MOLECULAR BASIS OF STAGE CONVERSION IN APICOMPLEXAN PARASITES

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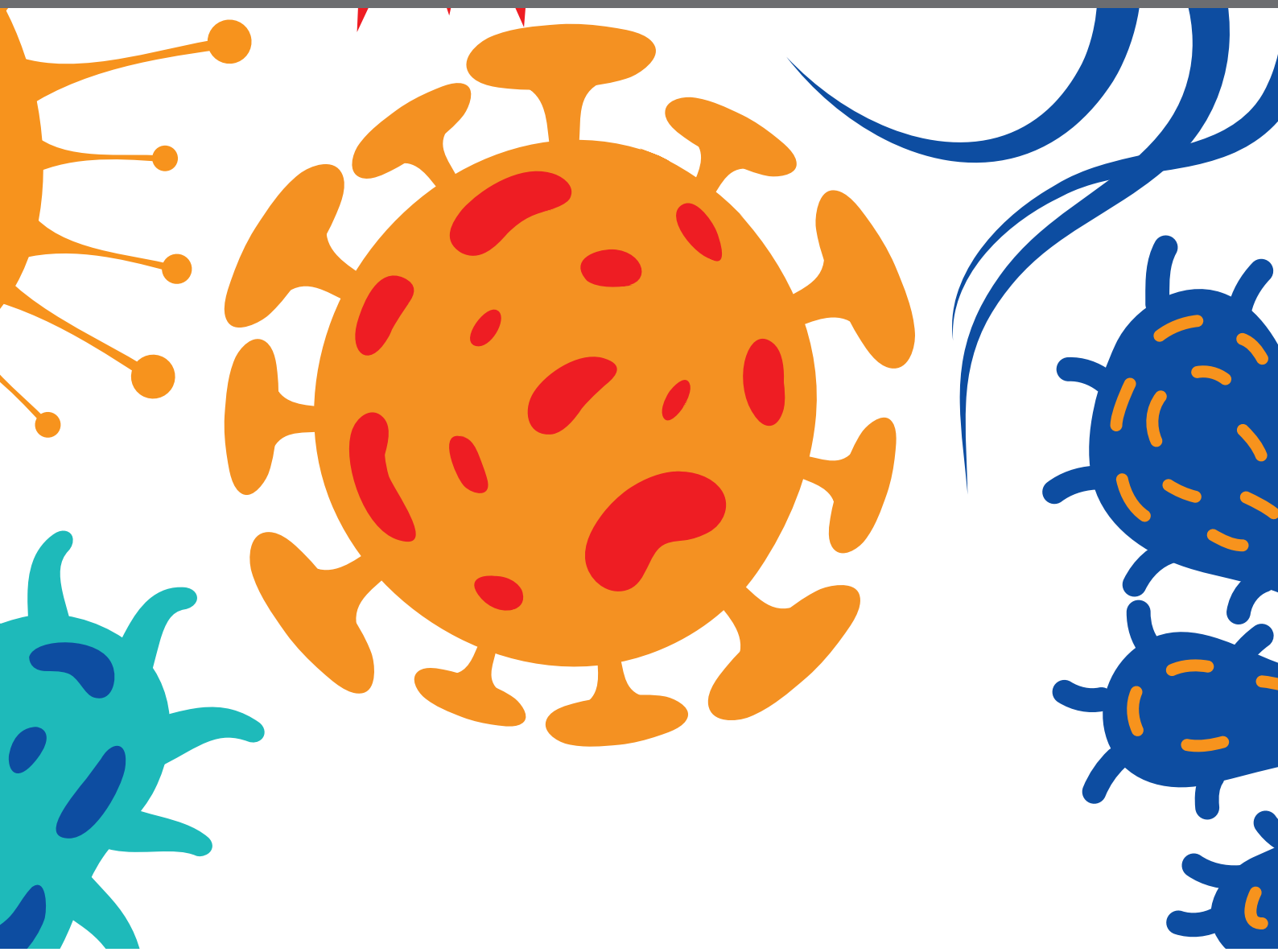

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MOLECULAR BASIS OF STAGE CONVERSION IN APICOMPLEXAN PARASITES

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Editorial: Molecular Basis of Stage Conversion in Apicomplexan Parasites

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Editorial on the Research Topic

Molecular Basis of Stage Conversion in Apicomplexan Parasites

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INTRODUCTION

The Apicomplexa is a large phylum of intracellular parasitic protozoa with more than 6000 species that present a set of invasion organelles organized in an apical complex (Adl et al., 2007). It is estimated that every metazoan can be infected by at least one species from this group (Morrison, 2009), indicating their diversity and adaptation to the parasitic lifestyle. Most of what we know about this phylum is based on the study of a subset of parasites of medical and veterinary importance, such as those causing malaria, cryptosporidiosis, neosporosis, and toxoplasmosis.

All Apicomplexa studied so far are characterized by complex life cycles, during which parasites transition through multiple life forms, often requiring intermediate hosts and vectorial transmission. This process involves multiple cell types and, consequently, differentiation and proliferation steps to adapt to the changing environment. Apicomplexa switch from intra- to extracellular environments; encysted slow-dividing forms, inaccessible to the immune system, to fast-dividing forms exposed to immunity; motile to non-motile. These transitions are all accompanied both by morphological and metabolic changes. The collection of articles in this Topic "Molecular Basis of Stage Conversion in Apicomplexan Parasites" reviews and updates current knowledge of the molecular mechanisms controlling stage conversion in some of the best-described Apicomplexa and presents new findings contributing to this field of study. It also integrates new insight into less-studied species, expanding our breadth of knowledge and understanding of the phylum as a whole.

Plasmodium spp. or *Toxoplasma gondii* parasites benefit from the improvement of a flexible genetic toolbox that permits in-depth dissection of stage transitions. Studies dissecting the regulation of gene expression and, ultimately, of stage transition have shown that a fine-tuned multilayer regulation of gene expression is necessary to control each transition. Understanding the dynamic morphological and metabolic changes at the structural and molecular level is paramount to developing effective prophylactic and treatment strategies as shown in Mévélec et al. In particular, stage-specific transcriptional regulation (involving epigenetic regulation, transcription factors, and chromatin organization) and translation repression are two mechanisms that shape the life cycle

progression in both *Plasmodium. spp* as shown in Briquet et al. and Hollin and Le Roch and *T. gondii* (Sokol-Borelli et al.). In that respect, the recent characterization of the RNA Binding Protein UIS12 provides further evidence of the importance of post-transcriptional mechanisms in the control of *P. berghei* sexual stage development.

In addition to *Plasmodium* spp. and *T. gondii*, a number of other apicomplexan parasites are now amenable to genetic manipulation which has expanded our understanding of stage conversion in this phylum. The molecular basis of stage transition of coccidian parasites such as *Eimeria* spp. are now being investigated, demonstrating how metabolic pathways can be reshaped during transitions and contribute to the parasite's adaptation to the environment as illustrated by Martorelli Di Genova and Knoll. Dissecting the biology of a wider selection of parasites from the phylum, such as *T. gondii's* closest relatives *Hammondia hammondi* and *Neospora caninum*, is also important as it can also contribute to our understanding of the evolution of adaptations of these parasites to specific biological niches and hosts. For example, Sokol-Borelli et al. show high similarity between parasite genomes and the presence of identified key factors for transition in *T. gondii, which* does not necessarily indicate that these same actors are controlling stage transition in other parasites.

However, despite this progress, some parts of the complex life cycles of Apicomplexa are still not experimentally amenable. The emergence of new *in vitro* models is now allowing for access to large parts of the complex life cycle of these parasites. For example, dissecting the biology of the *T. gondii* sexual cycle may now be within reach, as illustrated by Tomasina and Francia. Such fast progress in the field would not be possible without a number of technological advancements, including in particular improvements in the use of next-generation sequencing technologies. This approach is now powerful enough to investigate Apicomplexa species or life cycle stages that are difficult to access. For example, from an extensive collection of *Cryptosporidium* transcriptomics data, Li et al. produced a new bioinformatic pipeline that identified almost 400 developmentally regulated long non-coding RNAs. These RNAs cover ~10% of the protein-coding genome and are upregulated in time points corresponding to gametocytogenesis, suggesting a role in sexual development. Next-generation sequencing also provides an in-depth characterization of the *T. gondii* transcriptome. Markus et al. mapped transcriptional start sites (TSS) of *T. gondii* with high resolution and stage-specific alternative TSS were identified for bradyzoite genes. *T. gondii* TSS were also analyzed in reference to recently published nucleosome data and found to be located unusually deep into the nucleosome, suggesting a role of chromatin architecture in stage conversion. It is clear that non-coding elements play important roles in differentiation and require further exploration.

In conclusion, the collection of articles in this Research Topic highlight the contribution of the study of underrepresented species and life stages, as well as the new improved technologies and model systems to current understanding of the molecular basis of stage transitions in Apicomplexa.

AUTHOR CONTRIBUTIONS

MG wrote the manuscript. MEF, KKM, and MCP edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Key Limitations and New Insights Into the *Toxoplasma gondii* Parasite Stage Switching for Future Vaccine Development in Human, Livestock, and Cats

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Toxoplasmosis is a parasitic disease affecting human, livestock and cat. Prophylactic strategies would be ideal to prevent infection. In a One Health vaccination approach, the objectives would be the prevention of congenital disease in both women and livestock, prevention/reduction of *T. gondii* tissue cysts in food-producing animals; and oocyst shedding in cats. Over the last few years, an explosion of strategies for vaccine development, especially due to the development of genetic-engineering technologies has emerged. The field of vaccinology has been exploring safer vaccines by the generation of recombinant immunogenic proteins, naked DNA vaccines, and viral/bacterial recombinants vectors. These strategies based on single- or few antigens, are less efficacious than recombinant live-attenuated, mostly tachyzoite *T. gondii* vaccine candidates. Reflections on the development of an anti-*Toxoplasma* vaccine must focus not only on the appropriate route of administration, capable of inducing efficient immune response, but also on the choice of the antigen (s) of interest and the associated delivery systems. To answer these questions, the choice of the animal model is essential. If mice helped in understanding the protection mechanisms, the data obtained cannot be directly transposed to humans, livestock and cats. Moreover, effectiveness vaccines should elicit strong and protective humoral and cellular immune responses at both local and systemic levels against the different stages of the parasite. Finally, challenge protocols should use the oral route, major natural route of infection, either by feeding tissue cysts or oocysts from different *T. gondii* strains. Effective *Toxoplasma* vaccines depend on our understanding of the (1) protective host immune response during *T. gondii* invasion and infection in the different hosts, (2) manipulation and modulation of host immune response to ensure survival of the parasites able to evade and subvert host immunity, (3) molecular mechanisms that define specific stage development. This review presents an overview of

the key limitations for the development of an effective vaccine and highlights the contributions made by recent studies on the mechanisms behind stage switching to offer interesting perspectives for vaccine development.

Keywords: *T. gondii*, tachyzoite, brabyzoite, sporozoite, differentiation, immunity, vaccine

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that infects humans, domesticated and wild warm-blooded animals. As a result, the parasite has a world-wide distribution (Dubey, 2008). *Toxoplasma* infection is acquired by consumption of oocysts shed from cats in contaminated water or vegetables or by ingestion of tissue cysts contained in infected meat. *T. gondii* can undergo both asexual and sexual replications in cats and members of the feline family (definitive hosts), but can divide only asexually in all other warm-blooded mammals including humans (intermediate hosts). Tachyzoites, bradyzoites contained in tissue cysts and sporozoites contained in sporulated oocysts are the three infectious stages of *T. gondii.*

After ingestion of *T. gondii* oocysts or cysts by an intermediate host, the sporozoites or bradyzoites released into the lumen of the small intestine, pass through the intestinal epithelial barrier and rapidly undergo multiplication by endodyogeny within the parasitophorous vacuole (PV) inside various cell types. Tachyzoites disseminate throughout the organism after infecting circulating cells such as dendritic cells, natural killers, monocytes and macrophages (Courret et al., 2006; Persson et al., 2009). In addition, tachyzoites are capable of crossing the placental blood barrier to infect the fetus. Then, under the pressure of the host immune system, tachyzoites transform into bradyzoites, the slow replicating form of parasite. The encysted bradyzoites persist inside the host which correspond to establishment of chronic infection and are found in a variety of tissues including heart, skeletal muscle, lung and brain (Remington and Cavanaugh, 1965; Di Cristina et al., 2008). Upon immune suppression, bradyzoites will transform back into proliferating tachyzoites. In definitive host, bradyzoites invade the intestinal epithelium and differentiate into five morphologically distinct types of schizonts designated A through E (Dubey and Frenkel, 1972). Type E schizonts give rise to merozoites which differentiate into gametes. Males (microgametes) fertilize females (macrogametes) to produce diploid oocysts which develop thick impermeable walls and are shed in the feces (Tenter et al., 2000). Once sporulation occurs (1 to 5 days following secretion), oocysts are infectious for an extended period of time, depending on environmental conditions.

Felids acquire infection by carnivorism by ingesting prey tissue containing cysts or, more rarely, oocysts. From a systematic review and meta-analysis (from 1967 to 2017) the worldwide seroprevalence of *T. gondii* has been estimated to be 35% in domestic cat and 51% in wild felids respectively (Montazeri et al., 2020). *T. gondii* infections are highly prevalent in both sheep and goats and have been found in small ruminants worldwide (Stelzer et al., 2019). For example, in Europe seroprevalence values ranging from 24.5% to 89% have been reported in sheep (Stelzer et al., 2019). Ingestion of oocysts through contaminated fodder or water is the most important route of infection in small ruminants. Seroprevalences in pig varied according to management system in particular outdoor access is an important risk factor of infection, age, pig categories and geographic areas (Stelzer et al., 2019). Lower prevalence (<1%) is observed in pigs reared in indoor farms with control management conditions, whereas higher prevalence values (>60%) are found in farms without controlled conditions allowing outdoor access (De Berardinis et al., 2017). Most *T. gondii* infections in pigs are acquired by the ingestion of food/water contaminated with oocysts or by ingestion of intermediate hosts harboring tissue cysts (Stelzer et al., 2019). *T. gondii* is estimated to infect one-third of the human population with prevalence varying from 10% to over 50% according to geographic areas (Robert-Gangneux and Dardé, 2012). As omnivorous, humans are exposed to both *T. gondii* tissue cysts and oocysts in their diet through consumption of undercooked meat and water or vegetables contaminated with cysts or oocysts respectively. Isolation of viable parasites from tissues of pig and sheep confirm that these species represent a risk for human transmission (Halos et al., 2010; Alves et al., 2019; Miura et al., 2019). Recently, serologic tests able to distinguish oocyst- versus meat- induced infections, revealed that environmental oocyst transmission is a more important source of transmission than previously thought (Boyer et al., 2011; Hill et al., 2011).

In cat, *T. gondii* infection is generally asymptomatic. The host immunity is able to limit oocyst shedding to a short period usually 1–3 weeks (Dubey and Frenkel, 1974) and to protect against re-shedding after re-infection with homologous strain (Davis and Dubey, 1995; Dubey, 1995). However, partial or no protection against re-shedding is observed after second infection with heterologous strains (Zulpo et al., 2018). In sheep, acute toxoplasmosis is characterized by a brief episode of fever and lack of appetite (Stelzer et al., 2019). A primary maternal infection is a leading cause of stillbirth and preterm lamb loss. In Europe and USA, 10 to 23% of ovine abortions are caused by *T. gondii* (Dubey, 2009). In most cases, *T. gondii* infection in pig is subclinical, nevertheless, clinical disease in young animals and reproductive failure in sows have been reported worldwide (Stelzer et al., 2019). In healthy humans, primary infection with *T. gondii* is usually asymptomatic, nevertheless, flu-like symptoms can occur. *T. gondii* infections are more detrimental in immunocompromised individuals, such as those with HIV infection, patients receiving organ transplants or undergoing cancer treatment where primary infection or reactivation of latent tissues cysts results in encephalitis and pulmonary toxoplasmosis. A primary infection during pregnancy may result in congenital infection causing abortion or fetal

abnormalities, including hydrocephalus, intracranial calcifications and retinochoroiditis (Montoya and Liesenfeld, 2004). In the last years, ocular toxoplasmosis was identified not only following congenital infection but also in cases of postnatally acquired infections (Smith et al., 2020; Zhao and Ewald, 2020).

T. gondii disease severity is determined by many factors, including the host species and for each species the host genetic background, the genotype of the parasite and the stage that is acquired by the host, oocysts being more virulent than bradyzoites in tissue cysts (Lindsay and Dubey, 2020). It is now apparent that many atypical *T. gondii* genotypes exist besides the typical 3 genotypes (type I, type II and type III) first described in Europe and United States. In Europe, type II strains and type III strains are dominant, while types II, III, and 12 are dominant in North America. In Asia and Africa, (types II and III) and regional clonal lineages such as Chinese 1 in China or Africa 1 and Africa 3 in Africa are dominant. In contrast, the genetic diversity is much higher in South America, with no clear dominance of any particular genotype (Galal et al., 2019). An association between the *T. gondii* strains identified in South America and the incidence of severe ocular and systemic toxoplasmosis in immunocompetent patients has been made (Holland, 2003; Grigg et al., 2015).

Currently, drug therapies are ineffective against *T. gondii* cysts in tissues (Dunay et al., 2018). So, prophylactic strategies would be ideal to prevent infection. The observation that *T. gondii* induces a protective immunity in most hosts (animal species including people) suggest that an immunoprophylactic strategy is a realistic goal. In a One Health vaccination approach the objectives would be the prevention of congenital disease in both women and livestock species such as sheep and goats; prevention/reduction of *T. gondii* tissue cysts in food-producing animals; and prevention/reduction oocyst shedding in cats (Innes et al., 2019). Over the past 30 years, many vaccine candidates have been tested, mostly in inbred mice, using various strategies (reviewed in Faridnia et al., 2018; Dodangeh et al., 2019; Loh et al., 2019; Rezaei et al., 2019; Wang et al., 2019; Pagheh et al., 2020). Varying degrees of protective immunity have been achieved with most antigens tested. However, as the genetic background of mice influence the outcome of the vaccine trial, and as there is no standardized challenge protocol, comparison is difficult. Although promising, these strategies based on single or few antigens, are less efficacious than recombinant live-attenuated, mostly tachyzoite *T. gondii* vaccine candidates (**Table 1**).

New approaches to develop effective vaccines are needed and depend on a better understanding of host-parasite interactions and parasite biology. This review presents the progress on our knowledge of the (1) protective host immune response during *T. gondii* invasion and infection in the different hosts, (2) manipulation and modulation of host immune response to ensure survival of the parasites able to evade and subvert host immunity, (3) molecular mechanisms that define stages specific development. This review concentrates, where possible, on studies using the natural oral route of infection, either by feeding tissue cysts or oocysts of the relevant host.

IMMUNITY AGAINST *T. GONDII*: THE HOST PERSPECTIVE

Except in the congenital cases, natural infection occurs through the oral route after ingestion of tissue cysts or oocysts. Bradyzoites or sporozoites released from cysts or oocysts respectively invade intestinal cells and convert to tachyzoites, the fast multiplying stage leading to the rapid spread of the parasite throughout the whole body. Tachyzoites are not completely eliminated by the immune system, they differentiate into bradyzoites and persist as bradyzoite-containing cysts. In cats, both sexual and asexual reproductive stages occur after ingestion of tissue cysts or oocysts. The sexual cycle is restricted to the feline intestine. As a result, male and female gametes are formed and after fertilization immature oocysts are created and excreted into the environment *via* feces.

As immediate reaction to host cell invasion by the parasite, an innate immune response is activated followed by an adaptive immune response resulting in antigen presentation and activation of the antigen specific T and B cell response. In most cases the immune response is capable of controlling the acute phase of the infection but is unable to eradicate the tissue cysts and is protective against reinfection. A wealth of information has been obtained in murine model to study immune responses against *T. gondii*. All data acquired in mouse cannot be directly transposed to humans, cats and live stocks. However, due to the intracellular nature of the parasite, protective immunity to *T. gondii* is recognized to be primarily dependent on the cellular immunity mediated by both $CD4^+$ and $CD8^+$ T cells, interferon-gamma (IFN-γ) being identified as a key mediator of protection against both acute and chronic *T. gondii* infection in mice (Suzuki and Remington, 1988; Gazzinelli et al., 1991; Casciotti et al., 2002). *T. gondii* infection also promotes antibody responses in systemic and mucosal compartments. Although their action is limited to only extracellular parasites, antibodies can opsonize parasites for phagocytosis, block parasite cell invasion, and also activate the classical complement pathway. An interesting aspect is the specific IgA response in the gut mucosa that thought to be an important barrier to oral re-infection.

Cat Immunity

In the intestine of cat, 96 h post-oral infection with *T. gondii* cysts, upregulation of class I major histocompatibility complex (MHC) I related genes suggest that cats promote potent antigen-specific immunity to limit replication of the parasite (Wang M. et al., 2018). At the systemic level, peripheral blood mononuclear cells (PBMCs) isolated from blood cat samples produced IFN-γ as early as 4 days post-oral infection with *T. gondii* cysts when stimulated with *T. gondii* antigens (Yin et al., 2015). It has also been shown that IFN-γ and interleukin-12 (IL-12) genes are upregulated in cat mesenteric lymph nodes (MLNs) and spleen 7 days following oral infection (Koyama et al., 1999). More recently, RNA-sequencing was used to detect transcriptional changes, 7 days after cat oral infection with *T. gondii* cysts, in different cat tissues (small intestine, lung, heart, liver, spleen,

TABLE 1 | *T. gondii*, RH, PRU, ME49 attenuated live vaccines evaluated in mouse model.

Targeted gene	Immunizationmouse/ amount/route	Challenge*T. gondii*[1] / amount/route	Protection Acute[2]	Protection Chronic[3]	References
RH	**Tachyzoites**	**Type I**			
Carbamoyl phosphate synthetase II (CPSII)	BALB/c, 10^5, i.p.	RH I, 200 T, i.p.	100%		Fox and Bzik, 2002
	C57BL/6, 10^6, i.p	ME49 II, 100 C, oral	100%		Gigley et al., 2009
		ME49 II, 10 C, oral		99-93%	
MIC1+ MIC3	Swiss OF1, 20, i.p.	76K II, 45 C, oral		>96%	Cérède et al., 2005 Ismael et al., 2006
MIC2	BALB/c, $5x10^4$, i.p.	RH I, 150 T, i.p.	100%		Huynh and Carruthers 2006
Ribosomal protein (RPS13)	Swiss OF1, 10^5, i.p.	RH I, $2x10^3$ T, i.p.	100%		Hutson et al., 2010
		ME49 II, 50 C, i.p.		94%	
Ortidine monophosphate decarboxylase (OMPDC)	C57BL/6, 10^6, i.p.	RH I, 200 T, i.p.	100%		Fox and Bzik, 2010
OMPDC + Uridine phosphorylase (UP)	C57BL/6, 10^6, i.p.	RH I, 200 T, i.p.	100%		Fox and Bzik, 2010
Apical membrane antigen 1 (AMA1)	BALB/c, 10^5, i.p.	RH I, 10^5 T, i.p.	100%		Lagal et al., 2015
	C57BL/6J, 250, i.p.	RH I, 10^3 T, i.p.	100%		
	CD-1, 10^4, i.p.	RH I, 10^5 T, i.p.	100%		
	BALB/C, 10^5, i.p.	ME49 II, 10^3 T, i.p.		70%	
Phosphatidyl threonine synthase (PTS)	C57BL/6J, 500, i.p.	RH I, 50 T, i.p.	100%		Arroyo-Olarte et al., 2015
		ME49 II, 3 C, i.p.		ND[4]	
GRA17	Kunming, $5x10^4$, i.p.	PRU II, 20 C, oral		98%	Wang et al., 2017
		RH I, 10^3 T, i.p.	100%		
Novel Putative Transporter 1 (NPT1)	Kunming, 10^6, i.p.	RH I, 10^3 T, i.p.	100%		Yang et al., 2019
		PYS 9, 10^3 T, i.p.	100%		
		PRU II, 20 C, oral		98%	
GRA17+NTP1	Kunming, 10^6, i.p.	RH I, 10^3 T, i.p.	100%		Liang et al., 2020
		PYS 9, 10^3 T, i.p.	100%		
		PRU II, 100 C, oral	100%		
		PRU II, 100 O, oral	100%		
Tyrosine kinase–like 1 (TKL1)	Kunming, 10^6, i.p.	RH I, 10^3 T, i.p.	100%		Wang J. L. et al., 2020
		PYS 9, 10^3 T, i.p.	100%		
		PRU II, 100 C, oral	100%		
		PRU II, 100 O, oral	100%		
PRU	**Tachyzoites**	**Type II**			
OMPDC	C57BL/6, $3x10^6$, i.p.	RH I, 10^3 T, i.p.	100%		Fox and Bzik, 2015
		PRU II, $2x10^7$ T, i.p.		100%	
Lactate dehydrogenase (LDH1, 2)	BALB/c, 10^3, i.p	RH I, 100 T, i.p.	100%		Abdelbaset et al., 2017
Ca2+-dependent protein Kinase 2	Kunming, 500, i.p	RH I, 10^3 T, i.p.	100%		Wang J. L. et al., 2018
		PYS 9, 10^3 T, i.p.	100%		
		PRU II, 20 C, oral		98%	
GRA17	Kunming,, 10^6, i.p.	RH I, 10^3 T, i.p.	100%		Li et al., 2020
		PYS 9, 10^3 T, i.p.	100%		
		PRU II, 100 C, oral	100%		
		PRU II, 10 C, oral		98%	
ME49	**Tachyzoites**	**Type II**			
LDH1+ LDH2	ICR, 10^4, i.p.	ME49 II, 10^4 T, i.p.	100%		Xia et al., 2018
		VEG III, 10^4 T, i.p.	100%		
		C7719 Chinese 1,10^4 T, i.p.	100%		
OMPDC	ICR, 10^4, i.p	ME49 II, 10^4 T, i.p.	100%		Xia et al., 2018
Adenylosuccinate lyase (ADSL)	ICR, 100, i.p.	RH I, 500 T, i.p.	100%		Wang L. et al., 2020
		ME49 II, 10^3 T, i.p.	100%		

i.p., intraperitoneal route; T, tachyzoite; C, cyst; O, oocyst.

[1]*challenge: T. gondii strain and Type/genotype.*

[2]*protection against acute toxoplasmosis: non-vaccinated control groups : 100% mortality, except for Wang L. et al. (2020) where 80% mortality, was recorded following challenge with ME49 tachyzoites.*

% survival monitored more than 30 days post-challenge.

[3]*protection against chronic toxoplasmosis : % brain tissue cyst reduction/nonvaccinated control group.*

[4]*brain cysts were not detected (ND).*

brain). In almost all tissues, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, NOD-like receptor signaling pathway, NF-κB signaling pathway, MAPK signaling pathway, T cell receptor signaling pathway and the cytosolic DNA sensing pathway, were among the up-regulated immune pathways. Among co-expressed genes, IFN-γ induced Indoleamine 2,3-dioxygenase (IDO) was co-expressed in tissues: heart, liver, lung, small intestine, and spleen, but not in the brain tissue. IDO enzyme degrades L-tryptophan an amino acid for which *T. gondii* is auxotrophic. IFN-γ inducible Guanylate-binding protein (GBP) was detected in the heart, liver, lung, and spleen, but not in small intestine and brain.

GBP is a member of the GTPase family. GBPs accumulate on PVs of *T. gondii* to mediate cell autonomous immunity (Cong et al., 2018). After challenge *via* the carotid artery with *T. gondii* tachyzoites, precapsular or popliteal lymph nodes cytokine mRNA levels were quantitated by reverse transcription (RT)-quantitative competitive polymerase chain reaction (PCR) (Levy et al., 2004). Increased expression of IL-12, tumor necrosis factor alpha (TNF-α), IFN-γ, and IL-6 that peaked at 7 days post-challenge was observed. IL-2 expression increased earlier, at 3 days post-infection. An inflammatory response was found in naturally *T. gondii* infected cats, asymptomatic for toxoplasmosis (Faria et al., 2018). Serum levels of TNF-α, reactive oxygen species (ROS), and nitric oxide (NO) were higher in seropositive cats compared to seronegative cats.

T. gondii oral infection promotes antibody responses. Anti-*T. gondii* IgM and IgG antibodies were detected 10 and 14 days post-infection (Dubey et al., 1995). Serum IgA were detected later, on week 34 after infection (Burney et al., 1995). Anti-T. *gondii* IgA antibodies were also detected in the intestinal tract of *T. gondii* infected cats (Rush et al., 2001). IgA in aqueous humor were also detectable in some cats (Lappin et al., 1995).

Sheep Immunity

The few studies carried out to investigate the cellular immune response in *T. gondii* infected sheep, found that the early immune responses involved IFN-γ. Within 2 to 5 days post-infection IFN-γ was detected and persisted for 6 to 9 days in the efferent duct of a lymph node draining the site of subcutaneous inoculation with *T. gondii* tachyzoites (Innes et al., 1995a). T cells were the major population present in responding efferent lymph, the $CD4^+$ T cells initially being the predominant subpopulation. Days 9–10 after infection, $CD8^+$ T cells were the majority subpopulation. Efferent lymph cells produced IFN-γ when stimulated *in vitro* with *T. gondii* antigens from day 6 after infection (Innes et al., 1995b). Simultaneous IFN-γ and IL-12 responses were produced by *in vitro* stimulated mesenteric lymphocytes and splenocytes from sheep infected 4 days earlier with *T. gondii* cysts by the oral route (Verhelst et al., 2014). In PBMCs directly isolated from sheep infected orally with *T. gondii* cysts, a clear increase in IFN-γ mRNA expression, determined by quantitative RT-PCR, was observed 2 weeks post-infection which remained until 7 weeks post-infection, the end of the observation period. In contrast, IL-10 and IL-4 mRNA expression did not show a consistent increase in all animals (Verhelst et al., 2015). Similarly, IFN-γ production was found in supernatants from PBMCs stimulated with *T. gondii* antigens, collected from sheep infected orally with *T. gondii* oocysts, 2 weeks post-infection (Stanley et al., 2004).

Recently, the peripheral and placental immune responses in pregnant sheep after oral infection with *T. gondii* oocyst at different times of gestation, have been studied (Castaño et al., 2019). An early peripheral release of IFN-γ at the first week post-infection followed by a short peak of IL-10 and TNF-α at the second week post-infection was observed in maternal sera, without significant differences between infection at the first, second and last term of gestation, with the exception of TNF-α, which was higher on those animals infected at mid-gestation. Studying the cytokine transcript expression profile at the maternofetal interface, a mixed of T Helper 1 (Th1) and Th2 type placental immune response was detected. IFN-γ showed the highest fold increase after infection at the first, second and last term of gestation. IL-4 showed higher levels on the first and second terms, while IL-10 showed a clear increase at the second and third terms of gestation.

In addition to cellular mechanisms, *T. gondii* infection in sheep stimulates humoral immune responses. Sheep orally infected with oocysts mount an IgG response by 2 weeks post-infection. Maximum serologic titers are detected from 21 to 56 days post-infection (Tenter et al., 1992; Stanley et al., 2004; Lopes et al., 2009; Dos Santos et al., 2016). *T. gondii* specific IgM antibodies peaked at 3 weeks post-infection and preceded an IgG response (Trees et al., 1989).

Pig Immunity

Following oral infection of pig with *T. gondii* oocysts, a significant increase in IFN-γ, IL-15, TNF-α, and inductible-NO gene expressions, in lymph nodes draining sites of infection colonic lymph nodes (CLNs), jejunal Peyer's patches (PPs) and MLNs, was observed at 7 days post-infection. Commensurate with recovery from overt disease, the proinflammatory gene expression was reduced at day 14 post-infection. Along with the Th1-like pattern of gene expression an increased anti-inflammatory gene expression is also observed. The IL-10 gene expression is significantly increased at 7 days post infection in MLNs and CLNs. The expression of transforming growth factor-β significantly increased in the CLNs at 7 post-infection and increased in the MLNs at 14 post-infection. Peripheral IFN-γ in the serum was detected at 7 days post-infection (Dawson et al., 2005). The phenotype of PBMCs was characterized after oral infection with *T. gondii* oocysts. A temporal decrease in the percentage of $CD4^+$ cells on day 6 post-infection and a significant increase in the percentage of $CD8^+$ cells was observed in the second week of infection. An early activation of cells was also detected in the first week of infection by an increase in expression of activation markers CD25 and SLA-DQ (Solano Aguilar et al., 2001). At 6 weeks post-oral infection with *T. gondii* oocysts, transcription analysis in MLNs, retropharyngeal lymph node and spleen, showed significant increase of IFN-γ transcripts both in spleen and retropharyngeal lymph node and of the T-cell surface marker CD8α, the natural killer (NK) cell marker CXCR3 (C-X-C Motif Chemokine Receptor 3), and the adaptor protein MyD88 in the retropharyngeal lymph node (Bartley et al., 2019). Recently, transcriptome sequencing analysis of different pig tissues (liver, spleen, cerebral cortex, lung, and MLNs) was used to characterize the porcine tissue transcriptional landscapes 6 and 18 days post-oral infection with *T. gondii* oocysts (He et al., 2019). *T. gondii* infection causes differential expression of transcription factors, such as Irf1, Irf8, Stat1, and Stat3. Cytokines (38) and cytokine receptor-related transcripts (21) that were differentially expressed were also identified. Upregulation of these genes can increase chemotaxis of immune cells, including dendritic cells (DCs), NK cells, macrophages, and T cells that can be regulated by C-X-C Motif Chemokine Ligand (CXCL)9, CXCL10, and CXCR3

signaling pathways contributing to the pig immune response to *T. gondii* infection. Genomic hotspots encoding 24 host genes significantly correlated with *T. gondii* load were also identified some of them have known anti-*T. gondii* activity, such as Gbp1, Gbp2, Gbp7, Batf2, and Tap1.

Following oral infection of pig with *T. gondii* cysts, IFN-γ secretion by mediastinal and duodenal lymph node cells stimulated with *T. gondii* antigens was observed from day 8 post-infection onwards while jejunal and ileal lymph node cells did not secrete IFN-γ (Rahman et al., 2020). Restimulated PBMC cells secreted IFN-γ from day 14 post-infection onwards (Rahman et al., 2020). Similarly, IFN-γ mRNA expression was observed from week 2 to 5 post-oral infection with *T. gondii* cysts (Verhelst et al., 2011). During the same follow-up period, IL-10 and IL-4 responses were not increased (Verhelst et al., 2011; Verhelst et al., 2015). In a study from 1 month to 4 months post-oral infection with *T. gondii* cysts, PBMC cells restimulated with *T. gondii* antigens produced IFN-γ mRNA from 1 month to the end of the experiment, while IL-10, IL-12 and IL-17 mRNA productions were not detected (Jennes et al., 2017). The IFN-γ production by restimulated PBMCs was correlated with the infection dose and predominantly brought about by $CD3^+$ $CD4^-$ $CD8^+$ T lymphocytes (Jennes et al., 2017).

T. gondii oral infection with oocyst or cysts promotes mucosal and systemic humoral responses. The presence of anti-*T gondii* IgG antibodies in sera of pig infected orally with *T. gondii* oocysts or cysts was detected within 2 weeks post infection (Verhelst et al., 2011; Burrells et al., 2015; Basso et al., 2017; Campero et al., 2020; Rahman et al., 2020). Anti-*T. gondii* IgM antibodies were detected 2 weeks post-infection and disappeared by week 6 post-infection with *T. gondii* cysts (Verhelst et al., 2011). A specific IgG antibody response was found sixty days after oral infection with *T. gondii* oocysts in pig aqueous humor (Garcia et al., 2008). Anti-*T. gondii* IgA and IgG antibodies were also detected in oral fluid, a combination of saliva and serum transudates from capillaries in the oral mucosa and gingival tissues, 1.5 to 4 weeks post-infection with *T. gondii* oocysts (Campero et al., 2020).

Human Immunity

Analysis of immune responses in human is largely confined to the peripheral blood and, there is no way to examine immune responses at the site of initial infection. Identification of susceptibility alleles and human primary immune-deficiencies that are associated with *Toxoplasma* infection provide also information about what is needed for protection (Ngô et al., 2017; McLeod et al., 2020).

The innate immune system is the first to respond to infection with production of chemokines, interleukins and growth factors. Monocytes infected with *T. gondii* tachyzoites released Alarmin S100A11, leading to the RAGE dependent induction of the chemokine C-C Motif Ligand 2 (CCL2) a major chemokine required for monocyte-mediated immunity (Safronova et al., 2019). Additionally, in infected monocytes (isolated from healthy donors) the inflammasome sensor NLRP3 (Gov et al., 2017) is induced leading to IL-1β production. Moreover, the inflammasome sensor NLRP1 has also been implicated (Witola et al., 2011). Susceptibility to human toxoplasmosis was found to map to alleles of the NALP (NLRP1) gene and NALP1 was shown to contribute to the control of parasite growth in a human monocyte cell line (Witola et al., 2011). Monocytes and dendritic cells ($CD1c^+$) produced IL-12 and TNF-α in response to phagocytic uptake of live parasites (Tosh et al., 2016) whereas neutrophils produced both IL-12 and TNF-α in response to *T. gondii* antigen stimulation (Bliss et al., 1999).

Up-regulation of CD80, CD86, and CD40 on dendritic cells infected with *T. gondii*, have important implication for the initiation of a T cell response because T cell production of INF-γ in response to *T. gondii*-infected DC is dependent on CD40-CD40L and CD80/CD86-CD28 signaling (Subauste and Wessendarp, 2000). Recently, a morphological and immunohistochemical evaluation of inflammatory cells in axillary lymph nodes from a symptomatic acute toxoplasmosis which self-resolved, demonstrated the presence of M1 macrophages and of T helper 1 lymphocytes (De-Luca et al., 2018).

In chronic asymptomatic individuals, the PBMCs' immune responses against *T. gondii* antigens are predominantly characterized by high levels of IFN-γ (Prigione et al., 2006; Meira et al., 2015) and most *T. gondii*-specific $CD4^+$ T-cell clones obtained from PBMCs produced IFN-γ (Saavedra and Hérion, 1991; Daubener et al., 1995). Polymorphisms in the human endoplasmic reticulum-associated aminopeptidase (ERAAP) gene ERAP1 were associated with susceptibility to human congenital toxoplasmosis, emphasis the importance of MHC Class I antigen processing in humans in response to *T. gondii* (Tan et al., 2010).

