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REVIEW

A latent ability to persist: differentiation in *Toxoplasma gondii*

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Abstract

A critical factor in the transmission and pathogenesis of *Toxoplasma gondii* is the ability to convert from an acute disease-causing, proliferative stage (tachyzoite), to a chronic, dormant stage (bradyzoite). The conversion of the tachyzoite-containing parasitophorous vacuole membrane into the less permeable bradyzoite cyst wall allows the parasite to persist for years within the host to maximize transmissibility to both primary (felids) and secondary (virtually all other warm-blooded vertebrates) hosts. This review presents our current understanding of the latent stage, including the factors that are important in bradyzoite induction and maintenance. Also discussed are the recent studies that have begun to unravel the mechanisms behind stage switching.

Keywords *Toxoplasma* · Toxoplasmosis · Differentiation · Encystation · Tachyzoite · Bradyzoite · Latency · Gene regulation · Epigenetics · Immunity

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of infecting warm-blooded vertebrates worldwide [1]. Although the parasite cannot survive outside the host cell, it has a significantly high transmissibility. It is estimated that approximately one-third of the human population is infected with *Toxoplasma* [2]. It is one of the most widespread parasites due to its large range of host species and ability to transmit between hosts through diverse routes [3].

Toxoplasma belongs to the phylum *Apicomplexa,* which includes other important pathogens such as *Plasmodium, Eimeria*, *Babesia, Neospora, Sarcocystis,* and *Cryptosporidium* [4–6]. Upon initial infection of an immunocompetent host with *Toxoplasma,* an often asymptomatic acute infection is followed by establishment of chronic infection, which is marked by the formation of intracellular tissue cysts. These cysts can persist within the host tissues throughout the life of the host, maintained in the quiescent state by its immune system. Upon immune suppression, the bradyzoites in the cyst will transition back into proliferating tachyzoites and can lead to devastating tissue destruction. Reactivated toxoplasmosis is a major concern in HIV-infected individuals and other immunocompromised patients [7]. In most cases, due to the proclivity of the latent bradyzoite to reside in central nervous system tissues, encephalitis is usually the main cause of death after reactivation of infection [8]. In addition, *Toxoplasma* tachyzoites are capable of crossing the placental blood barrier to be transmitted vertically from mother to fetus. Vertical transmission can occur if an immunologically naïve woman becomes infected with the parasite during her pregnancy, and can cause serious congenital birth defects or spontaneous abortion [6, 9]. Ocular toxoplasmosis is also a common manifestation of *Toxoplasma*, in which the retina of the eye may be destroyed in both healthy and immunocompromised individuals [10]. Ocular toxoplasmosis was traditionally thought to stem from reactivation of a congenital infection, but acquired infection is now also appreciated to be a main cause of this disease [11], particularly in South America. *Toxoplasma* has

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also been linked with water borne outbreaks in humans and deaths in marine wildlife due to contamination of water with oocysts [12–14], which has led the parasite to being classified as a Category B Biodefense pathogen by NIAID.

A crucial event for parasite transmission and pathogenesis is the stage conversion between the actively replicating tachyzoites and the encysted bradyzoites. Conversion to a dormant tissue cyst augments parasite persistence inside the host, which, in turn, increases the chances of transmission through predation. Bradyzoites, therefore, provide *Toxoplasma* with extraordinary flexibility with respect to transmission, allowing the parasite to bypass its sexual stage to disseminate [3]. Of clinical relevance, the bradyzoite cysts appear to be refractory to current available drug treatments, giving rise to chronic infection that presently cannot be eradicated.

Characteristics of the bradyzoite cyst

Tissue cysts are intracellular structures that may develop in any visceral organ; however, they exhibit tropism for neural and muscle tissues. In vivo, cysts are most commonly detected in the brain, the eye, and in both skeletal and cardiac muscles [8]. Within the central nervous system (CNS), cysts have been detected in neurons, astrocytes, and microglia [15–17], although recent studies show that parasites primarily interact with neurons in mice [18, 19].

The size of immature cysts has been estimated to be about 5 µm with at least two parasites inside, indicating that cyst wall formation is an early event in bradyzoite development [20–23]. Furthermore, using bioluminescence imaging technology in combination with parasites expressing luciferase under the control of a bradyzoite-specific gene promoter, it was demonstrated that in vivo formation of bradyzoites commences as early as 1 day post-infection [24]. As the cyst matures, changes in its size may be observed due to bradyzoite replication within the cyst [25]. Mature tissue cyst size and shape is variable; on average, a mature brain cyst is spherical, about 10–70 µm in diameter and the number of parasites within the cyst may reach the thousands. In muscle tissues, cysts are generally more elongated in length and may reach a size up to 100 µm [8].

A number of major ultrastructural differences have been observed between the tachyzoite and bradyzoite stages. Bradyzoites are more slender than tachyzoites and have a pronounced crescent shape [26]. Bradyzoites contain a nucleus located at their posterior end and have electron dense rhoptry organelles and numerous micronemes [8, 16, 27]. Furthermore, bradyzoites contain amylopectin granules that stain red with periodic acid Schiff (PAS) [8]. It has been reported that in vitro differentiated bradyzoites may lose the parasite's apicoplast, a non-photosynthetic plastid-like organelle [22, 28]. Mature bradyzoites represent a growth-arrested stage of the parasite with a uniform 1 N content nuclear DNA [29]. Although this stage is generally considered to be quiescent, it may still be motile. In vitro bradyzoites have been observed exiting from an existing cyst to invade a new host cell and establish a new cyst [22]. This feature could explain the clusters of cysts that have been observed in vivo as well as the increase in cyst burdens in chronically infected mice. Recently, a novel method for isolating tissue cysts from brains of infected mice has been developed and has permitted a detailed study of in vivo cyst characteristics [30]. Analysis of these enriched cysts by immunostaining for a parasite surface protein found that clusters of parasites within a subset of cysts were undergoing cell division in an asynchronous manner, providing some of the first direct evidence that tissue cysts are more dynamic than previously believed [30].

Transformation of the parasitophorous vacuole membrane into the cyst wall

The formation of the cyst wall and the matrix material provide a physical barrier to host immune factors. The cyst wall is visible as a reinforcement of the parasitophorous vacuole membrane (PVM) that occurs beneath this membrane [16]. Typically, the PVM that contains the cyst is elastic and thin (< 0.5 µm), consisting of chitin or similar polysaccharides and glycoproteins that can be detected using various fluorescently labeled lectins [31–33]. The agglutinin from *Dolichos biflorus* (DBA) that recognizes α-linked *N*-acetyl galactosamine has high affinity for the bradyzoite cyst wall structure and is regularly used to detect cysts in tissue culture (Fig. 1). Currently, only a few components of the bradyzoite cyst wall have been identified and their molecular functions remain unclear. CST1, a glycoprotein recognized by DBA, localizes in the cyst wall under the PVM [33, 34]. CST1 was recently identified as being an *O*-glycosylated protein with a mucin domain, previously annotated as SRS44 and disruption of the gene results in impaired cyst formation and extremely fragile cysts [28]. Dense granule proteins (GRA proteins) have also been found in the bradyzoite cyst wall [34]. At the bradyzoite stage, GRA5 is predominantly localized to the modified PVM rather than the granular material that precipitates underneath this membrane [23, 34]. The other GRA proteins that have been observed in the cyst wall so far are GRA1, GRA2, GRA3, GRA6, and GRA7 [23, 34–36]. MAG1 (Matrix antigen 1) is another cyst wall and matrix-specific antigen [37]. Notably, MAG1 is also detected inside the parasitophorous vacuole of the tachyzoite stage but is present in significantly lower abundance, suggesting that it has an important role in the formation of the bradyzoite cyst wall [34, 38]. Recently, three novel cyst wall proteins were

