in abnormal parasite morphology as well as aberrant amylopectin accumulation. TgCDPK2 parasites shed high amounts of starch molecules into the residual body in the tachyzoite stage and harbored abnormally large amylopectin granules in the cytoplasm of bradyzoites, disfiguring their morphology. In addition, the authors found that the N-terminal carbohydrate-binding

domain as well as the catalytic kinase domain of TgCDPK2 are required for normal amylopectin storage. They demonstrated that TgCDPK2 regulated the activity of pyruvate phosphate dikinase, an enzyme involved in amylopectin generation. Lastly, TgCDPK2 parasites were shown to be unable to form cysts *in vivo* [105], providing evidence that regulation of amylopectin metabolism is required for successful chronic infection.

Recently, Sugi et al. characterized the role and regulation of glycogen phosphorylase 1 (TgGP1) in amylopectin metabolism of *Toxoplasma gondii* [106]. In other organisms, GP1's starch digestive functions and regulation through its N-terminal phosphorylation have been described [107,108]. In RH and Pru strains that had TgGP1 deletion, amylopectin, visualized by Periodic acid–Schiff staining, grossly accumulated in both the tachyzoite and bradyzoite stages [105,106]. To further understand the mechanism of how TgGP1 is regulated in the context of amylopectin catabolism, TgGP1 mutants were created that substituted glutamate for serine₂₅ to mimic phosphorylation and to create a hyperactive TgGP1 or substituted alanine for serine₂₅ to produce a phospho-null mutant that has ablated activity. The hyperactive state of TgGP1^{S25E} was not able to retain amylopectin while the phospho-null mutant TgGP1^{S25A} had abnormal accumulation of starch, similar to the TgCDPK2 and TgGP1 knock outs. While amylopectin dysregulation doesn't seem to affect tachyzoite replication, as they can discard excess starch polysaccharides within the residual body, having the correct amount of amylopectin and the ability to access these starch polymers for energy is crucial for bradyzoites during chronic infection. Dysregulation of TgGP1 leads to an imbalance of amylopectin stores, with too little or too much amylopectin impairing cyst formation within mice [105,106]. Thus, appropriate amylopectin regulation is needed for successful bradyzoite persistence *in vivo* as too much amylopectin may physiologically disrupt the bradyzoite and too little may not provide enough energy for bradyzoite replication [101] or production of new cysts [109].

While the kinase responsible for the direct phosphorylation of TgGP1 remains unknown, mammalian and yeast GP regulation by serine phosphorylation is primarily due to phosphorylase kinase (PhK) and cAMP-dependent protein kinase (PKA). While TgPKAc3 plays a role in bradyzoite differentiation [110], TgPKAc3-KO parasites do not show an abnormal amylopectin phenotype. This suggests that TgPKAc3 is not directly involved in phosphorylating serine₂₅ on TgGP1 though this phosphorylation event could have been compensated by other kinases. Because Uboldi et al. showed that TgCDPK2 parasites also accumulated abnormal amounts of amylopectin similar to the TgGP1 or TgGP1^{S25A} mutants, Sugi et al. also investigated the role of TgGP1 in the TgCDPK1 background. It was shown that TgGP1^{S25A} and TgGP1^{S25S} in TgCDPK2 parasites had excessive amylopectin accumulation while TgGP1^{S25E} displayed ablated amylopectin stores. Although the TgGP1^{S25E} mutant overcompensates for the abnormal amylopectin accumulation of the TgCDPK2 parasites, it does not rule out TgCDPK2 as a regulator of TgGP1. In addition, TgCDPK4-9 are also not likely to be the kinases that regulate TgGP1 as no defect in amylopectin storage was reported in TgCDPK4-9 knockout parasites [111,112]. While it has been shown that TgCDPK2 phosphorylates the catabolic enzymes of amylopectin upstream of TgGP1 [105], further studies on the kinases and phosphatases responsible for regulating TgGP1 is required to fully elucidate amylopectin catabolism.

These experiments provide evidence that post-translational modifications of enzymes involved in starch metabolism are mechanisms that *Toxoplasma gondii* utilizes to regulate amylopectin balance. Therapeutics that target enzymes that regulate amylopectin storage may be an avenue of eliminating cysts and chronic infection.

Cyst wall

One of the defining characteristics of bradyzoites is the presence of a cyst wall, a modified parasitophorous vacuole membrane, that envelopes the parasites. Under electron microscopy, the cyst wall can be seen as a 250 nm thick layer of electron dense material containing vesicles and tubular structures underneath the parasitophorous vacuole membrane [113,114]. The lumen of the cyst contains the matrix, which consists of tubules, vesicles, and amorphous proteinaceous material. Lemgruber et. al has shown that the cyst wall consists of a compact outer layer and a looser sponge-like layer which extends into the cyst matrix [113]. The matrix is in contact with the cyst wall, and it is likely that the vesicles and tubules are involved in the transport of materials from the bradyzoites to the cyst wall. While tachyzoite parasitophorous vacuole membrane is permeable for molecules up to 1291 Da [115], the cyst allows 10 kDa molecules to pass into the matrix, though with limited diffusion throughout the cyst [113]. The cyst aids the parasite in establishing chronic infection within its host, protects the parasite from the host immune responses, shields the parasites during ingestion from harsh digestive environments, and enhances transmissibility of the parasite to the predator of the host. Defining the mechanisms by which *T. gondii* creates the cyst wall will help explain the persistent nature of this parasite inside the host and unravel the function of the cyst. In addition, targeting the cyst wall may become one method to eliminate chronic infection from the host and prevent reactivation.