In addition to activating T cell-mediated immunity, IFN-γ activates different mechanisms to control intracellular *T. gondii* that seems to depend on specific host cell type. They include increased production of ROs (Murray et al., 1985), tryptophan limitation due to the upregulation of IDO (Pfefferkorn, 1984), the sequestration of iron (Dimier and Bout, 1998), K63-linked ubiquitination for endosomal destruction (Clough et al., 2016), noncanonical autophagy (Selleck et al., 2015). Recently, in IFN-γ-activated infected human macrophages, GBP1 recruitment to *T. gondii* PVs was shown to promote the lysis of *T. gondii* vacuoles and parasite membranes, releasing *T. gondii* DNA to trigger activation of the AIM2 inflammasome (Fisch et al., 2019).

Oral infection with *T. gondii* cysts or oocysts stimulates a humoral immunity used for the diagnosis of *Toxoplasma* infection. The humoral response involves IgG, IgM, IgA and IgE specific antibodies which may be detected in serum, tears, saliva, cerebrospinal fluid, colostrum and milk. Serum IgA and IgM antibodies specific to *T. gondii* are found in the serum during the first week following infection. Specific IgG antibodies may be detected 1 to 3 weeks after the initial rise in IgM levels and persists lifelong at residual titers (Correa et al., 2007; Robert-Gangneux and Dardé, 2012).

Mouse Immunity

The immune response to *T. gondii* has been extensively studied in mouse models. The mucosal immune system of the intestine is the front line of defense against *T. gondii*. Mucosal immunity involves the complex coordination of cells and cytokines to

enable control *T. gondii* infection without permitting emergence of proinflammatory immunopathology (Cohen and Denkers, 2015). Following oral infection, infected enterocytes secrete chemokines recruiting immune cell subsets. Neutrophils are one of the first cell types to arrive at the site of infection; they participate in the recruitment and activation of other immune cells such as macrophages and dendritic cells.

The innate event that initiates the production of IFN-I and proinflammatory cytokines including TNF-α and IL-12, is the recognition of *T. gondii via* pattern recognition receptors (PRRs). Among them, Toll-like receptors (TLRs) play a central role. Mice lacking MyD88, an adaptator molecule that acts downstream of TLR and IL-1 receptor family members are severely impaired in IL-12 production and failed to control the parasite after oral or systemic infection (Scanga et al., 2002; Sukhumavasi et al., 2008). Significant reduction of IFN-β was also observed in MLNs 5 days following oral infection with *T. gondii* cysts of mice lacking MyD88 (Han et al., 2014). *T. gondii* can stimulate innate immunity *via* multiple TLRs. *T. gondii* profilin is recognized by TLR 11/12, resulting in Myd88-dependent IL-12 production, a dominant mechanism driving IL-12 production in mice following intraperitoneal infection (Yarovinsky et al., 2005; Koblansky et al., 2013) or oral infection (Benson et al., 2009). TLR9, 2 and 4 have also been shown to be involved in the development of efficient IFN-γ responses by T cells in the lamina propria after oral infection with *T. gondii* cysts (Minns et al., 2006; Benson et al., 2009) and commensal bacteria are responsible for the activation of these TLRs (Benson et al., 2009). Another study, using deficient TLR4 or TLR2 deficient mice also showed that TLR4 deficient mice but not TLR2 deficient mice, had a higher number of brain cysts 35 days post oral infection with *T. gondii* cysts with a decrease of proinflammatory response 5 days after infection than wild type control mice. In contrast, following intraperitoneal infection TLR4 deficient mice had almost identical number of brain cysts and produced similar proinflammatory cytokines compared to wild type control mice (Furuta et al., 2006). As other *T. gondii* candidates able to activate TLRs, tachyzoite-derived GPIs have been shown to induce TLR2 and TLR4 dependent production of TNF-α in murine macrophages (Debierre-Grockiego et al., 2003) and recently microneme proteins 1 and 4 were also reported to stimulate IL-12 secretion in a TLR2/4-dependent manner in bone-marrow-derived DCs (Sardinha-Silva et al., 2019). In addition to the TLRs, *T. gondii* is also detected by C-C motif chemokine receptor 5 (CCR5) expressed on DC cells. *T. gondii* cyclofilin 18 is recognized by CCR5, stimulating IL-12 expression through activation of the G protein α I 1 family and MAPK (Aliberti et al., 2000). Furthermore, *T. gondii* triggers inflammasome activation *via* the inflammasome sensors NLRP1 and 3 in a caspase-1-dependent manner resulting in IL-1β and IL-18 production (Ewald et al., 2014; Gorfu et al., 2014; López-Yglesias et al., 2019). A recent study, demonstrated that *in vivo* inflammasome activation in response to *T. gondii* occurs in CD8α$^+$ DCs, inflammatory monocytes and neutrophils and suggested that a third sensor for *in vivo T. gondii* detection must exist in addition to NLRP1 and NLRP3 (Kupz et al., 2020).

The cytokine IL-12 controls the infection through initiation of IFN-γ production and is a key cytokine that links innate and adaptive immune system (Gazzinelli et al., 1994; Yap et al., 2000). Using bicistronic IL-12- yellow fluorescent protein reporter mice, it was shown that CD11c$^+$MHCII$^+$F4/80$^-$ dendritic cells, F4/80$^+$ macrophages, and Ly6G$^+$ neutrophils were the dominant cellular sources of IL-12 in the peritoneal cavity of mice infected intraperitoneally with *T. gondii* tachyzoites (Mercer et al., 2020). Dendritic cells contributed to the largest IL-12 positive population (Mercer et al., 2020). Furthermore, CD8α$^+$ DCs were identified as the key source of IL-12 as shown by the acute susceptibility and decreased IL-12 and IFN−γ production of *Batf3*$^{-/-}$ mice, which selectively lack only lymphoid resident CD8α$^+$ DCs and related peripheral CD103$^+$ DCs, during early systemic infection with *T. gondii* tachyzoites. This susceptibility was reversed by administration of IL-12. Oral infection of Batf3$^{-/-}$ mice demonstrated a similar susceptibility of these animals to *T. gondii* infection, with acute lethality and a failure to control parasite replication. Specifically, gut CD103$^+$ DCs are also absent in the Batf3$^{-/-}$ mice and may play a role during oral infection (Mashayekhi et al., 2011). A multiparameter flow cytometry identified in lymphoid tissues from intraperitoneally *T. gondii* infected mice an expansion and activation of both cDC subsets and plasmacytoid DCs and expansion of plasmacytoid DCs was also observed following oral infection (Pepper et al., 2008). Both plasmacytoid DCs maturation and IL-12 cytokine production are dependent on TLR11 (Pepper et al., 2008).

As a proinflammatory cytokine, IL-12 stimulates IFN-γ production, a key cytokine for control of acute and chronic infection (Suzuki et al., 1989; Suzuki and Remington, 1990; Gazzinelli et al., 1994). IL-12 stimulates intestinal innate lymphoid cells (ILC)-1 to produce IFN-γ and TNF-α in response to *T. gondii* oral infection. ILC1s are the main producers of IFN-γ and TNF-α compared to cNK cells and NKp46$^+$ NK1.1$^+$ ILC3s and mice lacking ILC1 (Tbx21$^{-/-}$) failed to control parasite replication in the small intestine and to recruit inflammatory monocytes (Klose et al., 2014). IL-12 triggers the production of IFN-γ later by T lymphocytes (Gazzinelli et al., 1994; Shirahata et al., 1994; Yap et al., 2000). CD4$^+$ T lymphocytes, recruited in dependence upon their expression of the chemokine receptor CXCR3 in the intestine, mediate activation of intestinal mucosa inflammatory monocytes *via* secretion of IFN-γ (Cohen et al., 2013).

An IL-12 independent production of IFN-γ by neutrophils that is regulated by TNF and IL-1β has been reported and selective elimination of neutrophils in TLR11 deficient mice infected intraperitoneally with *T. gondii* cysts resulted in acute susceptibility (Sturge et al., 2013). Inflammatory monocytes recruited by chemokine CCL3 produced in the intestinal mucosa by ICLs cells in dependence upon IL-18 in the intestine are associated with the ability to suppress early parasite replication at the site of infection (Dunay and Sibley, 2010). Furthermore, inflammatory monocytes have been identified as the major source of IFN-β in MLNs after oral infection with *T. gondii* cysts and mice lacking the receptor for type I IFN-1 showed higher parasite loads and reduced survival (Han et al., 2014). Expression of IFN-β by inflammatory

monocytes requires phagocytic uptake of parasites as well as signaling through TLR4 and MyD88 (Han et al., 2014).

A variety of cells respond to IFN-γ stimulation. IFN-γ signaling proceeds through activation of the signal transducer and activator of transcription 1 (STAT1). STAT1 upregulates the production of effector molecules such as NO and ROS in hematopoietic cells that lead to inhibition of intracellular parasite growth (Hunter and Sibley, 2013). IFN-γ also triggers the induction of immunity-related GTPase (IRG) proteins and GBPs, both in non-myeloid and myeloid cells to damage the PV membrane (PVM) (Howard et al., 2011; Kravets et al., 2016). IRG and GBP families are important factors in cell-autonomous immunity in mice. IFN-γ plays key roles not only in activating effector cells to kill the parasite but also in stimulating a production of mediators to recruit immune T cells from the periphery to the brain where tachyzoites transform into cysts to establish chronic infection (reviewed in Suzuki, 2020).

Adaptative immunity relies on the migration of antigen presenting cells (APCs) from the site of infection to secondary lymphoid organs and the activation of resident APCs through exposition to *T. gondii* antigens and on their ability to present antigens and to activate B cells, $CD4^+$ and $CD8^+$ T cells. Protection against *T. gondii* infection is mainly attributed to cell-mediated immunity and both $CD4^+$ and $CD8^+$ T cell subtypes are involved in the protection (Suzuki and Remington, 1988; Gazzinelli et al., 1991; Gazzinelli et al., 1992; Casciotti et al., 2002). Specific T lymphocytes act either as cytokine producer cells that help infected cells to kill the parasite or as cytotoxic cells that destroy infected cells. If IFN-γ production by infiltrated $CD4^+$ and $CD8^+$ T cells in the brain is required for the prevention of cerebral tachyzoite growth, anti-cyst activity of CD8+ T cells requires perforin in conjunction with microglia and macrophages help. CXCR3, CXCR6, and IL-18R are involved in recruitment and activation of microglia and macrophages to the T cell-attacked cysts for their elimination (Lutshumba et al., 2020). Peptide repertoire presented by MHC class I molecules to $CD8^+$ T cells is shaped by ERAAP. ERAAP-deficient H-2d mice generated significantly lower frequencies of immunodominant HF10-specific $CD8^+$ T cells and were consequently not protected from *T. gondii* infection (Blanchard et al., 2008).

Oral infection with *T. gondii* cysts also stimulates humoral immunity, in the digestive tract, the serum and milk (Chardes et al., 1990). The role of antibodies on resistance against *T. gondii* remained unclear until the use of B-cell-deficient mice (Kang et al., 2000; Johnson and Sayles, 2002). The results indicated the importance of the B-cell antibody response in preventing persistent proliferation of tachyzoites in the brain and lung during the chronic phase of infection.

T. gondii Antigens Recognized by Host Adaptive Immunity

Humoral Response

Infection is initiated by ingestion of oocysts or tissue cysts containing sporozoites and bradyzoites respectively. Around 2 days after ingestion and invasion of intestinal epithelial cells both convert into dividing tachyzoites which disseminate (acute infection), then tachyzoites convert to bradyzoites which persist the host's lifetime (chronic infection). Tachyzoites, sporozoites and bradyzoites have unique as well as shared antigens (Fritz et al., 2012; Nadipuram et al., 2020). Some antigens are no longer expressed by the parasite, in particular oocyst/sporozoite specific proteins in intermediate hosts. Specific bradyzoite proteins may be detected at the mucosal level at the beginning of infection and at the systemic level, latter during the induced chronic toxoplasmosis.

Serum IgG antibodies specific to *T. gondii* are mainly directed against tachyzoite/bradyzoite antigens. Most of the *T. gondii* antigens carrying B-cell epitopes have been identified after the screening of *T. gondii* cDNA libraries or high throughput protein microarray screening approaches (Beghetto et al., 2001; Hill et al., 2011; Liang et al., 2011; Döşkaya et al., 2014; Felgner et al., 2015; Döşkaya et al., 2018; Arranz-Solís et al., 2019) with sera from infected animals or infected humans. These antigens included rhoptry (ROP1, 2, 5, 6, 8, 18), dense granules (GRA1, 2, 3, 4, 5, 6, 7, 8, 9, 14), micronemes (MIC1, 2, 3, 4, 12), M2AP, AMA1, ROM5, MAG, P68, (Felgner et al., 2015; Döşkaya et al., 2018; Ybañez et al., 2020), surface proteins (SAG1, SAG2, SAG2A (SRS34A), SAG2C (SRS49D), SRS52A, SRS29A, SRS13). Serum IgG antibodies specific to bradyzoite BAG1 and MAG1 proteins, were detected early after infection in human sera (Di Cristina et al., 2004) and in mice orally infected with cysts (Hester et al., 2012). Serum IgG antibodies specific to oocyst/sporozoite proteins, namely TgOWP8, TgERP and TgCC5A were detected in sera from human and animals infected with oocysts (Hill et al., 2011; Santana et al., 2015; Liu et al., 2019). These proteins were proposed as new tools to identify the parasite stage of infection, allowing better understanding of sources of *T. gondii* infection in animals and humans.

The presence of mucosal antibodies measured in saliva, intestinal fluids, milk and tears has been reported (Correa et al., 2007). Salivary IgA antibodies specific to sporozoite TgERP protein, were detected in human infected with oocysts (Mangiavacchi et al., 2016). Oral infection with *T. gondii* oocysts induced production of anti-SAG1 IgG and IgA specific to tachyzoite stage in human saliva (Chahed Bel-Ochi et al., 2013) and oral fluids from pigs (Campero et al., 2020). In cat, oral infection with oocysts induced specific antibodies in sera, intestinal secretions, and fecal extracts not only to *T. gondii* tachyzoites, but also to bradyzoites, sporozoites, and enteroepithelial stages (Rush et al., 2001). The IgA from intestinal secretions bound to antigens on all enteroepithelial stages and to a less degree to bradyzoites and sporozoites but did not bind to tachyzoites whereas serum IgG bound to tachyzoites, bradyzoites, sporozoites, and enteroepithelial stages of *T. gondii* and serum IgA bound strongly to enteroepithelial stages but only weakly to tachyzoites and bradyzoites, suggesting a compartmentalization of the humoral response.

Cellular Response

Several endogenous $CD8^+$ *T. gondii*–derived epitopes have been identified to date (**Table 2**). Search for candidate $CD8^+$ T cell epitopes restricted by specific HLA molecules was based on several bioinformatic algorithms, and then binding affinity assays. The selected peptides were tested for their ability to elicit IFN-γ production by human PBMCs from seropositive (but not

TABLE 2 | HLA-restricted *T. gondii* $CD8^+$ T cell epitopes.

Gene ID[1]	Protein[2]	Peptide sequences	Immunogenicity[3] in mice (IFN-γ)	References
HLA-A2	**Average population**	**coverage: 25%**		
227280	GRA3 (T, B)	GRA3 $^{25–33}$ (FLVPFVVFL)	+	Cong et al., 2011
286450	GRA5 (T, B, SZ)	GRA5 $^{82-90}$ (GLAAAVVAV)	+	Cong et al., 2011
275440	GRA6 (T, B, SZ)	GRA6 $^{24–32}$ (VVFVVFMGV)	–	Cong et al., 2011
		GRA6 $^{29–37}$ (FMGVLVNSL)	+	Cong et al., 2011
233460	SAG1/SRS29B (T, SZ)	SAG1$^{289-297}$ (SPEKHHCTV	ND	Yano et al., 1997
207160	SAG2C/SRS49D (B)	SAG2C $^{38–46}$ (FLSLSLLVI)	+	Cong et al., 2011
207150	SAG2D/SRS49C (B)	SAG2D $^{180–189}$ (FMIAFISCFA)	+	Cong et al., 2011
207140	SAG2X/SRS49B (T)	SAG2X $^{44–52}$ (FVIFACNFV)	+	Cong et al., 2011
		SAG2X $^{351–359}$ (FMIVSISLV)	+	Cong et al., 2011
308020	SAG3/SRS57 (T, B, SZ)	SAG3 $^{375–383}$ (FLLGLLVHV)	+	Cong et al., 2011
		SAG3 $^{136–144}$ (FLTDYIPGA)	+	Cong et al., 2011
315320	SRS52A	SRS52A $^{12–20}$ (ITMGSLFFV)	+	Cong et al., 2011
291890	MIC1 (T, SZ)	MIC1 $^{9–17}$ (VLLPVLFGV)	+	Cong et al., 2011
214940	MIC2-M2AP (T, B, SZ)	MICA2P $^{11–19}$ (FAAAFFPAV)	–	Cong et al., 2011
295460	Got1 family protein	Got1 $^{26-34}$ (FLDRALLTL)	ND	Cardona et al., 2015
206350	Ter1p	Terp1 $^{184-192}$ (FLADLLHSV)	ND	Cardona et al., 2015
HLA-A3	**Average population**	**coverage: 24%**		
286450	GRA5 (T, B, SZ)	GRA5$^{89-98}$ (AVVSLLRLLK)	+	Cong et al., 2010
275440	GRA6 (T, B, SZ)	GRA6$^{164-172}$ (AMLTAFFLR)	+	Cong et al., 2010
203310	GRA7 (T, B, SZ)	GRA7$^{134-142}$ (RSFKDLLKK)	–	Cong et al., 2010
233460	SAG1/SRS29B (T, SZ)	SAG1$^{224-232}$ (KSFKDILPK)	+	Cong et al., 2010
207160	SAG2C/SRS49D (B)	SAG2C$^{13-21}$ (STFWPCLLR)	+	Cong et al., 2010
315320	SRS52A	SRS52A $^{250-258}$ (SSAYVFSVK)	+	Cong et al., 2010
HLA-B7	**Average population**	**coverage: 30%**		
227280	GRA3 (T, B)	GRA3 $^{27–35}$ (VPFVVFLVA)	–	Cong et al., 2012
203310	GRA7 (T, B, SZ)	GRA7 $^{20–28}$ (LPQFATAAT)	+	Cong et al., 2012

[1]*TGME49_Gene identifier according to ToxoDB.org. (ME49 type II strain).*

[2]*Identity of protein assigned by ToxoDB.org, stage specificity: tachyzoite (T), bradyzoite (B), sporozoite (SZ).*

[3]*Splenic T cells were isolated from HLA-A2, A3 or B7 supertype mice after peptide immunization and tested for their ability to generate IFN-γ response to peptide.*

ND, not done.

+, IFN-g production; -, no production of IFN-g.

seronegative) people using an IFN-γ ELISPOT assay. For this purpose, amino acid sequences from *T. gondii* tachyzoite, bradyzoite and sporozoite secreted or surface antigens were selected. These sequences included those of SAG1, SAG2C, SAG2D, SAG2X, SRS52A, SAG3, MIC1, MICA2P, GRA5, GRA6, and GRA3 for HLA-A02 specific peptide-binding, SAG1, SAG2C, SRS52A, GRA5, GRA6, and GRA7, for HLA-A03 specific peptide-binding and GRA3 and GRA7 for HLA-B07 specific peptide-binding (Tan et al., 2010; Cong et al., 2010; Cong et al., 2011; Cong et al., 2012). Two peptides for HLA-A02 specific peptide binding were also reported by Cardona et al. (2015), one from a putative ER-retrieval receptor (Ter1p) and another one from a Glutamicoxaloacetic transaminase (Got1). Both proteins have peptide signal and transmembrane domains and predicted to be cleaved at the proteasome. They are involved in parasite metabolic events. Furthermore a $CD8^+$ T cell clone derived from a person with chronic toxoplasmosis was shown to kill EBV-Ya cells infected with RH tachyzoites or pulsed with SAG1$^{289-297}$ (SPEKHHCTV) but not uninfected and non-pulsed EBV-Ya cells. The cytotoxicity was blocked with anti-HLA-A02 and anti-CD8 monoclonal antibodies (Yano et al., 1997).

In the search of parasite antigens involved in $CD4^+$ T cells response, T cell clones derived *in vitro* from chronically infected healthy persons or PBMCs were stimulated with a range of selected recombinant *T. gondii* proteins (or fragment of proteins). Proliferative responses and/or cytokine production were obtained for SAG1 and GRA2 in a HLA-DR-restricted manner (Prigione et al., 2000), for MIC2, MIC3, MIC4 and M2AP (Beghetto et al., 2005), for SAG1, GRA1, GRA6, and GRA7 (Fatoohi et al., 2002), SAG1, ROP1, GRA8 and MAG1 (Silva-Gutierrez et al., 2018) and for BAG1 and MAG1 (Di Cristina et al., 2004). In one study, the epitope peptide DPw4 restricted (ROP2$^{197-216}$: TDPGDVVIEELFNRIPETSV) recognized by a T cell clone specific for ROP2 was further defined using several algorithms followed by *in vitro* stimulation of the T cell clone (Saavedra et al., 1996).

To our knowledge, such approaches to identify T cell epitopes have not been undertaken for sheep, pigs and cats. Plenty of *T. gondii* antigens are recognized by specific antibodies, in all host species. As B cell activation and initiation of the humoral immune response to protein antigens requires both B and T cell involvement, this suggests that these targeted antigens potentially contain both $CD4^+$ T cell T and B epitopes.

IMMUNITY AGAINST *T. GONDII*: THE PARASITE PERSPECTIVE

The mechanisms of *T. gondii* persistence are studied mostly at the tachyzoite stage. *T. gondii* has developed a multitude of strategies partially strain-dependent, host cell-type specific and

host-species dependent (reviewed in Hakimi et al., 2017; Lima and Lodoen, 2019; Poncet et al., 2019; Zhu et al., 2019; Wong et al., 2020).

T. gondii manipulates host immunity through secretion of proteins, mainly ROP and GRA proteins that modify host transcriptional programs or signaling pathways. Through ROP18/ROP5/ROP17/GRA7 complex, type I strains of *T. gondii* inactivate host IRGs to evade the IFNγ-inducible GTPase-mediated host defense which plays a crucial role in the destruction of the PVM in a murine model. ROP54 in type II strain was also shown to interfere with the noncanonical IFN-γ dependent autophagy pathway by restricting immune loading of GBP2 to evade the GBP2-mediated immune response (Kim et al., 2016). Type I ROP18 has been shown to phosphorylate the transcription factor ATF6β leading to downregulation of CD8 T cell activation (Yamamoto and Takeda, 2012) and to inhibit the host NF-κB pathway by promoting p65 degradation (Du et al., 2014). The dense granule-resident effector, called TEEGR (Toxoplasma E2F4-associated EZH2-inducing Gene Regulator) has been shown to suppress NF-κB regulated TNF-α cytokine signaling in a type II strain (Braun et al., 2019). GRA18 (conserved across the *Toxoplasma* lineages) induces β-catenin-dependent genes associated with anti-inflammatory responses, including CCL17 and CCL22 chemokines in murine infected macrophages (He et al., 2018). *Toxoplasma* inhibitor of STAT1-dependent transcription (TgIST) (type I, II), which represses STAT1-dependent promoters (Gay et al., 2016; Olias et al., 2016) could be the effector responsible for the dysregulation of MHC II molecule expression in infected host cells (Poncet et al., 2019). ROP16 (type I, III) phosphorylates STAT3 and STA6, mainly leading to a decrease in IL-12 and Th1 response (Saeij et al., 2007; Yamamoto et al., 2009; Ong et al., 2010).

Contribution of these specific modulatory effectors in the chronic infection remains poorly investigated. Recently, Mayoral et al. (2020) showed that despite a decline in nuclear accumulation, TgIST still mediated suppression of IFN-γ signaling after prolonged infection with bradyzoites, and provided evidence that TgIST is exported beyond the early stages of host cell infection. TgPL1, a patatin-like phospholipase 1, localized within vesicles inside the tachyzoite, is localized to the PV and cyst wall in the encysted bradyzoite stage. TgPL1 was previously determined to be necessary for tachyzoites to suppress NO synthesis and prevent degradation in activated macrophages (Mordue et al., 2007; Tobin and Knoll, 2012). Contrary to mice infected with wild type parasites, mice infected with parasites lacking TgPL1 maintain high cytokine levels and do not display toxoplasmic encephalitis symptoms, suggesting that TgPL1 plays a role in the maintenance of chronic *T. gondii* infection (Tobin Magle et al., 2014).

Two specific modulatory effectors identified in type II strains namely GRA15 and GRA24 have an opposite effect, by promoting of a proinflammatory signal. GRA24 in type II strain activates p38 MAPK signaling pathway by prolong autophosphorylation and nuclear translocation of host cell p38a, leading to production of proinflammatory cytokine IL-12 in both infected macrophages and dendritic cells (Kim et al., 2005; Braun et al., 2013; Ten Hoeve et al., 2019). IL-12 produced by DCs plays a critical role in controlling acute *Toxoplasma* infection in mice (Mashayekhi et al., 2011), but at the same time, infected dendritic cell migration is maintained to contribute to efficient dissemination of the parasite (Ten Hoeve et al., 2019). GRA24 is not secreted beyond the PVM, is not localized to the host cell nucleus and instead accumulates within the *in vitro* tissue cysts (Krishnamurthy and Saeij, 2018). These observations suggest that GRA24 contributes to effective control of acute *T. gondii* infection and promotes the conversion of parasite into bradyzoites and persistence at the chronic infection stage. GRA15 type II activates the NF-κB pathway through interaction with tumor necrosis factor receptor associated factors (TRAFs), which are adaptor proteins functioning upstream of the NF-κB transcription factor (Sangaré et al., 2019), leading to human monocyte production of IL-1β (Gov et al., 2017), induction of IL-12 secretion by murine macrophages (Rosowski et al., 2011), and upregulation of the CD40 cell surface receptor on murine macrophages, which leads to IL-12 production (Morgado et al., 2014). The role of GRA15 during the chronic phase of infection has not been investigated.

The growing list of parasite effectors responsible for the activation, inhibition, and interaction of signaling pathways involved in the persistence of *T. gondii* in host cells will permit the identification of the strategies developed by *T. gondii* to limit its virulence thereby promoting the survival of its host and the formation of tissue cysts.

TOWARD EFFECTIVE VACCINES AGAINST *T. GONDII*

Livestock Animals and Cats

Live Vaccines in Livestock Animals and Cats

The recent development of new techniques based on genetic engineering allowed the explosion of strategies for vaccine development. Over the last decades, the field of vaccinology has been exploring safer vaccines by creation of recombinant immunogenic proteins, naked DNA and viral/bacterial recombinant vectors. Although promising, the strategies based on single or few antigens are less efficacious than attenuated live *T. gondii* strains. Recombinant *T. gondii* strains present all the diversity and complexity of *T. gondii* antigens, and enable appropriate processing and presentation of *T. gondii* antigens in the context of MHC class I antigens, $CD8^+$ T cells and IFN-γ being key elements of a protective immune response. Until now, most of the live attenuated *T. gondii* vaccines are tachyzoites, the only stage easy to produce *in vitro* and in large quantities.

The first one, the S48 strain live vaccine, resulted in one commercially available vaccine (Toxovax), for protection against congenital toxoplasmosis in sheep. The S48 strain has lost the ability to differentiate into bradyzoite and tissue cysts (Buxton, 1993) as a result of being passaged for many years in laboratory mice. In ewes, subcutaneous inoculation of 10^5 tachyzoites of the S48 strain, induced both cellular ($CD4^+$ and $CD8^+$ T cells) and humoral responses (Innes and Wastling, 1995; Wastling et al., 1995) and conferred significant protection against abortion after oral challenge with *T. gondii* oocysts (Buxton et al., 1993).

Studies have been carried out in lambs and pigs inoculated subcutaneously with the S48 strain, to evaluate its ability to reduce parasite burden in meat and thus improving food safety. It was found that the S48 strain protected against the development of tissue cysts following an oral challenge with *T. gondii* cysts (Katzer et al., 2014; Burrells et al., 2015). A chemically-induced mutant *T. gondii* strain that lacks the capacity to form oocysts in cat but undergoes both schizogony and gametogony in the intestine of cats after ingestion of tissue cysts (Dubey, 2017), the T-263 strain, was found to be effective in inducing immunity in cats (Frenkel et al., 1991; Freyre et al., 1993). Kittens orally inoculated with tissue cysts of the T-263 strain did not shed oocyst when challenged orally with tissue cysts of a heterologous normal strain (84% of the kittens) (Frenkel et al., 1991). The abilities of *T. gondii* T-263 tissue cysts, bradyzoites released from tissue cysts, and tachyzoites, to confer protection, were compared (Freyre et al., 1993). Orally administered bradyzoites of T-263 strain, either contained in intact tissue cysts or liberated from cysts, induced immunity to oocyst shedding. In contrast, tachyzoites did not completely protect against oocyst shedding, even when delivered directly to the duodenum (Freyre et al., 1993). A 3 years field trial on swine farms was conducted to determine the effectiveness of cat vaccination with bradyzoites of the T-263 strain, in reducing the prevalence of *T. gondii* in finishing pigs (Mateus-Pinilla et al., 1999). Under field conditions, cat vaccination was able to significantly reduce *T. gondii* infection in finishing pigs, suggesting that cat vaccination could contribute to improve food safety.

In recent years, the availability of the complete *T. gondii* genome sequence and genetic engineering methods allowed to develop genetically attenuated *T. gondii* strains, mostly by gene deletions. These new attenuated strains would be safer than S48 and T263 strains obtained after passage for many years in laboratory mice and chemical-induced mutations respectively. The first to be tested in sheep and cat was the strain called Mic1-3KO obtained by deletion of *MIC1* and *MIC3* genes in the *T. gondii* RH strain (Cérède et al., 2005). These genes encode microneme proteins involved in adhesion of the parasite to host cells (Fourmaux et al., 1996; Garcia-Réguet et al., 2000). The simultaneous disruption of the two genes was shown to impair invasion of cells *in vitro* and to impair virulence in mice (Cérède et al., 2005). The Mic1-3KO strain confers protection against chronic and congenital toxoplasmosis in outbred mice (Ismael et al., 2006). Ewes inoculated either subcutaneously or intraperitoneally with tachyzoites of the Mic1-3KO strain developed protection against *T. gondii* abortion following oral challenge with *T. gondii* oocysts (Mévélec et al., 2010). Mic1-3KO was as effective as the S48 strain. Moreover, preliminary results showed the potential of Mic1-3KO to reduce the development of tissue cysts in lambs born to vaccinated ewes. However, when administrated to cat subcutaneously or orally in a gastro-resistant solution to resist intestinal passage, Mic1-3KO did not prevent cats from oocysts shedding following oral challenge with *T. gondii* oocysts (Le Roux et al., 2020). Recently, a HAP2-deficient *T. gondii* strain was created using a CRISPR/Cas9 strategy in the CZ clone H3 *T. gondii* (type II strain). This HAP2-deficient *T. gondii* strain generates oocysts that fail to produce sporozoites (oocysts do not undergo meiosis). Oral administration of HAP2-deficient *T. gondii* tissue cysts to cats was shown to totally prevent oocyst excretion after oral infection with tissue cysts from wild-type *T. gondii*, demonstrating that this mutant could be a transmission-blocking vaccine (Ramakrishnan et al., 2019) (**Figure 1**).

Vaccination Perspectives for Cats and Livestock Animals

Oral vaccination of cats with bradyzoites of T263 strain led to effective prevention of oocyst excretion (Freyre et al., 1993). Bradyzoites that invade the feline intestinal epithelium differentiate into five morphologically distinct types of schizonts (Dubey and Frenkel, 1972). It is known that the enteric parasite cycle has a role in inducing immunity able to prevent oocyst shedding in cats (Dubey, 2017). Identification of the initial steps necessary for induction of protective immunity against oocyst shedding is necessary to move forward improved vaccination strategies.

Contrary to tachyzoites, bradyzoites are not easily manipulated *in vitro* and developing alternative systems to animal model is becoming a major issue in biology. *In vitro* differentiation models using stress conditions applied to tachyzoites have been demonstrated to induce cyst formation. These include alkaline stress, heat shock treatment, cytokine (IFN-γ), nutrient deprivation, and chemical/drug treatments. The most commonly used method is alkaline treatment and mostly fibroblasts and macrophages are used as host cells. The spontaneous *in vitro* differentiation of bradyzoites in the absence of extrinsic stress, offer new perspectives to develop more efficient differentiation models (Cerutti et al., 2020). Indeed, Paredes-Santos et al. (2016) demonstrated the ability of EGS tachyzoite strain to convert in intracellular cysts/bradyzoites, in different cell types and under physiological pH with a thick wall such as that of mature cysts isolated from mice. Moreover, this provides interesting perspectives to vaccinate livestock animals with bradyzoites through the oral route to induce efficient mucosal immune responses to tackle the parasite at its portal of entry. Additionally, recent advances in understanding the mechanisms behind stage switching provide potentially interesting perspectives for "spontaneous" *in vitro* switch from tachyzoite to bradyzoite stage. For example, parasites (type II or I) in which PKAc3, a protein kinase responsible for cAMP dependent tachyzoite maintenance while suppressing differentiation to bradyzoites, is deleted, have high rates of bradyzoite formation (CST1cyst wall positive vacuoles) (Sugi et al., 2016). Deletion of the transcription factor AP2IV-4 that has been shown to directly silence bradyzoite mRNA and protein expression in tachyzoite, improved also the *in vitro* switch from tachyzoite to bradyzoite stage (Radke et al., 2018). Bradyzoites generated in these models have not yet been fully characterized. They may be more heterogeneous and not equivalent to *in vivo* generated bradyzoites. We do not know if they are infectious by the oral route and able to initiate the enteric parasite cycle.

Until now, only recombinant attenuated tachyzoites were shown to be able to provide protection following challenge against chronic toxoplasmosis. Whether or not bradyzoites would be more efficient than tachyzoites in reducing brain cysts

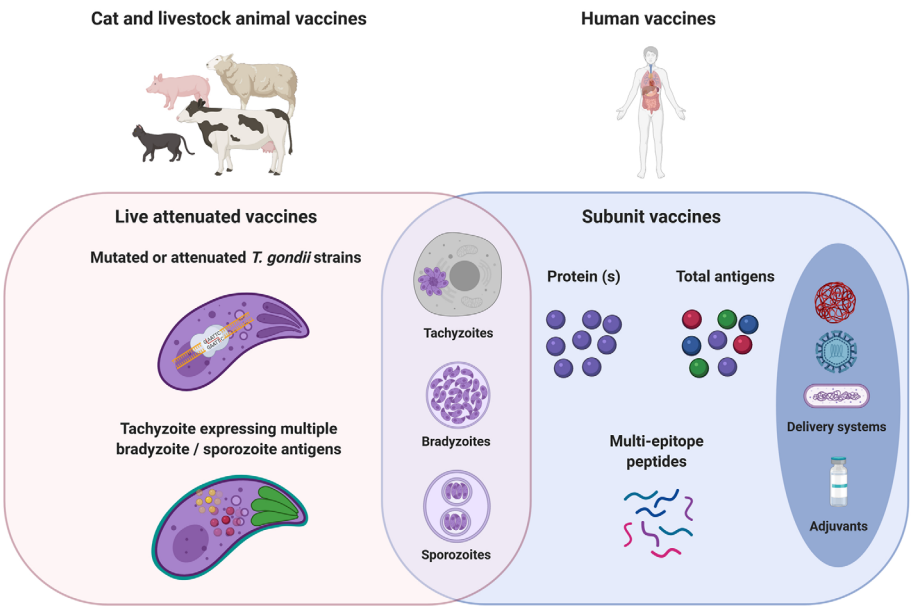

FIGURE 1 | Cat, livestock, and human toxoplasmosis vaccine strategies. *Toxoplasma* infection is acquired by three stage forms: tachyzoite, bradyzoite (cyst), or sporozoite (oocyst). In a one health vaccination strategy, the objectives would be the prevention of congenital disease in both women and livestock, the prevention/reduction of *T. gondii* tissue cysts in food-producing animals; and oocyst shedding in cats. Live mutated or attenuated *T. gondii* tachyzoite stage vaccines are mostly used for cat and livestock animals. The development of tachyzoites expressing multiple bradyzoite/sporozoite antigens could therefore improve the immunogenicity of live vaccines, whatever the *T. gondii* stage. Live vaccines would require passing considerable regulatory hurdles that may impede their use in human. Safer subunit vaccines have been explored by the generation of recombinant immunogenic proteins. The use of *T. gondii* extract allows vaccination with all the diversity and complexity of *T. gondii* antigens. Subunit vaccines need delivery systems and adjuvants to improve antigens bioavailability and stimulate protective cellular immune responses to *T. gondii.* Studies aiming at the identification of $CD8^+$, $CD4^+$, and B cell specific *T. gondii* epitopes are still needed to rationally design an efficient multi-epitope vaccine engineered to focus both T and B cells responses toward a set of critical epitopes selected from a wide range of antigens in order to diversify the scope of the induced immune responses.

loads has not been evaluated. The ability of bradyzoites to induce a bradyzoite specific immune response following oral infection with cysts remains controversial. In mice orally infected with *T. gondii* cysts, IgG directed against specific bradyzoite surface antigens SRS9, SAG2C, SAG2X, and SAG2Y were not detected (Kim and Boothroyd, 2005). However, BAG1, a bradyzoite antigen and MAG1 a matrix antigen, were shown to induce an early humoral and cell-mediated immune responses upon human infection (Di Cristina et al., 2004). In order to study the potential role of stage-specific expression and compartmentalization of antigens in the induction of a *T. gondii*-specific $CD8^+$ T cell response, a *T. gondii* strain engineering to express a secreted or cytoplasmic β-galactosidase either at the bradyzoite or tachyzoite stage was constructed. The frequencies and kinetics of β-galalactosidase-specific $CD8^+$ T cells in infected mice was monitored by MHC class I tetramer staining. Upon oral infection with *T. gondii* cysts, only β-galactosidase-secreting tachyzoites, but not bradyzoites secreting β-galactosidase, induced β-galactosidase-specific $CD8^+$ T cell in both the spleen and the brain of infected mice (Kwok et al., 2003). These results suggest that bradyzoites are not able to induce a specific $CD8^+$ T cell response at the systemic level. In another study to explore the role of stage-specific expression, parasites (Type II PRU strain) were engineering to express a specific bradyzoite surface protein (SRS9) at both tachyzoite and bradyzoite stages (or a specific tachyzoite surface protein (SAG1) at both tachyzoite and bradyzoite stages). In mice infected intraperitoneally with tachyzoites, expression of SRS9 at both tachyzoite and bradyzoite elicited a specific SRS9 humoral and cellular T cell response that was absent in wild type infection and resulted in reduced cyst burdens in brains of parasite-infected mice (Kim and Boothroyd, 2005). Both the amount and the systemic dissemination of tachyzoites may account for an efficient presentation of SRS9 by tachyzoites to the immune system (in the spleen and also in the brain). More recently, by the use of parasites expressing the CMHI peptide of OVA in fusion with the GRA6 protein at the tachyzoite stage or at both tachyzoite and bradyzoite stages, comparable OVA specific $CD8^+$ T cell responses were elicited when expressed only by tachyzoite or by both stages in the spleen and the brain of mice infected intraperitoneally with tachyzoites. Compared to the parental strain, both parasites expressing GRA6-OVA peptide at tachyzoite stage or at tachyzoite and bradyzoite stages reduced cyst burdens in the brains (Salvioni et al., 2019). These studies suggest that both the amount and the systemic dissemination of tachyzoites may account for an efficient presentation of bradyzoite specific or antigens shared by both tachyzoites and bradyzoites, by tachyzoites to the immune system (in the spleen and also in the brain). On the contrary, specific bradyzoite antigens seem poorly immunogenic due to the location and quiescent metabolic activity of bradyzoites along with the timing of the immune response that is able to see

them. Thus, a tachyzoite expressing multiple bradyzoite antigens could be a good candidate vaccine to induce efficient immune responses against both tachyzoite and bradyzoite stages. Indeed, intraperitoneal inoculation of tachyzoites expressing cyst wall proteins (e.g. BPK1, MCP4, CST1) and the bradyzoite surface antigen SRS9, by deleting the transcription factor AP2IV-4 that has been shown to directly silence bradyzoite mRNA and protein expression in tachyzoite, were unable to form cysts in brain tissue and a potent immune response characterized by increases inflammatory monocytes, IFN-γ and higher numbers of both $CD8^+$and $CD4^+$ T-cells was observed (Radke et al., 2018). These results suggest an increased parasite control by the mice compared to the parent strain. However, protection against an oral challenge with *T. gondii* cysts has not been performed in this study.