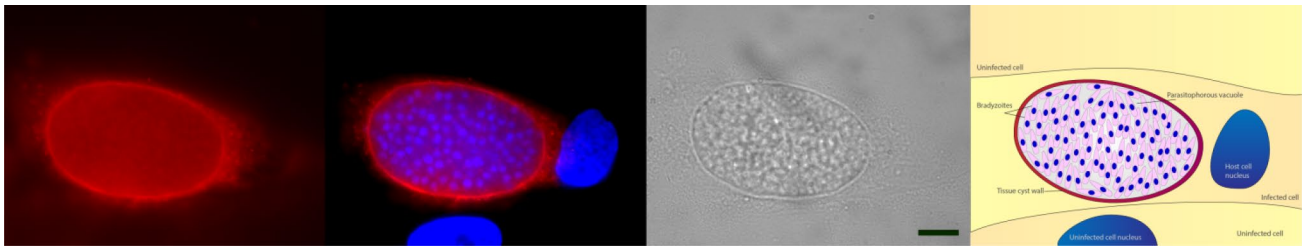

Fig. 1 5-day-old, in vitro bradyzoite cyst induced under alkaline conditions, stained with fluorophore-linked lectin from *Dolichos biflorus* (red), which binds to the polysaccharide-rich cyst wall. Blue: 4′,6-diamidino-2-phenylindole, DAPI (nucleus). Scale bar 10 µm. Labeled schematic depicts the stained tissue cyst in the infected host cell

identified: a bradyzoite pseudokinase (BPK1), a microneme adhesive repeat domain-containing protein 4 (MCP4), and a proteophosphoglycan [39–41], but the functions of these proteins have yet to be resolved. Further characterization of the cyst wall and its components are required to understand the molecular mechanisms of tissue cyst development.

Identification of genes contributing to bradyzoite development

Bradyzoites express certain genes exclusive to this stage of the life cycle. The findings that bradyzoites express specific antigens such as surface antigen 4 (SAG4); bradyzoite surface antigen, BSR4; surface antigen 2/2D (SAG2/2D), matrix antigen 1 (MAG1); 116-kDa cyst wall antigen (CST1); bradyzoite antigens 1–5 (BAG 1/5), specific metabolic enzyme isoforms (lactate dehydrogenase 2, LDH2; enolase 2, ENO1) [42–51] have led to extensive research focused on describing the function of bradyzoite-specific genes.

Initial attempts tried to identify genes that are specifically expressed or induced during *Toxoplasma* stage conversion. In an early study, a cDNA library was constructed from mRNA isolated from brain cysts in chronically infected mice. Screening of this expression library in bacteria with a pool of polyclonal antibodies against bradyzoite antigens led to the identification of several bradyzoite-related genes such as *mag1* [37], *bag1* [45], and *ldh2* [46, 47]. Parallel studies using material from in vitro differentiated bradyzoites confirmed the presence of the bradyzoite marker BAG1 [43] as well as other proteins, such as SAG4 [52]. Expressed sequence tag (EST) libraries from both in vitro- and in vivo-prepared bradyzoites have also been used for the detection of numerous stage-specific genes [53]. Another approach employed a subtractive cDNA library to identify genes that were exclusively expressed in bradyzoites, many of which are involved in crucial cellular processes such as metabolism, protein folding, and DNA repair [49]. Promoter trapping screens have also been effective for the identification of bradyzoite-specific genes [32, 54, 55]. These screens have led to the detection of several bradyzoite gene loci, for example the bradyzoite surface antigen *bsr4* (p36) [55]. More recently, deep RNA sequencing has allowed for a more global, non-biased approach to determining the patterns of gene expression in tachyzoites versus bradyzoites. Analysis of the total RNA from forebrains from both acute and chronically infected mice identified 547 parasite genes significantly regulated between the two stages of disease. In addition to previously reported alterations to metabolic gene expression, this approach also identified many novel stage-specific markers. From these results, it appears that the parasites downregulate one set of sag-related surface markers in the tachyzoite to upregulate another set of similar genes in the bradyzoite. A large proportion (~50%) of the significantly upregulated genes in the bradyzoite were hypothetical genes, hinting at a number of parasite-specific processes that could be targeted with inhibitors as therapies against chronic toxoplasmosis [56].

Improvements in the tools facilitating molecular genetics analyses in *Toxoplasma* have allowed the construction of an increasing number of bradyzoite-specific gene knockouts. The construction of the *bag1* knockout strain was one of the first attempts to disrupt a bradyzoite-specific gene [57, 58]. Other knockouts that have been described include the membrane proton-ATPase (PMA1) [59], the bradyzoite surface antigen SRS9 [60], the dense granule proteins GRA4 and GRA6 [61], the genetic locus ROP4/7 [61], the plant-like Apetala-2 transcription factors AP2XI-4, AP2IX-9, AP2IX-4, AP2IV-4 and AP2IV-3 [62–66], and the bradyzoite secreted pseudokinase 1 (BPK1) [39]. In all knockouts made to date, the production of cyst-like structures was observed in vitro, suggesting that individually these genes are not required for initiating bradyzoite switching. Nevertheless, defects in cyst morphology, cyst number, in vivo cyst formation, and transmission of infection indicate that many of these genes contribute significantly to cyst development.

In one study, chemical mutagenesis was used to identify tachyzoite to bradyzoite interconversion defective (Tbd^{-}) mutants [67]. Other approaches applied insertional

mutagenesis and reverse genetic screens [41, 68–70]. Finally, another study employed a signature-tagged insertional mutagenesis screen designed to isolate generally avirulent parasites. These clones were subsequently screened for reduced in vivo cyst formation to identify genes required only for pathogenesis [71]. Despite differences in the type of mutagenesis performed and the methods of selection of defective parasites, this work from different groups has collectively identified factors that may contribute to bradyzoite differentiation. However, most of the candidates have yet to be verified in independent studies as having a clear role in cyst formation. Moreover, some study designs may have isolated mutants resistant to in vitro differentiation stresses, and the genes may have more to do with stress resistance than differentiation. Despite these caveats, the genes and their associated bradyzoite phenotypes that have been identified from these screens are discussed in Table 1.

Further investigation is necessary to describe the molecular mechanisms of *Toxoplasma* differentiation. Individual studies of the genes implicated in bradyzoite development have been informative, but the complete picture of this developmental pathway is still elusive. Nevertheless, the advent of techniques such as mRNA deep sequencing will allow full-scale expression profiling to compare both the tachyzoite and bradyzoite developmental stages and will refine our current understanding of this clinically relevant developmental pathway. Importantly, new in vitro models need to be pursued to delineate the phenomenon of stress-induced and spontaneous differentiation, and candidate genes need to be supported by in vivo data.