A common method that is used to demonstrate the presence of the cyst wall is to use lectins that bind to sugars on the cyst wall. Such sugars include N-acetyl-D-glucosamine (detected by wheat germ agglutinin), alpha-methyl-mannoside (detected by Concanavalin A), N-acetyl-D-galactosamine (detected by soy bean agglutinin), and α -N-acetylgalactosamine (detected by lectins from *Dolichos biflorus*, *Vicia villosa*, *Artocarpus integrifolia*, and *Helix pomatia* species) [116–118]. These sugars play an important role in the biology of the cyst as disruption of glycosylation alters cyst biology resulting in a reduction in *in vivo* cyst numbers and/or fragile cysts. For example, deletion of the *Toxoplasma gondii* nucleotide-sugar transporter (TgNST1) results in the loss of α -N-acetylgalactosamine and N-acetyl-D-glucosamine in the cyst wall. The TgNST1 parasite has a reduction in cyst numbers within infected mouse brain [119]. These findings demonstrate an important role for post translational glycosylation of cyst wall proteins involved in formation of the cyst wall.

Toxoplasma gondii N-acetylgalactosaminyltransferases 1 to 3 have been recently characterized for their roles in hierarchical O-GalNAc glycosylation. Deletion of these glycosyltransferases resulted in more fragile cysts [118]. It has been demonstrated by Tomita et al. that these glycosyltransferases modify cyst wall protein 1 (CST1), a major structural component of the *T. gondii* cyst wall [118,120], in a hierarchical manner. Deletion of CST1 results in reduced cyst numbers in mice as well as cysts that are more fragile [60]. CST1 is probably a critical scaffolding protein for assembly of the cyst wall as evidence for CST1

having this function is seen in the “leaky cyst phenotype” with CST1 parasites (Figure 2). Using an antibody against an unknown matrix antigen, it can be seen that this matrix protein leaks into the host cell in the CST1 parasite, but is found in the cyst wall and matrix in wild type parasites.

Several dense granule (GRA) proteins have been shown to localize to the cyst wall although their staining within the cyst wall varies [59,121,122]. GRA proteins have been well characterized to play a role in the formation of the intravacuolar network within the parasitophorous vacuole [123]. It is tempting to speculate that the tubular structures seen within the cyst wall are derived from the intravacuolar nanotubules of tachyzoites since GRA proteins that form this structure are also found within the cyst wall layer and membrane [121,124,125]. Deletions of GRA4 and GRA6 (in a Type II Pru strain) have led to reduced cyst numbers in mouse brains [126], while deletion of GRA24 shows enhanced cyst wall formation *in vitro* [127]. Interestingly, deletion of aspartyl protease 5 (ASP5), a protein involved in the processing of several GRA proteins and the formation of the intravacuolar network, resulted in parasites that had irregular cyst wall staining [128]. More research on the roles of GRA proteins within bradyzoites and their contributions to the cyst wall is needed.

In addition to CST1 [118,120], other proteins discovered to be within the cyst wall include bradyzoite pseudokinase 1 (BPK1) [47,129], Microneme-Adhesive-Repeat domain containing protein 4 (MCP4) [47], matrix antigen 1 (MAG1) [130], proteophosphoglycan 1 (PPG1) [131], and brain colonization protein 1 (BCP1) [132]. BPK1 has been shown to be one component of the cyst wall that protects the parasite from pepsin-HCl treatment, which mimics the conditions in the stomach. Knockout of BPK1 shows a decrease in *in vivo* cyst formation after 8 weeks post infection and a decrease in cyst diameter, compared to the parental strain [129]. PPG1 knockout has been shown to downregulate BAG1 expression [131]. Interestingly, BCP1 localizes to the cyst wall under immunofluorescence within *in vivo* cysts but not *in vitro* derived cysts [132].

Further research is required to understand the interactions of the identified cyst wall proteins in the formation and function of the cyst wall. Furthermore, a detailed proteomic study of the cyst wall would provide the data needed to develop a comprehensive model of this crucial structure involved in latency and transmission.

CONCLUSIONS

There has been much progress in identifying the transcriptional and translational mechanisms that *Toxoplasma gondii* utilizes for development of its different stages. However, more research is still required to fully understand the genetic pathways and molecular mechanisms that drive tachyzoite to bradyzoite interconversion. Furthermore, more research needs to be conducted on the biology of the bradyzoite such as studying the regulation of amylopectin and the parasite’s utilization of these energy stores to help understand the energy demands the parasite requires to replicate within cysts. Lastly, studies on the cyst wall, especially its formation and its components, will facilitate our

understanding of how *T. gondii* achieves and maintains latency within its host cell and remains infective for transmission to its next host.

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Figure 1.
Localization of various tachyzoite and bradyzoite markers

Figure 2. Leaky cyst phenotype

CST1-knockout (*cst*) strain of *T. gondii* stained with mAb 4B8 that recognizes an unknown cyst wall/cyst matrix protein (A); co-localized with DAPI (B). Parental strain of *T. gondii* (Pru Ku80) stained with mAb 4B8 (C); co-localized with DAPI (D). *T. gondii* strains were cultured in bradyzoite conditions (pH 8.0, 0.5% CO₂).