For pigs especially in farms allowing outdoor access and sheep, infections occur after ingestion of *T. gondii* oocysts shed by cats in the environment. Vaccination through the oral route with oocysts/sporozoites would induce efficient mucosal immune responses to tackle the parasite at its portal of entry. The immune responses induced by oocysts at both mucosal and systemic levels remain poorly understood in livestock animals and even in mice. In humans and pigs, oral infection with *T. gondii* oocysts induced a systemic production of antibodies (IgG – IgM) directed against specific sporozoite/oocyst antigens (Hill et al., 2011; Santana et al., 2015; Liu et al., 2019). Salivary IgA against the sporozoite antigen TgERP were also detected in *T. gondii* infected human (Mangiavacchi et al., 2016). Moreover, in mice orally infected with *T. gondii* oocysts, serum IgG antibodies directed against the sporozoite specific CCp5A protein were detected (Santana et al., 2015). These observed humoral responses suggest that reactions involving sporozoite antigen specific B cells and follicular $CD4^+$ T cell can take place in germinal centers. The lack of knowledge, is due at least in part to technical difficulties to obtain purified oocysts. To date, oocysts are obtained from infected cats. Until recently, the molecular determinants that identify cats as the definitive host for sexual development were unknown. Martorelli Di-Genova et al. (2019) found that inhibition of murine delta-6-desaturase, an enzyme for the conversion of linoleic acid to arachidonic acid and supplementation of their diet with linoleic acid allowed *T. gondii* sexual development in mice. Mouse derived oocysts sporulate and are infectious. They also obtained sexual development in cell culture mouse cells supplemented with both linoleic acid and SC-26196 a specific inhibitor of the delta-6-desaturase enzyme, approximately 26% of the *T. gondii* vacuoles expressed both BRP1 and GRA11B. More recently, the *T. gondii* microrchidia (MORC) protein was identified as an upstream transcriptional repressor of sexual commitment (Farhat et al., 2020). Deletion of the MORC protein resulted in the induction *in vitro* of the expression of cyst wall and sporozoite proteins. These works open new perspectives for alternative *in vitro* production of sexual *T. gondii* stages and these new advances provide potentially interesting new perspectives for a better understanding of this life cycle stage, stage-host interactions and host immune responses.

If vaccination with sexual stages is not achievable, an alternative could be tachyzoites that express proteins, fragment of proteins, or chimeric proteins carrying T and B cell epitopes of antigens expressed by bradyzoite/sporozoite, to get homogeneous and reproducible parasite populations carrying these epitopes (**Figure 1**). The subcellular localization of these epitopes would be important to get the most immunogenic context. Using OVA as a model antigen, it has been shown that tachyzoites expressing secreted OVA in the vacuole but not those that express a cytosolic OVA stimulated OVA specific $CD4^+$ T cells (Pepper et al., 2004). In addition, tachyzoites expressing secreted OVA in the vacuole, and to a less degree those that express a surface membrane bound OVA stimulated OVA specific $CD8^+$ T cells. But tachyzoites expressing intracellular OVA (either in the cytosol, the mitochondrion or inner membrane complex) were unable to stimulate OVA specific $CD8^+$ T cells (Gregg et al., 2011). Thus, both specific $CD4^+$ and $CD8^+$ T cell responses would be promoted when the protein is secreted. Furthermore the fate of the secreted protein may also influence the $CD8^+$ T cell response. For example studies using GRA6, a *T. gondii* dominant antigen, showed that GRA6 MHC I presentation is favored when the protein is inserted in the PVM and when its C-terminal MHC I epitope is exposed to the host cell cytosol (Lopez et al., 2015; Buaillon et al., 2017). To increase MHC I presentation, strategies facilitating perturbation/disruption of the PVM to release PV localized antigens in the host cell cytosol may be developed. In previous studies, IRGs proteins were shown to enhance antigen presentation of *T. gondii* antigens in mouse embryonic fibroblasts (Lee et al., 2015). Recently, deletion of ROP5, ROP18 or GRA7, all implicated in resistance to IRGs that mediate parasite killing in mice, was shown to increase PV clearance and presentation of soluble PV antigen by MHC I molecules (Rommereim et al., 2019).

Another property of the recombinant live vaccine candidate would be the absence of persistent parasites in the vaccinated host. A growing list of bradyzoite or cyst wall specific gene knockouts, in attempts to block parasite conversion, can be found in the literature. The vast majority of knockouts resulted in reduced cyst burdens in chronically infected mice. For few of them, brain cysts were not detected. Loss of the Ca^{2+}-dependent protein kinase CDPK2 results in the hyperaccumulation of amylopectin in bradyzoites, leading to parasite death and abrogation of *in vivo* cyst formation (Uboldi et al., 2015). However, CDPK2 is not a major determinant of tachyzoite virulence, as CDPK2-deficient parasites exhibited no defect in acute virulence. GRA17 mediates the tachyzoite PVM permeability to nutrients, loss of GRA17 results in a failure of GRA17 deficient parasites to reach the brain and form cysts (brain cysts and *T. gondii* DNA were not detected). The viability of bradyzoites obtained from *in vitro* GRA17-deficient parasites was significantly reduced compared to wild-type bradyzoites. Furthermore, GRA17-deficient parasites were avirulent in mice (Paredes-Santos et al., 2019). Loss of the cyst wall GRA protein CST2 results in virulence defect and brain cysts were not detectable in mice with chronic infection (Tu et al., 2019). However, parasite load was not further investigated by PCR and CST2-deficient parasites are still able to form *in vitro* cysts. Recently, Waldman et al. (2020) identify a Myb-like transcription factor (BFD1) necessary for differentiation of tachyzoite to bradyzoite in cell culture and in mice. BFD1-

deficient *T. gondii* tachyzoites are unable to form cysts in mice, thus BFD1-deficient *T. gondii* tachyzoites could serve as a basis for developing live vaccine strain, capable of proliferating robustly yet unable to enter a chronic state.

Finally to improve the immunogenicity of a live vaccine, whatever the *T. gondii* stage, deletion of some genes used by the parasite to manipulate the host cell and/or overexpression of some genes that may potentiate the innate immune response may be achieved. For example, deletion of TgIST in a type II strain, a secreted effector conserved across strains, that has been shown to inhibit both STAT1 and STAT1/STAT2-mediated transcriptions and to block induction of type II and type I IFN- activated genes, results in increased parasite clearance in IFN-γ-activated cells and reduced mouse virulence (Gay et al., 2016; Olias et al., 2016; Matta et al., 2019). It has been shown that GRA15 from the type II strain activates NF-kB pathway while GRA15 from the type I does not. Expression of GRA15 type II in a type I strain successfully activated the signaling pathways of NF-kB and induced expression of CD40 on infected mouse macrophages and human THP-1 differentiated macrophages (Morgado et al., 2014).

Furthermore, as the primary infection with a specific strain does not confer complete protection against other *Toxoplasma* strains in both animal and human hosts (Jensen et al., 2015; Costa et al., 2018; Zulpo et al., 2018), the choice of the strain for a live vaccine will be dependent on its ability to induced heterologous immune response and could be adapted to its geographical use. If in Europe and North America, the *T. gondii* strains clonal lineages (types II, III and 12) predominate, non-clonal genotypes are abundant and more genetically diverse in South and Central America (Galal et al., 2019).

Humans

Human Vaccine Development

Numerous vaccine trials have been conducted in mice yielding important insights into toxoplasmosis vaccine development. However, predictive protectivity estimated in mice may be not extrapolated to human, considering the differences in immune responses between mouse and human. Sheep and pig may be more relevant animal models than the mouse to test potential human vaccines. Both sheep and pig have clinical similarities responses to *T. gondii* compared to human (Nau et al., 2017; Betancourt et al., 2019). In particular, pigs like humans are exposed to both *T. gondii* cysts and oocysts (Stelzer et al., 2019). In addition, the physiology and the immune system of pigs are closely that of humans (Meurens et al., 2012). A key difference between mice and humans, sheep and pigs, is the innate immune response. In mice, TLR11 and TLR12 recognition of *T. gondii* profilin induced IL-12 production. Humans, pigs and sheep do not have functional equivalents to murine TLR11 and TLR12. In addition, humans lack most IRGs, contrary to mice where the IFN-γ inducible IRGs proteins play an essential role in IFN-γ-mediated immunity to *T. gondii*. Another species-specific immune defense mechanism involves granulysin. Granulysin (GNLY), an antimicrobial peptide contained in cytotoxic granules in human natural killer cells and cytolytic T lymphocytes but not in mouse cells, has been shown to mediate *T. gondii* death *in vitro* and GNLY-transgenic mice are protected against infection by *T. gondii*, and survive infection that are lethal to wild-type mice (Dotiwala et al., 2016).

To stimulate protective cell mediated immune responses to *T. gondii*, in particular a $CD8^+$ T cell protective immune response, live vaccines are likely effective for generating such a response. Furthermore, live vaccines did not need adjuvant as immunity booster and they give the opportunity to immunize with all the diversity and complexity of *T. gondii* antigens. However, live vaccines would require passing considerable regulatory hurdles that may impede their use in human. One possibility to immunize with all the diversity and complexity of *T. gondii* antigens is the use of *T. gondii* extract. The second one is the use of delivery systems and adjuvants that improved antigen bioavailability and elicited cellular immune responses, especially Th1 type (**Figure 1**). Such an approach has been applied to sheep. Intranasal immunization with *T. gondii* tachyzoite antigens (crude extract) encapsulated into poly(d,l-lactide-co-glycolide) (PLG) micro and nanoparticles plus cholera toxin, induced both systemic and mucosal humoral responses, and cell-mediated responses. However, only a slight modification of the febrile response to oral challenge with sporulated *T. gondii* oocyst was observed in animals immunized with particulate *T. gondii* antigens and cholera toxin compared to control group (Stanley et al., 2004). Intranasal vaccination of sheep with a total extract of *T. gondii* tachyzoite proteins associated with maltodextrin nanoparticles (DGNP), generated specific Th1-cellular immune responses and led to a marked decrease of brain cysts compared with the non-vaccinated group after oral challenge with sporulated *T. gondii* oocysts (Ducournau et al., 2020). To our knowledge, *T. gondii* extracts from *T. gondii* bradyzoite or sporozoite stages have not been investigated in sheep and pigs. The technical challenges to obtain such extracts in large quantities have hindered their use in vaccination trials on large animals.

Despite significant differences between mice and humans, HLA transgenic mice may be considered as good approximation of human responses. To develop peptide-based vaccines created with identified endogenous *T. gondii*–derived epitopes recognized by human $CD8^+$ T cells, HLA A02, A03, and B07 transgenic mice have been use. These HLA molecules are present in more than 90% of the human population. The selected peptides, derived from tachyzoites, bradyzoites or sporozoites antigens administrated subcutaneously with a universal helper T cell epitope, PADRE, and adjuvants (GLA-SE combined or not with Pam2Cys, to adjuvant MHC Class I restricted $CD8^+$ T cells) elicited IFN-γ and reduced parasite burden in HLA A02, A03, and B07 transgenic mice following intraperitoneal challenge with type II tachyzoites that express firely luciferase for *in vivo* detection (Cong et al., 2010; Tan et al., 2010; Cong et al., 2011; Cong et al., 2012; El Bissati et al., 2016). To further increase the immunogenicity of the peptides, multi-epitopes vaccines instead of pool peptides have been constructed by El Bissati et al. (2017). They included 5 HLA A03 restricted epitopes (from SAG1, SAG2C, SRS52A, GRA5 and GRA6), a universal $CD4^+$ T cell epitope PADRE, all arranged to optimize proteasomal cleavage and flagellin as a scaffold and TLR5 agonist to create Self-Assembling Protein Nanoparticles (SAPNs). These SAPNs known to improve the immune response due to the

repetitive display of antigens were also adjuvanted with TLR4 ligand emulsion adjuvant (GLA-SE). With this formulation, $CD8^+$ T cells were activated and a significant brain cyst reduction in A03 transgenic mice was observed following intraperitoneal challenge with tachyzoites that express firely luciferase (El Bissati et al., 2017).

Vaccination Perspectives for Humans

Until now only few studies have addressed the identification of epitopes derived from *T. gondii* proteins presented by HLA major histocompatibility complex molecules I and relatively few *T. gondii* protective epitopes have been identified to date. As *T. gondii* has a large genome (around 65 Mb), screening with synthetic peptides spanning all the proteins encoded in the genome with $CD8^+$ T cells from immune individuals is not applicable. As experiments using parasites engineered to express model antigen have previously shown that transgenic antigen-specific $CD8^+$ T cells are detected in transgenic parasite-infected mice only if the transgenic antigen is expressed as a secreted/membrane bound protein, a set of secreted/membrane bound proteins were selected and bioinformatics algorithms have been used to identify MHC binding peptides. In these studies, a protective immunity was obtained in transgenic HLA mice that could be improved with additional $CD8^+$ T cell peptide candidates from the different stages of *T. gondii*, and also by the addition of $CD4^+$ T cell-eliciting epitopes. The immunogenicity of predicted *T. gondii* $CD4^+$ epitopes in transgenic mice has not been evaluated to date. Transgenic mice expressing both MHC class I and MHC class II molecules from human origin could be useful in epitope screening and vaccine trials. For example, HLA-A02.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice have been used in DNA vaccination trials with a hepatitis B antigen and proved to be efficient in inducing a protective cellular and humoral response. This response was more efficient than the responses induced in transgenic mice expressing either HLA-0A2 or HLA-DR1 alone (Pajot et al., 2004). An immunosense vaccine for humans encompassing CD8, CD4, and B cell epitopes from different stages of *T. gondii* and tested in transgenic mice expressing both MHC class I and II from human origin will be interesting to look at although engineering these mice with different variant HLAs will be challenging and hard to manage. Furthermore, the pathways by which parasite antigens may be acquired and processed for MHC presentation are multiple, depending notably on the location, trafficking of the protein within parasites and host cells (Jensen, 2016; Tsitsiklis et al., 2019). Further studies are needed to understand the detailed mechanisms of these pathways, from entry to early dissemination with cysts or oocysts at mucosal sites. Until now, most studies focused on tachyzoite infected cells.

Protection against *T. gondii* mainly depends on a cellular-mediated immune response, nonetheless antibodies may contribute to protection. They can act on the extracellular tachyzoites by opsonization, complement fixing, neutralization/inhibition of host cell invasion. Those directed against antigens that are implicated in attachment to, or penetration in the host cells of the different *T. gondii* stages are of particular interest. Numerous linear B cell epitopes have been identified to date and are useful for *T. gondii* serological diagnosis (Maksimov et al., 2012; Arranz-Solís et al., 2019). Noteworthy, most of naturally recognized epitopes are discontinuous and are more challenging to identify. Analysis of the three-dimensional (3D) X-ray structure of an antigen by using bioinformatical software programs is needed for the search of these conformational epitopes.

Studies aiming at the identification of $CD8^+$, $CD4^+$, and B cell specific *T. gondii* epitopes are still needed to rationally design an efficient multi-epitope vaccine engineered to focus both T and B cells responses toward a set of critical epitopes selected from a wide range of antigens in order to diversify the scope of the induced immune responses. In parallel, knowledge of new adjuvants and vaccine delivery systems will be useful in the development of effective vaccines.

CONCLUSIONS

Recent advances in deciphering the *T. gondii* life stage conversions including those throughout the sexual cycle, pave the way for *in vitro* studies to tackle the biological questions related to *T. gondii* and will hopefully open new perspectives to move toward effective vaccines. As *T. gondii* biology is intimately linked to the immune response, identification of the initial steps necessary for induction of protective immunity in the relevant host is also critical. Intestinal organoid models that are able to reproduce the *in vivo* situation, offer the opportunity to provide insights into these early host parasite interactions (Betancourt et al., 2019). New tools aiming to identify protein composition of the different *T. gondii* stages including oocyst and cyst walls and stage conversions such as interactome constructions using proteins identified through BioID or RNA single cell sequencing (Xue et al., 2020) will help to better understand the parasite biology and to identify possible new vaccine candidates. In addition, recently, a comprehensive genomescale metabolic model (GEM) of the *T. gondii* tachyzoite metabolic network that incorporates genetic, transcriptomic, and metabolomic data has been developed (Krishnan et al., 2020), should be applied to *T. gondii* bradyzoite or sexual stages to decrypt/to decipher metabolic cues and molecular signaling cascades that trigger bradyzoite transformation and switching to the sexual stages. Our progress toward understanding the biology of *T. gondii* and new tools and technologies enable new opportunities in vaccine development.

AUTHOR CONTRIBUTIONS

M-NM and ID-P conceptualized the content and wrote the manuscript. ZL made changes and clarified the document. M-NM generated the tables and ZL generated the figure with the aid of BioRender (©biorender.com.). All authors contributed to the article and approved the submitted version.

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REVIEW
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A Comparison of Stage Conversion in the Coccidian Apicomplexans *Toxoplasma gondii*, *Hammondia hammondi*, and *Neospora caninum*

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Stage conversion is a critical life cycle feature for several Apicomplexan parasites as the ability to switch between life forms is critical for replication, dissemination, pathogenesis and ultimately, transmission to a new host. In order for these developmental transitions to occur, the parasite must first sense changes in their environment, such as the presence of stressors or other environmental signals, and then respond to these signals by initiating global alterations in gene expression. As our understanding of the genetic components required for stage conversion continues to broaden, we can better understand the conserved mechanisms for this process and unique components and their contribution to pathogenesis by comparing stage conversion in multiple closely related species. In this review, we will discuss what is currently known about the mechanisms driving stage conversion in *Toxoplasma gondii* and its closest relatives *Hammondia hammondi* and *Neospora caninum*. Work by us and others has shown that these species have some important differences in the way that they (1) progress through their life cycle and (2) respond to stage conversion initiating stressors. To provide a specific example of species-specific complexities associated with stage conversion, we will discuss our recent published and unpublished work comparing stress responses in *T. gondii* and *H. hammondi*.

Keywords: ***Toxoplasma gondii*, *Hammondia hammondi*, *Neospora caninum*, bradyzoite, tissue cysts**

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INTRODUCTION

Eukaryotic parasites with multi-host life cycles have the unique challenge of persisting in a variety of different environments. Parasites of this nature must exist in a life form that is permissible and optimized for growth, survival, and transmission in a given host. The ability to adopt these specific life forms is especially critical for tissue cyst forming coccidia in the Sarcocystidae family which includes *Sarcocystis, Frenkelia, Besnoitia, Cystoisopora, Toxoplasma, Hammondia, and Neospora* species (Smith, 1981; Duszynski et al., 2018). Here, we will discuss the current knowledge about how three closely related parasites from this family, *Toxoplasma gondii*, *Hammondia hammondi*, and *Neospora caninum*, approach the challenges associated with multi-host life cycles. These three mammalian parasites follow heteroxenous, two host life cycles (Frenkel et al., 1970; Frenkel and

Dubey, 1975; McAllister et al., 1998; Dubey, 2009; Dubey and Ferguson, 2015) where they must survive in a variety of cell types with varying immune pressures. In order to be successful in their hosts, these parasites are capable of converting to life forms that each serve an important function for survival and fitness. Although these parasites exhibit the same infectious life forms, they have fundamental differences in their life cycles and stage conversion strategies. This review will focus on the critical differences in stage conversion between tachyzoites, the rapidly replicating life form, and bradyzoites, the slower growing parasites comprising tissue cysts, in *T. gondii, H. hammondi*, and *N. caninum.*

T. gondii, *H. hammondi*, and *N. caninum* are all closely related (**Figure 1A**), obligate intracellular parasites that follow two-host life cycles (See detailed description in section 2). These parasites species also share the same class of organelles and have several shared genomic features. *T. gondii* and *H. hammondi* share >95% of their genomes in near perfect synteny (Walzer et al., 2013), while *T. gondii* and *Neospora caninum* genomes are >81% syntenic (Adomako-Ankomah et al., 2014). Conservative predictions have identified 7,095 shared orthologs between *H. hammondi* and *T. gondii* and 6,308 orthologs between *T. gondii* and *N. caninum* (Lorenzi et al., 2016). Despite these genetic similarities, they exhibit substantial differences in pathogenesis, host range, and life cycles.

Toxoplasma gondii has a broad host range and can infect all warm-blooded animals including birds (Tenter et al., 2000). *T. gondii* is responsible for the human disease toxoplasmosis and has infected approximately 2 billion people worldwide (Furtado et al., 2011). Although infections are often asymptomatic, *T. gondii* infections persist in the host in the form of tissue cysts, the life stage that contributes to chronic infection (Remington and Cavanaugh, 1965). Tissue cysts cannot be cleared by the host immune response nor can they be eliminated by known antiparasitic drugs (Tomavo and Boothroyd, 1995; Sullivan and Jeffers, 2012). *T. gondii* infections are thought to be long term, as bradyzoite containing tissue cysts reside in host tissue for extended periods of time and can reactivate causing clinical disease (Tenter et al., 2000; Weiss and Kim, 2000; Rougier et al., 2017). *T. gondii* tissue cysts can cause severe complications for immunocompromised individuals, such as HIV/AIDS and organ transplant patients, when latent infections reactivate into highly replicative life forms and result in tissue damage (Derouin et al., 1987; Derouin et al., 2008). Furthermore, bradyzoite containing tissue cysts maintained in the animal population contributes to the spread of *T. gondii* to both animals and humans, as *T. gondii* is the leading cause of death caused by a food borne illness in the United States (CDC - Toxoplasmosis; Tenter et al., 2000; Tenter, 2009). Individuals, even those who are immunocompetent, infected with *T. gondii* can also develop ocular toxoplasmosis. *T. gondii* is capable of invading and replicating in the retina which can result in severe retinal damage that can lead to blindness (Ozgonul and Besirli, 2017).

Despite being the nearest living relative of *T. gondii*, *H. hammondi* is avirulent in comparison to *T. gondii*. *H. hammondi* is not known to infect humans and is not known to cause natural disease in any animal model (Dubey and Sreekumar, 2003). *H. hammondi* has a restricted host range when compared to *T. gondii*, as its only known natural intermediate hosts are rodents (Mason, 1978), roe deer (Entzeroth and Scholtyseck, 1978), and goats (Shimura and Ito, 1987). Despite having a limited natural host range, *H. hammondi* has been shown to experimentally infect monkeys (Dubey and Wong, 1978), dogs (Dubey, 1975), rabbits, and pigs (Dubey and Sreekumar, 2003), yet is unable to infect birds (Wallace, 1975; Dubey and Streitel, 1976). Despite this slight expansion in experimental host range, nonmurine animals are considered poor hosts for *H. hammondi* as infections do not result in robust chronic infection as *H. hammondi*-infected tissues from these animals produce fewer oocysts compared to *H. hammondi*-infected tissues from murine hosts upon sexual reproduction (Dubey and Streitel, 1976).

In stark contrast to the avirulent nature of *H. hammondi*, *N. caninum* is the major cause of bovine abortion, resulting in losses of over a billion dollars worldwide in cattle industries (Dubey et al., 2007; Goodswen et al., 2013). Like with *T. gondii*, the formation of tissue cysts is also critical for the transmission and survival of *N. caninum.* The reactivation of bradyzoite-containing

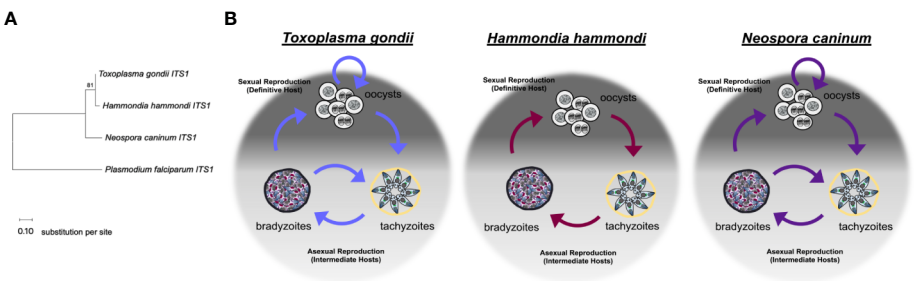

FIGURE 1 | Relationships between *Toxoplasma gondii*, *Hammondia hammondi*, and *Neospora caninum.* **(A)** Neighbor-joining tree depicting relationship between the ITS1 (internal transcribed spacer 1) sequences of *T. gondii*, *H. hammondi*, and *N. caninum*. *P. falciparum* is used as an outgroup. ITS1 sequences were chosen to highlight the relationship between these parasite species due to high variability often attributed to these non-coding sequences. Sequences were obtained from GenBank. Bootstrap values for 1000 replicates are indicted. Scale bar represents the substitutions per site. **(B)** Diagrams showing the life cycle of *T. gondii*, *H. hammondi*, and *N. caninum.*

tissue cysts into fast replicating tachyzoites during bovine pregnancy results in efficient transmission to the fetus. Efficient transmission, persistence, and recrudescence in asymptomatic cows is a significant biotic constraint in these agriculturally important animals, and currently there is no treatment or vaccine for bovine neosporosis (Buxton et al., 2002; Dubey et al., 2006; Williams and Trees, 2006; González-Warleta et al., 2018). While cattle are considered the major intermediate host, *N. caninum* can also infect a number of other domestic and wild ruminant species where its infection can result in disease (Dubey et al., 2013).

LIFE CYCLES OF *TOXOPLASMA GONDII, HAMMONDIA HAMMONDI*, AND *NEOSPORA CANINUM*

Toxoplasma gondii follows a facultative homoxenous/heteroxenous two host life cycle (**Figure 1B**). Sexual reproduction of *T. gondii* occurs in its definitive hosts which include members of the *Felidae* genus and produces millions of orally infectious oocysts that are environmentally stable. Each oocyst contains 8 sporozoites contained within 2 sporocysts that are surrounded by an oocyst wall (Frenkel et al., 1970; Dubey, 1998; Dubey, 2009). When oocysts are ingested by an intermediate host, the sporozoites will excyst within the host digestive system and invade the intestinal epithelium. Once in the intestinal epithelium, sporozoites differentiate into tachyzoites, which are the fast growing life stage responsible for acute infection. During a primary infection of a naïve intermediate host, *T. gondii* tachyzoites are capable of crossing the placenta, which can result in vertical transmission of this parasite (McAuley, 2014). Eventually, *T. gondii* tachyzoites differentiate into bradyzoites, the slow growing life stage associated with chronic infection. Bradyzoites are found within tissue cysts which typically reside in the central nervous system or in skeletal or cardiac muscle (Dubey et al., 1998). Bradyzoite tissue cysts are orally infectious to the definitive host. When ingested by a definitive host, tissue cysts will differentiate into the sexual stages resulting in the production of millions of oocysts, thus completing the life cycle (Dubey and Frenkel, 1972). Additionally, cats have been described as complete hosts for *T. gondii* because they support proliferation of tachyzoites and bradyzoites in extra-intestinal tissues in addition to supporting sexual life stages and sexual reproduction in intestinal tissues (Frenkel, 1977). One of the most critical components of the *T. gondii* life cycle is reactivation. Reactivation can occur when 1) a chronically infected host becomes immunocompromised which results in rapid proliferation and dissemination of tachyzoites or 2) when a naïve intermediate host ingests *T. gondii* tissue cysts, the bradyzoites contained within these tissue cysts are released during digestion and differentiate back to tachyzoites, which then disseminate and cause acute infection in the host before encysting during chronic infection. Reactivation results in expanded transmission for *T. gondii*, as it allows for the parasites to be continually propagated through asexual reproduction in intermediate hosts and underlies disease progression in immunocompromised hosts (Dubey et al., 1998). To date, the molecular determinants of this unique ability have not been elucidated, but it has likely had a dramatic impact on the global distribution and broad host range of *T. gondii*.

In comparison to *T. gondii*, *Hammondia hammondi* follows a strict obligate heteroxenous life cycle (**Figure 1B**). Sexual reproduction only occurs within the intestinal epithelium of the definitive host, which, like *T. gondii*, includes felids. *H. hammondi* oocysts, like *T. gondii* oocysts, are orally infectious to intermediate hosts (Frenkel and Dubey, 1975; Dubey and Ferguson, 2015). Unlike *T. gondii* infections, feline hosts can only support *H. hammondi* enteroepithelial stages and do not support extraintestinal infections (Dubey and Sreekumar, 2003). Furthermore, during asexual reproduction *H. hammondi* remain infectious to intermediate hosts until they terminally differentiate into tissue cyst stages (Dubey and Sreekumar, 2003; Sokol et al., 2018) that are only orally infectious to definitive feline hosts. It is hypothesized that terminally differentiated *H. hammondi* parasites are incapable of reactivation, however, this has yet to be tested in head-to-head comparisons with *T. gondii*. Furthermore, *H. hammondi* is not known to be capable of vertical transmission (Dubey and Streitel, 1976).

Like *T. gondii, N. caninum* also follows a complex facultative heteroxenous life cycle (**Figure 1B**). As described for *T. gondii* and *H. hammondi*, sexual reproduction for *N. caninum* infections in the definitive host (in this case canines rather than felines) results in oocyst production and subsequent excretion in host feces. *N. caninum* oocysts are environmentally stable and contain sporozoites that are orally infectious to intermediate hosts. *N. caninum* undergoes sexual reproduction in members of the *Canis* genus (Donahoe et al., 2015) and it is presumed that sexual reproduction occurs in enteroepithelial cells similar to *T. gondii* and *H. hammondi* in felid infections, however, little is known about the sexual life cycle of *N. caninum* infections. The first report of enteroepithelial developmental stages of *N. caninum* was published in 2015 and identified oocyst and schizont-like structures in epithelia in a naturally infected dog (Kul et al., 2015) *N. caninum* tachyzoites and bradyzoites can be found in both intermediate and definitive host tissue, primarily in the central nervous system (Dubey et al., 2002; Dubey et al., 2007). *N. caninum* infections in canids are similar to *T. gondii* in felids in that dogs can be considered complete hosts for *N. caninum* and support replication of all three infectious life stages. As with *T. gondii*, transmission via carnivory of infectious tissue cysts is important for horizontal transmission of *N. caninum* to the definitive host. Although experimental infections demonstrate *N. caninum* tissue cysts are orally infectious (Lindsay and Dubey, 1990; Lindsay et al., 2001), the ingestion of sporulated oocysts is the only known natural mode of horizontal transmission to cattle (De Marez et al., 1999; Kano et al., 2005; Eastick and Elsheikha, 2010). Vertical transmission is the most frequent mode of transmission in bovine hosts and both endogenous (reactivated) and

exogenous (primary) transplacental infections occur (Williams et al., 2009). During vertical transmission, *N. caninum* converts into fast-replicating tachyzoites which cross the placenta to transmit to offspring often resulting in abortion. Following transmission, *N. caninum* converts to bradyzoites to evade the host immune responses and tissue cysts form. It is thought the interconversion from bradyzoite to tachyzoite, allows for one infected animal to transmit the parasite to offspring repeatedly. In support of this, recrudescence and transmission have been described in cattle (Williams et al., 2009), sheep (Gutiérrez-Expósito et al., 2020) and in dogs (Cole et al., 1995; Barber and Trees, 1998; Heckeroth and Tenter, 2007). As for *T. gondii*, the means by which *N. caninum* is capable of moving both forwards and backwards in its life cycle is unknown.

Regardless of parasite species, the formation of bradyzoites is a critical step in the transmission of *T. gondii*, *H. hammondi*, and *N. caninum*, despite clear differences in their life cycles. Bradyzoites are necessary for transmission as their cyst wall provides protection during passage through the mammalian gut (Jacobs et al., 1960), and they are also required for sexual reproduction which dramatically expands the number of infectious oocysts available to infect new hosts. Even though the definitive hosts for *T. gondii* and *N. caninum* (felines and canines, respectively), are complete hosts (host that can support both sexual and asexual replication), bradyzoite formation within these animals is still needed in order for sexual reproduction to occur. Bradyzoites also allow the parasites to remain in a host for extended period of time as they cannot be cleared by the host immune system. Finally, interconversion that occurs in *T. gondii* and *N. caninum* allows for these parasites to be transmitted completely independently of the definitive host, a remarkable trait given the vastly different species that can serve as intermediate hosts for these parasites. For these reasons, understanding the mechanisms driving the conversion of tachyzoites to bradyzoites and how it compares between species is necessary to manage the many manifestations of disease caused by these organisms. Moreover, by identifying the developmental sensors, triggers, and components in these species and comparing their activities in each may lead to a better understanding of how this process is regulated on the molecular level.

INDUCERS OF BRADYZOITE DEVELOPMENT

While environmental signals are likely at the heart of stage conversion in *T. gondii* and its near relatives, the precise mechanisms used to respond to these signals have remained elusive. However, the effect of various signals on inducing life stage development, specifically tissue cyst formation, has been well studied in *T. gondii.* Furthermore, recent work has begun to identify distinct responses to these triggers in *H. hammondi*, indicating the presence of a divergent stress response in this species.

Toxoplasma gondii

Bradyzoite formation in *T. gondii* has been extensively studied. An important, but underappreciated, fact is that most strains of *T. gondii* are capable of spontaneously converting into bradyzoites when grown in vitro in a variety of different host cells (Lindsay et al., 1991; Lindsay et al., 1993). When *T. gondii* infections are initiated with parasites derived from sporozoites or from tachyzoites or bradyzoites that have not be extensively passaged following isolation, the parasites will first grow as tachyzoites but will later form tissue cysts that express bradyzoite markers and/or are able to induce oocyst shedding when fed to cats (Lindsay et al., 1991; Jerome et al., 1998). This phenomenon of spontaneous stage conversion in *T. gondii* was most thoroughly studied in *T. gondii* strain VEG. When infections are initiated with *T. gondii* VEG sporozoites, the parasites differentiate into a rapidly growing tachyzoite stage resembling many lab-adapted strains like *T. gondii* RH, and after ~20 divisions they begin to differentiate into slower growing parasites that express bradyzoite markers, suggesting that there may be a developmental clock controlling this spontaneous conversion (Jerome et al., 1998). Taken together, these findings suggest that there is some type of parasite intrinsic factor that enables these parasites to transition to bradyzoites following initial tachyzoite expansion.

Host factors, specifically the differentiation state of a given host cell, can also impact *T. gondii* cystogenesis. *T. gondii* forms tissue cysts when grown in mouse primary skeletal muscle cells that have been differentiated into polynucleated myotubes, which are withdrawn from cell cycle progression (Ferreira-da-Silva et al., 2009; Swierzy and Lüder, 2015). When these host cells are genetically manipulated to knock-down Testis-specific Y-encoded-like protein 2 (Tspyl2), a negative cell cycle regulator that contributes withdraw from host cell cycle progression in these myotubes, *T. gondii* fails to form tissue cysts in these cells (Swierzy and Lüder, 2015). Additionally, expression levels of the host gene human cell division autoantigen-1 (CDA1) are important for bradyzoite development. *T. gondii* has been shown to grow slower and express bradyzoite specific genes when grown in host cells treated (pre-treatment or continuous treatment) with the trisubstituted pyrrole small molecule Compound 1, which upregulates expression of host CDA1 (Radke et al., 2006). CDA1 is a negative regulator of cell growth and has regions with homology to testis protein TSPY (Chai et al., 2001). Additionally, *T. gondii* forms tissue cysts when grown in HeLa cells that overexpress a transgene encoding CDA1 (Radke et al., 2006). The ability of cells that have withdrawn from their cell cycle to promote tissue cyst formation in *T. gondii* is a likely contributor to the preference *T. gondii* shows for muscular tissue and the tissues of the central nervous system.

Several well characterized stressors are known to induce *T. gondii* tachyzoite-to-bradyzoite stage conversion in vitro (summarized in **Table 1**). Perhaps the most well-known stressor that leads to robust formation of *T. gondii* tissue cysts in vitro is alkaline pH (pH ~8 as compared to the

TABLE 1 | Summary of exogenous stressors that induce bradyzoite development in *Toxoplasma gondii*.