Metabolism in the bradyzoite stage

As the parasites transform from replicating tachyzoites to latent bradyzoites, changes in parasite metabolism have been observed. It may be assumed that these changes allow the parasites to remain dormant for long periods of time and are mediated by upregulation of bradyzoite-specific forms of various metabolic enzymes, particularly those involved in dealing with oxygen radical metabolism and carbohydrate metabolism. The formation of the amylopectin storage granules upon differentiation to bradyzoites indicates that the parasites modulate carbohydrate metabolism during stage switching. Tight regulation of amylopectin levels is required for maintaining chronic infection in mice [30]. Site-directed mutagenesis of an enzyme responsible for amylopectin digestion, the *Toxoplasma* glycogen phosphorylase (TgGP), was performed to determine the importance of amylopectin metabolism in both tachyzoite and bradyzoite stages. Mutation of a phosphorylated serine to inactivate (S25A) TgGP led to excessive accumulation of amylopectin. Conversely, mutation of this residue to a phosphoserine-mimetic (S25E) caused amylopectin deficiency. Although both mutant lines were capable of differentiation in vitro, they both displayed significantly reduced cyst burdens in the brains of chronically infected mice compared to the parental line, indicating that maintaining the appropriate levels of amylopectin is necessary for establishing a persistent chronic infection [30, 72].

It also appears that bradyzoites rely on anaerobic glycolysis for generation of ATP, since they do not retain a functional TCA cycle [48]. Further evidence for changes in carbohydrate metabolism comes from differential regulation of bradyzoite-specific isoforms of lactate dehydrogenase (LDH2), glucose-6-phosphate dehydrogenase (G6-PI), and enolase-1 (ENO1), all of which display different stability and enzymatic properties compared to their tachyzoite counterparts [48, 73]. The bradyzoite-specific LDH2 isoform is hypothesized to be important for establishing chronic infection in the host, since mice infected with LDH2-knockdown parasites displayed much lower numbers of brain cysts compared to the parental line [74].

Persistence of chronic *Toxoplasma* infection also relies on continued autophagy and turnover of old organelles or cellular components. Cathepsin protease L is not required for tachyzoite replication or the initial stages of bradyzoite development, but is critical for viability of the mature tissue cyst. Genetic or chemical ablation of cathepsin protease activity caused an accumulation of undigested autophagosomes and loss of bradyzoite viability, indicating that the parasites must recycle proteins and organelles to persist [75].

The long-lived latent cysts must also be capable of dealing with exposure to various radicals and reactive metabolic byproducts; consequently, enzymes that cope with this stress are upregulated during bradyzoite formation, e.g., thioredoxin and glutathione peroxidase [53].

Experimental models of cyst formation

Strain differences

Genetic analysis of *Toxoplasma* isolates from Europe and North America has identified three predominant lineages of the parasite designated Types I, II, and III, which resulted from a single genetic cross approximately 10,000 years ago [76]. The three strains differ in their growth rate, virulence, their ability to traverse or to pass through epithelial layers (transmigration), and their capacity to form cysts [77, 78].

Type I strains generally replicate faster and, consequently, are more virulent in mice. A Type I strain isolated in 1939, designated "RH" after the patient from which it was isolated [79], is the most common laboratory strain in use. Today's RH strain has a rapid replication rate and is highly amenable to genetic manipulation, making it a favored tool

Table 1 Genes associated with bradyzoite development or maintenance in *Toxoplasma*

Name	ToxoDB ID	Function	References
CST1	TGME49_264660 (SRS44)	Cyst wall glycoprotein, recognized by *Dolichos biflorus* lectin	[28, 33, 34]
MAG1	TGME49_270240	Cyst wall and matrix protein, bradyzoite-specific marker	[37]
BPK1	TGME49_253330	Cyst wall protein. In Δ*bpk1* strain cysts are smaller and more sensitive to pepsin-acid treatment; Δ*bpk1* strain has reduced ability to cause oral infection	[39]
MCP4	TGME49_208730	Cyst wall protein	[40]
PPG1	TGME49_297520	Serine/proline-rich proteophosphoglycan (GU182879), this gene has a role in cyst wall formation	[41]
SAG4 (or SRS35B)	TGME49_280570	SAG-family-related surface protein of bradyzoite	[52]
BSR4 (or SRS16C)	TGME49_320180	SAG-family-related surface protein of bradyzoite	[55]
SAG2C (or SRS49D)	TGME49_207160	SAG-family-related surface protein of bradyzoite	[188, 189]
SAG2D (or SRS49C)	TGME49_207150	SAG-family-related surface protein of bradyzoite	[188, 189]
SAG2Y (or SRS49A)	TGME49_207130	SAG-family-related surface protein of bradyzoite. The transcript is upregulated in bradyzoites. Δ*sag2y* forms cysts both in vitro and in vivo but demonstrates a defect in persistence in vivo	[190]
SAG2X (or SRS49B)	TGME49_207140	SAG-family-related surface protein of bradyzoite. The transcript is upregulated in bradyzoites. Δ*sag2x* forms cysts both in vitro and in vivo but demonstrates a defect in persistence in vivo	[190]
SRS9 (or SRS16B)	TGME49_320190	SAG-family-related surface protein of bradyzoite	[60]
BRP1	TGME49_314250	Bradyzoite rhoptry protein 1, localized in rhoptries of bradyzoites and found to be secreted in PV of in vitro bradyzoites Δ*brp1* strain develops and establishes both acute and chronic infection normally	[191]
HSP60	TGME49_247550	Mitochondrial heat shock protein, member of mitochondria chaperone family. HSP60 transcript is highly increased in bradyzoites. The protein is localized in two vesicular bodies distinct from the mitochondrion in encysted bradyzoites	[50]
hsp30/BAG1 (BAG1)	TGME49_259020	Small heat shock protein, bradyzoite-specific marker, not necessary for cyst formation	[43, 45]
LDH2	TGME49_291040	Lactate dehydrogenase 2 is a glycolytic enzyme upregulated in bradyzoites. Δ*ldh2* parasites form fewer brain cysts	[46, 47, 74]
ENO1	TGME49_268860	Bradyzoite-specific Enolase 1, functions in glycolysis	[73]
PMA1	TGME49_252640	P type ATPase, upregulated in bradyzoite stage. The protein is localized in the membrane of mature bradyzoites purified from brain tissue cysts of chronically infected mice. The Δ*pma-1* strain has reduced ability to form bradyzoites in response to a number of different in vitro triggers, but is still capable of establishing chronic infection in mice	[51, 59]
AP2XII-6	TGME49_249190	Putative transcription factor. The insertional mutant on this locus is defective in upregulating bradyzoite markers during in vitro induction	[82]
AP2XI-4	TGME49_315760	Putative transcription factor. Δ*ap2xi-4* parasites are unable to express many genes including some bradyzoite markers during pH stress differentiation, and have defects in cyst formation in mice	[63]
AP2IX-9	TGME49_306620	Transcription factor that represses bradyzoite formation. Δ*ap2ix-9* parasites have enhanced ability to form cysts in vitro. Overexpression of AP2IX-9 inhibits bradyzoite formation	[62, 65]