Stressor	Parasite life stage used for infection	Parasite strain	Host cell	Method used to determine bradyzoite formation	Citation
Alkaline pH (treatment of infected host cells)	Sporozoites	VEG	Human foreskin fibroblasts (HFFs)	Bradyzoite-specific antibodies	(Jerome et al., 1998)
	In vivo tachyzoites	RH	HFFs; Vero cells	Bradyzoite-specific antibodies	(Soête et al., 1994)
Alkaline pH (extracellular parasites)	*In vitro* tachyzoites	ME49	HFFs	Bradyzoite-specific antibodies	(Weiss et al., 1998)
Heat Shock (43 degrees C)	*In vivo* tachyzoites	RH	Vero cells	Bradyzoite-specific antibodies	(Soête et al., 1994)
Sodium arsenite	*In vivo* tachyzoites	RH	Vero cells	Bradyzoite-specific antibodies	(Soête et al., 1994)
Sodium nitroprusside (SNP) (extracellular parasites)	*In vitro* tachyzoites	ME49	HFF	Bradyzoite-specific antibodies	(Weiss et al., 1998)
SNP (infected host cells)	*In vitro* tachyzoites	NTE	Murine bone marrow-derived macrophages (BMDM)	Bradyzoite-specific antibodies	(Bohne et al., 1994)
Interferon gamma	*In vitro* derived tachyzoites	NTE	Murine peritoneal macrophages	Bradyzoite-specific antibodies	(Bohne et al., 1993)
	In vitro tachyzoites	NTE	Murine BMDM	Bradyzoite-specific antibodies	(Bohne et al., 1994)
Antimycin A	*In vitro* tachyzoites	NTE	Murine BMDM	Bradyzoite-specific antibodies	(Bohne et al., 1994)
Oligomycin	*In vitro* tachyzoites	NTE	Human fibroblasts	Bradyzoite-specific antibodies	(Bohne et al., 1994)
Atovaquone	*In vitro* tachyzoites	PLK	HFFs	Bradyzoite-specific antibodies	(Tomavo and Boothroyd, 1995)
Arginine starvation	*In vitro* tachyzoites	RH; PLK	HFFs	Dolichos biflorus agglutinin	(Fox et al., 2004)
Pyrimidine starvation	*In vitro* tachyzoites	RHδUPRT	HFFs	Bradyzoite-specific antibodies	(Bohne and Roos, 1997)
Cholesterol depletion (Lipoprotein depleted serum)	*In vitro* tachyzoites	ME49	Chinese hamster ovary cells	Bradyzoite-specific antibodies	(Ihara and Nishikawa, 2014)
Compound 1	*In vitro* tachyzoites	ME49B7; Pru; VEG; CTG	HFFs	Bradyzoite-specific antibodies; Dolichos biflorus agglutinin	(Radke et al., 2006)

standard pH growth conditions of 7.2–7.4). Multiple groups have shown that alkaline pH induces *T. gondii* cyst development either when applied to host cells after infection (Soête et al., 1994) or when applied to extracellular parasites prior to infection (Weiss et al., 1998). Despite robust induction of *T. gondii* tissue cyst development, the exact mechanism remains unknown. It is possible that alkaline pH derived stress induces a myriad of both host and parasite derived signals that are needed in order to initiate bradyzoite development in *T. gondii.* Furthermore, treatment of infected host cells with pH 6.8 media has also been shown to induce bradyzoite development (Weiss et al., 1995). In addition to changes in pH, heat shock (43°C as opposed to 37°C) and sodium arsenite treatment of infected host cells have also been shown to induce stage conversion in *T. gondii*, however heat shock is not an optimal method for inducing bradyzoite development as it often results in decreased parasite invasion, parasite killing, and loss of host cell viability (Soête et al., 1994). Nutrient starvation can also induce stage conversion in *T. gondii*. Pyrimidine starvation achieved via deletion of the uracil phosphoribosyl transferase (UPRT) gene in combination with growth in atmospheric CO_2 (0.03% compared to 5%) also leads to a parasite growth reduction and expression of bradyzoite markers (Bohne and Roos, 1997). Arginine starvation also decreases the replication of *T. gondii* and induces tissue cyst formation (Fox et al., 2004). Cholesterol depletion via growth in media supplemented with lipoprotein depleted serum (as compared to growth in 5% fetal bovine serum) has also been shown to induce bradyzoite gene expression (Ihara and Nishikawa, 2014). Additionally, interferon gamma (IFN-γ) treatment of *T. gondii* infected macrophages results in expression of bradyzoite specific antigens (Bohne et al., 1994), however IFN-γ is not capable of inducing expression of bradyzoite specific antigens in human fibroblasts (Suzuki et al., 1989; Bohne et al., 1993; Soête et al., 1994; Weiss et al., 1995). Other in vitro stressors that may mimic IFN-γ-driven immune pressure also induces *T. gondii* cystogenesis, such as the production of nitric oxide. Exogeneous nitric oxide produced from sodium nitroprusside (SNP) treatment can induce bradyzoite development (Bohne et al., 1994). Exogenous nitric oxide likely inhibits proteins involved in the electron transport chain, thus treatment with mitochondrial inhibitors oligomycin, antimycin A, (Bohne et al., 1994), and atovaquone (Tomavo and Boothroyd, 1995) can also induce expression of bradyzoite antigens in *T. gondii.* Overall, the numerous and diverse exogenous stressors capable of inducing bradyzoite development in *T. gondii* suggest that multiple signals can be used as triggers to induce the fundamental process of bradyzoite formation.

In comparison to in vitro systems, in vivo factors that induce cystogenesis are much less clear. Tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) may play a role in restricting cystogenesis, as TNF receptor p55- and p75-deficient mice and iNOS deficient mice develop more tissue cysts

in the brain compared to wild type mice despite relatively equivalent parasite burden in peritoneal cells early in infection. Interestingly, mice deficient for TNF-α receptors or iNOS succumb to chronic infection while the WT parasites survive (Scharton-Kersten et al., 1997; Yap et al., 1998). It is challenging to determine if the increase in tissue cysts in the brains of these mice is a result of TNF-α and iNOS restricting tissue cyst development, or if more parasites make it to the brain prior to tissue cyst formation. Future experiments using live imaging that quantifies parasite burden could be useful to test these hypotheses. Additionally, CD4+ and CD8+ T-cells are important for the maintenance of chronic infection characterized by the bradyzoite/tissue cyst life stage. Depletion of these cells with neutralizing antibodies leads to reactivation of *T. gondii* infection leading to parasite proliferation (Gazzinelli et al., 1992). It is also thought that IFN-γ may play a critical role in cystogenesis. It is hypothesized that IFN-γ contributes to the initiation of tissue cyst formation in vivo, as it can induce cystogenesis in vitro (described above), however IFN-γ knockout mice fail to control acute proliferation of parasites and succumb to acute infection (Suzuki et al., 1988; Suzuki et al., 1989) even when infected with avirulent strains of *T. gondii* (Coombs et al., 2020) making it difficult to determine if IFN-γ induces cystogenesis in vivo. It is also possible that *T. gondii* spontaneously forms tissue cysts during in vivo infections, however this spontaneous development is again challenging to observe experimentally due to the lethality of *T. gondii* infections in mice with disrupted immune systems. Linking what is known about in vitro cyst development in *T. gondii* to what happens in vivo is a significant, but important, knowledge gap in the field that will require new technological innovation to fill.

Hammondia hammondi

In comparison to *T. gondii*, little is known about bradyzoite and tissue cyst formation in *H. hammondi*. When grown in vitro, *H. hammondi* fails to grow in continuous culture and spontaneously undergoes a terminal differentiation process where it completely converts to tissue cysts that are only infectious to definitive feline hosts (Sheffield et al., 1976; Riahi et al., 1995; Dubey and Sreekumar, 2003; Sokol et al., 2018). The timing of tissue cyst formation corresponds to when *H. hammondi* parasites lose their ability to infect a new host cell (Sokol et al., 2018), demonstrating that *H. hammondi* follows a strict obligate heteroxenous life cycle even in in vitro growth conditions (a sharp contrast to both *T. gondii* and *N. caninum)*. Furthermore, comparative transcriptomic analysis between replicating *T. gondii* and *H. hammondi* showed that the *H. hammondi* transcriptional profile is enriched for genes that are typically reserved for expression during bradyzoite and sexual stages occurring in feline intestinal cells in *T. gondii* (Sokol et al., 2018). It is likely that *H. hammondi* follows a strictly regulated life cycle where it is poised to convert to its next life stages after a pre-defined time as a given life form.

Even though *H. hammondi* completely and spontaneously forms tissue cysts when grown in vitro, it cannot be induced to form tissue cysts with alkaline pH, a robust inducer of stage conversion in *T. gondii*, at early time points following sporozoite-initiated infection (Sokol et al., 2018). This discovery was important because it suggested that the ability to constitutively respond to alkaline pH in *T. gondii* was a derived trait. Since this initial work we have also investigated if *H. hammondi* is eventually able to form tissue cysts in response to alkaline pH derived stress applied at later stages in its in vitro cycle. Interestingly, we have found that when alkaline pH derived stress is applied for 48 h at later developmental time points (Day 13 post sporozoite-derived infection), significantly more tissue cysts form in response to alkaline pH treatment than occur spontaneously at this time (**Figure 2**). This finding suggests that as *H. hammondi* progresses through a predefined developmental program it differentiates from a life form that is incapable of responding to alkaline pH (unable to sense and/or respond) to a life form that is capable of sensing and/or responding. This hints at a conserved linkage between alkaline pH responsiveness and the tachyzoite to bradyzoite stage conversion. Future work investigating the differences in gene expression between these time points could be promising in uncovering additional components of the mechanisms that these parasites use to sense and respond to their environment. It is currently unknown as to how *H. hammondi* responds to other stressors known to induce tissue cyst development in *T. gondii*.

Neospora caninum

Like *T. gondii*, *N. caninum* alternates between two life stages presumably to survive host immune responses. The mechanisms underlying *N. caninum* tachyzoite to bradyzoite conversion remain largely unknown (to an even greater extent than for *H. hammondi*) due to difficulties in developing in vitro models for bradyzoite development. Methods used to obtain *T. gondii* bradyzoites in vitro are ineffective or inefficient for *N. caninum* cyst formation. Although nitric oxide treatment of murine keratinocytes infected with *N. caninum* tachyzoites yields cysts, they are surrounded by thick keratin filament bundles and thus impede parasite purification processes (Vonlaufen et al., 2002). Very few studies have investigated the effects of stress on stage conversion for *N. caninum*. One study suggests that nitric oxide, increasing pH, or increasing temperature can increase tachyzoite to bradyzoite conversion in *N. caninum* (Weiss et al., 1999). SNP has also been shown to increase expression of *N. caninum* bradyzoite and cyst wall markers (Risco-Castillo et al., 2004). However, there are no studies investigating any difference in gene expression during the tachyzoite to bradyzoite conversion process in *N. caninum.* Substantial work with regards to how to identify *N. caninum* bradyzoites and reliably produce this life stage in vitro and in vivo is still needed in order to expand our understanding of how stage conversion occurs in *N. caninum.*

PARASITE INTRINSIC MOLECULAR MECHANISMS OF STAGE CONVERSION

Chromatin

Stage conversion in eukaryotic parasites is accompanied by significant changes in patterns of gene expression (Gomez et al., 2010; Chen et al., 2018). The chromatin landscape of a

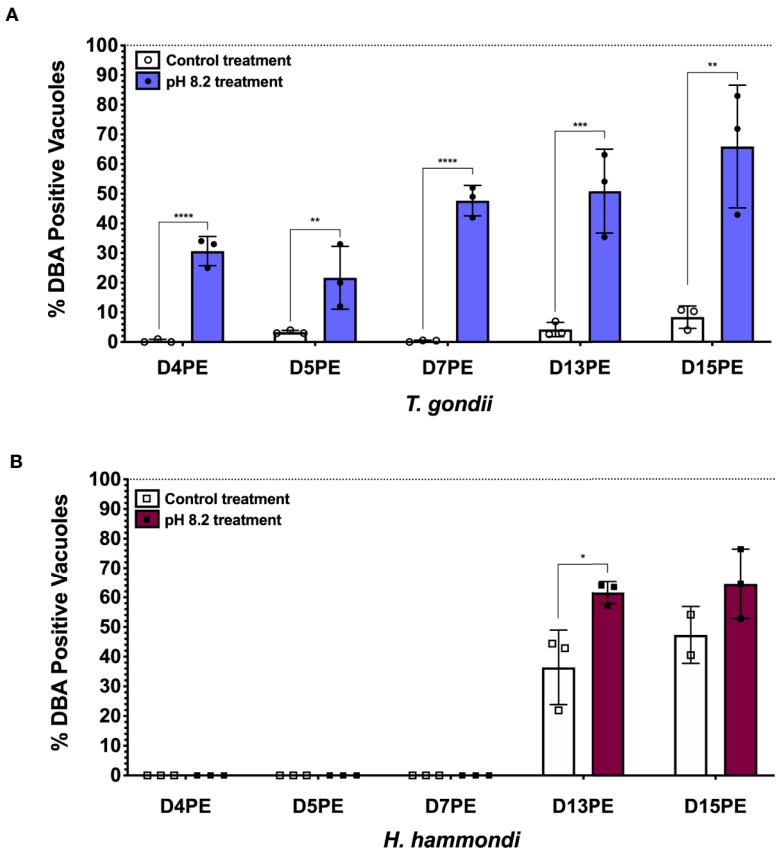

FIGURE 2 | *H. hammondi* can respond to stress conditions when exposed to alkaline pH 13 days post excystation (DPE) from oocysts. **(A)** Percentage of DBA positive vacuoles observed following 48 h of alkaline pH stress initially applied at D4, 5, 7, 13, and 15PE for *T. gondii* VEG. **(B)** Percentage of DBA positive vacuoles observed following 48 h of alkaline pH stress initially applied at D4, 5, 7, 13, and 15PE for *H. hammondi* American. Statistical significance was determined by 2way ANOVA with Sidak's multiple comparisons test of arcsine transformed data. (N = 2–3 biological replicates, *P = 0.03, **P < 0.01, ***P < 0.001, and ****P < 0.0001) This experiment, with the exception of time of alkaline stress application at the time points mentioned above, was performed as previously described (Sokol et al., 2018).

given life stage plays a large role in what genes are expressed, therefore playing a critical role in stage conversion. Several chromatin remodeling factors have been identified in *T. gondii* that play a role in altering the chromatin landscape during different life stages. One such factor is Histone Deacetylase 3 (HDAC3), which is a component of *T. gondii's* corepressor complex. HDAC3 is associated with bradyzoite specific promoters (Saksouk et al., 2005) and when its activity is inhibited with the compound FR235222, expression of bradyzoite genes increases (Bougdour et al., 2009). These findings suggest that histone deacetylation via HDAC3 functions to repress the expression of bradyzoite genes and keep *T. gondii* in a tachyzoite life form. HDAC3 has been shown to work with the recently discovered *T. gondii* microrchidia (MORC) protein. MORC interacts with several AP2 transcription factors (Farhat et al., 2020) [including AP2IX-4 and AP2XII-2 discussed below (Srivastava et al., 2020)] and recruits HDAC3 to chromatin, enabling the generation of hypo-acetylated chromatin which represses gene expression. When MORC is depleted in *T. gondii*, the parasites begin to express transcripts typically expressed in other life stages, such as merozoite and oocysts specific genes, that are restricted to the *T. gondii* sexual stages (Farhat et al., 2020). These findings suggest that MORC functions as a repressor of sexual development associated gene expression through chromatin modification in *T. gondii*. Our transcriptional data from replicating *H. hammondi* on Day 4 (D4) and Day 15 (D15) post sporozoite infection, does not show any changes in transcriptional abundance of either of *H. hammondi's* orthologs of HDAC3 or MORC (**Figure 3**). However, in comparison to *T. gondii*, *H. hammondi's* transcriptional profile is enriched for genes that are associated with sexual development in *T. gondii* (Sokol et al., 2018), a process which has been shown to be repressed by MORC (Farhat et al., 2020). Because of this similarity, we hypothesized that transcript abundance of MORC repressed genes would be enriched in *H. hammondi* at D15 compared to D4. To test this hypothesis, we conducted pre-ranked gene set enrichment analysis (GSEA) as previously described (Subramanian et al., 2005) and found significant enrichment for MORC repressed transcripts [gene sets derived

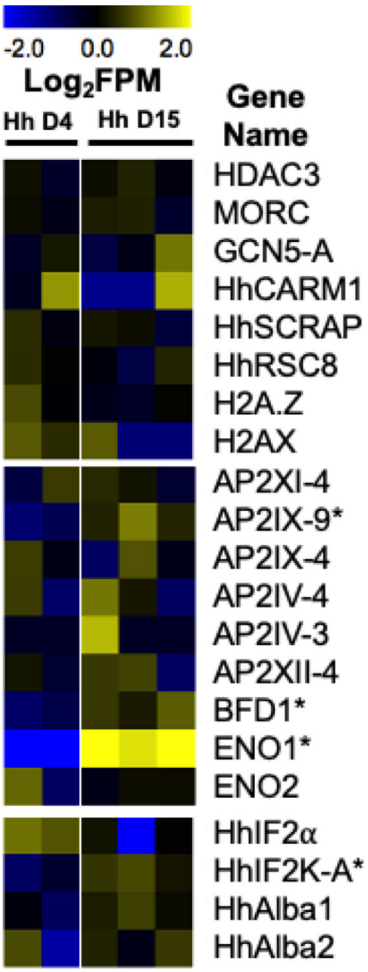

FIGURE 3 | Heatmap representing *H. hammondi* transcriptional abundance during spontaneous development of known regulators of bradyzoite formation. Heatmaps show mean centered Log_2FPM values. Asterisk (*) represent genes with significant differences between D4 (N = 2 biological replicates) and D15 (N = 3 biological replicates) samples. Significance is defined as $|Log_2$ Fold Change$| > 1$ and $P_{adj} < 0.01$. *T. gondii* gene IDs for these genes and the gene IDs for each orthologs in *H. hammondi* and *N. caninum* are found in **Table 2**. The *H. hammondi* transcriptional data used to generate this figure was obtained from (Sokol et al., 2018).

from data published in (Farhat et al., 2020)] (NES= -10.88, FDRq = ~0.00) in D15 *H. hammondi* (**Figure 4**). This analysis suggests that transcriptional changes in spontaneously developing *H. hammondi* resemble some of the transcriptional changes observed in MORC depleted *T. gondii*. It would be interesting to further investigate if altering MORC levels could alter *H. hammondi's* terminal differentiation phenotype.

Another histone remodeling enzyme found in *T. gondii* is GCN5-A, a lysine histone acetyltransferase. This enzyme plays an oppositional role to HDAC3. GCN5-A is found at tachyzoite promoters when *T. gondii* is grown under tachyzoite growth conditions (Saksouk et al., 2005). Additionally, ChIP qPCR experiments have shown that when *T. gondii* is exposed to alkaline pH stress conditions, GCN5-A occupancy is enriched in the promotors of bradyzoite specific genes that are upregulated in response to stress. When GCN5-A is knocked out in *T. gondii*, the parasites are unable to upregulate 74% of known bradyzoite genes in response to alkaline pH induced stress (Naguleswaran et al., 2010). Together, these findings demonstrate that GCN5-A plays a critical role in *T. gondii's* ability to alter its gene expression in response to alkaline pH stress.

Additional chromatin remodeling enzymes that play a role in the alteration of stage conversion associated gene expression have also been identified in *T. gondii*. These include TgCARM1 (Saksouk et al., 2005), TgSCRAP (Sullivan et al., 2003), and TgRSC8 (Craver et al., 2010; Rooney et al., 2011). TgCARM1 is a histone arginine methyltransferase protein that is essential for parasite replication. When N-methyltransferase activity is inhibited with the small molecule AMI-1 via pretreatment of extracellular parasites, *T. gondii* forms significantly more bradyzoites compared to a vehicle control (Saksouk et al., 2005). TgSCRAP, a Snf2-related CBP activator protein and SNF/SWI chromatin remodeler, upregulates the expression of the known bradyzoite gene bradyzoite antigen 1 (BAG1) during alkaline pH induced stress, and has been shown to enhance CREB (cAMP response element binding protein) mediated transcription, which may suggest a role in the protein kinase A signaling pathway that has also been implicated in bradyzoite development for *T. gondii* (Sullivan et al., 2003). Another chromatin remodeling complex that contributes to bradyzoite development is TgRSC8, a homolog of the nucleosome remodeling complex Rsc8p protein in *Saccharomyces cerevisiae*. When this protein is mutated in *T. gondii*, some bradyzoite genes showed a significant reduction in transcript abundance when exposed to alkaline pH stress conditions. However, these mutants did not show reduced Dolichos biflorus agglutinin (DBA) staining (Rooney et al., 2011), which specifically recognizes the glycosylated cyst wall protein CST1 (Zhang et al., 2001). In addition to chromatin remodelers, histone variants have also been implicated in stage conversion in *T. gondii*. The histone variant H2A.Z is expressed in mature, in vivo bradyzoites and H2AX is also expressed in mature, in vivo bradyzoites but also displays increased expression in vitro during alkaline pH stress (Dalmasso et al., 2009). All together, these findings suggest that the alteration of chromatin is an important contributor to stage conversion associated gene expression in *T. gondii*.

Despite having orthologs of all of these chromatin remodeling enzymes and histone variants, our understanding of their role in bradyzoite formation in *H. hammondi* and *N. caninum* is mostly unclear. Our transcriptional data from replicating *H. hammondi* show no significant changes in the transcript abundance for any of the *H. hammondi* orthologs during spontaneous development (**Figure 3**). However, these findings could suggest that transcriptional abundance of these genes is needed similarly in all life stages and that the mechanisms controlling their translation, activation, or their recruitment to specific genes are important for initiating the tachyzoite to bradyzoite developmental transitions.

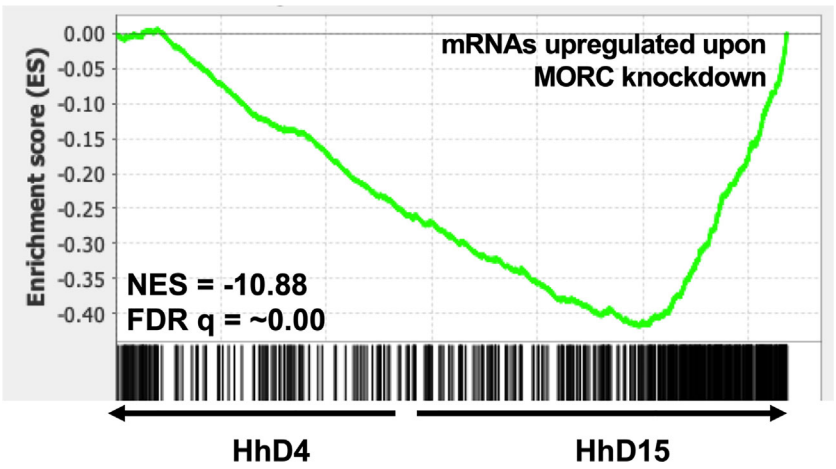

FIGURE 4 | Spontaneously developing *H. hammondi* transcriptional abundance is enriched for genes regulated by MORC. Preranked GSEA, comparing D4 and D15 HhEth1, ranked listed was calculated from Log_2 fold change between D4 and D15 spontaneous developing HhEth1 samples. The data used to generate this figure was obtained from (Sokol et al., 2018). Gene sets were created with data from (Farhat et al., 2020).

Transcription Factors

Transcription factors are an important class of proteins that play a significant role in the control of stage conversion specific gene expression and have long been investigated for their role in stage conversion associated gene expression in *T. gondii.* One of the first major classes of transcription factors investigated for their role in stage conversion in *T. gondii* were the AP2 transcription factors. AP2 factors were first identified in Apicomplexans in 2005. These transcription factors share the Apetala2 (AP2) integrase DNA binding domain typically found in transcription factors of numerous plant species (Balaji et al., 2005). AP2 transcription factors are known to play a major role in stage conversion in *Plasmodium* species (Painter et al., 2011). There are currently 67 AP2 factors (66 annotated as AP2 domain transcription factors and 1 annotated as an AP2 domain-containing protein) in the *T. gondii* ME49 genome (Gajria et al., 2008). However, only 6 of these AP2 transcription factors have been tied to bradyzoite development in *T. gondii*. The AP2 transcription factor AP2IX-9 functions mainly as a repressor of bradyzoite development, maintaining parasites in an intermediate, pre-bradyzoite state. AP2IX-9 binds to the CAGTGT motif and functions to repress transcription (Radke et al., 2013). Furthermore, deletion of AP2IX-9 results in increased tissue cyst formation in parasites cultivated in normal growth conditions (Hong et al., 2017). During spontaneous development (D4 versus D15) in *H. hammondi*, we see significant increases in transcriptional abundance of the *H. hammondi* ortholog of AP2IX-9 ($|Log_2$ Fold Change$|$ = 2.21, P_{adj} = 0.009). Since AP2IX-9 is known to keep parasites in a pre-bradyzoite state, this data could indicate that D15 *H. hammondi* are being maintained in a pre-bradyzoite like state, prior to their complete terminal differentiation which is first observed at D23 post sporozoite derived infection (Sokol et al., 2018). Another AP2 factor, AP2XII-2, has been shown to interact with the MORC protein and results in increased tissue cyst formation in vitro upon knockdown in *T. gondii*, suggesting that this factor may be important for maintaining tachyzoites (Srivastava et al., 2020). Additionally, AP2 transcription factors have been identified in *T. gondii* that are involved in promoting bradyzoite development. These include AP2IV-4 (Radke et al., 2018), AP2IV-3 (Hong et al., 2017), AP2IX-4 (Huang et al., 2017), and AP2XI-4 (Walker et al., 2013). When these AP2 transcription factors are deleted, parasites have a decreased ability to form tissue cysts in vitro and/or in vivo. Furthermore, alteration (either deletion or overexpression) of these factors results in significant differences in transcript abundance of known bradyzoite genes (Walker et al., 2013; Hong et al., 2017; Huang et al., 2017; Radke et al., 2018). Our *H. hammondi* transcriptional data does not show any significant differences in transcript abundance in these AP2 factors during spontaneous development in *H. hammondi* (**Figure 3**).

In addition to AP2 transcription factors, *T. gondii* has additional transcription factors that are critical for stage conversion. One such transcription factor is Bradyzoite Formation Deficient 1 (BFD1). BFD1 was recently identified as a master transcriptional regulator of bradyzoite development in *T. gondii* using a large-scale genetic screen. BFD1 is a nuclear localized Myb-like DNA binding protein that binds to the CACTGG motif near that transcriptional start site of differentially regulated genes. When BFD1 is deleted in *T. gondii*, these parasites fail to form tissue cysts in vitro in response to alkaline pH derived stress and treatment with Compound 1. These knockout parasites also fail to form tissue cysts in vivo. Furthermore, BFD1 knockout parasites fail to express several bradyzoite specific genes when treated with alkaline pH, suggesting that BFD1 plays a major role in initiating stage conversion associated gene expression (Waldman et al., 2020). Transcriptional abundance of the *H. hammondi* ortholog of BFD1 is modestly but significantly upregulated ($|Log_2$ Fold Change$|$ = 1.47, P_{adj} = <0.001) during

TABLE 2 | Summary of genes known to play a role in stage conversion in *T. gondii*.

Gene Name	Role in stage conversion associated gene expression	Gene ID *T. gondii (from Toxodb.org)*	Gene ID *H. hammondi* ortholog *(from Toxodb.org)*	Gene ID *N. caninum* ortholog *(from Toxodb.org)*
HDAC3	Represses transcription	TgME49_227290	HHA_227290	NCLIV_045860
MORC	Works with HDA3 and AP2 factors to repress transcription	TgME49_305340	HHA_305340	NCLIV_043930
GCN5-A	Activates transcriptions	TgME49_254555	HHA_254555	NCLIV_008840
CARM1	Activity needed to maintain tachyzoites	TgME49_294270	HHA_294270	NCLIV_001020
SCRAP	Upregulates BAG1 expression	TgME49_280800	HHA_280800	NCLIV_019390
RSC8	Regulates transcription of bradyzoite genes	TgME49_286920	HHA_286920	NCLIV_013840
H2A.Z	Expressed in bradyzoites; function in stage conversion is unknown	TgME49_300200	HHA_300200	NCLIV_064530
H2AX	Expressed in bradyzoites; function in stage conversion is unknown	TgME49_261580	HHA_261580	NCLIV_025910
AP2XI-4	Promotes bradyzoite formation	TGME49_315760	HHA_315760	NCLIV_058430
AP2IX-9	Maintains pre-bradyzoites	TgME49_306620	HHA_306620	NCLIV_044800
AP2IX-4	Promotes bradyzoite formation	TgME49_288950	HHA_288950	NCLIV_041340
AP2IV-4	Promotes bradyzoite formation	TgME49_318470	HHA_318470	NCLIV_011080
AP2IV-3	Promotes bradyzoite formation	TgME49_318610	HHA_318610	NCLIV_010930
AP2XII-4	Interacts with MORC, maintains tachyzoites	TgME49_217700	HHA_217700	NCLIV_062490
BFD1	Required for bradyzoite formation	TgME49_200385	HHA_200385	NCLIV_038230
ENO1	Promotes bradyzoite formation	TgME49_268860	HHA_268860	NCLIV_037490
ENO2	Expressed in tachyzoite nuclei, function in stage conversion is unknown	TgME49_268850	HHA_268850	NCLIV_037500
IF2a	Phosphorylated in bradyzoites, leads to increase in bradyzoite specific gene expression	TgME49_258740	HHA_258740	NCLIV_027770
IF2K-A	Phosphorylates IF2a, activity promotes bradyzoite formation	TgME49_229630	HHA_229630	NCLIV_030460
Alba1	Promotes bradyzoite formation	TgME49_221380	HHA_221380	NCLIV_004920
Alba2	Promotes bradyzoite formation	TgME49_218820	HHA_218820	NCLIV_061560

spontaneous development (**Figure 3**), suggesting that increases in BFD1 expression in *H. hammondi* may play an important role in *H. hammondi's* terminal differentiation phenotype.

Additional *T. gondii* proteins that may play a role in stage conversion are the glycolytic enzymes, Enolase1 (ENO1) and Enolase 2 (ENO2). ENO1 is specifically expressed in bradyzoites and is a known bradyzoite specific gene. ENO2 is expressed in tachyzoites and can be found localized in the cytoplasm and nucleus (Ferguson et al., 2002). When ENO1 is deleted in *T. gondii*, the ability of the parasite to form in vivo cysts is impaired. Both ENO1 and ENO2 have been shown to bind promoters in ChIPseq and ChIP qPCR experiments and ENO1 has been shown to bind the ENO2 promoter. Furthermore, analysis of the ENO1 promoter revealed a stress response element that could be activated with nuclear extracts purified from bradyzoites (Kibe et al., 2005). Together, this data demonstrates a role as transcription factors implicated in stage conversion for ENO1 and ENO2. ENO1 is significantly upregulated during spontaneous development in *H. hammondi* (**Figure 3**), however it is possible that this increase could also be due to ENO1's metabolic role for bradyzoites.

RNA-Binding Proteins

While stage conversion is typically accompanied by global changes in transcript abundance in Apicomplexan parasites, translation control is an additional component needed for stage conversion associated gene expression. Several RNA binding proteins have been identified in *T. gondii* and some have been implicated in the conversion between tachyzoites and bradyzoites. Such factors include TgIF2α/eIF2 (eukaryotic initiation factor 2 alpha) and TgIF2K-A (initiation factor 2 kinase -A). TgIF2K-A is responsible for phosphorylating and activating TgIF2α, which functions as an inhibitor of translation initiation. TgIF2α is phosphorylated in response to stress derived from both alkaline pH and heat shock (Sullivan et al., 2004). TgIF2α phosphorylation is maintained in bradyzoites. When dephosphorylation of TgIF2α is inhibited, parasites both increase the transcriptional abundance of known bradyzoites genes and form DBA positive tissue cysts (Narasimhan et al., 2008). Furthermore, when the activity of TgIF2K-A is inhibited in *T. gondii*, thus preventing TgIF2α phosphorylation, these parasites form significantly fewer tissue cysts when exposed to alkaline pH stress (Augusto et al., 2018). Our transcriptional data from replicating *H. hammondi* shows that transcript abundance of HhIF2K-A is significantly upregulated during spontaneous development (($|\text{Log}_2$ Fold Change$|$ = 1.22, P_{adj} = <0.001) (**Figure 3**) (Sokol et al., 2018). This increase in transcriptional abundance could correlate to increased IF2α phosphorylation and translational inhibition in *H. hammondi*. Additional experiments investigating HhIF2α phosphorylation and HhIF2K-A activity during bradyzoite formation in *H. hammondi* would be helpful in identifying translation control as a conserved mechanism of stage conversion.

Additionally, *T. gondii* encodes two RNA binding proteins related to Alba proteins in archaea, TgAlba1 and TgAlba2. In response to alkaline pH derived stress, both TgAlba1 and

TgAlba2 colocalize with RNA granules. These proteins bind greater than 30 RNAs, including their own. TgAlba1 binds to the promoter of TgAlba2. Upon deletion of TgAlba1, TgAlba2 is no longer translated. Furthermore, deletion of TgAlba1 in *T. gondii* also leads to decreased tissue cyst formation in vitro (in response to alkaline pH stress) and in vivo (Gissot et al., 2013). However, we do not see significant changes in transcriptional abundance in the *H. hammondi* orthologs of TgAlba1 and TgAlba2 (**Figure 3**). Together, these examples of RNA binding proteins found in *T. gondii* demonstrate that translational control plays a critical role in stage conversion associated gene expression.

CONCLUSIONS

The tachyzoite to bradyzoite transition is a fundamental developmental process for the success of *T. gondii* and its closest relatives *H. hammondi* and *N. caninum*, as it is necessary for the survival and transmission of these parasite species. Some similarities in exogenous stressors that induce bradyzoite formation exist between species, indicating that there is some conservation in the way that these parasites sense stress. Moreover, the genetic components involved in the mechanisms governing stage conversion between tachyzoite and bradyzoite life stages are only starting to be uncovered for *T. gondii*, but so far include several factors that are needed for both transcriptional and translational control. Both *H. hammondi* and *N. caninum* have syntenic orthologs of all of these genetic components identified in *T. gondii* (summarized in **Table 2**), suggesting that they may also play a role in stage conversion in these species as well. However, only a few of these genes have altered transcriptional abundance in spontaneously differentiating *H. hammondi*. These observations from *H. hammondi* suggest that the mechanisms controlling stage conversion may function differently in *H. hammondi* and may rely on more than transcriptional regulation alone in order to induce bradyzoite development, which is not surprising given the importance of the tachyzoite to bradyzoite transition and the complexity of stage conversion associated gene expression. Together, these studies indicate that future work aimed at linking these factors in gene regulatory networks, in addition to identifying new factors, is needed to contribute to a better understanding of how these parasites initiate the global changes in stage conversion associated gene expression on a mechanistic level.

AUTHOR CONTRIBUTIONS

SS-B, RC, and JB conceptualized, wrote, and edited this manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Structural and Molecular Underpinnings of Gametogenesis in *Toxoplasma gondii*

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Toxoplasma gondii is a widely prevalent protozoan parasite member of the phylum Apicomplexa. It causes disease in humans with clinical outcomes ranging from an asymptomatic manifestation to eye disease to reproductive failure and neurological symptoms. In farm animals, and particularly in sheep, toxoplasmosis costs the industry millions by profoundly affecting their reproductive potential. As do all the parasites in the phylum, *T. gondii* parasites go through sexual and asexual replication in the context of an heteroxenic life cycle involving members of the Felidae family and any warm-blooded vertebrate as definitive and intermediate hosts, respectively. During sexual replication, merozoites differentiate into female and male gametes; their combination gives rise to a zygotes which evolve into sporozoites that encyst and are shed in cat's feces as environmentally resistant oocysts. During zygote formation *T. gondii* parasites are diploid providing the parasite with a window of opportunity for genetic admixture making this a key step in the generation of genetic diversity. In addition, oocyst formation and shedding are central to dissemination and environmental contamination with infectious parasite forms. In this minireview we summarize the current state of the art on the process of gametogenesis. We discuss the unique structures of macro and microgametes, an insight acquired through classical techniques, as well as the more recently attained molecular understanding of the routes leading up to these life forms by *in vitro* and *in vivo* systems. We pose a number of unanswered questions and discuss these in the context of the latest findings on molecular cues mediating stage switching, and the implication for the field of newly available *in vitro* tools.

Keywords: *Toxoplasma gondii*, sexual cycle, molecular cues, sexual differentiation, parasite dissemination

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INTRODUCTION

Infecting about a third of the world's population *Toxoplasma gondii* is a widely prevalent protozoan parasite, member of the phylum Apicomplexa, and the etiological agent of toxoplasmosis (Dubey, 2013; Torgerson and Mastroiacovo, 2013; Sherri et al., 2017). Its success as a pathogen is partly owed to its proficiency in invading virtually any nucleated cell in an immense range of warm-blooded hosts, ranging from humans and farm animals, to birds and marine species. Its main mechanism of pathogenesis is a consequence of its obligate intracellular lifestyle, which inevitably culminates in the

lysis of the infected cell as the parasite replicates and expands. Notwithstanding, its ability to persist without killing the host is owed to its ability to switch from a fast dividing, disease-causing form, known as the tachyzoite, to a slow dividing, encysted/latent form, known as the bradyzoite. While the former is responsible for acute toxoplasmosis, the latter can persist chronically in its host. Acute toxoplasmosis most notably causes reproductive failure in humans, but it can also cause a range of clinical manifestations from eye to neurological disease in chronically infected immunocompromised individuals. Ocular toxoplasmosis is also frequent in congenitally or chronically infected immunocompetent individuals (Commodaro et al., 2009). In farm animals, and particularly in sheep, toxoplasmosis costs the industry millions by affecting reproductive outcomes (Dubey et al., 2020). Likewise, toxoplasmosis has been shown to greatly affect sensitive populations of wild animals, with particular effects on marine life and devastating ecological outcomes (Shapiro et al., 2019). A common route of infection for humans and other carnivores is the consumption of undercooked meat infected with bradyzoites lodged as cysts in skeletal muscle or the brain (Pinto-Ferreira et al., 2019). Vertical transmission is also possible when the infection is acquired by a naive host during pregnancy. Transmission is also possible by the serendipitous interaction with infective oocysts, shed by feline species in their feces.

Felines are *T. gondii*'s definitive host, whereby the parasite undergoes its sexual cycle. Sexual differentiation entails the formation of macro and a microgametes, and zygote formation can occur upon their combination. This process is key to the generation of genetic diversity as this is the only stage at which genetic admixing can occur as any other zoite life stage of this parasite is haploid. Equally important, this process is central to dissemination, as a single felid can shed hundreds of millions of oocysts, and these are stable in the environment for at least a year. The annual oocyst burden has been estimated to range from 3 to 434 oocysts per square foot in different community surveys in the United States. Oocyst burden is concentrated in areas where cats preferably defecate, this is places with loose soil, such as gardens, children's play areas, and sandboxes (Torrey and Yolken, 2013). An astonishing example of how potentially harmful oocysts can be in the environment is showcased by the toxoplasmosis epidemic in sea otters in coastal California. Infectious oocysts shed by domestic cats, located many miles away, are dragged along by the river that flows into the otter's natural aquatic habitat (Miller et al., 2002; Conrad et al., 2005; Shapiro et al., 2012; Vanwormer et al., 2013). Close to 9% of their mortality can be ascribed to meningoencephalitis caused by *Toxoplasma* (Thomas and Cole, 1996; Shapiro et al., 2019).

Understanding the biology of the life forms that precede the formation of infective oocyst, is, in addition to a fascinating ill-understood source of biological data, central to developing efficient intervention strategies to prevent their formation and spread. Here, we review the current state of the art on gametogenesis, integrating the plethora of fantastic structural insight acquired through classical microscopy with exciting new molecular insights provided by *in vitro* and *in vivo* systems on the molecular cues facilitating stage switching.

THE LIFE STAGES OF *TOXOPLASMA GONDII*

The life forms of *T. gondii* can be generally grouped in two; the ones that replicate clonally and those that are generated by combination of gametes.

The asexually or clonally replicating forms develop in warm-blooded intermediate hosts whereby two distinct forms can be identified; the tachyzoite and the bradyzoite. The tachyzoite is a highly proliferative form commonly associated with acute infection, reactivation, and vertical transmission. The bradyzoite, on the other hand, is a latent, albeit metabolically active, slow growing encysted form. Bradyzoites are commonly associated with the chronic stages of toxoplasmosis, persistence, immune evasion, and are refractory to currently available anti-toxoplasmosis pharmacotherapies (Weiss and Kim, 2000; Lyons et al., 2002; Dzierszinski et al., 2004; Sullivan and Jeffers, 2012; Knoll et al., 2013; Watts et al., 2015; Sinai et al., 2017; Cerutti et al., 2020). Evasion is mediated by cell type tropism and sequestration to immune-privileged sites such as the brain and skeletal muscle, as well as the process of encystation (Sullivan and Jeffers, 2012; Knoll et al., 2013). Both tachyzoites and bradyzoites follow a cell division scheme known as endodyogeny consisting of a single round of DNA replication by semi-closed nuclear mitosis. The internal assembly of two daughter cells occurs concomitantly with nuclear mitosis, inside the mother cell (Francia and Striepen, 2014; White and Suvorova, 2018; Gubbels et al., 2020). Tachyzoite cell division is rapid, generating two new cells per mother cell every 6–8 h. Bradyzoites instead divide slower but can assemble and sustain between 1000–2000 bradyzoites per cyst (Weiss and Kim, 2000; Knoll et al., 2013).

When a feline eats undercooked meat infected with a cyst containing bradyzoites, most commonly from an infected mouse, the parasite can gain access to the definitive host's gastrointestinal tract [**Figure 1A** (Dubey, 2006)]. Here, it can develop into either one of two life-forms: the tachyzoite or a merozoite. As in other hosts, tachyzoites can disseminate throughout the feline body and switch to bradyzoite residing in immune-privileged sites. However, clinical signs of toxoplasmosis are rarely observed in cats. The disease is more likely to occur in cats with suppressed immune systems, including young kittens and cats with feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV). Toxoplasmosis is more severe in transplacentally infected kittens which can develop a variety of clinical manifestations ranging from hepatitis to cholangiohepatitis, pneumonia, and encephalitis (Dubey and Carpenter, 1993a; Dubey and Carpenter, 1993b; Webb et al., 2005; Calero-Berna and Gennari, 2019). More frequently, however, bradyzoites will differentiate into merozoites within the feline enterocytes (**Figures 1B, C**) (Frenkel, 1973; Dubey et al., 1998; Weiss and Kim, 2000; Webb et al., 2005; Dubey, 2009; Dubey, 2013). This transformation initiates with several rounds of rapid asexual expansion within the intestinal epithelium as merozoites, initiating the sexual replication track.

While tachyzoites invade and replicate extra-intestinally, merozoites develop only within enterocytes. Recent work by Hehl and col. identified important gene expression differences between tachyzoites and merozoites that could underlie this cell

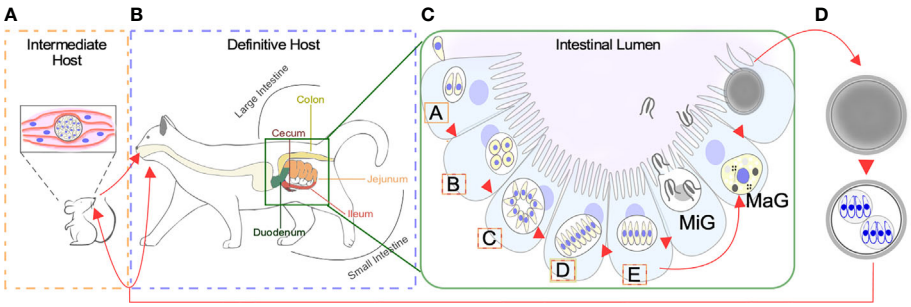

FIGURE 1 | Life Stages of *Toxoplasma gondii*. **(A)** Warm blooded animals can act as intermediate hosts. Slow dividing bradyzoites can persist both in the brain and skeletal muscle within a tissue cyst. Transmission of bradyzoites can occur through carnivorism. Intermediate hosts can be infected by sporulated oocyst contaminating the environment. **(B)** Species from the Felidae family act as definitive hosts. Infection can occur by consuming tissue cysts containing bradyzoites, or sporozoites contained within sporulated oocysts. Differentiation of bradyzoites into asexually expanding merozoites happens along the intestinal tract. **(C)** Bradyzoites will sequentially differentiate into A-E merozoites prior to giving rise to macro (MaG) and micro (MiG) gametes. The intestinal segment where each meront is found more often is color coded according to B. **(D)** Combination of MaG and MiG gives rise to a unsporulated oocysts which mature in the environment becoming sporulated, sporozoite containing, infective oocysts.

type tropism. A subset of well characterized dense-granule (GRA), microneme (MIC), and rhoptry (ROP) genes, known to be involved in attachment, invasion, and host cell modification to allow intracellular replication, are exclusively expressed in tachyzoites, and notably downregulated in merozoites. Merozoites seem to preferentially express a large subset of surface membrane proteins, known as SAG1-related sequences (SRS) for their homology to the surface antigen protein TgSag1, making them enterocyte-interaction competent (Speer and Dubey, 1998; Ferguson, 2004; Hehl et al., 2015). This is consistent with findings in *Plasmodium* whereby gamete-gamete interaction, recognition, and fertilization is mediated by a family of proteins, known as the Pfs-230-related 6-Cys adhesins, characterized by the presence of the SRS fold (Arredondo and Kappe, 2017). In addition, SRS proteins have been proposed to stimulate immune responses stimulating molecules, playing a role in intestinal inflammation and diarrhea, thereby potentially contributing to the dispersal of oocysts from felines (Hehl et al., 2015).

STRUCTURAL ASPECTS OF *T. GONDII* GAMETES AND PRE-GAMETE STAGES

Only 2 days after tissue cysts are ingested by the cat, micro and macrogametes are formed initiating the sexual cycle (Dubey et al., 1998). Our knowledge on the ultrastructure of pre-sexual stages is set on outstanding electron microscopy studies done in the 60's and 70's (Colley and Zaman, 1970; Melton and Sheffield, 1970; Pelster and Piekarski, 1971; Frenkel and Dubey, 1972; Sinden et al., 1976). Work by Dubey & Frankel systematically recorded the morphological details of five pre-gamete stages, named A to E, sequentially formed upon colonization of the epithelial cells of the small and large cat's intestine (Dubey and Frenkel, 1972) (**Figures 1B, C**). Of these, only D or E meronts may differentiate into sexual gametes. Type A merozoites are readily observed, 12–18 h post infection. Their shape is round and they are commonly found singly or as a collection of 2 or 3. A meronts are commonly found in the jejunum, at the surface of the epithelium and in the lamina propria. How these meronts replicate exactly, remains elusive (Dubey and Frenkel, 1972). Type B meronts are observable 12–54 h post infection, are ovoid in shape, and commonly found in the jejunum, the ileum, at the surface of the epithelium (near the base of villi) and in the lamina propria, and more rarely in the glandular epithelium. B meronts replicate by endodyogeny and endopolygeny (Dubey and Frenkel, 1972). C meronts are observable 24–54 h post infection. Their shape is elongated, and they are found at the same locations as B meronts. C meronts are thought to replicate by schizogony following a rosette pattern (Dubey and Frenkel, 1972). Type D meronts are observable between 40 h and 15 days after infection. Their shape is elongated, and they are commonly found in the jejunum, ileum and the colon (Dubey et al., 1998). Type D meronts can be further classified in three subtypes; subtype I, found 48–72 h post infection, divides by endopolygeny and is found in groups of 2-4. Subtype II is found 3–15 days post infection, divides by schizogony and is found in groups of 5-35 merozoites. Subtype III merozoites albeit ill-defined, are thought to arise from "splitting"; a sort of modified schizogony whereby a large uninucleated cytosolic mass parcels into single meronts (Dubey and Frenkel, 1972). Finally, type E meronts appear 3–15 days after infection. Their shape is elongated, and they are commonly found in the jejunum, ileum, and seldomly in the colon. They replicate by schizogony (Dubey and Frenkel, 1972).

Upon asexual differentiation of meront type (A-E), gametogenesis is preceded by the formation of macro and micro gamonts. Gamonts are detectable 3–15 days after experimental inoculation of kittens with infective oocyst (Dubey et al., 1998). Gamonts are found throughout the small intestine, especially in the ileum. The precursor of the male gametocyte, the microgamont, is of ovoid shape and represents 2%–4% of the mature gametocyte population. Microgamonts can yield 6-21 microgametes, with a mean of 12. They are ellipsoidal,

with the nucleus occupying much of the cell body. The anterior end is a pointed structure called the perforatorium, within which reside two microtubule organizing centers (MTOCs) acting as basal bodies organizing two posterior flagella (Dubey et al., 1998; Ferguson and Dubremetz, 2013; Francia et al., 2016; Morlon-guyot et al., 2017). On the other hand, macrogamonts are subspherical containing a single nucleus. Each macrogamont will give rise to a single macrogamete (Dubey and Frenkel, 1972). The cytosol of macrogamonts is populated with protein-rich wall forming bodies, polysaccharide granules and lipid droplets, which will all contribute to wall formation by sequentially releasing their content during oocyst maturation (Ferguson et al., 1975; Freppel et al., 2019). The oocyst wall starts forming within the host cell before the macrogamete is released, implying that fertilization has to take place inside the host cell (Freppel et al., 2019).

Macro and microgamete formation are not equilibrated in output. Every round of gametogenesis produces 1 macrogamete, and 15–30 microgametes. The number of macrogametes exceeds the number of microgametes by an average of 19-fold (Ferguson, 2002). Given that *T. gondii* can efficiently proliferate asexually, it has been proposed that sexual proliferation only provides a real advantage when the possibility of producing genetic diversity between different strains infecting the same feline exists. When a single strain is involved in this process, zygote formation based on fertilization has been argued as an extremely inefficient process (Ferguson, 2002). Quantification of the relative frequencies obtained from genetic crosses between two parental strains with distinct drug resistance phenotypes, suggest that sexual differentiation yields Mendelian inheritance patterns in the progeny, arguing that oocysts are only formed by fecundation (Pfefferkorn and Pfefferkorn, 1980). However, the only physical evidence supporting fertilization that has been generated, to our knowledge, is an electron micrograph showing several microgametes attached to an extracellular macrogamete, taken by Ferguson [Figure 4 in (Ferguson, 2002)]. Based upon this, Ferguson proposed that the ample efficiency with which oocyst are shed could be explained by the formation of oocysts from macrogametes alone through parthenogenesis (Ferguson, 2002). Though this hypothesis has not yet been experimentally tested, the unbalanced output of gametogenesis together with the long journey that every microgamete has to do to fertilize one macrogamete, has complicated the visualization of the process and its definitive understanding. Hence, how, when and where fertilization happens in the gut of the felids is still to be deciphered (Ferguson and Dubremetz, 2013).

Unsporulated oocyst are shed by cat in their feces (**Figure 1D**). These sporulate 1–5 days after shedding, forming a mature infection-competent oocyst. Sporulation is temperature-dependent; it is optimally efficient at temperatures closer to 25°C; delayed at temperatures lower than 15°C and inhibited at 37 or 4°C. Presence of oxygen is also required for oocyst sporulation and infectivity (Dubey et al., 1970). Each sporulated oocyst contains two sporocyst with four sporozoites each (Dubey et al., 1970). This stage is equipped with a multilayer wall that protects it from adverse environmental conditions, this being the only stage of the whole cycle of this parasite that develops outside the host cell (Ferguson and Dubremetz, 2013) (Ferguson et al., 1975).

MOLECULAR AND STRUCTURAL LINKS BETWEEN ASEXUAL AND SEXUAL REPLICATION

From Cell Division Control to Orchestrating Motility: The Microtubule Organizing Centers of *T. gondii* in the Context of Microgamete Formation

Toxoplasma gondii assembles two flagella per cell only when differentiated to microgamete. For fertilization to take place, every microgamete leaves the host cell where it was generated, swims across the intestinal lumen, finds a host cell containing a macrogamete, crosses the cell membrane, and fecundates the macrogamete (Ferguson, 2002). Because fertilization of a macrogamete requires the microgamete to be able to move, the flagella play a pivotal role in licensing this step. Ultimately, if oocyst formation relies on fertilization, then microgamete mobility is paramount to *T. gondii's* environmental dissemination.

The flagellar axoneme consists of 9 microtubule doublets and a central pair of microtubules, and it is nucleated from a centriole-based basal body, formed by two microtubule-based barrels known as centrioles (Dubremetz, 1971; Dubremetz, 1973; Morrissette and Sibley, 2002a; Morrissette and Sibley, 2002b; Francia et al., 2016). In related coccidian species, such as *Eimeria* and *Sarcocystis*, basal body structure has been proposed to consist of either 9 + 0 or 9 + 2 singlets microtubules, or a canonical basal body, akin to what is observed in metazoan, formed by 9 + 0 triplet microtubules. The exact basal body structure of *T. gondii* remains unclear. However, electron micrographs suggest that it could consist of a 9 + 1 singlet microtubule structure (Pelster and Piekarski, 1971; Scholtyseck et al., 1972; Vetterling et al., 1973; Ferguson et al., 1974; Mehlhorn and Heydorn, 1979; Mueller et al., 1981; Morrissette and Sibley, 2002a; Walker et al., 2013; Francia et al., 2016; Morrissette and Gubbels, 2020).

The centrosome is the main microtubule organizing center from which most basal bodies derive during flagellar assembly in other species. In *T. gondii* the centrosome of asexual stages consists of two 200 nm x 200 nm parallel centrioles with a 9 + 1 singlet microtubule geometry, a morphology that is notably distinct from canonical centrosomes and basal bodies (Dubremetz, 1971; Dubremetz, 1973; Morrissette and Sibley, 2002a; Francia and Striepen, 2014; Suvorova et al., 2015; Francia et al., 2016). Because the geometries and microtubule composition of the *T. gondii* basal body are ill-defined, it is still unclear whether it assembles from the asexual stage centrioles, or whether it is assembled *de novo*. *De novo* basal body synthesis has been proposed for *Plasmodium* sexual stages, as the asexual stages organize their asexual cell division through a "centriolar plaque" which consist of a few conserved components of other microtubule organizing centers, such as the centrosome, but lacks centrioles (Francia et al., 2016).

Canonical centriole biogenesis involves the hierarchical and sequential layout of structural components, catalyzed by

overlapping regulatory signals in coordination with cell cycle progression. In metazoan, specialized kinases, such as PLK-4, Zyg1, or PLK1 trigger the centriole assembly cascade. An evolutionary conserved and ancestral protein module (known as UNIMOD; which includes SAS-6, SAS4/CPAP, and SAS5/STIL) whose occurrence is correlated with presence or loss of centrioles has been proposed to control centriole biogenesis, and to have emerged in the LECA (last eukaryotic common ancestor). However, homologs for the kinases, STIL, and other UNIMOD components, are not encoded for in the genome of *T. gondii*. Interestingly, the genome of *T. gondii* encodes for two SAS6 homologs (de Leon et al., 2013). SAS6 is a highly conserved and it is responsible for the cartwheel assembly, crucial for establishing centriole and basal body geometry. One of the homologs is found at the centrosome (TgSAS6) while the other one, named SAS6-Like (TgSAS6L), is located at the conoid, a structure proposed to have emerged from an ancestral MTOC whose original function was to nucleate flagella (Francia et al., 2012; de Leon et al., 2013; Back et al., 2020; O'Shaughnessy et al., 2020). Indeed, de Leon, and col., showed that a SAS6L homolog localizes to the basal body of the flagellated trypanosomatid, *Trypanosoma brucei*, leading them to propose that SAS6L could exhibit a similar localization in *T. gondii* (de Leon et al., 2013).

Canonical centriole biogenesis encompasses different isotypes of tubulins (involved in microtubule nucleation). Many of the homologs of these proteins were identified in *T. gondii*, while others are seemingly missing (Suvorova et al., 2015; Morlon-guyot et al., 2017). The presence of three α- and β-tubulin isotypes genes, δ- and ϵ-tubulin, SAS6 and SAS4 coding genes, indicate that this parasite could potentially assemble a canonical 9 + 0 triplet microtubule basal body (Nagel and Boothroyd, 1988; Garreau de Loubresse et al., 2001; Dupuis-Williams et al., 2002; Dutcher et al., 2002; Marshall and Rosenbaum, 2003; Xiao et al., 2010; Ross et al., 2013). In line with this, recent work by Ramakrishnan and col. found that the protein bearing the highest degree of similarity to ϵ-tubulin present in *T. gondii* genome, (originally annotated as β3-tubulin, but reclassified as ϵ-tubulin by Morlon-guyot et al., 2017), is indeed expressed in the enteroepithelial stages (Ramakrishnan et al., 2019).

T. gondii also encodes for a homolog of the centrosomal protein 164 (Cep164); homologs of this protein form appendages, a molecular signature that distinguishes the mother centriole from the daughter centriole in a basal body. Appendages serve to anchor the mother centriole to the membrane, enabling it to nucleate the flagellar axoneme. Though its role has not been explored, TgCEP164 could potentially be important for basal body anchoring (Morrissette, 2015; Morlon-guyot et al., 2017). Consistently, this protein does not express in aflagellated tachyzoites or bradyzoites as determined by asexual stages transcriptomic and proteomic experiments (Morlon-guyot et al., 2017). In contrast, evidence for TgCEP164 expression in enteroepithelial stages supports its functional role in sexual stages (Ramakrishnan et al., 2019).

Once flagella have been built, their maintenance requires several proteins. Intraflagellar Transport (IFT) is required to move structural and signaling components of the axoneme from the cell body to the tip and vice versa. IFTs and motor proteins such as kinesin and dynein coding genes can be identified in the *Toxoplasma gondii* genome (Farhat et al., 2020). However, their role in flagellar maintenance and parasite biology has not been explored.

Where Am I? Molecular Cues Directing Gamete Formation Only in the Cat

The use of cats as experimental models has obvious ethical implications, it is costly and requires specialized animal housing facilities unavailable in the vast majority of research institutes. Notwithstanding, work done based on oocysts produced this way has been crucial in uncovering phenomena central to *T. gondii* dissemination and pathogenesis, including a plethora of structural studies (some, detailed above), as well as functional genomic and proteomics assays. Nonetheless, a long standing question in the field has been; why cats? In other words, what are the molecular cues that elicit the differentiation of *T. gondii* from bradyzoites into merozoites and then into gametocytes, exclusively at the cat's intestinal epithelia. How are these signals interpreted and translated within the parasite?

Approaching this question has been technically challenging, as in principle it requires the use of companion animals as experimental models, and the temporal resolution of distinct stages is complicated by their overlap in *in vivo* infections. However, more cost-efficient, animal-friendly alternatives have been developed allowing researchers to dive into questions that were once inaccessible. One of such milestones has been the work done on enterocyte 2D cell cultures, and the generation of 3D enteroids, as models to study the interactions between the parasite and enterocytes (Moura et al., 2009; Luu et al., 2019). Though these approaches have greatly contributed, these can only partially replicate stage conversion.

More recently, an *in vitro* approach aimed at replicating the intestinal gut environment in 2D cultures has significantly contributed in this sense. Breakthrough work by Martorelli Di Genova and colleagues, approached a long-standing mystery in the field: what is the biochemical footprint that makes felines the selected definitive host? Briefly, the authors identified that host-specificity to felines in the sexual stage is owed to the accumulation of linoleic acid, given by the lack of delta-6-desaturase activity in their intestines, (Di Genova et al., 2019). Felines are the only mammals that lack this enzyme activity in their intestines. Remarkably, inhibiting this enzyme's activity alone, and supplementing mice diet with an excess of linoleic acid, allowed them to now become *T. gondii* infectious oocyst spreaders (Di Genova et al., 2019).

The flip side of the question (i.e. what is happening within the parasite to elicit sexual differentiation)? was also recently approached and teased out. Farhat and colleagues found that a microrchidia homologue, named MORC1, assembles a complex with histone deacetylase 3 (HDAC3) and a number of apicomplexan transcription factors well known to influence stage transitions, known as ApiAP2s (Balaji et al., 2005; Farhat et al., 2020). MORC1 also regulates HAP2, a gene known to be required for macrogamete fertilization by the microgamete both in *T. gondii* and in *Plasmodium* (Liu et al., 2008; Angrisano et al., 2017; Ramakrishnan et al., 2019; Farhat et al., 2020).

Interestingly HAP2 depletion leads to oocysts that are unable to sporulate. (Ramakrishnan et al., 2019).

Though formation of oocysts was not reported, conditional depletion of MORC1 was shown to elicit *in vitro* sexual differentiation by evidencing the formation of flagellar like structures, labeled by the expression of epitope tagged PF16, an armadillo repeat protein essential for flagellar function, in tissue culture. Therefore, MORC1 was put forward as a master regulator, controlling different transcriptional programs involved in stage transitions of sexual development (Farhat et al., 2020). Consistent with its proposed role, MORC1 depletion elicits gene expression of bradyzoite specific genes, 85% of the merozoite-specific transcripts, including those coding for proteins that are important for merozoite development like GRA11a,GRA11b, and merozoite-restricted surface proteins (SRS), as well as putative flagellar and basal body assembly proteins of the microgamete; six IFTs, nine kinesins, 11 dyneins, one basal body protein, and three radial spoke proteins (Farhat et al., 2020). Furthermore, MORC1 depletion derepresses the expression of 89% oocyst specific genes and almost all proteins present in fully sporulated oocyst (Farhat et al., 2020). MORC1 depletion also triggered expression of AP2IX-9, an ApiAP2 shown to prevent merozoites from switching back to bradyzoite, keeping the unidirectionality of the sexual stage development (Farhat et al., 2020). Downregulation of the tachyzoite invasion machinery was shown to accompany these processes (Hehl et al., 2015; Farhat et al., 2020).

CLOSING REMARKS

Understanding the biochemical footprint that made felines the selected host by *T. gondii* to complete its sexual differentiation constitutes a milestone in our comprehension on the biology of this parasite and a major technical breakthrough. The use of *in vitro* models for the study of sexual stages has opened the possibility for large scale molecular, dynamic and structural studies, avoiding the ethical considerations and technical limitations of using domestic animals as experimental models. No longer requiring the use of companion animals to quantitatively access sexual stages provides the scientific community with an unprecedented opportunity to dive into once unattainable biological questions relevant to persistence and dissemination such as those pertaining to basal body and flagellar assembly.

Understanding the molecular pathway leading up to committing to sexual stages is equally important, providing the potential of developing strategies to fight this parasite at the very origin of differentiation. These findings pave the way for generating genetically admixed strains *in vitro* opening new avenues for vaccine development, and new cell culture-based drug screening platforms for drugs aimed at sexual stages and the oocyst.

AUTHOR CONTRIBUTIONS

RT and MF wrote the manuscript and designed the Figure. All authors contributed to the article and approved the submitted version.

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frontiers in Cellular and Infection Microbiology

REVIEW
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Comparisons of the Sexual Cycles for the Coccidian Parasites *Eimeria* and *Toxoplasma*

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Toxoplasma gondii and *Eimeria* spp. are widely prevalent Coccidian parasites that undergo sexual reproduction during their life cycle. *T. gondii* can infect any warm-blooded animal in its asexual cycle; however, its sexual cycle is restricted to felines. *Eimeria* spp. are usually restricted to one host species, and their whole life cycle is completed within this same host. The literature reviewed in this article comprises the recent findings regarding the unique biology of the sexual development of *T. gondii* and *Eimeria* spp. The molecular basis of sex in these pathogens has been significantly unraveled by new findings in parasite differentiation along with transcriptional analysis of *T. gondii* and *Eimeria* spp. pre-sexual and sexual stages. Focusing on the metabolic networks, analysis of these transcriptome datasets shows enrichment for several different metabolic pathways. Transcripts for glycolysis enzymes are consistently more abundant in *T. gondii* cat infection stages than the asexual tachyzoite stage and *Eimeria* spp. merozoite and gamete stages compared to sporozoites. Recent breakthroughs in host-pathogen interaction and host restriction have significantly expanded the understating of the unique biology of these pathogens. This review aims to critically explore advances in the sexual cycle of Coccidia parasites with the ultimate goal of comparing and analyzing the sexual cycle of *Eimeria* spp. and *T. gondii*.

Keywords: coccidia, *Toxoplasma*, *Eimeria*, sexual reproduction, merozoite, schizont

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INTRODUCTION

Coccidia is a subclass of the phylum Apicomplexa that includes a wide range of obligatory intracellular parasites. Coccidia pathogens were first described in 1879 by Leuckart as ovoid cells found in a patient sample (Dobell, 1922). Many global pathogens, like *Toxoplasma gondii* and *Eimeria* spp., belong to the Coccidia (Robben and Sibley, 2004). All species in the phylum Apicomplexa, including the subclass Coccidia, reproduce sexually (Smith et al., 2002; Walker et al., 2013). As obligatory intracellular pathogens, Coccidia must infect and parasitize a host to complete their life cycle (Entzeroth et al., 1998; Gupta et al., 2012). The host range is determined by the possible different species that a pathogen can parasitize (Reid et al., 2012), giving species-specific parasites a limited host range. The host range of most *Eimeria* spp. is limited to one single species, whereas only the sexual cycle of *Toxoplasma gondii* has a narrow host range only including species of the family Felidae (Kogut, 1990).

Even though all Coccidia have a sexual cycle, the most molecular characterization has been performed on *T. gondii* and *Eimeria* spp.; therefore, they are the focus of this review. Pioneering work has also been done in *Hammondia*, *Besnoitia*, *Neospora*, and *Sarcocystis* (García-Lunar et al., 2014; Blazejewski et al., 2015; Sokol et al., 2018; Horcajo et al., 2018; García-Sánchez et al., 2019; Jiménez-Meléndez et al., 2020) and further molecular characterization of these parasites will be fundamental for further understanding of sexual regulation and commitment in Coccidia. In this review, we highlight the most recent findings regarding sexual development for *T. gondii* and *Eimeria* spp. For example, the differences in transcript expression between sexual and asexual stages revealed many stage-specific genes in *T. gondii* and *E. tenella* (Walker et al., 2015; Hehl et al., 2015; Su et al., 2017). During merogony and sexual differentiation, the expression of genes responsible for fundamental cellular processes, such as metabolism and host-pathogen interaction, changes widely (Behnke et al., 2014; Reid et al., 2014; Walker et al., 2015; Hehl et al., 2015; Su et al., 2017; Ramakrishnan et al., 2019). In this review, these differences will be explored and correlated to additional discoveries in the sexual cycles of *T. gondii* and *Eimeria* spp.

TOXOPLASMA AND EIMERIA LIFE CYCLES

Both *T. gondii* and *Eimeria* spp. undergo sexual reproduction with a restricted host range during their life cycle (Kogut, 1990; Reid et al., 2012). Despite the host restriction in the sexual cycle, *Eimeria* spp. and *T. gondii* have fundamental differences in their life cycles. The genus *Eimeria* contains around 1,700 described species (Walker et al., 2013; Clark et al., 2017). In general, *Eimeria* spp. are monoxenous parasites, as they complete their life cycles in a single host (Barta, 1989; Walker et al., 2013; Clark et al., 2017). The genus *Toxoplasma* only contains one species, *T. gondii*, and its sexual cycle is restricted to felines (Martorelli Di Genova et al., 2019). For its asexual cycle, *T. gondii* can infect many warm-blooded animals, including several species of mammals and birds. In the intermediate host, *T. gondii* replicates asexually and differentiates into persistent tissue cysts, containing the stage called bradyzoites (Ong et al., 2011).

T. gondii is classified as a cyst forming Coccidia because it develops into tissue cysts in the intermediate hosts during the asexual life cycle (Dubey, 2020). This asexual cycle can be completed successively, as these tissue cysts can be passed indefinitely within intermediate hosts. After ingestion, *T. gondii* bradyzoites can either differentiate into a tachyzoite to start the asexual cycle or a pre-sexual stage to start the sexual cycle, depending on whether the host is a non-feline or feline, respectively (Martorelli Di Genova et al., 2019). However, that is not the case for *Eimeria* spp. (**Figure 1**). *Eimeria* spp. do not form tissue cysts and do not have intermediate hosts because their whole life cycle is complete within the same host (Lal et al., 2009; Walker et al., 2013). *Eimeria* spp. sporozoites always differentiate into pre-sexual stages *in vivo* (Walker et al., 2013; Walker et al., 2015).

The life cycle of *Eimeria* spp. starts when a new host ingests oocysts by fecal-oral contact or through contaminated food or water (Robben and Sibley, 2004). In the case of *T. gondii*, the sexual cycle usually starts upon the ingestion of tissue cysts containing bradyzoites (Martorelli Di Genova et al., 2019). There is experimental evidence that *T. gondii* tachyzoites or oocysts can start the sexual cycle in cats; however, oocyst shedding is significantly delayed (Dubey, 2005). The prepatent period is 3–8 days when cats are fed bradyzoite cysts, but 5–34 days when cats are fed tachyzoites and 18–41 days when cats are fed oocysts. This delay likely indicates that additional developmental steps occur before the tachyzoites or sporozoites can undergo sexual development, perhaps even becoming transitioning through the bradyzoite stage. For both *T. gondii* and *Eimeria* spp., the oocyst or cyst wall is digested in the stomach, and the parasites are liberated to infect the intestine (Russell and Sinden, 1981). While *T. gondii* sexual development exclusively occurs in the feline small intestine (Martorelli Di Genova et al., 2019), different *Eimeria* species have distinct host tissue tropism for their sexual cycles, such as the liver or gallbladder, but most occur within the intestinal tract (Dubey, 1986; Walker et al., 2013; Walker et al., 2015; Sivajothi et al., 2016).

In both *T. gondii* and *Eimeria* spp., merozoites can either continue replicating or differentiate into sexual stages (**Figure 1**) (Su et al., 2017). For Eimeria spp., the number of rounds of merozoite replication is genetically determined and differs between the species and isolates and can respond to artificial selection (Montes et al., 1998; Pakandl, 2005). There are two distinct sexual stages: macrogamete and microgamete. Macrogametes, or female gametes, remain intracellular while the microgametes, or male gametes, can swim in the extracellular environment *via* flagellum until they invade a new cell (Walker et al., 2015). Upon the invasion, fusion of microgamete with macrogamete forms a diploid zygote (**Figure 1**), followed by the formation of a protective wall, resulting in an unsporulated oocyst (Walker et al., 2013). The newly formed oocysts are shed within the host feces (Bussière et al., 2018). Once released into the environment, oocysts may sporulate depending on the conditions (Zhou et al., 2016). During oocyst sporulation, the diploid zygote replicates by meiosis, generating haploid sporozoites (**Figure 1**) (Striepen et al., 2007; Dubey, 2019).

HOST SPECIFICITY

The extensive speciation in *Eimeria* is remarkable, as this genus contains around 1,700 described species (Clark et al., 2017). Most described *Eimeria* spp. are thought to be restricted to a single host species; however, experimental evidence suggests that some *Eimeria* spp. that infect rodents have a broader host range (Clark et al., 2017; Mácová et al., 2018; Jarquín-Díaz et al., 2020). The biological mechanisms underlying *Eimeria* speciation and host restriction are unknown. Two independent studies did not find evidence of coevolution between *Eimeria* spp. and their

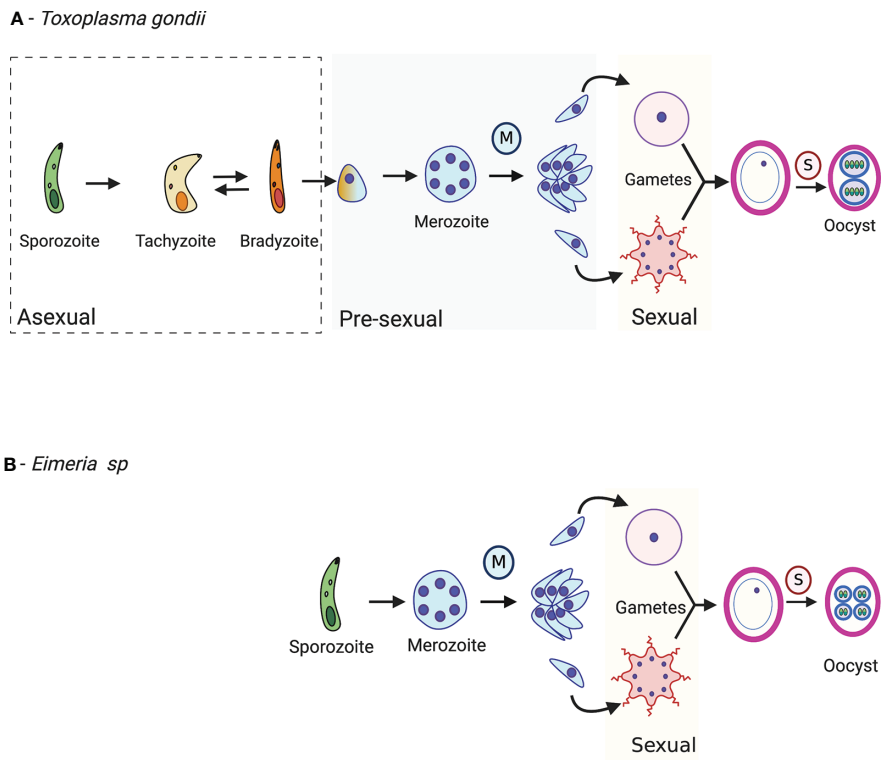

FIGURE 1 | The life cycle of *T. gondii* **(A)** and *Eimeria* spp. **(B)**. After host cell invasion, *T. gondii* bradyzoites may either differentiate into a pre-sexual stage or a tachyzoite. *T. gondii* tachyzoites are a fast-replicative stage and disseminate the infection in the host. *Eimeria* sporozoites replicate asexually and differentiate into pre-sexual stages. The pre-sexual stages for *T. gondii* and *Eimeria* spp. are schizonts and merozoites because they precede the sexual stages and are committed to sexual development. During merogony (blue M), nuclei replication form multi-nucleated cells, schizonts, that post cytoplasmic division, generates merozoites. Merozoites can either keep replicating or differentiate into sexual stages. The two gametes, macrogamete, and microgamete fuse and generate oocyst. Sporulation (pink S) occurs in the early oocyst, generating sporozoites. Lastly, *T. gondii* sporozoite differentiates into tachyzoites after an oocyst infects the host.

respective host species (Kvičerová and Hypša, 2013; Vrba and Pakandl, 2015). One of these studies suggests that *Eimeria* specification is likely caused by the adaptation of the parasite to its host, rather than a cophylogenetic process (Kvičerová and Hypša, 2013).

The sexual cycle, but not the asexual cycle of *T. gondii*, presents a host range restricted to felines (Dubey, 2020). Felines are exclusive carnivores and are auxotrophic for both taurine and desaturated fatty acids with more than two double bonds, such as arachidonic acid (Sinclair et al., 1979; Rentschler et al., 1986). Feline arachidonic acid auxotrophy is due to the lack of delta-6-desaturase (D6D) activity in their intestines (Sinclair et al., 1979). The absence of this enzyme activity is not observed in other mammals. D6D adds additional double bonds to fatty acids, and it is fundamental for arachidonic acid synthesis (Obukowicz et al., 1998). This phenotype results in the systemic accumulation of linoleic acid (LA) in felines (Sinclair et al., 1979). Our group showed that high levels of LA are required for *T. gondii* bradyzoites to differentiate into pre-sexual stages (Martorelli Di Genova et al., 2019). We showed that supplementation of mice diet with LA and a D6D inhibitor is enough to promote the sexual cycle of *T. gondii* in a rodent, breaking the species barrier (Martorelli Di Genova et al., 2019). It remains unknown the reason why LA is crucial for *T. gondii* sexual development. In fungi, LA has been shown to interfere with *Aspergillus* and *Ophiostoma* cellular development (Calvo et al., 1999; Naruzawa et al., 2015). In these fungal species, oxylipins derived from LA were characterized as signaling molecules with important roles in the fungi sexual and asexual differentiation (Naruzawa et al., 2015; Fischer and Keller, 2016). These findings suggest the possibility of LA, or derivatives molecules, having signaling roles during *T. gondii* sexual development.

Pre-sexual stages for both *Eimeria* spp. and *T. gondii* replicate in a process known as merogony (Ramakrishnan et al., 2017; Ma et al., 2019). During merogony, parasite cells replicate their nuclei at a high rate (Dubey et al., 2017) forming cells with many nuclei sharing the same cytoplasm (Sheffield, 1970; Dubey, 2017). Merozoites are developed by the end of this process, and they can differentiate into gametes after replicating asexually (Ferguson and Dubremetz, 2020). The replication rate by which the genetic material and the parasites themselves replicate is presumed to be higher for pre-sexual stages than any other Coccidia life stage (Hehl et al., 2015).

TRANSCRIPTOMIC ANALYSIS OF METABOLIC PATHWAYS

Independent transcriptomes of *E. tenella* and *T. gondii* have shown the upregulation of a significant number of metabolic pathways in the pre-sexual stages compared to other developmental stages (Behnke et al., 2014; Reid et al., 2014; Walker et al., 2015; Hehl et al., 2015; Ramakrishnan et al., 2019). Analysis of the transcriptome datasets for *E. tenella* and *T. gondii* (Gajria et al., 2008) shows enrichment for different metabolic pathways (**Figure 2**). The datasets used in this analysis were obtained from published data (Behnke et al., 2014; Reid et al., 2014; Walker et al., 2015; Hehl et al., 2015; Ramakrishnan et al., 2019) and were all previously normalized. We analyzed only the three datasets available for *T. gondii* cat infection stages that were simultaneously compared to tachyzoites, as well as two independent studies of *E. tenella* merozoite and gamete stages compared to sporozoites. Apart from the variability observed among the datasets, there are important trends shared between the different experiments.