Table 1 (continued)

Name	ToxoDB ID	Function	References
AP2IX-4	TGME49_288950	Cell cycle-regulated transcription factor that represses expression of bradyzoite genes. *Δap2ix-4* parasites have reduced ability to form cysts under alkaline stress in vitro	[64]
AP2IV-3	TGME49_ 318610	Transcription factor that is induced during bradyzoite development and promotes expression of bradyzoite-specific genes. Overexpression of TgAP2IV-3 enhances bradyzoite formation in vitro	[65]
AP2IV-4	TGME49_318470	Cell cycle-regulated transcription factor that represses bradyzoite-specific transcripts during tachyzoite development. Parasites lacking this factor do not form cysts in mouse brain tissue	[66]
GRA1	TGME49_270250	Dense granule protein. In the cyst, GRA1 is localized in the cyst wall and the cyst matrix. Unknown function	[23, 34, 35]
GRA3	TGME49_227280	Dense granule protein. In the cyst, GRA3 is localized in the cyst wall. Unknown function	[23, 34, 35]
GRA5	TGME49_286450	Dense granule protein. In the cyst, GRA5 is localized at the cyst wall membrane. Unknown function	[23, 34, 42, 192]
GRA6	TGME49_275440	Dense granule protein. In the cyst, GRA6 is localized in the cyst wall. Unknown function	[23, 34, 35]
GRA7	TGME49_203310	Dense granule protein. In the cyst, GRA6 is localized in the cyst wall. Unknown function	[36]
Splicing factor, arginine/serine rich	TGME49_213635	Putative splicing factor. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
OWP1	TGME49_204420	Annotated as putative oocyst wall protein. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
Replication factor A protein 3	TGME49_238110	Function unknown. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
PUS1	TGME49_202640	Pseudouridine synthase homolog. Two insertional mutants on this locus are defective in tachyzoite-to-bradyzoite differentiation in vitro. Infection with Δ*pus-1* parasites results in higher mortality and increased cyst burden	[70]
ZFP1	TGME49_218362	Nucleolar CCHC motif zinc-finger protein 1. Both the insertional mutant and the Δ*zfp1* strain have impaired differentiation in vitro and fail to upregulate bradyzoite-specific transcripts	[69]
Tg-ncRNA-1	TGME49_238110	Non-coding RNA 1. Insertional mutants on this locus show differentiation defects and reduced cyst loads	[71, 193]
TgRRS1	TGME49_320450	Ribosome biogenesis regulatory protein 1 that it localized to the nucleolus. Two insertional mutants in *tgrrs1* are defective in bradyzoite formation in vitro	[194]
DNA primase subunit, large subunit	TGME49_297840	Function unknown. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
TBC domain-containing protein	TGME49_285730	Function unknown. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
HECT-domain (ubiquitin-transferase) domain-containing protein	TGME49_270580	Function unknown. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
Supt5	TGME49_233300	Rho-GAP domain-containing protein, transcription elongation factor. The insertional mutant on this locus is defective in bradyzoite differentiation in vitro	[82]
TgSRCAP	TGME49_280800	SWI/SNF-like factor, which is upregulated during bradyzoite differentiation	[184]

Table 1 (continued)

Name	ToxoDB ID	Function	References
TgRSC8	TGME49_286920	SWI/SNF-like factor. An insertional mutant with reduced protein levels of TgRSC8 expresses lower levels of some bradyzoite-specific transcripts during in vitro induction	[41, 185]
eIF2-α	TGME49_286420	Homologue of the alpha subunit of the eukaryotic initiation factor 2. eIF2-α is highly phosphorylated in bradyzoites. Important factor in regulation of bradyzoites differentiation and maintenance as well as in reactivation of bradyzoites to tachyzoites	[87, 155, 156, 195]
HDAC3	TGME49_227290	Histone deacetylase 3, the protein is highly enriched in promoters of silent genes. Chemical inhibition of HDAC3-induced bradyzoite differentiation in vitro	[172, 174]
GCN5a	TGME49_254555	Histone lysine acetyltransferase A. Transcriptional analysis of Δ*gcn5a* parasites demonstrates a role for TgGCN5a in upregulating bradyzoite transcripts in vitro	[172, 173]

for the study of tachyzoite biology. However, RH parasites are limiting in the study of conversion from tachyzoite to bradyzoite. Although it has been reported that, under stress conditions, RH tachyzoites will upregulate specific bradyzoite genes and, in some reports, produce bradyzoite-specific proteins or cyst wall components [80–82], the parasites are largely incapable of forming mature bradyzoite cysts either in vitro (in cell culture) or in vivo (during mouse infection). However, low-passage type I strains are capable of switching to bradyzoites [83], which means that extensive passage in vitro is associated with the loss of developmental competency [84]. Type II and Type III strains replicate at a slower rate, readily convert to bradyzoites, and are hypovirulent in mice, but these characteristics change with prolonged in vitro passage. Differences in the developmental capacity among these strains are influenced by adaptation to in vitro growth in HFF cells.

Genome sequence data from parasite lines of all three types have been collected and can be accessed at the *Toxoplasma* genome database (http://www.toxodb.org). This genome information has proven essential in dissection of the mechanisms and pathways for conversion from tachyzoite to bradyzoite forms.

Stress-induced bradyzoite formation

The early stages of the transition from tachyzoite to bradyzoite may be studied using in vitro culture and conditions shown to induce the conversion. These methods have been used to examine potential pathways associated with the early stages of bradyzoite development. Different kinds of stress applied to proliferating tachyzoites have been demonstrated to induce cyst formation.

The most commonly used method to induce bradyzoite cysts for in vitro culture is continuous alkaline treatment of parasite-infected host cells by adjusting the growth medium to pH 8.0–8.2 [80]. A brief alkaline treatment of extracellular tachyzoites prior to invasion has also been demonstrated to increase frequency of cyst development [85]. A number of other methods that involve adjustments to the culture medium or culture conditions to trigger stress-induced bradyzoite formation include various forms of nutrient deprivation through arginine starvation [86], axenic incubation [49], and depletion of pyrimidine in uracil phosphoribosyltransferase (UPRT)-deficient parasites grown in 0.03% CO_2 [22, 81].

Bradyzoite formation may also be enhanced using heat shock as an exogenous stress [80]. Soete et al. [80] observed bradyzoite formation after heat shocking host cells at 43 °C before allowing parasites to invade at 37 °C and applying an additional heat shock to the infected cells.

Chemical treatment of the parasites and host cells has also been used to apply stress and trigger cyst formation. Tunicamycin treatment of parasite-infected cells causes endoplasmic reticulum (ER) stress and subsequent bradyzoite development [87]. Chemical stress may also be applied using sodium arsenite [80]. Chemical inhibition of calcium-induced egress with the herbicide fluridone has also been demonstrated to result in a larger proportion of bradyzoite cysts in culture [88].

Compounds that inhibit mitochondrial function and subsequently induce oxidative stress (i.e., sodium nitroprusside or atovaquone) will also trigger bradyzoite development [89, 90]. Interferon-gamma (IFN-γ) treatment of tachyzoite infected fibroblasts will not induce bradyzoite development [80], but, when murine macrophages are infected with *Toxoplasma* and subsequently treated with IFN-γ, bradyzoite development commences, likely as a result of nitric oxide production from the activated macrophage [91].