Many metabolic pathways are enriched across the independent datasets. Central carbon, especially glycolysis, and amino acid metabolism pathways are enriched among all datasets for the pre-sexual stages of both organisms. These findings are corroborated by a proteomics study of *E. tenella* that reported the overexpression of several metabolic enzymes associated with oxidative phosphorylation in the pre-sexual stages compared to other life stages (Lal et al., 2009). Amino acid pathways are also enriched in pre-sexual and sexual stages (Behnke et al., 2014; Reid et al., 2014; Walker et al., 2015; Hehl et al., 2015; Ramakrishnan et al., 2019). Apart from *E. tenella*, transcriptomics of other poultry infecting species *Eimeria maxima* and *Eimeria necatrix* as well as the mouse infecting species *Eimeria falciformis* also suggested higher metabolic needs in pre-sexual and sexual stages compared to sporozoites (Su et al., 2017; Ehret et al., 2017; Hu et al., 2018), highlighting an overall trend of higher metabolic demand in the pre-sexual stages of Eimeria spp. For *Eimeria bovis*, pre-sexual stage replication *in vitro* is directly impacted by host cellular sterol profile, demonstrating the intrinsic parasite dependency on the host metabolism and nutrient availability (Taubert et al., 2018). The variability between pre-sexual stages datasets might be a consequence of the multiple differentiation steps that precede sexual differentiation. Also, it appears that the same pathways upregulated in the pre-sexual stages are still upregulated in sexual stages (**Figure 2**). Because it is not possible yet to purify *T. gondii* gametes or merozoites, the data sets from these samples likely had a combination of both, so it is unclear how similar the metabolism between pre-sexual and sexual stages is. An overall similarity was previously observed between *T. gondii*, *E. tenella*, and *E. falciformis* pre-sexual and sexual stages transcripts expressions, further supporting our analysis (Ehret et al., 2017).

It is known that *T. gondii* tachyzoites usurp and modify host metabolism to fulfill their metabolic needs and replicate intracellularly (Krishnan et al., 2020; Olson et al., 2020). It has been hypothesized that the massive replication during merogony has a higher energy demand than tachyzoite replication (Behnke et al., 2014; Hehl et al., 2015). Additionally, critical metabolites and metabolic pathways could be required for the sexual cycle of these parasites. This hypothesis is corroborated by the specific requirement of LA, but not oleic acid, for sexual differentiation of *T. gondii* (Martorelli Di Genova et al., 2019). In higher eukaryotes, it has been shown that differences in metabolism are responsible for cellular differentiation (McGraw and Mittal, 2010; Gatie and Kelly, 2018). Therefore, the overall metabolic upregulation could be intrinsically related to the parasite differentiation during pre-sexual development and not simply a consequence of accelerated replication. It is fundamental to highlight that transcriptomic data is not a measure of metabolite abundance, and direct analysis of metabolites is needed to characterize pre-sexual and sexual stage metabolism fully.

PRE-SEXUAL STAGES

Apart from metabolic pathways, many other cellular functions are likely altered in the pre-sexual stages. Transcriptional analysis of *T. gondii* pre-sexual stages reveals changes in the expression of transcripts critical for host-pathogen interaction (Behnke et al., 2014; Hehl et al., 2015; Ramakrishnan et al., 2019). Independent transcriptomes showed that pre-sexual and sexual stages downregulate the expression of most rhoptry and granular protein genes that are upregulated in tachyzoites (Hehl et al., 2015; Ramakrishnan et al., 2019). Similarly, the expression of rhoptry kinases is higher in sporozoites than pre-sexual stages for *E. falciformis* (Heitlinger et al., 2014). In *T. gondii* tachyzoites, rhoptry proteins have important known functions, including the biogenesis of the moving junction, which is required for invasion (Dlugonska, 2008; Pernas and Boothroyd, 2010). Some rhoptry proteins are secreted into the host cell and can associate with host mitochondria, endoplasmic reticulum, or be translocated to the host nucleus (Dlugonska, 2008; Pernas and Boothroyd, 2010). Some rhoptry proteins are also involved in the biogenesis of the parasitophorous vacuole, while dense granule proteins are generally responsible for its architecture and function (Pernas and Boothroyd, 2010; Gold et al., 2015).

For *T. gondii*, only two pre-sexual stage effector proteins have been characterized, TgGRA11B and TgBRP1. TgGRA11B is exclusively expressed in *T. gondii* pre-sexual stages but changes its localization from the dense granules to the parasitophorous vacuole membrane as the pre-sexual stages develop (Hehl et al., 2015; Ramakrishnan et al., 2017). *T. gondii* pre-sexual stages also express TgBRP1, a known bradyzoite rhoptry protein (Schwarz et al., 2005). The role of TgBRP1 and TgGRA11B is still unclear during pre-sexual development, but these findings show that the pre-sexual forms have unique interactions with the host compared to the asexual stages of infection. A transcriptomic analysis also showed that *T. gondii* pre-sexual stages overexpress different surface antigens linked to GPI, further suggesting differences in its interaction with the host (Hehl et al., 2015). *E. tenella* also expresses pre-sexual stage surface antigens. These

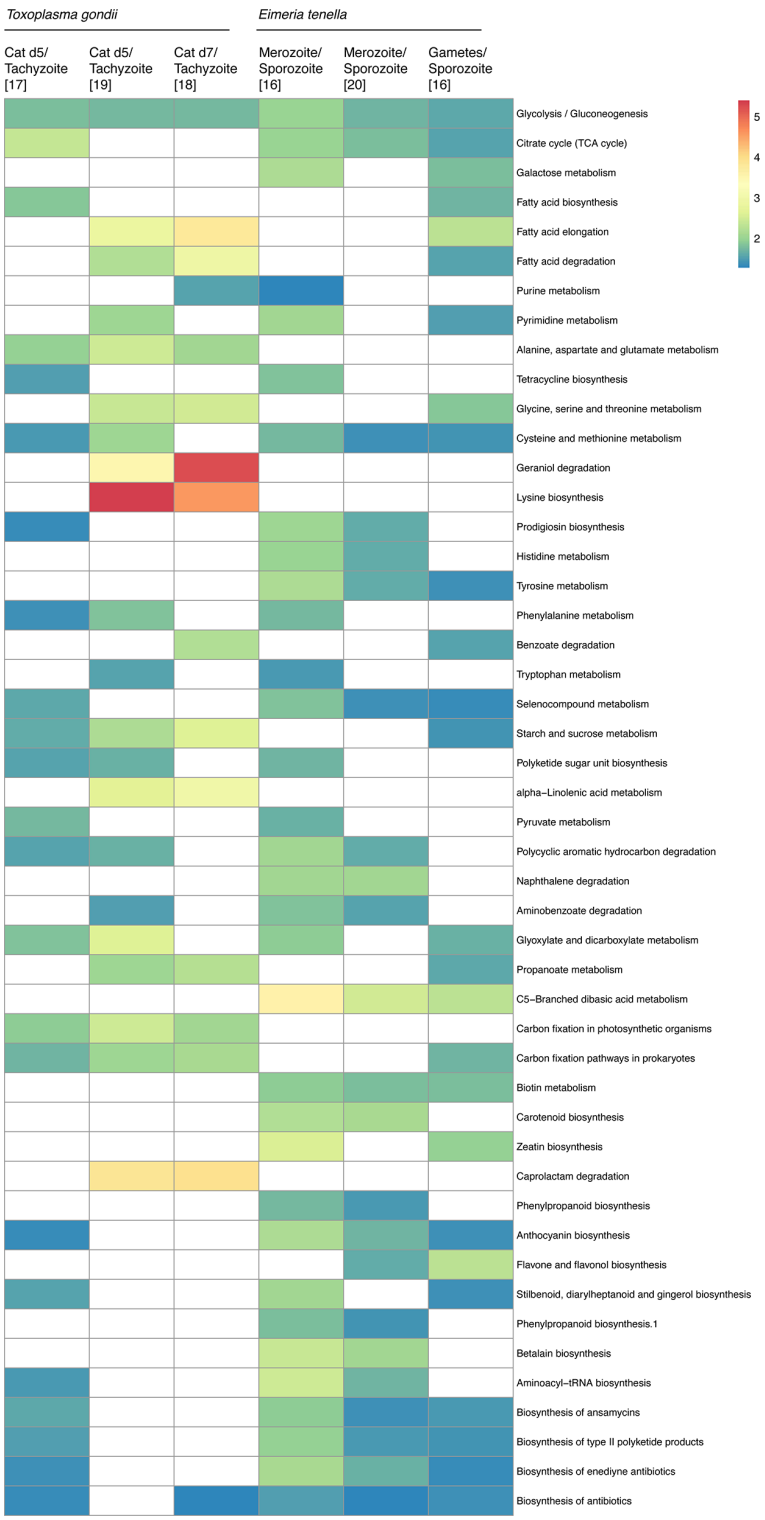

FIGURE 2 | Metabolic pathway enrichment from transcriptional analysis of pre-sexual and sexual stages. Pre-sexual stage transcriptomics was compared using KEGG pathway analysis on ToxoDB (Gajria et al., 2008). The heatmap shows how many times each metabolic pathway is enriched in pre-sexual stages compared to asexual stages for each dataset. Pathways that were not enriched are marked as white. All pathways presented were enriched in at least two independent datasets. Figure created using ClustVis (Metsalu and Vilo, 2015). Tg, *Toxoplasma gondii*; Et, *Eimeria tenella.* For *T. gondii* sexual stages, only sample EES5 was used for the comparison.

stages overexpress many surface antigens (SAG) linked to glycosylphosphatidylinositol (GPI) in comparison with sporozoites (Reid et al., 2014). Another study using these *E. tenella* pre-sexual overexpressed SAGs showed that some of them suppressed expression of interferon-gamma and IL-12 in macrophages *in vitro* and induced expression of IL-10 (Chow et al., 2011). Another subset of these SAGs induced nitric oxide production in macrophages, potentially being related to the inflammatory response caused by *E. tenella* infection (Chow et al., 2011).

Another important side of host-pathogen interaction during pre-sexual development is the host immune response to the infection. Transcriptomic analysis of *T. gondii* infected cats showed a high expression of transcripts crucial for the immune response (Cong et al., 2018). Examples of these transcripts with high expression in infected cats include interferon-gamma and different cytokines (Cong et al., 2018). Most reports describe that toxoplasmosis infection causes mild symptoms in immunocompetent felines, suggesting that the immune system can clear the infection or at least cause the parasites to differentiate into the chronic infection bradyzoite stage (Calero-Bernal and Gennari, 2019). Host features may vary the *T. gondii* oocyst shedding rates, including cat breed as a potential factor (Must et al., 2017).

In contrast to *T. gondii* infection in cats, the *Eimeria* spp. infection often harms the host. The infection by *Eimeria* spp. typically results in malabsorption, consequential weight loss, and mortality in-floor raised chickens (Quiroz-Castañeda and Dantán-González, 2015). Increased intestinal IL-10 expression during *Eimeria* spp. infection in chickens is suggested to be related to the symptoms (Arendt et al., 2019). On the other hand, interferon-gamma seems protective against avian coccidiosis, as shown in many studies (Kim et al., 2019).

Limited tissue culture options are one major roadblock for studying pre-sexual and sexual stages and their interaction with the host. Since the 1960s, efforts have been made to maintain Coccidia *in vitro* (Strout et al., 1965). A recent finding shows that chicken epithelial cell line supports *E. tenella* sexual development, from sporozoites to gametes (Bussière et al., 2018). For *T. gondii*, LA supplemented feline or D6D-inhibited mouse organoid support pre-sexual and sexual stages development, but without infectious oocyst production (Martorelli Di Genova et al., 2019).

SEXUAL COMMITMENT AND DIFFERENTIATION IN COCCIDIA

Commitment to sexual differentiation is a key process during the life cycle of Apicomplexa parasites. After commitment, the parasite developmental program will culminate with its differentiation into a sexual stage (Bechtsi and Waters, 2017). For example, sexual commitment in the Apicomplexa parasite *Plasmodium falciparum* is a well-described process. The sexual commitment of *P. falciparum* parasites relies on the expression of a specific ApiAP2 factor, named PfApiAP2-g. ApiAP2s are transcriptional factors found in Apicomplexa that have been shown to regulate virulence and cellular development in these parasites (Jeninga et al., 2019). The name derives from the apetala transcription factors 2 (AP2), a family of transcription factors discovered in plants (Jeninga et al., 2019). Single-cell transcriptomics of PfApiAP2-G conditional depleted parasites revealed that this factor upregulates the expression of other transcription factors and histone modifiers, and defined PfApiAP2-G as a master regulator of gametogenesis (Poran et al., 2017). The expression of PfApiAP2-G is regulated by histone methylation and acetylation, demonstrating a critical epigenetic regulation of the parasite differentiation (Bechtsi and Waters, 2017).

Previous studies have described that *T. gondii* has 67 ApiAP2 factors, and many of them are stage-specific according to transcriptomic studies (Hehl et al., 2015; Kim et al., 2020). Seminal work showed that the differentiation of *T. gondii* tachyzoites into bradyzoites is tightly regulated by multiple ApiAp2, demonstrating a central role of these transcription factors in this parasite's development (Radke et al., 2013; Hong et al., 2017). The microrchidia protein (MORC) in a complex with ApiAP2 factors is responsible for repressing sexual commitment in tachyzoites (Farhat et al., 2020). The complex MORC-ApiAP2 is responsible for recruiting a histone deacetylase, TgHDAC3, that promotes specifically the deacetylation of bradyzoite and pre-sexual and sexual stage-specific genes, further disrupting their expression (Farhat et al., 2020). The addition of a histone deacetylase inhibitor, FR235222, to tachyzoites induces their differentiation into bradyzoites in fibroblasts in tissue culture (Bougdour et al., 2009). These findings suggest that epigenetics might play a critical role in *T. gondii* differentiation, as observed in the sexual commitment of *P. falciparum* (Bechtsi and Waters, 2017).

The differentiation of *T. gondii* intro bradyzoites is orchestrated by a Myb-like transcription factor named BFD1 (Waldman et al., 2020). BFD1 induced bradyzoite differentiation is independent of a change in the media pH or any other cellular stress (Mayoral et al., 2020). In the life cycle of *T. gondii*, when a feline consumes an intermediate host, the bradyzoite is the developmental stage that precedes the pre-sexual ones. It would be relevant to explore if BFD1 and MORC together regulate bradyzoite development and commitment to the sexual cycle.

The number of ApiAP2 factors varies from 44 to 54 in *Eimeria* spp. infecting chickens (Reid et al., 2014), while the mouse infecting species E. *falciformis* contains only 17 genes containing an AP2 domain (Ehret et al., 2017). As observed in *T. gondii*, cellular differentiation is likely regulated by ApiAP2 factors in *Eimeria* spp. Transcriptional analysis of the pre-sexual stages of *E. tenella* revealed that ApiAP2 expression profile changes during pre-sexual development (Su et al., 2017). Individual deletion of 10 out of 33 ApiAP2 factors was successful in a screening performed in sporozoites of *E. tenella* (Hu et al., 2020). This finding indicates that these 10 ApiAP2 are likely dispensable in *E. tenella*. Lastly, sexual commitment in *Eimeria* spp. has been proposed to be genetically programmed and not reliant on environmental clues, as observed in

P. falciparum and likely the case for *T. gondii* (Smith et al., 2002; Walker et al., 2013).

Final Steps of the Sexual Cycle: Gametogenesis and Oocyst Biology

Transcriptomics of *E. tenella* showed upregulation of over 800 transcripts during gametogenesis compared to sporozoites, identifying many gamete-specific transcripts (Walker et al., 2015). Macrogamete specific transcripts are intrinsically related to oocyst wall biogenesis. According to this study, distinct wall protein transcripts and transcripts surface antigens are upregulated in macrogametes. Macrogametes also upregulate proteases, oxidoreductases, glycosylation, and fatty acid metabolism transcripts. This finding gives an initial insight into the mechanisms for the oocyst wall biosynthesis.

The same study shows that microgametes upregulate axonemes, flagella, DNA condensation, and transcripts likely related to gamete fusion (Walker et al., 2015). Additionally, a study using *E. falciformis* confirmed the trends observed for *E. tenella* gametes upregulated transcripts (Ehret et al., 2017). These putative transcripts might play a significant role in microgamete locomotion and fusion to the macrogamete. HAPLESS2 (HAP2) is a known microgamete protein in *P. falciparum* with a role in fertilization (Vega-Rodriguez et al., 2015). The *E. tenella* (Walker et al., 2015) and *T. gondii* (Ramakrishnan et al., 2019) orthologues are highly expressed during the sexual development of both species. The deletion of TgHAP2 results in non-infectious oocysts with aberrant morphology and generates a putative anti-*Toxoplasma* vaccine strain (Ramakrishnan et al., 2019). Gamete fusion and meiosis in *T. gondii* was indirectly demonstrated by crossing two single drug-resistant strains and obtaining haploid strains resistant to both drugs (Pfefferkorn and Pfefferkorn, 1980). Remarkable electron microscopy work showed *T. gondii* microgametes attached to a macrogamete or early oocysts, suggesting the occurrence of fertilization (Ferguson and Dubremetz, 2020). Fertilization in *E. maxima* appears to be affected by heat stress (Schneiders et al., 2020). In this study, heat-stressed parasites presented different transcriptional profile compared to the control; EmHAP2 transcript expression, for example, was significantly decreased (Schneiders et al., 2020). Another pioneering study showed that cross-fertilization is common in polyclonal infections of *E. tenella*, further demonstrating the occurrence of fertilization in this parasite (Blake et al., 2015).

After fertilization of gametes, oocysts form within the host cells and are subsequently shed within the host feces. Coccidia oocysts are resistant to environmental stress, surviving extreme conditions, such as UV (Ware et al., 2010), hypochlorite, and ozone (Wainwright et al., 2007). Oocysts remain viable, and likely infectious, for many months after shedding (Dubey, 2019). The structure and chemical composition of the oocyst wall is responsible for the oocyst resilience (Dumètre et al., 2013).

The oocyst wall is composed of two distinct structures: outer and inner walls (Ferguson and Dubremetz, 2020). The outer wall surrounds the oocyst, while the inner wall is physically attached to the outer wall and in contact with the cellular membrane (Mai et al., 2009; Bushkin et al., 2013). Despite the interaction between inner and outer walls, they are independent, as hypochlorite treatment strips off only the outer wall while the inner one remains intact. In *E. tenella* and *E. maxima*, proteins correspond to at least 90% of the outer wall composition weight, and lipids and carbohydrates are responsible for 10% of the weight, 8%, and 2%, respectively (Mai et al., 2009). Bushkin et al. showed that lipids present in the oocyst wall of *T. gondii* and *E. tenella* wall were a complex mixture of triglycerides, similar to *Mycobacterium* walls, and could be acid-fast stained (Bushkin et al., 2013).

One interesting transcript upregulated in *E. tenella* macrogametes is amiloride-sensitive amine oxidase (EtAO2). The protein coded by this gene contains an extracellular adhesive MAM (meprin, A5, μ domain) domain that likely plays a significant role in oocyst wall biogenesis. AO2 activity catalyzes the cross-linking of tyrosine rich peptides, forming dityrosine bonds that are involved in the oocyst wall hardening (Belli et al., 2003; Walker et al., 2015).

The presence of dityrosine bonds in the oocyst wall was first indicated by a seminal study using *E. maxima* as a model. This study identified tyrosine-rich glycoproteins, EmGAM56, and EmGAM82, and showed that they are proteolytically processed and incorporated in *E. maxima*'s oocyst wall (Belli et al., 2003). Dityrosine bonds are also present in the *T. gondii* oocyst wall (Wang Z. T. et al., 2017). *T. gondii* transcriptomics showed upregulation of TgAO2 and transcripts from tyrosine-rich glycoproteins orthologues in the late stages of sexual reproduction (Ramakrishnan et al., 2019). The expression of TgAO2 in macrogametes was demonstrated by immunofluorescence, suggesting a conserved mechanism for Coccidia wall biogenesis (Walker et al., 2015). The presence of dityrosine bonds in the oocyst wall of *Eimeria* and *T. gondii* confers the oocyst wall autofluorescent when excited with blue light (Belli et al., 2003; Ramakrishnan et al., 2019).

A subset of proteins named oocyst wall proteins (OWP) is transcriptionally expressed in both *E. tenella* and *T. gondii* (Walker et al., 2015; Ramakrishnan et al., 2019). Orthologs of these cysteine-rich OWPs were first characterized in *Cryptosporidium* (Templeton et al., 2004). There are 7 cysteine-rich OWPs in *T. gondii* (named OWP1-7), and they localize to the oocyst wall (Possenti et al., 2010). OWPs play a role in the oocyst wall structure and have a central role in the oocyst wall of many Apicomplexan (Templeton et al., 2004; Possenti et al., 2010). While no further characterization of the OWPs has occurred yet for *E. tenella*, one study showed that two OWPs are expressed during sporulation and present in the sporocyst wall in *Eimeria nieschulzi* (Jonscher et al., 2015) further suggesting additional roles for OWPs in Coccidia parasites.

CONCLUDING REMARKS

Transcriptional studies of *Eimeria* spp. and *T. gondii* revealed many key components that are shared in their sexual cycle. The

upregulated metabolism into the pre- and sexual stages may explain the complexity in reproducing these stages *in vitro*. A central hypothesis is that without the fulfilled metabolic needs, Coccidia differentiation in pre-sexual and sexual stages is reduced or completely ablated. This hypothesis is further suggested by the sexual cycle of *T. gondii*, relying on the LA concentration (Martorelli Di Genova et al., 2019). Along with lipid metabolism, other intestinal physiology factors might be critical to consider. The presence of bile salts, hypoxia, high osmotic pressure, and the microbiota are all intestinal conditions that need to be considered (Boyer, 2013; Guinane and Cotter, 2013). Primary cells and organoid models can support partial sexual development of Coccidia parasites, but infectious oocyst production has not yet been observed in cell culture (Bussière et al., 2018; Martorelli Di Genova et al., 2019). However, *in vitro* fertilization of *Cryptosporidium* gametes has occurred in some cell lines, highlighting that while it is challenging to reproduce the Apicomplexa life cycle in tissue culture, it is not impossible (Heo et al., 2018; Wilke et al., 2019). One significant difficulty is to obtain sporozoites from Coccidia oocysts and culture them. Recently improved techniques might significantly improve this procedure and maximize tissue culture yields (Sokol et al., 2020).

Although there are similarities between the sexual cycles of *T. gondii* and *Eimeria* spp., there are also substantial differences. Likely these life cycle differences result in unique sexual commitment, regulation, and development between the two Coccidia genera. Conversely, the *T. gondii* sexual cycle appears to be triggered by host factors. This hypothesis is corroborated by the presence of two distinct masters of *T. gondii* differentiation, BFD1 and MORC (Farhat et al., 2020; Waldman et al., 2020). Furthermore, epigenetics is likely to play a central role in *T. gondii* development, as exemplified by a study using histone deacetylase inhibitors and its effect on development (Bougdour et al., 2009). Additional studies are necessary to define further how *T. gondii* and *Eimeria* spp. parasites commit to the sexual cycle and whether each genus has a unique sexual regulation mechanism.

While sexual reproduction is a common trait in the phylum Apicomplexa (Smith et al., 2002), *T. gondii* and closely related organisms from the Sarcocystidae family have an independent asexual cycle. After these pathogens infect an intermediate host, they develop into tissue cysts. As tissue cysts are infectious for both intermediate and definitive host, Sarcocystidae parasites can infect intermediate hosts indefinitely, skipping the definitive host, and sexual reproduction as a result. It is unclear how long *T. gondii* strains replicate asexually in nature and what the evolutionary consequences are of continually missing sexual reproduction.

Chronic toxoplasmosis is still a morbidity factor for immunocompromised individuals (Wang Z.-D. et al., 2017). Both Coccidia pathogens put at risk the economy, especially regarding livestock and agriculture. Toxoplasmosis causes recurrent abortions in sheep (Shahbazi et al., 2019). Avian coccidiosis from Eimeria spp. is responsible for increases in weight loss and mortality in chickens resulting in significant economic losses for the poultry industry yearly (Noack et al., 2019). Overall, to study these pathogens, it is necessary to comprehend both basic biology concepts as well to generate medical and economic relevant knowledge.

AUTHOR CONTRIBUTIONS

BG wrote the review and generated the figures. LK edited the review and figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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From Genes to Transcripts, a Tightly Regulated Journey in *Plasmodium*

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Over the past decade, we have witnessed significant progresses in understanding gene regulation in Apicomplexa including the human malaria parasite, *Plasmodium falciparum*. This parasite possesses the ability to convert in multiple stages in various hosts, cell types, and environments. Recent findings indicate that *P. falciparum* is talented at using efficient and complementary molecular mechanisms to ensure a tight control of gene expression at each stage of its life cycle. Here, we review the current understanding on the contribution of the epigenome, atypical transcription factors, and chromatin organization to regulate stage conversion in *P. falciparum*. The adjustment of these regulatory mechanisms occurring during the progression of the life cycle will be extensively discussed.

Keywords: *Plasmodium*, gene regulation, epigenetics, chromatin, AP2-G, sexual commitment

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INTRODUCTION

Malaria affected 228 million people and 405,000 deaths in 2018 (WHO, 2019) and remains one of the major global health problems. In sub-Saharan Africa, this disease is responsible for ~20% of all-cause mortality among children under 5 years old. The causative agent is a protozoan parasite, *Plasmodium*, belonging to the phylum Apicomplexa. Although, five plasmodial species can infect human, *P. falciparum* is associated with the greatest morbidity and mortality.

The life cycle of *P. falciparum* is complex and can be divided in two parts: the sexual phase in mosquito vector and asexual phase in human liver and red blood cells. After a bite by an infected female Anopheles, sporozoites are injected into the human and invade hepatocytes. This asymptomatic phase leads to the multiplication of parasites, which are released into the bloodstream and initiate the intraerythrocytic developmental cycle (IDC) inside red blood cells. During this cycle, merozoite develop to immature ring and progress to mature schizont, before dividing into 16 to 32 new merozoites, which immediately invade new erythrocytes. A portion of these parasites differentiate and mature into male and female gametocytes, and are ingested by another female mosquito. Thousands of sporozoites will be produce and migrate to the salivary glands to complete the cycle.

This parasite life cycle progression is accompanied by important transcriptional changes at the population level (Bozdech et al., 2003; Le Roch et al., 2003; Lemieux et al., 2009; Otto et al., 2010; López-Barragán et al., 2011; Hoo et al., 2016; Toenhake et al., 2018). Recently, advances in single-cell RNA sequencing provided new insights in our understanding of gene expression across different stages and *Plasmodium* species (Reid et al., 2018; Walzer et al., 2018; Howick et al., 2019; Sà et al., 2020). To ensure this dynamic gene expression, adjustable according to environmental factors and the parasite development, a tight and coordinated regulation is fundamental at each stage. Despite recent progress, a complete understanding of all mechanisms regulating gene expression is still

lacking and several biological questions remained to be answer if we want to find new way to hamper parasite proliferation in a specific manner. Here, we discuss the current knowledge on the involvement of regulatory mechanisms in gene expression across the parasite development and stage conversion. The contribution of epigenetics and chromatin-associated proteins as well as the role of chromatin organization will be detailed, with a particular interest in *P. falciparum*.

OVERVIEW OF CHROMATIN STRUCTURE AND GENE REGULATION IN PLASMODIUM

In this section, we will describe the different regulatory mechanisms of gene expression identified so far in *Plasmodium*. Examples and specific features will be depicted in details thereafter.

Overview of the Nucleosome Landscape

In eukaryotic cells, genomic DNA is wrapped around nucleosomes leading to the formation of a compact structure, the chromatin. A nucleosome is constituted of a histone octamer containing two units of H2A, H2B, H3, and H4. These histones can be exchanged with several histone variants that have been identified in *P. falciparum*, H2A.Z, H2B.Z, CenH3, and H3.3, and which provides an additional level of regulation (Sullivan, 2003; Miao et al., 2006; Bártfai et al., 2010). These histone variants seem to have specific features such as H2A.Z, which is associated with AT-rich sequences found in intergenic regions of *P. falciparum* (Hoeijmakers et al., 2013; Petter et al., 2013) or PfCENH3 positioned at the centromeres (Hoeijmakers et al., 2012). In addition to its packaging role, nucleosomes also play a crucial role in regulation and changes in occupancy control multiple biological processes, including gene expression. In general, active promoters of *P. falciparum* show a nucleosome-depleted region (NDR) upstream of the transcription start site (TSS), the binding site of the preinitiation complex (PIC), while silenced genes exhibit a higher nucleosome occupancy in their promoter regions hampering the interaction of the PIC (Ponts et al., 2011; Bunnik et al., 2014; Kensche et al., 2015). In model organisms, this NDR is flanked by well-positioned nucleosomes (-1 and +1) (Jiang and Pugh, 2009), but a strongly positioned nucleosome +1 is lacking in *P. falciparum* (Ponts et al., 2011; Bunnik et al., 2014). Intergenic regions display a poor nucleosome occupancy, unlike the start and end of coding regions (Westenberger et al., 2009; Ponts et al., 2011; Bunnik et al., 2014; Kensche et al., 2015), and are enriched in H2A.Z and H2B.Z (Hoeijmakers et al., 2013; Petter et al., 2013). These findings indicate that the nucleosome landscape of *Plasmodium* correspond to the initial layer in the control of gene expression.

Overview of Epigenetic Regulation and Histone Modifiers

Although, nucleosome structure and positioning along the chromatin provide one of the first layer to control gene expression, a variety of post-translational modifications (PTMs) on the protruding N-terminal tails of histones supplement this regulatory mechanism. Histone modifications are relatively well conserved in eukaryotes including *Plasmodium*. Histone acetylation, phosphorylation and methylation are the major PTMs and can affect the interaction of the nucleosomes with DNA and the overall chromatin structure. Using such mechanism, the accessibility of promoter regions by the transcriptional machinery can be modulated, promoting or inhibiting gene expression. Several PTMs were initially detected in *P. falciparum* by mass spectrometry (Miao et al., 2006; Salcedo-Amaya et al., 2009; Trelle et al., 2009; Coetzee et al., 2017). A more recent comprehensive study identified over 230 PTMs in asexual blood stages of which 160 had never been detected in *Plasmodium* and 88 had never been identified in any other species (Saraf et al., 2016). Quantitative and dynamic profiles of histone PTMs as well as combinatorial associations indicate an unusual chromatin organization with parasite-specific histone modifications that may be directly related to transcriptional activity, DNA replication, and cell cycle progression. Overall, this data suggest that the malaria parasite has a unique histone modification signature that correlates with parasite virulence. Additional ChIP-seq experiments to identify the genome wide distribution of histone PTMs demonstrate that the histones H3K9me3 and H3K36me3 are associated with heterochromatin and silenced genes (Salcedo-Amaya et al., 2009; Trelle et al., 2009; Bunnik et al., 2018; Fraschka et al., 2018), and are mutually excluded from H3K9ac and H3K4me3, which are enriched in euchromatin and active promoters (Cui et al., 2007; Bártfai et al., 2010; Ruiz et al., 2018). These histone marks are crucial in maintaining the transcriptional state and their modification can lead to a change in the steady state. Hence, the histone PTMs can be reversed and are strictly placed under the control of a multitude of histone modifiers, the writers and erasers. The writers are enzymes capable of laying down a histone mark such as histone acetyltransferases (HATs), including GNAT and MYST proteins, and histone lysine methyltransferases (HKMTs) such as SET proteins (Cui et al., 2007; Cui et al., 2008). Their antagonists, the erasers, remove the marks placed by the writers and are composed, among others, of histone deacetylases and sirtuins (HDACs), and histone lysine demethylases (HKDMs) (Cui et al., 2008; Chaal et al., 2010). These proteins are themselves subject to PTMs, which could modulate their activity during the life cycle (Solyakov et al., 2011; Lasonder et al., 2012). Inhibition of HDACs or HATs lead to deregulation of gene expression in *P. falciparum* (Cui et al., 2007; Chaal et al., 2010), making these histone modifiers intensively studied as potential therapeutic targets in *Plasmodium* (Coetzee et al., 2020). As generally conserved across the eukaryotic phylum, additional work will be required to identify inhibitors that will target parasite enzymes with limited toxicity against their human homologs.

In addition to the histone modifiers, epigenetic marks are recognized and interpreted by proteins designated as readers, stabilizing the recruitment of specific protein complexes involved in various biological functions. Recently, several readers have been identified in *P. falciparum* using histone peptide pull-down coupled to quantitative mass-spectrometry (Hoeijmakers et al., 2019). Among them, they detected the Heterochromatin Protein 1 (HP1), well known to be associated with H3K9me3 and mediating the

formation of heterochromatin (Pérez-Toledo et al., 2009). They also noticed an enrichment of bromodomain proteins PfBDP1 and PfBDP2 on acetylated H2B.Z while PfGCN5-PfADA2, members of the transcriptional coactivator complex (Cheon et al., 2020), are associated with H3K4me2/me3. Deciphering the function of writers, erasers, and readers and how they work, and form dynamic and specific complexes could be an important step in improving our understanding of gene regulation in *Plasmodium*.

Recently, the importance of noncoding RNAs (ncRNAs) as epigenetic regulators has emerged in most eukaryotes including protozoan parasites. By definition, ncRNAs are split into two groups, small (sncRNA) and long ncRNA (lncRNA) based on their respective length. In higher eukaryotes, lncRNAs influence various essential cellular processes such as chromosome maintenance, epigenetic remodeling, transcription, translation and control of protein activity (Marchese et al., 2017). In *P. falciparum*, thousands of ncRNAs have been identified but few of them have been characterized (Raabe et al., 2009; Broadbent et al., 2015). Among them, a family of telomere-associated lncRNAs (TAREs) participate in telomere maintenance and regulation of virulence gene (Broadbent et al., 2011; Sierra-Miranda et al., 2012). We describe below in more details, the role of lncRNA in the regulation of *var* genes and gametocyte commitment. Collectively, this data validates the fundamental role of epigenetic in regulation of gene expression in *Plasmodium*, through the involvement of histone marks, proteins modifiers and ncRNAs.

Overview of Chromatin Organization

After decades of work, new evidences indicate that the chromatin architecture also plays a crucial role in eukaryotic gene expression including *Plasmodium*. Initially, fluorescence microscopy and fluorescence *in situ* hybridization (FISH) techniques have been used to investigate the chromatin organization in the parasite nucleus. Chromosome conformation capture (3C) methodology, was developed several years ago to reveal the spatial chromatin structure and nearby genomic loci (Dekker et al., 2002). The limits of this technique led to the development of derivative methods such as chromosome conformation capture-on-chip (4C) (Simonis et al., 2006; Simonis et al., 2007) and chromosome conformation capture carbon copy (5C) (Dostie et al., 2006; Ferraiuolo et al., 2012). However, to date, Hi-C is still the most used methodology to study the 3D organization of the chromosomes since it allows the identification of chromatin interactions in an "all-vs-all" manner (Lieberman-Aiden et al., 2009; van Berkum et al., 2010). Several Hi-C experiments were generated in different *Plasmodium* stages and species (Lemieux et al., 2013; Ay et al., 2014; Bunnik et al., 2018; Bunnik et al., 2019) and demonstrated distinct chromatin features at each stage of the parasite life cycle progression (see following sections for detailed characteristics). Globally, the different methods used in *P. falciparum* identified a repressive cluster at the nucleus periphery that includes telomeres and heterochromatin, while centromeres are in general found at the opposite side of the nucleus with an exception of the sporozoite stage (Freitas et al., 2005; Lopez-Rubio et al., 2009; Hoeijmakers et al., 2012; Ay et al., 2014; Bunnik et al., 2018). Although, no classical topologically associating domains (TADs) (Dixon et al., 2012; Sexton et al., 2012) were identified in *Plasmodium*, the parasite chromatin exhibits specific structure linked to virulence genes. In addition to this particular feature, a correlation between the chromatin 3D structure and gene expression profile was observed across the entire genome from the telomere to the centromere throughout the *P. falciparum* life cycle. A similar gene expression gradient was also observed in *Plasmodium knowlesi* (Bunnik et al., 2019). Conversely, no correlation between chromatin structure and gene expression profile was observed in *Babesia microti* and an inverse correlation from the centromere to the telomere was noticed in *Toxoplasma gondii*. Collectively, the spatial organization of the *Plasmodium* genomes is controlled at a higher level when compared to other Apicomplexa investigated and its dynamic throughout the parasite life cycle provides most likely a particular complementary way to control gene regulation.

Overview of the Transcription Machinery

The three different layers of regulatory mechanisms, described above, strongly participate in the regulation of the transcription in malaria parasites, promoting or inhibiting the binding of the transcriptional machinery on the promoter and TSS regions. Indeed, in eukaryotes, the synthesis of mRNA required the recruitment of the PIC on the TSS. This complex is generally composed of the TATA-binding proteins (TBPs), TBP-associated factors (TAFs), the general transcription factors TFII, and the RNA polymerase II (RNA pol II) (Luse, 2014). In *Plasmodium*, despite the presence of most of the components of this complex, some subunits have not yet been identified suggesting a partial adaptation of the parasite to ensure a proper transcription (Callebaut et al., 2005). Moreover, some of the components of the transcriptional machinery such as PfTBP and PfTFIIE have been detected upstream of inactive genes lacking acetylated histones (Gopalakrishnan et al., 2009). Similarly, RNA pol II has been detected in early and late asexual stages on active and inactive promoters (Rai et al., 2014). It has been suggested that pausing mechanism of the RNA pol II at these stages could be a major way to control transcriptional activation and elongation (Lu et al., 2017). These initial results indicate that PIC occupancy is not clearly associated with the transcriptional status and multiple mechanisms may be involved to activate the *Plasmodium* transcriptional machinery.

In addition to the PIC, specific transcription factors are key proteins promoting or inhibiting gene's transcription. Through their DNA-binding domains, they are able to bind specific DNA motifs, identified as enhancers or promoter regions to recruit chromatin modifying and remodeling complexes as well as the PIC when needed. The ratio of the total number of genes to the predicted number of specific transcription factors in *P. falciparum*, is one of highest in eukaryotes indicating a relative paucity of transcription factors (Templeton et al., 2004). The discovery of the 27 members of the ApiAP2 transcription factor family or AP2s, specific to apicomplexan parasites and analogous to Apetala-2 in plant, was instrumental to our understanding of gene regulation in *Plasmodium* (Balaji et al., 2005; Campbell et al., 2010). Due to their specificity, they have been the subject of particular attention to comprehend how so few transcription factors could tightly control the expression of 5,500 coding genes during the entire life cycle. One

hypothesis is that these AP2s operate as master regulators and transcribe hundreds of genes at specific stages. This has been specifically demonstrated for the transcription factors involved in stage transition such as AP2-G and AP2-G2 in gametocytes (Sinha et al., 2014; Yuda et al., 2015), AP2-O in ookinetes (Yuda et al., 2009), AP2-SP in sporozoites (Yuda et al., 2010) and AP2-L in liver stages (Iwanaga et al., 2012). Recently, knock-out screenings in rodent *Plasmodium* have showed that these AP2s are essential to these particular stages during parasite development (Modrzynska et al., 2017; Zhang et al., 2017). However, a recent machine learning model demonstrated that at least in asexual stages, the identified AP2 DNA-binding motifs, may play a limited role in erythrocytic transcriptional regulation suggesting that while AP2 may interact with some promoters to either act as a repressor or activator such interaction can only happen in association with several chromatin-associated proteins and a favorable epigenetic environment to ensure transcription (Read et al., 2019). Taken together, *Plasmodium* parasites have developed some particular features to compensate for their lack of transcription factors.