Cyclic adenosine monophosphate (cAMP)-mediated signaling regulates a number of parasite processes including differentiation from tachyzoite to bradyzoite [92]. Using an engineered optogenic *Toxoplasma* strain in which cAMP levels could be controlled by photo-activation, Hartmann et al. [92] demonstrated that a minimum level of intra-parasitic cAMP was required for initiation of bradyzoite formation, but increased levels of cAMP impaired the process, indicating that cAMP-responsive factors are involved in the differentiation signaling pathway. The importance for tightly regulated cAMP levels in parasite differentiation is underscored by a recent study on a cAMP-dependent protein kinase A homologue, TgPKA3. Knockout of the TgPKA3 catalytic subunit led to a growth defect in tachyzoites and an increase in spontaneous cyst formation, in both Type I and Type II backgrounds. The role of TgPKA3 in cAMP levels was confirmed by rescue of the aberrant growth and differentiation phenotypes with the compound 3-isobutyl-1-methylxanthine, which restores cAMP levels in the parasite [93].

Host cell determinants and spontaneous bradyzoite development

Factors in the host cell may also act as determinants of cyst development, reviewed in [94]. *Toxoplasma* tachyzoites replicate rapidly and require sufficient levels of nutrients that are scavenged from the host cell to support this proliferation. If host cell metabolic conditions limit the supply of essential nutrients to the parasites, it will slow the replication rate and favor differentiation to the bradyzoite stage [91, 95]. This has been demonstrated in in vitro parasite culture when removal of essential nutrients such as arginine or lipoprotein (a source of cholesterol) from the culture medium favors bradyzoite differentiation [86, 96]. The host cell glycolytic flux can also influence the rate of parasite initiation of differentiation. Dividing host cells in high glucose conditions have an enhanced rate of glycolysis. This generates higher levels of lactate which was shown to favor tachyzoite replication and to inhibit conversion to bradyzoites [97]. Treatment of human host cells with a trisubstituted pyrrole called "Compound 1" prior to tachyzoite infection slowed parasite replication and led to the upregulation of bradyzoite-specific gene expression [98]. Although compound 1 was originally developed as an inhibitor of cyclic guanosine monophosphate (cGMP) kinase [99, 100], it was found to exert its effect through host cell factors [98]. The same study also demonstrated that pre-stressing host cells with alkaline pH before parasite infection was capable of enhancing cyst formation [98].

While cellular stress is a strong mediator of tachyzoite-to-bradyzoite conversion, other factors may play a role in initiating the development of cysts. Tachyzoites of cystogenic Type II and Type III strains will spontaneously convert to bradyzoites in in vitro culture, particularly when host cells have been inoculated with a low multiplicity of infection (MOI) or if egressed tachyzoites are continuously removed from the culture [101]. Although *Toxoplasma* tachyzoites are capable of infecting any nucleated cell from a warm-blooded animal, certain cell types are more conducive to cyst formation than others [102]. Transcriptomic analysis of parasite gene expression during infection of a number of different cell types indicated greater bradyzoite-specific gene expression in certain cell types such as neurons or muscle cells, even in the absence of extrinsic stress. These data suggest that a subset of cell types is more conducive to bradyzoite formation [103]. The cell cycle stage of host cells that are more permissive to bradyzoite formation may be a strong influence. Terminally differentiated mouse myotubes (skeletal muscle fibres) restricted tachyzoite replication and promoted bradyzoite differentiation compared to their dividing myoblast progenitors [104]. Neurons are in a similar state of terminal differentiation to skeletal muscle cells and it is tempting to speculate that this explains the enrichment of tissue cysts in the CNS and muscle tissue in infected animals.

Host immunity factors and tissue cysts

Since the initiation of cyst development has been largely studied in the context of stress, it has been proposed that in vivo stress applied by immune system factors during host infection is crucial in triggering tachyzoites to transform into bradyzoites [105]. The host immune system can effectively control *Toxoplasma* infection (for a recent review, see [106]), but it appears that rather than specific immune effectors actively inducing chronic infection, a functional immune response maintains parasite in the latent bradyzoite state, and that a dysfunctional immune response simply allows acute infection to go unchecked.

Cell-mediated immunity is vital for the restriction of *Toxoplasma* and the development of chronic infection in an immunocompetent host (reviewed in [106, 107]). Increased susceptibility to *Toxoplasma* has been observed in patients with impaired T-cells as well as in mice that lack B-cells, $CD8^+$, or $CD4^+$ T-cells [108–110]. $CD4^+$ cells, which can activate $CD8^+$ cells, have been proven to be necessary for the maintenance of $CD8^+$ functions during the chronic stage of infection [111, 112].

Notably, it appears that highly virulent *Toxoplasma* strains have evolved strategies to inhibit the functions of $CD8^+$ cells. A recent study showed that $CD8^+$-mediated immunity is seriously compromised during infection by the RH strain [113]. The kinase activity of ROP18 in the virulent Type I strain was found to target the host activating transcription factor 6β (ATF6-β) for degradation [114], which resulted in downregulation of $CD8^+$ cell-mediated immune responses. Although RH parasites are capable of developing

tissue cysts, infection never progresses to the chronic stage, because the host immune system is rapidly overwhelmed by ROP18 function and tachyzoite proliferation leads to systemic infection, organ failure, and death.

The major product of activated $CD8^+$ [115], $CD4^+$ T-cells [116] as well as of non-T and non-NK cells [117–119] is IFN-γ, a cytokine that is well documented to be a key mediator of *Toxoplasma* infection. In contrast to wild-type mice that survive and develop chronic infection, IFN-γ knockout (KO) mice succumb to infection with avirulent *Toxoplasma* strains [120]. Moreover, chronic parasite infection does not develop in mice that have been treated with monoclonal antibodies against IFN-γ [115, 121]. Depending on the host species and the cell type, IFN-γ has been proven to activate a variety of effector mechanisms that are required for resistance during chronic infection.

After *Toxoplasma* infection, IFN-γ induces the formation of reactive oxygen and nitric oxide (NO) metabolites in macrophages, microglia, and astrocyte cells of mice and humans [122–124]. It appears that the NO by-product is a key factor for suppressing parasite replication and inducing bradyzoite development [89, 125–127], similar to in vitro induction of bradyzoites with sodium nitroprusside, as previously discussed. However, NO is not an essential factor for in vivo formation of bradyzoites. Tissue cysts could develop in inducible nitric oxide synthase KO mice [120] as well as in mice that had been treated with aminoguanidine, the inhibitor of the enzyme that produces NO [128].