GENE REGULATION DURING INTRAERYTHROCYTIC DEVELOPMENTAL CYCLE

Transcriptional Status Across the Intraerythrocytic Developmental Cycle

The IDC of *P. falciparum* has been well studied over the years since it is responsible for most symptoms observed in humans. Typically, this cycle is defined by rings, trophozoites, and schizonts but correlating these phenotypic stages to classical eukaryotic cell cycle phases (G1, S, G2, and M) has been quite challenging (Arnot et al., 2011).

In ring stage, the nucleus is globally compacted (Weiner et al., 2011; Ay et al., 2014) and chromosomes are enriched in nucleosomes (Ponts et al., 2010; Bunnik et al., 2014) (**Figure 1**). This condensed environment may inhibits transcription and result in low transcriptional activity observed at this stage (Bozdech et al., 2003; Le Roch et al., 2003). To complement nuclear compaction, the number of nuclear pores detected is low and their size relatively small, which correlate with low transcriptional activity and RNA import/export (Weiner et al., 2011). Moreover, pausing of RNA pol II has been detected at this specific stage suggesting that while the polymerase is positioned on promoter regions in anticipation of the trophozoite stage, it is not activated for transcriptional initiation and elongation processes (Lu et al., 2017).

The trophozoite is considered as the principal stage of development with significant morphological changes and remodeling of the infected red blood cell. During this step, the volume of the genome increases considerably indicating a more open chromatin structure (Ay et al., 2014), in correlation with an active transcription status (Bozdech et al., 2003; Le Roch et al., 2003) (**Figure 1**). This is also accompanied by a notable increase in the number of nuclear pores, which exhibit a specific distribution and are enriched in transcriptionally active compartments to facilitate RNA export (Weiner et al., 2011; Dahan-Pasternak et al., 2013). To ensure an intense transcriptional activity (Lu et al., 2017), this stage undergoes drastic chromatin rearrangement such as decrease of nucleosome occupancy in promoter regions (Ponts et al., 2010; Bunnik et al., 2014; Kensche et al., 2015) and modifications of histone marks with presence of H3K9ac and H3K4me3 in promoter of active genes (Cui et al., 2007; Bártfai et al., 2010; Ruiz et al., 2018) (**Figure 1**). Conversely, genes known to be critical in sexual and mosquito stages exhibit a high nucleosome occupancy and repressive histone PTMs.

During the schizogony, nucleosomes are repacked promoting the compaction of the chromatin, required for the formation of daughter cells and the next invasion (Ponts et al., 2010; Ay et al., 2014; Bunnik et al., 2014). Global transcription is also highly reduced and a decrease in the size and number of nuclear pores is observed (Weiner et al., 2011) (**Figure 1**). Despite intensive nuclear compaction, genes involved in invasion, such as erythrocyte binding antigens, merozoite surface proteins, and rhoptry associated proteins, are highly transcribed at this stage (Le Roch et al., 2003; Lu et al., 2017). This transcription is controlled by PfBDP1, in association with the transcription factor AP2-I, and the acetylated histones enriched in their promoter regions (Josling et al., 2015; Santos et al., 2017). Analysis of the schizont transcriptome from field and laboratory strains showed a differential expression of these invasion genes, suggesting the adaptation of parasites to successfully invade red blood cells (Tarr et al., 2018). Machine learning model suggested that high levels of H3K9ac and H4K20me3 marks correlate strongly with high expression in schizonts, while H3K4me3 marks correlate to active genes in ring and trophozoite stages (Read et al., 2019). After a schizont burst, the merozoites are released into the bloodstream and invade new red blood cells, which perpetuates the erythrocytic cycle. Although few studies are available on gene regulation at this stage, we can assume that the genome is highly compacted, as the function and size of these parasites seem to indicate. The lowest level of transcription in the entire life cycle was also detected at the population and single-cell levels and also corresponds to invasion genes (Bozdech et al., 2003; Le Roch et al., 2003; Howick et al., 2019). Altogether, the data validate a strong and tight gene regulation across the IDC consistent with the role of these four asexual stages.

Transcriptome analyzes of resistant strains showed that parasites can adjust their gene expression profile and life cycle in response to drugs by slowing or arresting their cell cycle to protect their survival (Adjalley et al., 2015; Shaw et al., 2015; Rocamora et al., 2018). Recent studies using RNA-seq on field isolates causing more severe malaria showed a distinct transcriptome with upregulation of genes involved in multiples pathways such as pyrimidine metabolism, tricarboxylic acid cycle and GTPase activity while *var* genes were down-regulated (Tonkin-Hill et al., 2018). Collectively, these studies indicate that environment changes can have a significant effect on *P. falciparum* gene expression across its life cycle.

Regulation of Virulence Genes

One of the most interesting features of the parasite is its ability to evade the host's immune system. For this purpose, *P. falciparum* possesses several clonally variant gene families such as *var*, *rifin*, *stevor*, and *Pfmc-2TM*, and most of these genes are located in the

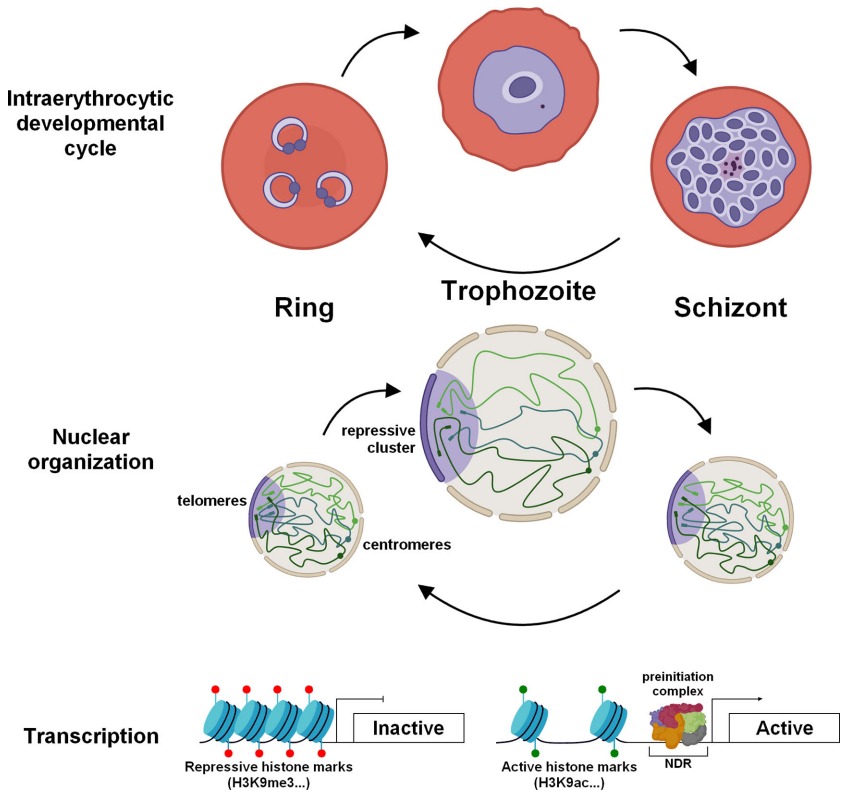

FIGURE 1 | Dynamic chromatin architecture and epigenetic regulation in asexual blood stages of *P. falciparum*. During the IDC, drastic re-organization of the chromatin is observed to promote the transcriptional burst in trophozoite stage. The chromatin is lightly packed and enriched in active histone marks. The size of the nucleus increases as well as the number of nuclear pores. Throughout the IDC, centromeres (spheres) are clustered and located at the periphery of the nucleus while telomere regions (rectangles) form a repressive cluster on the opposite side.

subtelomeric regions. The most studied family is that of *var*, which contains ~ 60 genes encoding PfEMP1 antigen, mediating cytoadherence and infected red blood cells sequestration. Only one copy of PfEMP1 is expressed at a time and exported at the surface of the infected red blood cell, limiting the exposure of PfEMP1 variants to the host immune system. Thus, this mutually exclusive expression requires a tight regulation to ensure that all *var* genes are repressed, while only one *var* gene is actively transcribed and translated. In asexual blood stages, inactive *var* genes are enriched in H2A as well as the repressive marks H3K9me3 and HP1 (Freitas et al., 2005; Chookajorn et al., 2007; Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009; Trelle et al., 2009; Ukaegbu et al., 2014). These genes are present at the periphery of the nucleus, and regrouped at the repressive cluster(s) (Ralph et al., 2005; Lopez-Rubio et al., 2009; Ay et al., 2014; Bunnik et al., 2019) (**Figure 2**). Conversely, the active *var* gene is also present at a perinuclear location, permissive for transcription, and showed a high distribution of H3K9ac and H3K4me3 marks, and H2A.Z/H2B.Z histone variants (Freitas et al., 2005; Lopez-Rubio et al., 2007; Petter et al., 2011; Petter et al., 2013). Moreover, histone PfH3.3 is associated with the active promoter and could play a role in memory for the next generation of parasites (Fraschka et al., 2016). While most of the *var* genes are localized in telomeric regions of all chromosomes, some can be found in the core of the chromosomes. For those, the formation of large chromatin loops facilitate their clustering and interaction with all subtelomeric *var* loci (Lemieux et al., 2013; Ay et al., 2014).

Several histone modifiers have been described to be directly involved in modifications of these histone marks localized on the *var* gene loci (**Figure 2**). The histone deacetylases PfSir2A, PfSir2B, and HDA2 and the methyltransferases, PfSET10 and PfSET2, have been identified to be essential in the poise of *var* expression (Duraisingh et al., 2005; Freitas et al., 2005; Lopez-Rubio et al., 2009; Volz et al., 2012; Jiang et al., 2013; Coleman et al., 2014; Ukaegbu et al., 2014). An alternative regulatory mechanism was also identified with the involvement of sense and antisense lncRNAs transcribed from the intron and extending to exons 1 and 2 (Epp et al., 2009) (**Figure 2**). The antisense lncRNA is associated with its locus to activate the gene while its interference causes a decrease of active *var* gene expression, promoting a switching (Amit-Avraham et al., 2015; Jing et al., 2018). Furthermore, a family of GC-rich ncRNA is known to act in *trans*- and *cis*- to regulate the repressed *var* genes as well as the active locus (Wei et al., 2015; Guizetti et al., 2016).

The family of clonally variant gene *Pfmc-2TM* is also placed under the control of these GC-rich regulatory elements (Barcons-Simon et al., 2020) while the transcription factor AP2-exp has been recently implicated in the expression of *rifin*

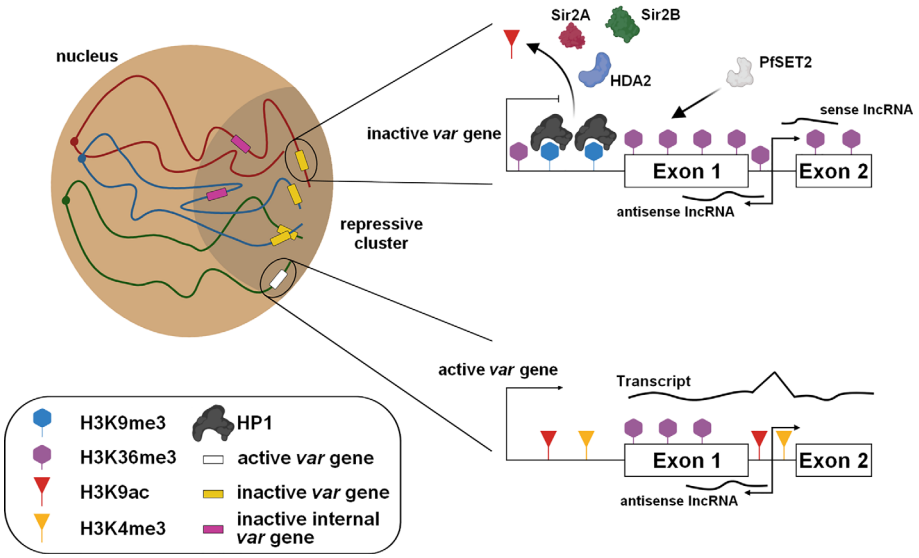

FIGURE 2 | Regulation of the mutually exclusive *var* gene expression. The subtelomeric and internal *var* genes are grouped in the repressive cluster and maintained in a silent state with the presence of repressive marks and the expression of specific lncRNAs. For the active *var* gene present at the nuclear periphery, epigenetic changes occur and require the involvement of protein modifiers.

and *stevor* (Martins et al., 2017). Altogether, the data showed that the mutual exclusive expression of virulence genes, required to escape the immune response, is tightly regulated by epigenetic factors and chromatin architecture.

Mutual exclusive expression is also described in invasion genes such as clag3.1 and clag3.2, for which histone marks and noncoding RNAs are involved (Cortés et al., 2007; Rovira-Graells et al., 2015) and this epigenetic memory seems to be erased during the conversion into transmission stages (Mira-Martínez et al., 2017).

COMMITMENT TO SEXUAL STAGES

During the IDC, a proportion of parasites differentiate into sexually mature male and female gametocytes. As these forms are essential for transmission of the parasite into mosquitoes, interest has grown over the years to better understand the molecular mechanisms driving the conversion and the development of gametocytes with the hope that researchers will identify new drugs or vaccine strategies to block disease transmission. A few years ago, AP2-G was identified and validated as a master transcription factor for sexual commitment in *Plasmodium* spp. (Kafsack et al., 2014; Sinha et al., 2014; Kent et al., 2018). In the asexual cycle, *ap2-g* is repressed and its promoter enriched in repressive histone marks (e.g H3K9me3) as well as HP1 (Brancucci et al., 2014; Coleman et al., 2014; Filarsky et al., 2018) (**Figure 3**). Various environmental factors such as food, lysophosphatidylcholine restriction, and high parasitemia have been described to be preponderant in the sexual development (Brancucci et al., 2017), but others factors, signaling pathways, and metabolites remain most likely to be discovered. Despite uncertainties, several studies have demonstrated the crucial role of Gametocyte development protein 1 (GDV1) during sexual differentiation. In asexual blood stages, the expression of GDV1 is repressed by its own antisense RNA, while in stress conditions, an unknown mechanism restrains the function of the inhibitory RNA leading to GDV1 expression (**Figure 3**). This protein triggers HP1 eviction on the *ap2-g* promoter, which destabilizes silencing leading to de-repression and activation of *ap2-g* expression (Broadbent et al., 2015; Filarsky et al., 2018). After a first peak, a rapid drop of AP2-G transcripts abundance is observed before a new wave of expression (Poran et al., 2017). A positive transcriptional feedback loop of AP2-G participates in this stabilization and ensure a bistability switch (Poran et al., 2017), a model of all-or-none expression described in several cell differentiations (Xiong and Ferrell, 2003; Wang et al., 2009; Bhattacharya et al., 2010). The ApiAP2 transcription factor, PF3D7_1222400, adjacent to *ap2-g* locus, appears to participate in this switch as well as the helicases ISWI and SNF2L, whose the expression is correlated with the de-repression of *ap2-g*, and could contribute to the accessibility of the locus (Poran et al., 2017). If this stabilization occurs in early ring stage, the parasite can directly start its differentiation in gametocyte, designated as Same Cycle Conversion (SCC) route (Bancells et al., 2019). Otherwise, the Next Cycle Conversion (NCC) is observed and parasite pursues its development until schizogony and invasion before to initiate the sexual development. Regarding *ap2-g* locus, Hi-C result indicates that the gene is no longer associated with the repressive territory in early gametocytes (Bunnik et al., 2018). We can assume *ap2-g* leaves this region upstream the sexual commitment to facilitate its expression (**Figure 3**).

The binding of AP2-G to specific motifs of hundreds of active promoters designates it as the master regulator of gametogenesis (Campbell et al., 2010; Kafsack et al., 2014; Sinha et al., 2014; Poran et al., 2017; Kent et al., 2018; Josling et al., 2020). These

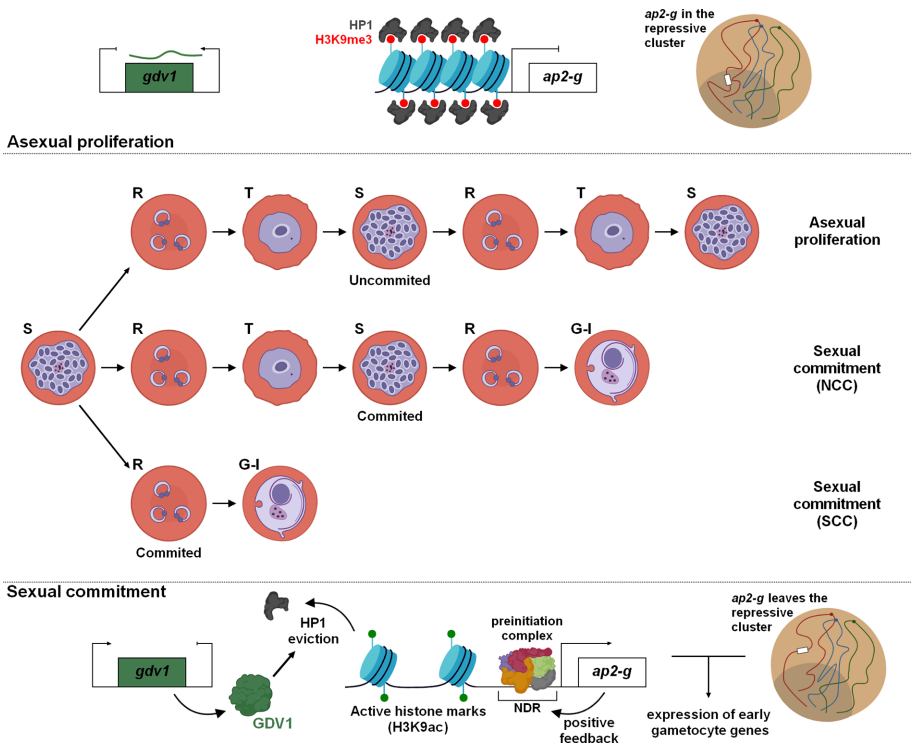

FIGURE 3 | Sexual commitment in *Plasmodium falciparum*. Cellular and molecular mechanisms maintain in a poised state the sexual and asexual proliferation. Epigenetic regulation and global changes of chromatin structure are fundamental for the expression of PfAP2-G and initiation of gametocytogenesis. Depending on the timing of PfAP2-G stabilization, two differentiation pathways have been identified: NCC, Next Cycle Conversion and SCC, Same Cycle Conversion.

target genes are mostly considered as early gametocyte genes or secreted proteins involved in erythrocyte remodeling (Kafsack et al., 2014; Poran et al., 2017). In committed schizonts, AP2-G was also detected upstream of some invasion genes such as *sera*, *eba175*, and *ron5*, occasionally in cooperation with AP2-I (Poran et al., 2017; Josling et al., 2020). Further investigations could provide valuable information to decipher this conversion mechanism and facilitate the identification of potential therapeutic targets to hamper the parasite transmission.

GENE REGULATION DURING GAMETOCYTOGENESIS

Once parasites are committed, immature forms develop into male and female gametocytes during the gametocytogenesis. The duration of this process differs between the different species of *Plasmodium*. The maturation of gametocytes takes 24–48h in the rodent parasites, while 8-12 days are necessary for *P. falciparum*, divided in 5 stages (I to V), morphologically distinguishable (Gautret and Motard, 1999).

Although, the telomeres and centromeres are still grouped in their respective cluster, a reorganization of the chromosomes is detected by Hi-C in gametocytes (Bunnik et al., 2018). The heterochromatin cluster is expanded and contains virulence and invasion genes as well as the genes not required for the gametocytogenesis and an enrichment of the repressive marks H4K20me3, H3K27me3 and H3K36me2 is observed in stage I-III gametocytes (Coetzee et al., 2017). Interestingly, some histone modifications are described to be specific to gametocytes such as H3K36me2/me3, H3K27me2/me3, and H3K79me3 while H3K9me2, H3K18me1, and H3K4me2/me3 are specific of asexual stages (Coetzee et al., 2017).

In late gametocytes, interactions between *ap2-g* locus and virulence genes were detected by Hi-C, suggesting the gene has regained its place in the repressive cluster, at least partially (Bunnik et al., 2018). Another transcription factor, AP2-G2, has a specific function in gametocytes by repressing genes required for asexual proliferation since mutant parasites in *P. berghei* are able to differentiate in gametocytes but cannot fully mature (Yuda et al., 2015).

Hi-C experiment identified additional large chromosome rearrangements on chromosome 14, with the formation of two super domains (Bunnik et al., 2018), similar to what is observed during the inactivation of one of the X chromosome in human and mouse (Rao et al., 2014; Deng et al., 2015). Near the boundary of these two super domains, *ap2-o3* and *ptpa* have been identified and both are involved in sexual development suggesting this large rearrangement may promote their active transcription. The transcription factor AP2-O3 is described as specific of female gametocytes and is required for normal ookinete formation in rodent parasites (Modrzynska et al., 2017; Zhang et al., 2017) while

PTPA regulates the activity of the phosphatase PP2A and participates in the regulation of the *P. falciparum* cell cycle (Vandomme et al., 2014).

During the maturation process, parasites differentiate sexually in phenotypically distinguishable male and female gametocytes. It is still unclear whether the sexual determination occurs with the early sexual commitment or if it is happening downstream (Tadesse et al., 2019). Interestingly, the sex ratio seems to fluctuate according to environmental conditions and *Plasmodium* species, but overall, the balance is in favor of females (Tadesse et al., 2019). Thereafter, male gametocytes will be activated and form eight microgametes in the mosquito midgut, counterbalancing the previous imbalance (Kuehn and Pradel, 2010).

Despite differences in their respective transcriptomes and proteomes (Khan et al., 2005; Walzer et al., 2018), few epigenetic changes were observed between female and male gametocytes in *P. berghei* (Witmer et al., 2020). The histone mark H3K9ac is associated with active transcription in asexual blood stages and male gametocytes, while female gametocytes do not show a higher occupancy and a low abundancy in histone variants H2A.Z and H2B.Z is also detected (Witmer et al., 2020). This result could be explained by the storage of the mRNAs in a messenger ribonucleoprotein complex and the involvement of mRNA binding proteins to regulate transcription at the post-transcriptional level at this particular stage. Additionally, a transcription factor, identified as AP2-G3 or AP2-FG, has been described as female specific and demonstrated to regulate over 700 genes (Yuda et al., 2019). Collectively, different regulatory changes were observed during the gametocytogenesis and participate in both sexual development and differentiation between female and male gametocytes.

GENE REGULATION IN MOSQUITO AND LIVER STAGES

The immature gametocytes, stages I to IV of *P. falciparum*, are mainly sequestrated in bone marrow and cannot be detected in peripheral circulation in infected humans (Gardiner and Trenholme, 2015). Mature stage V gametocytes re-enter into the bloodstream and can be ingest by a mosquito during a blood meal. Once gametocytes reach the mosquito's midgut, environmental signals activate the gametogenesis (Kuehn and Pradel, 2010) and formation of zygote, ookinete, and then oocyst. Due to the difficulty to isolate these stages, few studies are available. Epigenetic analyzes in oocysts of *P. falciparum* and *P. berghei* showed the heterochromatin distribution is globally conserved and H3K9ac and H3K27ac are highly correlated with active transcription (Gómez-Díaz et al., 2017; Witmer et al., 2020). An interesting example of genetic regulation concerns the protein Cap380, a marker of oocyst development, whose gene is not expressed during IDC and gametocytes (Itsara et al., 2018). In oocysts, an enrichment in H3K9ac was observed in the promoter region of *pbcap380*, in correlation with a higher abundance of *cap380* transcripts (Witmer et al., 2020).

Several ApiAP2 transcription factors have been demonstrated as essential for the parasite development such as AP2-O and AP2-O2 in *P. falciparum* and rodent parasites (Yuda et al., 2009; Kaneko et al., 2015; Modrzynska et al., 2017; Zhang et al., 2017). In *P. berghei*, AP2-O was detected by ChIP-seq in promoter regions of more than 500 genes, including *cap380*, confirming the role of this protein as master regulator (Kaneko et al., 2015).

In *P. falciparum* and *P. vivax* sporozoites, Hi-C experiments indicated that invasion and virulence genes are strongly associated with the repressive cluster (Bunnik et al., 2018). These genes showed an enrichment of H3K9me3 as well as telomeric and subtelomeric regions while H3K9ac and H3K4me3 are present along the chromosomes, especially outside the coding regions for *P. vivax* (Gómez-Díaz et al., 2017; Zanghì et al., 2018; Vivax Sporozoite Consortium, 2019). Interestingly, one *var* gene (PF3D7_1255200) seems to play a specific role at this stage. Compared to other virulence genes, *pf3d7_1255200* showed low level of H3K9me3 and an AP2-exp binding motif, known to control expression of a subset of clonally variant gene families (Gómez-Díaz et al., 2017; Martins et al., 2017). In addition, the expression of the lncRNA antisense correlates with the expression of this gene confirming the involvement of lncRNA in the *var* regulation (Gómez-Díaz et al., 2017).

Hi-C experiment in P. falciparum sporozoites demonstrated a chromatin rearrangement with loops and long-range interactions for csp, trap, and spect1 (Bunnik et al., 2018). Likewise, PbAP2-SP was detected in promoter regions of sporozoite-specific genes such as spect1, trap, and sera, and its disruption prevented sporozoite formation confirming the essentiality of this factor (Yuda et al., 2010). Additional transcription factors were identified to play a crucial role in sporozoite development such as AP2-SP2 and AP2-SP3 (Modrzynska et al., 2017; Zhang et al., 2017). Rearrangements are also observed with the loss of the centromeres cluster and the A-type rDNAs showed a large increase in contacts with the repressed virulence genes, transcriptionally inactive in mosquito stages (Bunnik et al., 2018). Altogether, to ensure the transcription of genes required for cell traversal and hepatocyte invasion, chromatin is significantly reorganized to facilitate the involvement of specific transcription factors.

Due to the difficulty of working at the liver stage, regulation of gene expression has been very little studied. In *P. cynomolgi*, responsible of malaria in various macaque monkeys, the use of a histone methyltransferase inhibitor in liver culture was able to promote hypnozoite activation, suggesting that epigenetic marks could be involved in parasite reactivation, although its direct effect has not yet been demonstrated (Dembélé et al., 2014). One AP2 transcription factor, PbAP2-L, was demonstrated as not essential for liver invasion but crucial for the expression of several genes and maturation of the parasite inside hepatocytes (Iwanaga et al., 2012). Further experiments are needed to decipher the exact mechanisms regulating gene expression in liver stage and highlight possible specific features.

DISCUSSION

In this review, we covered the recent progress on regulation of gene expression in *P. falciparum*. This regulation encompasses local and global chromatin structure changes across the

whole life cycle to ensure the proper transcription and stage conversion. Local modifications play a primordial role with the involvement of nucleosome occupancy, epigenetic modifications, protein modifiers, and lncRNAs promoting gene activation or silencing. Heterochromatin is enriched in HP1, H3K9me3, and H3K36me3 marks while euchromatin exhibits H3K9ac and H3K4me3 marks and a lower nucleosome occupancy facilitating the binding of the general transcriptional machinery in the NDR. During the IDC, drastic chromatin remodeling has been observed in relation to the transcriptional status of each stage. Unlike compact chromatin at the ring and schizont stages, an open chromatin was observed in trophozoites by Hi-C and FISH experiments promoting the transcriptional burst. The exploration of the regulation of virulence genes and in particular the mutually exclusive expression of *var* genes highlighted how genes can be controlled by a combination of different and complementary mechanisms. Although the overall architecture is similar in gametocytes, few exceptions were detected as the presence of specific histone marks and the remodeling of the *ap2-g* locus. This master transcription factor is essential for the sexual commitment and epigenetic modifications are required on its locus and that of *gvd1*, as well as a re-organization from the repressive cluster. In mosquito and liver stages, few studies have been conducted but particular features have been observed such as the loss of the centromere clustering in sporozoites. However, our understanding of gene regulation in *Plasmodium* is far from complete and further experiments are still require to decrypt all molecular components and specific features involved in controlling parasite development, in particular in stages that are under investigated such as in mosquito or liver.

AUTHOR CONTRIBUTIONS

TH and KLR conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Dynamic Roles of the Inner Membrane Complex in the Multiple Stages of the Malaria Parasite

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Apicomplexan parasites, such as human malaria parasites, have complex lifecycles encompassing multiple and diverse environmental niches. Invading, replicating, and escaping from different cell types, along with exploiting each intracellular niche, necessitate large and dynamic changes in parasite morphology and cellular architecture. The inner membrane complex (IMC) is a unique structural element that is intricately involved with these distinct morphological changes. The IMC is a double membrane organelle that forms *de novo* and is located beneath the plasma membrane of these single-celled organisms. In *Plasmodium* spp. parasites it has three major purposes: it confers stability and shape to the cell, functions as an important scaffolding compartment during the formation of daughter cells, and plays a major role in motility and invasion. Recent years have revealed greater insights into the architecture, protein composition and function of the IMC. Here, we discuss the multiple roles of the IMC in each parasite lifecycle stage as well as insights into its sub-compartmentalization, biogenesis, disassembly and regulation during stage conversion of *P. falciparum*.

Keywords: malaria, *Plasmodium*, Apicomplexa, Alveolata, inner membrane complex, membrane dynamics

INTRODUCTION

The Apicomplexa represent a phylum of eukaryotic, single celled organisms that include human (i.e., *Plasmodium* spp., *Toxoplasma gondii*, and *Cryptosporidium* spp.) and livestock (i.e., *T. gondii*, *Eimeria* spp., and *Babesia* spp.) parasites with a severe impact on global health and socio-economic development. Human malaria parasites, *Plasmodium* spp., are the most medically important member of this distinct phylogenetic group and cause more than 400,000 deaths per year (WHO, 2018). Antimalarial resistant *P. falciparum*, the most lethal human malaria parasite species, are spreading (Dondorp et al., 2009; Imwong et al., 2017; Woodrow and White, 2017) and no efficacious vaccine has been developed to date. The devasting impact on endemic communities due to malaria has the potential to worsen with climate change and disruption of malaria control measures from outbreaks of other infectious diseases such as SARS-CoV-2 and Ebola (Rogerson et al., 2020; Sherrard-Smith et al., 2020).

Human malaria infection begins after a bite of a female *Anopheles* spp. mosquito that injects the parasite into the skin (**Figure 1**). These parasites, called sporozoites, actively enter blood vessels and reach the liver where they invade hepatocytes. Within hepatocytes, the elongated sporozoites transform into spherical hepatic stages that replicate *via* multiple fission events and produce thousands of merozoites contained in host-cell derived vesicles known as merosomes (Stanway et al., 2011). The merozoites are released from the liver before they rapidly invade and multiply within red blood cells (RBCs); with this occurring repeatedly every ~48 h for *P. falciparum*. Mass proliferation in blood stages is responsible for the clinical manifestations of malaria. Some RBC-infecting parasites differentiate into sexual forms called gametocytes, which can be taken up during the blood meal of another mosquito. Male and female gametocytes fuse within the mosquito midgut and form a motile ookinete that transmigrates the mosquito midgut epithelium and differentiates into an oocyst. Oocyst maturation results in the formation of up to 2,000 sporozoites (Stone et al., 2013), which in turn are transmitted to the host during another blood meal of the mosquito.

In this review we walk through the malaria parasite's lifecycle and focus on the role of the inner membrane complex (IMC) as the parasite transitions between various host cells such as RBCs, hepatocytes and mosquito midgut cells. The double membrane IMC organelle underlies the plasma membrane (PM) and is present in four of the morphologically distinct stages of the parasite's lifecycle. These different parasite stages are not only characterized by their distinct physiology, but also by dramatic changes in size, shape and cellular architecture, facilitating growth and multiplication in diverse cellular environments. Here we primarily focus on the IMC of the human pathogen *P. falciparum*, with additional data from other *Plasmodium* spp. and *T. gondii* included to expand and sharpen our functional understanding of this unique membranous system.

THE INNER MEMBRANE COMPLEX: A CHARACTERISTIC CELLULAR STRUCTURE SHARED ACROSS THE ALVEOLATA

Plasmodium spp., along with *T. gondii*, ciliates, and dinoflagellates are members of the Alveolata, a group of diverse unicellular eukaryotes. Evidence for a relationship between the diverse organisms that make up this phylogenetic supergroup only became clear from rRNA-sequence derived phylogenetic analysis (Wolters, 1991). Identification of a double membraned organelle below the PM (together called a pellicle) as a synapomorphic morphological feature, originating in ancestral Alveolata and shared across the supergroup, supports the evolutionary relationship between these unicellular eukaryotes (Cavalier-Smith, 1993).

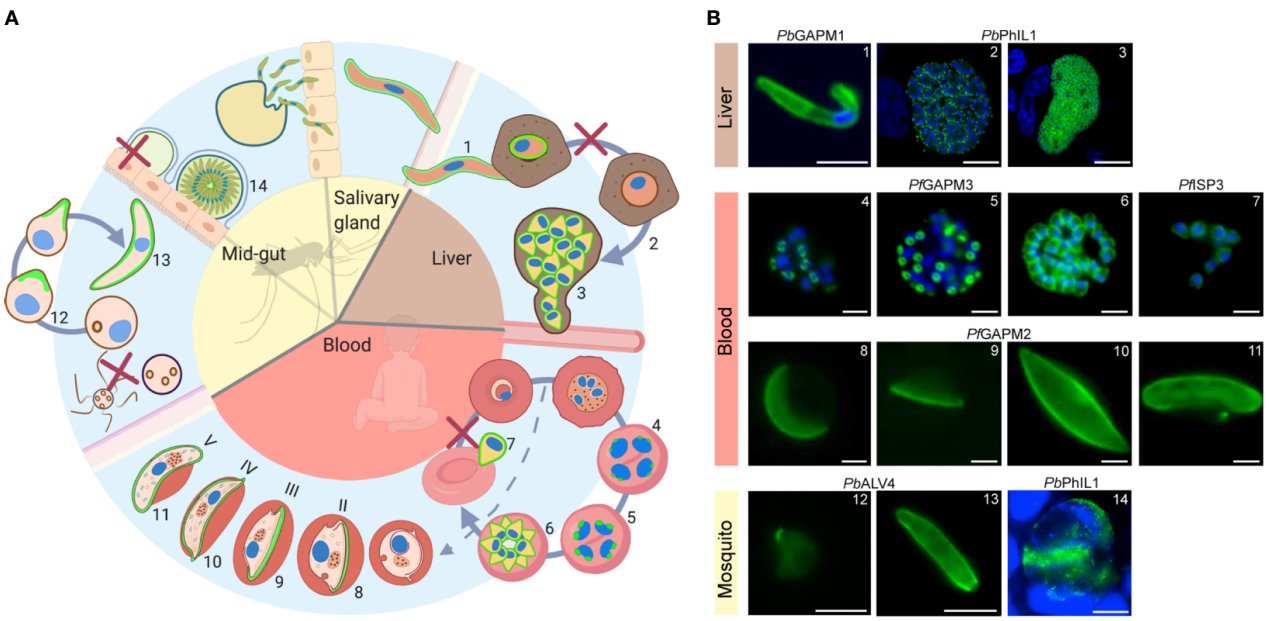

FIGURE 1 | IMC dynamics through the malaria parasite life cycle. **(A)** Schematic representation of the lifecycle through the mosquito and human host. The IMC in different stages is highlighted in green. IMC disassembly is marked with a red cross. **(B)** Fluorescent microscopy images showing the IMC (green) at different stages in the parasite lifecycle. Specific IMC-GFP fusion proteins are indicated above the images. Blue; DAPI stain. Numbers in B refer to those in **(A)** 1 = Sporozoite (Kono et al., 2012), 2 = IMC biogenesis during liver cell schizogony (Saini et al., 2017), 3 = mature liver schizont (Saini et al., 2017), 4–6 = IMC biogenesis during schizogony in erythrocytes (Kono et al., 2012), 7 = free merozoites (Hu et al., 2010), 8–11 = developing IMC during gametocytogenesis (Kono et al., 2012), 12–13 = IMC biogenesis during ookinetogenesis (Kono et al., 2012), 14 = oocyst (Saini et al., 2017). Scale bars, 2 µm (micrographs 4–11) and 5 µm (micrographs 1, 12, 13, 14) 10 µm (micrographs 2, 3).

The double membrane organelle underlying the PM, consisting of a series of flattened vesicles and interacting proteins, termed alveoli in ciliates, amphiesma in dinoflagellates and the IMC in the Apicomplexa, fulfills various functions depending on the organism's diverse lifestyle and habitat. In aquatic ciliates and dinoflagellates, the alveoli play a structural role and can serve as a calcium storage organelle (Länge et al., 1995; Kissmehl et al., 1998; Ladenburger et al., 2009). In the endoparasites belonging to the Apicomplexa, the IMC has evolved additional functions that facilitate the unique strategies the parasites use to survive. As in the ciliates and dinoflagellates, the IMC of apicomplexan parasites provides structural support. However, the IMC also acts as a scaffold during the formation of daughter cells (Kono et al., 2012) and anchors the actin-myosin motor used for the gliding motility that powers host-cell invasion of these parasites, known as the glideosome (Keeley and Soldati, 2004). Like the secretory organelles (Rhoptries, Micronemes, and dense granules)—that form the core of the apical complex and have roles in motility/host cell invasion—the IMC is formed *de novo* during cytogenesis, starting at the apical pole of the parasite, and is most likely derived from Golgi vesicles (Bannister et al., 2000).