An IFN-γ-mediated tryptophan degradation response has been observed in human brain endothelial cells, HFF cells, and macrophages infected with *Toxoplasma* [129–131]. In addition, IFN-γ-treatment of *Toxoplasma*-infected cells results in upregulation of indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2), which are host enzymes that are responsible for degradation of tryptophan [132–134]. Since *Toxoplasma* is auxotrophic for tryptophan, upregulation of IDO1 and IDO2 restricts availability of this essential amino acid and thus inhibits parasite replication [135]. Parasite replication may be restored with the addition of exogenous tryptophan [136]. In vivo inhibition of IDO1 and IDO2 with a chemical inhibitor did not affect the levels of IFN-γ produced after *Toxoplasma* challenge, but treatment of chronically infected mice with IDO inhibitors led to reactivation of the disease and increased *Toxoplasma* cyst burden, demonstrating an important role for IDO during latent infection [134]. Notably, IDO has also recently been shown to be involved in host immunity against *Trypanosoma cruzi* and malaria [137, 138].

The host immune effector Tumor Necrosis Factor α (TNFα) has also been shown to play an important role in establishing chronic *Toxoplasma* infection [139, 140]. IFN-γ may function in synergy with TNFα, since treatment of *Toxoplasma*-infected murine macrophages induces the expression of bradyzoite markers [89]. Interleukin 6 (IL-6) is another inflammatory cytokine that causes the increase of cyst numbers produced in vitro in HFF cells [141]. It also appears that control of both acute and chronic stages of infection depends on IL-12, a cytokine that drives the production of IFN-γ [115, 142, 143].

In addition to host cell background influencing spontaneous bradyzoite conversion, the particular in vivo distribution of cysts in brain and muscle tissues may be facilitated by specific local immune responses [144, 145] or potentially ineffective immune responses within these tissues [146]. Alternatively, organ or cell-type-specific factors that trigger high levels of stage conversion and tissue cyst formation may contribute to enhance parasite persistence in these tissues. As previously mentioned, treatment of host cells with Compound 1 modulates host cell factors, making them more permissive to bradyzoite formation [98]. Treatment of host cells with Compound 1 prior to infection with *Toxoplasma* induced the upregulation of the human cell division autoantigen-1 gene (CDA-1). The increased expression levels of CDA-1 affected parasite replication, arresting the parasite cell cycle and inducing bradyzoite gene expression, directly implicating host cell factors in the differentiation process [98]. Furthermore, it was recently noted that the metabolic state of a cell, as well as non-immune secreted metabolites, could designate host cells as either permissive or resistant to bradyzoite development [97, 147].

Although the host immune responses play critical roles in elimination of tachyzoites and contribute to host environmental changes that foster tachyzoite to bradyzoite conversion and maintenance of tissue cysts, it remains unclear how these signaling pathways compare to induction methods currently used for in vitro differentiation models. The growing convenience of RNA sequencing will be valuable for comparing the transcriptional responses of parasites during the early stages of bradyzoite development during in vivo infection and the commonly used in vitro induction models. Pinpointing the commonalities within the different models will identify the core signaling molecules that drive the transition to the chronic stage.

Reactivation of infection: bradyzoites to tachyzoites

The tissue cysts remain largely quiescent for the life of a host, but tachyzoites can re-emerge and cause toxoplasmosis in immunocompromised patients. The reactivation mechanisms have not been studied extensively, but it is generally assumed that abrogation of the host immune response results in bradyzoite-to-tachyzoite conversion [105]. Human immunodeficiency virus (HIV) patients with reduced numbers of $CD4^+$ cells acquire toxoplasmosis [7, 148]. A similar course of infection occurs in chronically infected mice that lack $CD4^+$ cells [109], further supporting that cell-mediated

immunity is important for suppressing bradyzoite reactivation. In addition, it has been demonstrated that in vivo depletion of $CD8^+$ and $CD4^+$ cells in chronically infected mice results in reactivation of *Toxoplasma* infection [149]. Suppressing IFN-γ production also facilitates bradyzoite reactivation during chronic infection in mice [150–152]. In long-term murine astrocyte cultures of brain-derived *Toxoplasma* cysts, the presence of IFN-γ is necessary to suppress reactivation. Removal of IFN-γ from the culture, 120 day post-infection, resulted in cyst rupture and the appearance of cells heavily infected with tachyzoites predominating in the culture [153]. One current hypothesis of the mechanism that links IFN-γ with suppression of reactivation is that IFN-γ may stimulate continuous anti-parasitic activity in the astrocytes, causing cell cycle arrest, which has been shown to be associated with bradyzoite differentiation [29]. A direct role of the host immune system in repression of reactivation remains elusive, since, even in healthy mice, tissue cysts can occasionally rupture, releasing invasive parasites that infect new cells [17].

Molecular mechanisms

Translational control—downregulation of global translation and preferential translation of transcription factors

Given that virtually any type of cellular stress can induce bradyzoite differentiation, and can do so independently of the host cell, attention has been directed to the study of stress response pathways in the parasite to illuminate the mechanisms driving stage conversion. A common response to cellular stress in all eukaryotic organisms is the control of global translation via phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α). Depending on the type of stress the cell is experiencing, one of a number of specific kinases is activated to phosphorylate eIF2α, leading to global downregulation of translation with concurrent, preferential translation of a subset of mRNAs that code for factors required to deal with the stress insult [154]. Recent evidence points to translational control via the *Toxoplasma* homologue of eIF2α, TgIF2α, as a contributing factor in regulating the transition of tachyzoite to bradyzoite, maintenance of the dormant bradyzoite, as well as the reactivation of bradyzoites into proliferating tachyzoites, reviewed in [155]. When *Toxoplasma* tachyzoites are subjected to different cellular stresses that are known to induce bradyzoite development, TgIF2α becomes phosphorylated [156]. In contrast to intracellular replicating tachyzoites, TgIF2α is phosphorylated to a high degree in mature bradyzoites [87]. Dephosphorylation of mammalian eIF2α is specifically inhibited by treatment with salubrinal or guanabenz [157, 158]. These compounds also inhibit dephosphorylation of TgIF2α and treatment of tachyzoites with either salubrinal or guanabenz triggers bradyzoite differentiation in vitro [87, 159]. Furthermore, cultured bradyzoite cysts treated with salubrinal or guanabenz were no longer able to reactivate into replicating tachyzoites [159].

Once TgIF2α has been phosphorylated, translation is dramatically downregulated [87]. The decrease in protein synthesis conserves resources and allows the parasite to reprogram its genome to respond accordingly to the stress, which, in the case of *Toxoplasma*, may include switching to the latent bradyzoite stage [3]. Discerning which mRNAs are preferentially translated, while TgIF2α is phosphorylated, would be of great use in understanding the precise sequence of events and the essential components required for the transition from tachyzoite to bradyzoite. Polysome profiling has been used to identify the mRNAs that are undergoing translation at a specific time point. We have developed a polysome fractionation protocol for *Toxoplasma* combined with microarray analyses, which allowed the identification of a subset of mRNAs that are suggested to undergo preferential translation in parasites experiencing ER stress as a result of tunicamycin treatment [160]. A number of these preferentially translated mRNAs encode proteins containing plant-like AP2 domains, which have been identified as the major family of transcription factors in *Apicomplexa* [161]. This set of preferentially translated transcription factors are prime candidates for the main effectors upregulating bradyzoite-specific genes and the transition to the dormant cysts.

Transcriptional regulation of bradyzoite-specific genes

Once the switch to cyst development has been triggered, upregulation of bradyzoite-specific genes has been observed with a concurrent downregulation of tachyzoite-specific genes. A number of studies using different techniques have sought to identify the genome-wide changes in gene expression during the bradyzoite developmental program [40, 53, 56, 162, 163]. Cascades of gene expression are observed as the parasites transition from one stage to the next, indicating that transcriptional regulation plays an important role in the regulation of stage switching. A number of these studies also report distinct functional groups of genes that are differentially regulated, with surface antigens, metabolic proteins, and secreted proteins distinguished with significant differences in expression levels [40, 162].

The initial results from genome sequencing of *Toxoplasma* and related apicomplexan parasites suggested a surprisingly small number of putative transcription factors, prompting focus on epigenetic means for gene regulation [164]. However, a growing body of evidence indicates that gene expression is regulated by the conventional eukaryotic

mechanisms. Much of the conventional general transcriptional machinery appears to be conserved in *Toxoplasma* [165]. There also appears to be specific DNA motifs for recruitment of factors controlling stage-specific gene expression. The promoters of 16 bradyzoite-specific genes were mapped to high resolution, which led to the identification of *cis*-acting elements that appear to be specific to genes induced during differentiation. These elements from the two bradyzoite-specific genes *bag1* and *B-NTPase* were inserted into the constitutive DHFR-TS promoter and converted it into a promoter that enhanced expression of a reporter under bradyzoite-induction conditions [83].

To identify DNA-associated factors that could function as repressors of bradyzoite gene expression during tachyzoite proliferation, pull-downs using the bradyzoite-specific promoter *ENO1* as bait were performed. This study identified different regulatory factors such as an RNA helicase, kinases, phosphatases, and heat shock proteins, as well as a number of hypothetical proteins containing domains with high homology to known chromatin-association factors. One of the proteins pulled down was an FK506-binding protein (FK506BP) homologue called *Toxoplasma* nuclear factor 3 (TgNF3). TgNF3 displays a nuclear localization in tachyzoites, but was found to be predominantly cytoplasmic in bradyzoites. TgNF3 was shown to associate with both nucleosomes and free histones to repress ENO1 and 18S ribosomal RNA genes [166].

The characterization of the ApiAP2 family of plant-like transcription factors in *Toxoplasma* and related apicomplexan parasites [161] has prompted efforts to identify the specific AP2 factors that may be responsible for coordinating the response to the different signals that trigger bradyzoite differentiation (reviewed in [167]). Collation of the various gene expression analyses that have been performed on differentiating parasites has identified a handful of AP2 factors that are specifically upregulated during bradyzoite development and are dampened once the mature tissue cyst is formed [65, 168]. Genetic disruption of these AP2 factors is revealing unexpected complexity in the stage transition from tachyzoite to bradyzoite, which appears to be mediated by interplay between both activators and repressors of bradyzoite genes.

At least two of these AP2 factors, AP2IV-3 and AP2XI-4, function as activators of bradyzoite genes [63, 65]. Deletion of AP2XI-4 in both Type I and Type II backgrounds generated mutant lines that were unable to initiate expression of bradyzoite marker genes during stress [63]. The Type II mutant also displayed a significant defect in cyst formation during in vivo infection in mice [63]. AP2IV-3 is also induced early during bradyzoite development to promote expression of bradyzoite genes [65]. Knockout of AP2IV-3 reduced the rate of cyst formation in response to alkaline stress, while inducible overexpression of this factor increased the rate of cyst formation, even in the absence of extrinsic stress. Gene expression analysis of the AP2IV-3 overexpressing parasites confirmed upregulation of a subset of known alkaline stress-responsive genes [65].

Conversely, another AP2 factor that is upregulated early during bradyzoite development AP2IX-9 acts as a repressor of bradyzoite formation. AP2IX-9 knockout parasites have a higher rate of spontaneous cyst conversion in normal growth medium and overexpression of AP2IX-9 decreased the rate of differentiation under alkaline stress [62, 65]. Both AP2IV-3 and AP2IX-9 appear to regulate the same target genes and were found to directly associate with the chromatin in at least one promoter region (*bag1*); however, further studies are required to understand how the action of these antagonistic factors culminates in a decision to either continue replication as a tachyzoite, or commit to bradyzoite development.

Adding to the complexity of tissue cyst development at the molecular level is findings on two other AP2 factors that are cell cycle regulated in tachyzoites. Although not essential for tachyzoite replication, AP2IX-4 knockout mutants displayed a defect in cyst formation in vitro and in vivo [64]. Localization of AP2IX-4 protein revealed that it is cell cycle regulated and expressed only in dividing parasites, both tachyzoites and bradyzoites. Gene expression analysis reveals that AP2IX-4 operates as a repressor of bradyzoite genes during alkaline stress, consistent with AP2IX-4 being present in dividing parasites [64]. Similarly, another cell cycle-regulated factor that peaks at the S/M stage in tachyzoites, AP2IV-4, was also identified as a repressor of bradyzoite genes when gene expression analysis on the knockout parasites revealed many bradyzoite-specific transcripts to be upregulated [66]. Although this mutant line displayed a comparable cyst formation rate to the parental line under alkaline stress in vitro, it was completely deficient in tissue cyst formation during mouse infection, suggesting that the aberrant expression of bradyzoite proteins early during infection primed the host immune response for efficient clearing of the parasite load before chronic infection could be established [66]. Collectively, these studies argue against a single master regulator of bradyzoite differentiation and suggest that multiple transcription factors are involved in fine-tuning gene expression at multiple checkpoints during the transition to latency.

The cell cycle and bradyzoite formation

The cyclic regulation of many of the regulators involved in bradyzoite formation suggests that this differentiation process is intimately linked to the parasite cell cycle. Early experiments showed that when the cell cycle is blocked, differentiation does not proceed [169]. Subsequent studies have attempted to elucidate the point in the cell cycle in

which the tachyzoite switches to the bradyzoite developmental program and which factors play a role at this transition point. Gene Set Enrichment Analysis (GSEA) on a number of independent gene expression data sets from differentiation experiments provides strong evidence that bradyzoite formation and cell cycle regulation are coupled. Genes associated with the S/M phase of the cell cycle were more highly enriched in bradyzoites, while genes corresponding to the G1 phase were more enriched in tachyzoites [170, 171]. Cell cycle comparison between replicating and differentiating tachyzoites identified an enrichment of parasites in late S and M stages when bradyzoite development was triggered [95]. Concurrent with this observation, microarray analysis at different points in the tachyzoite replication cycle found that the levels of a number of bradyzoite-specific transcripts peaked at the late mitotic phase of the cell cycle [168]. This was observed in unstressed parasite populations, suggesting that even under normal conditions, certain factors that are capable of initiating bradyzoite development are present, but the master signal for the transition is either absent or overpowered by other regulators that drive tachyzoite replication. This evidence supports a model whereby tachyzoites pass through a point in the cell cycle in which they are poised for development into a bradyzoite. If the signal for bradyzoite development is present, the gene expression program for cyst formation proceeds, but, in the absence of such stress signals, tachyzoite replication continues.

Changes in the epigenetic landscape for tachyzoite versus bradyzoite genes

A series of studies have demonstrated that epigenetics and histone post-translational modifications (PTMs) play a vital role in protozoan parasite biology [63]. More specifically, *Toxoplasma* epigenetics has been linked with the regulation of genes expressed during oxidative stress responses, cell cycle, and DNA repair mechanisms, as well as during tachyzoite-to-bradyzoite stage conversion [172–177].

Mining of the *Toxoplasma* genome has identified a wealth of factors homologous to the chromatin remodeling machinery of other well-characterized eukaryotes [164, 176]. In addition, chromatin immunoprecipitation coupled to microarray chip (ChIP-chip) experiments have been utilized to determine that H3K4 tri-methylation and H3K9 acetylation are markers of active promoters in *Toxoplasma* [178]. The first evidence that linked epigenetic regulation with bradyzoite development was the observation that nucleosomes present in inactive bradyzoite-specific promoters are hypoacetylated during the tachyzoite stage but become acetylated under bradyzoite differentiation conditions [172, 179]. The converse was shown for tachyzoite-specific genes, whereas constitutively expressed genes were hyperacetylated in both developmental stages [172]. The differential association of histone deacetylase TgHDAC3 and histone acetyltransferase TgGCN5a at stage-specific promoters was also observed using ChIP assays [172]. Specifically, TgGCN5a is highly enriched at the promoter regions of transcribed genes [172, 173, 180], while TgHDAC3 is found at promoter regions of bradyzoite-specific genes that are silent during tachyzoite replication [172]. Moreover, analysis of the transcriptional profile of a TgGCN5a KO strain suggested a role for TgGCN5a in triggering bradyzoite gene expression in response to alkaline stress. TgGCN5a KO parasites under alkaline treatment did not upregulate *bag1* or *ldh2*, and these parasites were deficient in stress recovery [173]. *Toxoplasma* also expresses a second GCN5 histone acetyltransferase homologue, TgGCN5b, which appears to be crucial during tachyzoite replication, but a role for this enzyme in mediating the transition to bradyzoite cyst has yet to be determined [29]. The involvement of specific HDACs in differentiation was revealed through the use of the compound FR235222, which specifically inhibits TgHDAC3 [174]. Treatment of *Toxoplasma*-infected cells with FR235222 induced bradyzoite differentiation [174]. In addition, comparisons of the transcriptional profiles of tachyzoites in the presence or absence of apicidin, another histone deacetylase inhibitor, suggested that the tachyzoite-to-bradyzoite conversion is regulated in part through histone acetylation and deacetylation modifications [181]. While these studies support the role of histone modifying enzymes in bradyzoite differentiation, it should be noted that "histone" modifying enzymes have numerous other substrates whose activity could be regulated by acetylation [182, 183].

Chemical inhibitors have also been used to determine the role of methyltransferases in *Toxoplasma* stage differentiation. Specifically, pre-treatment of extracellular tachyzoites with AMI-I, an inhibitor of the arginine methyltransferase TgCARM1, induced cyst formation [172]. ChIP assays were used to confirm that AMI-I treatment resulted in a reduction of H3R17 methylation in the nucleosomes at promoter regions of tachyzoite-specific genes, suggesting an important function of the H3R17 mark for tachyzoites [172]. However, methylation of H3R17 can also be observed in in vitro bradyzoites, pointing to a key function for TgCARM1 in both stages. A significant gene duplication event and subsequent divergence in the SET-containing domain histone lysine methyltransferases has been reported in *Toxoplasma* [175]. TgSET8 was shown to be enriched in heterochromatic regions such as silenced rRNA genes, satellite repeats, and telomeric sites, with the repressive methylation marks H3K9 and H4K20 [175]. Notably, increased H4K20me1 levels have been detected in bradyzoite containing cysts purified from mice [175], indicating that TgSET8 may facilitate the maintenance of the chronic stage inside the host.

An interesting family of chromatin remodelers that has not been studied extensively, but has been linked to

epigenetic regulation of stage conversion, is the SWI/SNF ATP-dependent nucleosome remodeling complexes. Intriguingly, the *Toxoplasma* genome has 17 predicted members of this family [176]. The mRNA from one member of this family, *TgSRCAP* (Snf2-Related CBP Activator Protein), is upregulated during in vitro bradyzoite stage conversion [184]. As previously mentioned, another member of this family, *TgRSC8*, was identified in an insertional mutagenesis screen designed to select for mutants defective in bradyzoite formation [41, 185].

Changing nucleosome composition is an additional mechanism that may be used to control gene expression during stage conversion in *Toxoplasma*. In addition to the canonical histones, H2A and H2B, the *Toxoplasma* genome encodes a number of histone H2 variants, denoted H2AX, H2AZ, and H2Bv [164]. H2Bv forms dimers with H2A.Z on the promoter regions of active genes [186]. Notably, the incidence of H2A.Z changes in relation to the transcriptional status of a gene; preliminary data showed that H2A.Z is positioned within the coding region of silent bradyzoite-specific genes and within promoter regions but not coding regions of actively expressed genes [187]. In contrast, H2AX is enriched at promoters of repressed genes as well as within silent genomic regions in tachyzoites [186]. Furthermore, the expression levels of H2AX are increased during in vitro bradyzoite formation, suggesting that H2AX may facilitate global gene repression during the latent bradyzoite stage [186].

Multiple approaches have demonstrated the importance of epigenetic regulation during the transition from tachyzoite to bradyzoite. Many of the fundamentals of epigenetic regulatory mechanisms appear to be well preserved in parasites. However, there is a surprisingly high diversity within the proteins that constitute the chromatin remodeling machinery in *Toxoplasma.* PTM-histone mapping studies [187] as well as characterization of "species-specific" and "stage-specific" epigenetic modifications of *Toxoplasma* will assist in elucidating the complete mechanism of the complex developmental pathway driving bradyzoite formation.

Conclusion and future directions

The ability of *Toxoplasma* to form latent cysts and persist in the host for many years is the key to the parasite's successful dissemination throughout a large proportion of the human population and numerous animal hosts worldwide.

We are beginning to understand the early stages of the transition from tachyzoite to bradyzoite induced by stress, but further research is required to flesh out the mechanisms involved. Importantly, these mechanisms need to be compared to newer models of in vitro differentiation that do not require the application of exogenous stress (i.e., spontaneous differentiation). Recent advances have identified a number of AP2 and chromatin remodeling factors that are responsible for controlling the gene expression program as the parasites switch from tachyzoite to bradyzoite, but how they are engaged and how they interplay remains largely unresolved. Many elements are at work in this complex, multifactorial process, and advances in the molecular tools for cystogenic *Toxoplasma* strains will help to address remaining questions.

An area that has been largely neglected to date is the process of reactivation and how the parasite re-enters the proliferative tachyzoite cycle after dormancy. Phosphorylation of TgIF2α appears to play a crucial role in perpetuating quiescence of the bradyzoites within the cyst and tantalizing new studies demonstrate that pharmacological interference with translational control can impede tachyzoite growth and the reactivation of infection [159].

Ultimately, we will require a detailed understanding of the signaling processes required for bradyzoite formation to interrupt the tachyzoite–bradyzoite transition. Definition of these pathways may enable us to chemically target central mediators of the differentiation and latency processes, and allow development of novel therapies for the individuals at risk of toxoplasmosis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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