The large diversity in the functions and physiology of the pellicle are not limited to just the groups across the Alveolata, as the biology of the IMC also differs markedly between different apicomplexan parasites. Two examples of this can be found when comparing the IMC of the well-studied parasites *P. falciparum* and *T. gondii*. In the asexual cycle of *P. falciparum* malaria parasites, IMC biogenesis occurs *de novo* during schizogony (Kono et al., 2012). In *T. gondii*, the IMC is recycled from the mother cell into the forming daughter cells along with *de novo* biogenesis of new IMC double membrane during endodyogeny (Ouologuem and Roos, 2014). In *T. gondii*, the IMC and its associated glideosome are important in egress (Frénal et al., 2010) while this does not appear to be the case in *Plasmodium* spp. (Perrin et al., 2018).

The diversity in the IMC goes further with differences seen between parasites in different *Plasmodium* species (Ngotho et al., 2019) and beyond this to large morphological and functional differences in the IMC between each unique *P. falciparum* parasite stage as we discuss in this review.

DEFINING THE INNER MEMBRANE COMPLEX: PROTEIN COMPOSITION AND COMPLEX FORMATION

To date ~45 IMC proteins have been identified in *Plasmodium* spp. (**Table 1**). Phylogenetic profiling of known IMC proteins has shown that the evolution of the IMC involved the repurposing of several ancestral eukaryotic proteins, such as

TABLE 1 | *Plasmodium* spp. IMC proteins and other apicomplexan IMC proteins with *P. falciparum* homologs.

Short ID	Gene ID	TM	Functional domains	References
ALV1/IMC1a	PF3D7_0304000	No	Alveolin repeat	Khater et al., 2004[Pb]; Gould et al., 2008; Al-Khattaf et al., 2017[Pb]
ALV2/IMC1e	PF3D7_0304100	No	Alveolin repeat	Kono et al., 2012; Tremp et al., 2014[Pb]
ALV3/IMC1h	PF3D7_1221400	No	Alveolin repeat	Kono et al., 2012; Volkmann et al., 2012[Pb]; Coghlan et al., 2019[Pb]
ALV4/IMC1g	PF3D7_0525800	No	Alveolin repeat	Hu et al., 2010; Kono et al., 2012; Gao et al., 2018[Py]
ALV5/IMC1c	PF3D7_1003600	No	Alveolin repeat	Hu et al., 2010; Kono et al., 2012; Tremp et al., 2014; Tremp et al., 2017[Pb]
ALV6/IMC1f	PF3D7_1351700	No	Alveolin repeat	Al-Khattaf et al 2015[Pb]; Gao et al., 2018[Py]
ALV7/Pf77/IMC1j	PF3D7_0621400	No	Alveolin repeat	Kono et al., 2012; Gao et al., 2018[Py]
IMC1b	PF3D7_1141900	No	Alveolin repeat	Tremp et al., 2008[Pb]
IMC1d	PF3D7_0708600	No	Alveolin repeat	Al-Khattaf et al., 2015[Pb]
IMC1i	PF3D7_0823500	No	Alveolin repeat	Kaneko et al., 2015[Pb]; Gao et al., 2018[Py]
IMC1k	PF3D7_1341800	No	Alveolin repeat	Al-Khattaf et al., 2015[Pb]; Gao et al., 2018[Py]
IMC1l	PF3D7_1417000	No	Alveolin repeat	Al-Khattaf et al., 2015[Pb]; Gao et al., 2018[Py]; Gao et al., 2018; Kumar et al., 2019[Py]
IMC1m	PF3D7_1028900	Yes	Alveolin repeat	Al-Khattaf et al., 2015[Pb]; Gao et al., 2018[Py]
ELC	PF3D7_1017500	No	EF Hand domain	Green et al., 2017
GAP40	PF3D7_0515700	Yes (10)	None identified	Green et al., 2017
GAP45	PF3D7_1222700	No	None identified	Baum et al., 2006
GAP50	PF3D7_0918000	Yes (2)	Metallo-dependent phosphatase	Baum et al., 2006; Yeoman et al., 2011
GAPM1	PF3D7_1323700	Yes (6)	None identified	Bullen et al., 2009; Hu et al., 2010; Kono et al., 2012
GAPM2	PF3D7_0423500	Yes (6)	None identified	Bullen et al., 2009; Hu et al., 2010; Kono et al., 2012
GAPM3	PF3D7_1406800	Yes (6)	None identified	Bullen et al., 2009; Kono et al., 2012
MTIP	PF3D7_1246400	No	EF-hand domain	Bergman et al., 2003[Py]; Green et al., 2006; Dearnley et al., 2012
MyoA	PF3D7_1342600	No	Myosin	Baum et al., 2006; Siden-Kiamos et al., 2011; Robert-Paganin et al., 2019; Blake et al., 2020[Pb]

(Continued)

TABLE 1 | Continued

Short ID	Gene ID	TM	Functional domains	References
BCP1	PF3D7_1436200	No	None identified	Rudlaff et al., 2019
BTP1	PF3D7_0611600	Yes (4)	None identified	Kono et al., 2016
BTP2	PF3D7_0704100	Yes (7)	None identified	Rudlaff et al., 2019
CINCH	PF3D7_0407800	No	None identified	Rudlaff et al., 2019
HADO/HAD2	PF3D7_1205200	No	HAD phosphatase	Akinosoglou et al., 2015; Engelberg et al., 2016[Pb]
Morn1	PF3D7_1031200	No	MORN repeats	Ferguson et al., 2008; Kono et al., 2016; Rudlaff et al., 2019
ATRP	PF3D7_0410600	No	Armadillo repeats	Amlabu et al., 2020
Coronin	PF3D7_1251200	No	WD40 repeats	Bane et al., 2016
DHHC1	PF3D7_0303400	Yes (5)	DHHC Palmitoyltransferase, ankyrin repeats	Wetzel et al., 2015
DHHC2	PF3D7_0609800	Yes (4)	DHHC Palmitoyltransferase, palmitoylated	Wang et al., 2020[Py]; Santos et al., 2015
DHHC3	PF3D7_1121000	Yes (4)	DHHC Palmitoyltransferase	Frénal et al., 2013; Hopp et al., 2016[Pb]
DHHC9	PF3D7_1115900	Yes (4)	DHHC Palmitoyltransferase	Tay et al., 2016
G2	PF3D7_0929600	Yes (1)	EF hand domains, palmitoylated	Tremp et al., 2013[Pb]
GAMER	PF3D7_0805200	No	None identified	Kaneko et al., 2015[Pb]
ISP1	PF3D7_1011000	No	None identified	Wetzel et al., 2015; Poulin et al., 2013[Pb]; Gao et al., 2018[Py]
ISP3	PF3D7_1460600	No	None identified	Hu et al., 2010; Poulin et al., 2013[Pb]; Gao et al., 2018[Py]; Kono et al., 2012; Wetzel et al., 2015
NIPA	PF3D7_0522600	Yes (8)	Magnesium transporter	Hu et al., 2010; Kono et al., 2012
NN	PF3D7_1345600	No	None identified	Hu et al., 2010; Kono et al., 2012
NN	PF3D7_1445700	No	None identified	Birnbaum et al., 2017
PhIL1	PF3D7_0109000	No	None identified	Kaneko et al., 2015; Schneider et al., 2017; Campelo Morillo et al., 2020[Pb]; Saini et al., 2017[Pb]
PIP1	PF3D7_1355600	No	None identified	Schneider et al., 2017
PIP2	PF3D7_1431100	No	None identified	Kono et al., 2012; Schneider et al., 2017
PIP3	PF3D7_1430800	No	None Identified	Kono et al., 2012; Schneider et al., 2017
SIP	PF3D7_0510300	No	None identified	Lentini et al., 2015[Tg]
IMC20	PF3D7_1447500	No	None identified	Chen et al., 2015[Tg]
IMC27	PF3D7_0518900	No	None identified	Chen et al., 2015[Tg]
FBXO1	PF3D7_0619700	No	F-box domain	Baptista et al., 2019[Tg]
AC5	PF3D7_0722900	No	None identified	Chen et al., 2015[Tg]
ISC1	PF3D7_1341500	No	Palmitoylated	Chen et al., 2015[Tg]
ISC3	PF3D7_1431900	Yes (8)	Choline transporter	Chen et al., 2015[Tg]
MSC1b	PF3D7_1407700	No	None identified	Lorestani et al., 2012[Tg]
PP7/AC6	PF3D7_1423300	No	Serine/Theronine phosphatase, EF hand domains	Chen et al., 2015[Tg]

A list of the currently identified IMC proteins in Plasmodium spp., along with the P. falciparum homologs of proteins that have been localized to the IMC in other apicomplexans. Proteins are grouped by location (blue, Alveolins; red, Glideosome; green, Basal complex; yellow, IMC) and ordered within groups by their name to emphasize proteins with potentially related functions/localization. In gray are Plasmodium falciparum homologs of IMC proteins identified in Toxoplasma gondii (Tg) but which have yet to be confirmed as IMC localized in Plasmodium spp. Transmembrane domain and functional domain data is based on predictions from PlasmoDB (Aurrecoechea et al., 2009). All references where work was not carried out in P. falciparum are noted (Pb, Plasmodium berghei; Py, Plasmodium yoelii). NN, not named. Additional information including the results of random mutagenesis of IMC proteins covered in this table are available at the online database www.plasmoddb.org.

coronin, together with the innovation of alveolate-specific proteins that occurred early in the Alveolata lineage such as the alveolins (Kono et al., 2012). More recent evolution of *Plasmodium* spp. specific proteins such as PF3D7_1345600 has led to a set of taxon-specific IMC proteins that facilitate the adaptation of the IMC to taxon-specific niches and functions. This evolutionary diversity mirrors the spectrum of functionalities provided by the IMC, from key roles in basic structural integrity for all Alveolates to the machinery that allows endoparasites to invade into and divide within specific host cells.

The best studied group of IMC proteins are components of the motor complex that drives the locomotion of all motile parasite stages—also referred to as the "glideosome" (**Table 1**) (Webb et al., 1996; Opitz and Soldati, 2002; Baum et al., 2006). This actin-myosin motor complex powers the motility needed for transmigration, gliding, invasion and potentially egress (Frénal et al., 2010); the physiological trademark of the motile merozoite, sporozoite and ookinete stages of parasite development. Although multiple components of the glideosome such as the glideosome-associated proteins GAP40

GAP45, GAP50, GAP70, and GAPMs (Gaskins et al., 2004; Bullen et al., 2009; Frénal et al., 2010; Yeoman et al., 2011) together with Myosin A (MyoA) (Pinder et al., 1998; Baum et al., 2006; Ridzuan et al., 2012), the myosin A tail-interacting protein [MTIP in *Plasmodium* spp., or myosin light chain (MLC1) in *T. gondii*] (Herm-Götz et al., 2002; Bergman et al., 2003) and the "essential light chain 1" protein (ELC1) (Green et al., 2017) have been identified, a comprehensive and systemic identification of all components of the glideosome has not been performed. Recent years have uncovered some structural insights into this protein complex, but this improved understanding is still limited to individual components (Bosch et al., 2006; Bosch et al., 2007; Bosch et al., 2012; Boucher and Bosch, 2014) or to sub-complexes such as the trimeric structure composed of MyoA, ELC1 and MLC1 (Moussaoui et al., 2020; Pazicky et al., 2020) with no structural information on the entire glideosome complex.

Another interesting group of proteins are the so called alveolins, an Alveolata specific and conserved multi-protein family encompassing at least 13 proteins (**Table 1**) (Gould et al., 2008; Kono et al., 2012; Volkmann et al., 2012; El-Haddad et al., 2013; Tremp et al., 2014; Al-Khattaf et al., 2015). A representative alveolin was initially identified in *T. gondii* and named Inner Membrane Complex Protein 1 (TgIMC1) (Mann and Beckers, 2001). Alveolins are peripheral membrane proteins that constitute a subpellicular network of proteins located at the cytoplasmic face of the IMC. They are characterized by the presence of one or more highly conserved domains composed of tandem repeat sequences and were recognized as a unique protein family shared across all alveolates (Gould et al., 2008; Kono et al., 2012; El-Haddad et al., 2013; Al-Khattaf et al., 2015). Members of this family show stage specific expression patterns in *P. falciparum* and have been implicated in parasite morphogenesis and gliding motility at least in sporozoites and ookinetes, although the precise molecular details for these roles have yet to be elucidated for alveolins of *Plasmodium* spp. (Khater et al., 2004; Tremp et al., 2008; Volkmann et al., 2012). Stage specific transcriptomics in *P. falciparum* (López-Barragán et al., 2011; Gómez-Díaz et al., 2017; Zanghì et al., 2018) show that some alveolins are expressed across all lifecycle stages (e.g., IMC1a/ALV1, IMC1c/ALV5) while others seem to be absent in specific stages (e.g., IMC1f/ALV6 in gametocytes, IMC1l in asexual blood stages) or are exclusively expressed in one specific stage (e.g., IMC1i in ookinetes). A comprehensive detailed functional mapping of this multi-gene family across different stages of the *P. falciparum* lifecycle will be instrumental to understand the precise function of individual alveolins.

Beside the alveolins, other additional IMC associated peripheral membrane proteins like the IMC sub-compartment-proteins (ISPs) (Beck et al., 2010; Hu et al., 2010) have been characterized (Tonkin et al., 2004; Fung et al., 2012; Poulin et al., 2013; Gao et al., 2018; Wang et al., 2020). In contrast to integral membrane proteins of the IMC that are most likely trafficked *via* the ER and Golgi to their final destination (Yeoman et al., 2011), the majority of these peripheral membrane proteins are linked to the IMC membrane by their lipid anchors. For example, the ISP proteins as well as GAP45 depend on their N-terminal palmitoylation and myristoylation motif for membrane association (Wetzel et al., 2015; Wang et al., 2020). This occurs *via* co-translational and post-translation modification by the cytosolic N-myristoyltransferase (Gunaratne et al., 2000) and IMC embedded palmitoyl acyl transferases (Beck et al., 2013; Frénal et al., 2013; Wetzel et al., 2015). The alveolins, however, do not display a dual acylation motif and how they are trafficked to and interact with the IMC has yet to be described.

INNER MEMBRANE COMPLEX STRUCTURE AND FUNCTION THROUGHOUT THE PLASMODIUM LIFE CYCLE

The Asexual Blood Stage

Each individual *P. falciparum* merozoite within the schizont is surrounded by the double-membrane IMC, which sits approximately 20 nm below the PM (Morrissette and Sibley, 2002). The IMC is described as a mono-vesicle in this stage and appears to be continuous in fluorescence microscopy images, although gaps or discontinuities in the merozoite IMC have been observed in cryo-preserved TEM images (Hanssen et al., 2013; Riglar et al., 2013). At each pole of the merozoite is a defined sub compartment of the IMC. At the apical end of the merozoite, the IMC is supported by subpellicular microtubules (SPMs, 2-3 per merozoite) which are anchored in and organized by a series of rings termed polar rings (Morrissette and Sibley, 2002). At the basal end of the merozoite an additional structure, referred to as the basal complex is located which was first described in *T. gondii* (Ferguson et al., 2008; Hu, 2008; Lorestani et al., 2010).

The *de novo* biogenesis of the IMC has been studied in some detail in *P. falciparum* by fluorescence microscopy (Yeoman et al., 2011; Dearnley et al., 2012; Kono et al., 2012; Ridzuan et al., 2012). This process occurs during schizogony where a new IMC is formed for each of the 16–32 individual daughter merozoites in a schizont (**Figure 1**). The IMC of the growing daughter cells is established at the apical end of each new merozoite prior to nuclear division. The nucleation of the nascent IMC is closely associated with the centrosome placing it in the center of the developing IMC structure (Kono et al., 2012). The budding IMC grows from a point at the apical end into a ring until, at the end of schizogony; it has expanded to completely cover the newly formed daughter cell. During this process, the basal complex marks the leading edge of the growing IMC and migrates from the apical pole of the daughter cell to the basal pole where it resides after the completion of schizogony (Kono et al., 2016). PfMORN1 (Ferguson et al., 2008; Kono et al., 2016), PfBTP1 (Kono et al., 2016), PfBTP2 (Rudlaff et al., 2019), PfBCP1 (Rudlaff et al., 2019), PfHAD2a (Engelberg et al., 2016), and PfCINCH (Rudlaff et al., 2019) are well established basal complex markers. Parasites deficient in PfCINCH have impaired segmentation of daughter cells, suggesting this protein has an essential role in contraction of the basal

complex and pinching off of newly developed daughter cells (Rudlaff et al., 2019) and highlighting the important role of the IMC during cytokinesis. While in *P. falciparum*, how this contraction works remains unknown, in *T. gondii*, the apparent role of the myosin, MyoJ, in the contraction implies a mechanism involving the actin-myosin motor (Frénal et al., 2017).

Another process that remains mechanistically completely unknown is the rapid disassembly of the IMC after successful re-invasion of the merozoite into a new RBC. Once invasion is complete, the IMC as a central structure for cytokinesis and motility has outlived its purpose and needs to be disassembled to allow for growth and division of the parasite. The disassembly of the entire IMC may happen in as little as 15 min (Riglar et al., 2013) but definitely appears to be completed within 1 h. How this task is achieved, how it is regulated and what happens to the lipids and proteins from the now superfluous double-membrane structure remains to be explored.

The Sexual Blood Stage: Gametocytes

To achieve transmission from the vertebrate host to the mosquito the parasite produces male and female gametocytes. Sexual stage conversion in *P. falciparum* is characterized by drastic morphological changes of the parasite that occur over approximately 10–12 days and includes five (I to V) morphologically distinct stages (**Figure 1**) (Fivelman et al., 2007). Fully mature gametocytes display a falciform shape, from which *P. falciparum* derives its name. During their maturation, gametocytes lengthen significantly with fully mature gametocytes being 8–12 μm in length, approximately six times bigger than merozoites. Early in gametocytogenesis, their distinct morphology permits the gametocyte to sequester and develop within the bone marrow. Once fully mature, gametocytes migrate back into circulation where their morphology enables them to avoid immune clearance during passage through splenic sinuses unlike asexual parasites (Joice et al., 2014; Lee et al., 2018; De Niz et al., 2018; Obaldia et al., 2018).

The double membrane IMC below the PM in gametocytes was first identified 40 years ago (Smalley and Sinden, 1977). In *P. falciparum* it is organized in distinct vesicles or plates numbering between 11 (Meszoely et al., 1987) and 13 (Kono et al., 2012; Schneider et al., 2017) per gametocyte. The IMC plates are thought to be connected *via* proteinaceous "sutures", although no direct correlation between the distinct delineating lines seen in fluorescence microscopy and structures in electron microscopy have been made to date. The sutures are hypothesized to support the alignment of microtubules (Kaidoh et al., 1993; Dearnley et al., 2012; Kono et al., 2012) or to connect the IMC with the PM (Meszoely et al., 1987). Presently, two proteins (Pf3D7_1345600 and DHHC1) have been localized to the sutures in gametocytes (Kono et al., 2012; Wetzel et al., 2015), although no functional data connecting these proteins to their distinct localization is available.

Maturation of *P. falciparum* gametocytes can be accurately assessed by IMC formation. Stage I gametocytes are only distinguishable from asexual trophozoites on the molecular level, through expression of specific marker proteins such as Pfs16 (Bruce et al., 1994) with no IMC proteins detectable at this stage (Dearnley et al., 2012; Kono et al., 2012). Stage II gametocytes are characterized by elongation of the transversal microtubules and the start of IMC biogenesis (Sinden, 1981). IMC marker proteins such as PhIL1 (Schneider et al., 2017), GAP45 (Dearnley et al., 2012) and GAPM2, ISP3 and PF3D7_1345600 (Kono et al., 2012) appear as a spine-like structure with longitudinal orientation in close association with an array of microtubules. Three-dimensional SIM immunofluorescence microscopy shows a ribbon-like arrangement of PhIL1 around the microtubules (Schneider et al., 2017). Although still unclear, it is likely that microtubules provide a scaffold for the placement of the Golgi-derived IMC-bound vesicles that fuse to form the IMC. During stage III, the IMC membranes further wrap around the parasite and show thickened areas at the growing edge (Schneider et al., 2017). This then proceeds to stage IV where the parasite is maximally elongated and has a "banana-like" structure with the IMC completely surrounding the parasites. The final stage V is characterized by disassembly of the microtubule network until only small patches are observed below the IMC, a process initiated by an unknown signal. In parallel with this process, the parasite rounds up and the sutures become less pronounced (Dearnley et al., 2012; Schneider et al., 2017). Disassembly of the microtubule network leads to changes in the rigidity and deformation of the parasite (Dearnley et al., 2012) that promotes the reentering of these parasites from the bone marrow into the peripheral blood circulation and their uptake by the mosquito.

During uptake by the mosquito, the gametocytes are activated by external stimuli (reduced temperature, xanthurenic acid and pH rise), round up, egress from the RBC and transform into female macrogametes and exflagellated male microgametes. This transformation happens very rapidly (~2 min) (Sologub et al., 2011) and is highly dependent on disassembly of the IMC as a prerequisite for rounding up. Again it is unclear how this process is initiated, what its molecular basis is, or the fate of the membrane following disassembly. An interesting observation is the formation of nanotubules between forming gametes after gametocyte activation (Rupp et al., 2011). These are membranous cell-to-cell connections that may facilitate mating of the male and female gametes. The authors provide an intriguing hypothesis, which proposes that these may be formed out of the recycled IMC-vesicles as their appearance is coincident with the disappearance of the IMC (Rupp et al., 2011). Interestingly, a recent study in *P. berghei* suggests that active trans-endothelial migration of gametocytes (De Niz et al., 2018) exhibit an as yet uncharacterized mode of actin-dependent deformability and/or motility that could also be dependent on the IMC.

Taken together, in contrast to its central role in motility in invasion-competent merozoites, ookinetes and sporozoites, the IMCs most prominent role during gametocytogenesis appears to be a structural one which drives this stage's significant changes in size and shape (Dixon et al., 2012; Kono et al., 2012; Schneider et al., 2017).

In the Mosquito: From Zygote to Ookinete

Following fusion of the activated male and female gametes in the midgut of mosquitoes, a diploid, non-motile zygote is formed which undergoes meiosis and transforms into immature ookinetes known as retorts (reviewed in Bennink et al., 2016). These intermediate cells develop and 20 h post gametocyte fusion are fully transformed into motile ookinetes (Siciliano et al., 2020), which transmigrate through the epithelial cells to settle beneath the basal lamina of the mosquito midgut (**Figure 1**).

Like in merozoite maturation in the asexual blood stage, the IMC is formed again *de novo* during ookinetogenesis, starting as a patch at the apical end of the zygote (**Figure 1**) (Tremp et al., 2008). The round zygotes undergo huge and rapid morphological changes, gaining a protrusion, elongating and maturing to finally form an elongated, crescent-shaped ookinete (Guttery et al., 2015; Bennink et al., 2016). The ookinete's apical rings are formed at the same time and organize the SPM of > 40 microtubules (Bertiaux et al., 2020) under the IMC. These are initially seen at the apical protrusion of the zygote but extend during zygote elongation into a "dome-like" structure under the IMC (Wang et al., 2020). The dual acetylated peripheral IMC proteins ISP1 and 3 appear to serve here as a tether linking the SPM with the IMC in order to maintain a proper pellicle cytoskeleton. Congruently, ΔISP1 and ΔISP3 parasites show a reduced rate of zygote to ookinete differentiation, indicating that the IMC has an important role in transitioning from a round zygote to a crescent shaped ookinete (Wang et al., 2020).

Together with its role in mediating some of the large morphological changes that occur during the transformation from round, fertilized zygote to an elongated ookinete, another obvious role of the IMC is the facilitation of ookinete motility. Again, the IMC acts to anchor the glideosome, providing a stable structure from which to produce and exert forward motion. Motility is important for crossing the midgut and establishment of the replicative oocyst stage in the mosquito. Knock out of the IMC protein IMC1b/ALV5 led to reduced ookinete motility and therefore fewer oocysts produced in the mosquito (Tremp et al., 2008). After crossing the mosquito midgut, the ookinete differentiates into an oocyst. The transition from ookinete to early oocyst is also associated with loss of the IMC (Harding and Meissner, 2014) prior to sporogony. No details of this process are known yet, including what triggers and mediates disassembly and what happens to the large amount of the lipid and protein.

Relatively little is known about the architecture and protein composition of the IMC in these mosquito infecting stages. However, freeze fracture SEM images of ookinetes from the poultry parasite *P. gallinaceum* provide a glimpse of what appears to be a largely distinct IMC morphology from other stages (Alavi et al., 2003). The micrographs show a single vesicle punctured by multiple large pores. The pores have an external diameter of 43 nm and 12-fold symmetry. The single IMC vesicle appears to have a zig-zagged row of proteins connecting it along one edge, which has been termed a suture, as in gametocytes. What these pores are composed of, their function and why they have not been observed in other stages is currently unclear.

Out of the Mosquito: Sporozoite Formation and Motility

Once in the basal lamina the ookinete develops into an oocyst (**Figure 1**). Sporogony leads to thousands of sporozoites forming within the ~14 days of oocyst development. During sporogony, again, the IMC is formed *de novo* around each new sporozoite. It appears to be a complex process as the parasite grows larger, transforms from a "solid phase" to a vacuolated phase, undergoes multiple nuclear divisions and forms multiple segregated sporoblasts (Terzakis et al., 1967). Each sporoblast within the oocyst acts as a center for the synchronous budding of hundreds of new sporozoites (Sinden and Matuschewski, 2005). At the initiation of budding, the microtubule organizing center is positioned at the cortex. In a process similar to IMC formation in the merozoite, the apical rings and microtubules are in place and the IMC grows toward the basal end of each elongating parasite until a final contraction at the basal end completes sporogony (Schrevel et al., 2008). The versatile sporozoite emerges from the oocyst (Frischknecht and Matuschewski, 2017), and makes its way to the mosquito salivary glands where it invades first the basal lamina and then the acinar cells (Stering and Aikawa, 1973).

The elongated sporozoite (10–15 μm) is highly motile and moves at speeds of over 2 μm/s (Münter et al., 2009) for multiple minutes. Motility remains after injection into the dermis by the mosquito (Vanderberg, 1974; Amino et al., 2006) until the parasites invade a blood vessel, travel to the liver and finally settle in a hepatocyte. The distinctive crescent sporozoite shape may be due to a linkage of myosin to the subpellicular network (SPN) below the IMC (Khater et al., 2004; Kudryashev et al., 2012). In turn, this shape is likely to contribute to the distinct spiral gliding trajectories of the sporozoite (Akaki and Dvorak, 2005). This unusual gliding motility (Frischknecht and Matuschewski, 2017) depends on proteins of the TRAP (thrombspondin related anonymous protein) family (Sultan et al., 1997). TRAP proteins span the PM and possess a cytoplasmic tail domain that is believed to interact with actin, since mutations in this region led to defects in active locomotion (Ejigiri et al., 2012). TRAP proteins are released from micronemes to the sporozoite surface (Gantt et al., 2000; Carey et al., 2014) and serve as an adaptor and force transmitter between the target cells and the sporozoite.

The sporozoite stage has been studied by cryo-electron tomography, which allows a view of the IMC without artefacts from staining or slicing (Kudryashev et al., 2010). In these cells, the IMC sits approximately 30 nm below the PM and appears to be a highly homogenous flattened vesicle with minor discontinuities noted (Kudryashev et al., 2012).

Into the Blood: Hepatic Schizogony and Merozoite Formation

After invasion of the hepatocyte, the sporozoite dedifferentiates from the elongated motile cell into a rounded cell, starting at its center. This rounding is accompanied by the disassembly of the IMC as well as the sporozoite's subpellicular network (Jayabalasingham et al., 2010). Intriguingly, the rounding of the

sporozoite and disassembly of the IMC does not require a host cell as it can also be triggered outside the cell at 37°C in serum (Kaiser et al., 2003). Early EM images of *P. berghei* in hepatocytes show the shrinkage of the IMC to cover half the cell at 24 hpi, with just a small section covered at 28 hpi (Meis et al., 1985). This view of IMC disassembly after sporozoite invasion of hepatocytes is supported by fluorescence microscopy studies showing the shrinkage of the IMC to one side of the cell (Kaiser et al., 2003). More recently, EM images appear to show the dispersal of a membrane thought to be the IMC into the parasitophorous vacuole (PV) in hepatocytes infected with *P. berghei*, which the authors suggest could be a rapid mechanism for clearing this organelle and allowing for the fast growth and multiplication of the parasite (Jayabalasingham et al., 2010). Importantly, IMC disassembly is a prerequisite for the first mass-proliferative stage of schizogony within the host (Meis et al., 1985; Stanway et al., 2011) resulting in up to 29,000 exoerythrocytic merozoites in *P. berghei* (Baer et al., 2007) and up to 90,000 in *P. falciparum* (Vaughan et al., 2012). How exactly the disassembly or expulsion of the IMC occurs and what controls it remains to be determined.

OUTLOOK

The IMC is a unique structural compartment that undergoes continuous and profound reconfiguration during the parasite's lifecycle, mirroring the changes in stage specific architecture and motility that are reliant on IMC function. Although our understanding of the IMC has continuously increased over the years driven by molecular studies in *Plasmodium* spp. *as well as in T. gondii*, there are still large gaps in our knowledge and many unanswered questions in the field.

Many evolutionary questions still remain unanswered such as what selection pressures may have led to the evolution of the IMC in the ancestor of alveolates and what contribution did the IMC play in speciation. Clearly, the IMC was repurposed into a useful scaffold from which to drive cell invasion of apicomplexans. How this complex motor came to interact with the IMC scaffold may become apparent as we learn more about the early apicomplexan parasites and their move toward intracellular parasitism.

Many functional questions about the IMC in different stages of parasite development remain open. These include how IMC architecture at molecular resolution differs between stages, what the stage specificity of proteomes is and whether sub-compartmentalization and regulation of IMC assembly and disassembly is different in each stage. Another open question is how the different subcompartments of the IMC switch functions depending on their requirements. While some subcompartments and protein complexes, such as the basal complex and glideosome, have been partially characterized across different stages of the life cycle, such detailed studies have not yet been undertaken for others. Additional intriguing structures such as the apical annuli have been described in *T. gondii* that appear to be embedded in the IMC (Hu et al., 2006; Beck et al., 2010; Engelberg et al., 2020). Although there is currently no experimental evidence for the presence of such a structure in *Plasmodium* spp., a homolog of an apical annuli protein has been identified in the genome of *P. falciparum* that might serve as an "IMC pore" (Engelberg et al., 2020).

Another missing link is between the formation of the IMC and nuclear division. During mitosis, there appears to be a switch from local control of asynchronous rounds of nuclear division to global control. This final, global round of nuclear division occurs in parallel to IMC formation as well as with other processes of daughter cell formation. The molecular drivers of this process remain to be determined. In *T. gondii*, the bipartite centrosome acts as a signalling hub and coordinates the separation of nuclear division and daughter cell formation (Suvorova et al., 2015). However, although it can be speculated that a similar mechanism is likely to exist in *P. falciparum*, it has yet to be demonstrated.

A key question for future study is how the IMC's large quantity of lipid and protein gets recycled or remade in each stage of the lifecycle. Other than a few hints from electron micrographs showing pieces of membranes below the PM in young rings (Riglar et al., 2013) or the apparent expulsion of membrane in hepatocytes (Jayabalasingham et al., 2010), neither of which have been confirmed to be of IMC origin, we have no clear understanding of what happens to the IMC after it is no longer needed at different stages of *Plasmodium* spp. development. A few different hypotheses could be imagined, including the IMC merging with the PM after selective proteolysis of IMC proteins by the ubiquitin-proteasome system, bulk degradation by macroautophagy or bulk expulsion of the whole organelle. Degradation followed by *de novo* biogenesis in each stage is energy intensive, making it tempting to imagine a mechanism where the lipids and proteins are recycled. However, whether and how the IMC double membrane and protein is recycled or synthesized across different stages of malaria parasite development, often within a very short time-frame (minutes), is another mystery of this versatile organelle that remains to be elucidated.

Finally, with recent advances in techniques, particularly of imaging methods, we expect that answering the questions outlined above is already or will soon become feasible. As the IMC lies so closely below the PM (~20–30 nm) and its two membranes are extremely close together (~10 nm), few tools have high enough resolution to distinguish the three membranes. Although data from FIB-SEM of plastic embedded samples has not yet reached the resolution required to clearly resolve the IMC in published data, this technique has huge potential for providing 3D overviews of cell morphology (Rudlaff et al., 2020). To obtain more detailed information and easily resolve the three membranes, cryo electron tomography of FIB-milled samples will be necessary. Although EM techniques provide the spatial resolution needed, they only give a snapshot in time. The processes that we have described here are dynamic and vary in their speed. They therefore also need to be studied with tools which provide superior spatial and temporal resolution over, e.g., the period of IMC assembly, motile function and disassembly. The implementation of novel imaging techniques into the malaria field, such as lattice light sheet microscopy, is

promising and has the potential to provide high-resolution, temporal insights into the dynamic IMC.

AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Analysis of Long Non-Coding RNA in *Cryptosporidium parvum* Reveals Significant Stage-Specific Antisense Transcription

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Cryptosporidium is a protist parasite that has been identified as the second leading cause of moderate to severe diarrhea in children younger than two and a significant cause of mortality worldwide. *Cryptosporidium* has a complex, obligate, intracellular but extra cytoplasmic lifecycle in a single host. How genes are regulated in this parasite remains largely unknown. Long non-coding RNAs (lncRNAs) play critical regulatory roles, including gene expression across a broad range of organisms. *Cryptosporidium* lncRNAs have been reported to enter the host cell nucleus and affect the host response. However, no systematic study of lncRNAs in *Cryptosporidium* has been conducted to identify additional lncRNAs. In this study, we analyzed a *C. parvum in vitro* strand-specific RNA-seq developmental time series covering both asexual and sexual stages to identify lncRNAs associated with parasite development. In total, we identified 396 novel lncRNAs, mostly antisense, with 86% being differentially expressed. Surprisingly, nearly 10% of annotated mRNAs have an antisense transcript. lncRNAs occur most often at the 3′ end of their corresponding sense mRNA. Putative lncRNA regulatory regions were identified and many appear to encode bidirectional promoters. A positive correlation between lncRNA and upstream mRNA expression was observed. Evolutionary conservation and expression of lncRNA candidates was observed between *C. parvum*, *C. hominis* and *C. baileyi*. Ten *C. parvum* protein-encoding genes with antisense transcripts have *P. falciparum* orthologs that also have antisense transcripts. Three *C. parvum* lncRNAs with exceptional properties (e.g., intron splicing) were experimentally validated using RT-PCR and RT-qPCR. This initial characterization of the *C. parvum* non-coding transcriptome facilitates further investigations into the roles of lncRNAs in parasite development and host-pathogen interactions.

Keywords: lncRNA, RNA regulation, Apicomplexa, parasite development, stranded RNA-Seq, transcriptome

INTRODUCTION

Cryptosporidium is an obligate protist parasite that causes a diarrheal disease called cryptosporidiosis which spreads via an oral-fecal route. Human cryptosporidiosis, mainly caused by *Cryptosporidium parvum* and *Cryptosporidium hominis*, is typically self-limited and causes 1~2 weeks of intense watery diarrhea in people with healthy immune systems. However, the illness may be lethal among the immunocompromised including individuals with AIDS, cancer, and those receiving transplant anti-rejection medications. In recent years, several *Cryptosporidium* species, predominantly *C. hominis*, have been identified as the second most prevalent diarrheal pathogen of infants globally after rotavirus (Bouzid et al., 2013; Kotloff et al., 2013; Painter et al., 2015; Platts-Mills et al., 2015; Sow et al., 2016) and a leading cause of waterborne disease among humans in the United States (Centers for Disease Control and Prevention)[1]. Thus far, Nitazoxanide, the only FDA-approved drug is not effective for use in infants or those with HIV-related immunosuppression (Amadi et al., 2009) i.e. the most susceptible populations, and no vaccine is available (Amadi et al., 2002).

Cryptosporidium has a complex lifecycle in a single host. The *Cryptosporidium* oocyst which is shed in feces is a major extracellular lifecycle stage. It can stay dormant and survive in the environment for months (Drummond et al., 2018). After ingestion of oocysts through contaminated water or food, sporozoites are released which are capable of invading intestinal epithelial cells where both asexual and sexual replication occur. Following invasion, sporozoites develop into trophozoites and undergo asexual replication to generate type I meronts and type II meronts. Type I meronts are thought to be capable of reinvading adjacent cells generating an asexual cycle (Fayer, 2008), while Type II meronts contribute to the formation of microgamonts (male) or macrogamonts (female) to complete the sexual stages (Bouzid et al., 2013). Conventional monolayer cell culture does not permit completion of the life cycle much beyond 48 h post-infection (hpi) (**Figure 1A**), for as of yet poorly understood reasons but gametogenesis does occur (Tandel et al., 2019). The lack of *in vitro* culture has historically impeded the development of new drugs and vaccines for this medically important parasite. Recently, there have been several breakthroughs including genetic manipulation (Vinayak et al., 2015; Sateriale et al., 2020) and lifecycle completion. Several promising approaches have been developed including using a cancer cell line as host (Miller et al., 2018), biphasic and three-dimensional (organoid) culture systems, (Morada et al., 2016; DeCicco RePass et al., 2017; Heo et al., 2018; Cardenas et al., 2020), hollow fiber technology (Yarlett et al., 2020), and air-liquid interface (ALI) cultivation system (Wilke et al., 2019; Wilke et al., 2020). These breakthroughs are enabling better, much needed, studies of the parasite's full life cycle. A better understanding of the conditions and regulatory processes necessary for *Cryptosporidium* development are essential and will prove beneficial for the identification of drug and vaccine targets.

[1] https://www.cdc.gov/parasites/crypto/index.html.

The first genome sequence of *C. parvum* was published in 2004 with a genome size of ~9 Mb and ~3800 protein-encoding genes annotated (Abrahamsen et al., 2004). Since this milestone, our understanding of the parasite and its biology have progressed remarkably. Early *in vitro* transcriptome analyses using semi-quantitative RT-PCR over a 72 h post-infection (pi) time course during *in vitro* development revealed complex and dynamic gene expression profiles. Adjacent genes are not generally co-regulated, despite the highly compact genome (Mauzy et al., 2012). Under UV irradiation, *C. parvum* oocysts have shown a vital stress-induced gene expression response according to microarray data (Zhang et al., 2012). mRNA expression related to gametocyte and oocyst formation were studied using RNA sequencing of sorted cells (Tandel et al., 2019). Yet, little is known about the regulation of key developmental transitions. How the parasite regulates gene expression in order to invade hosts, amplify, evade the immune system and interact with their host awaits further discovery.

Most canonical eukaryotic enhancer proteins are not detected in Apicomplexa, the phylum that *Cryptosporidium* belongs to (Iyer et al., 2008), except for the transcriptional activators Myb and zinc finger proteins C2H2 (Cys2His2) and two additional transcription factor families. Instead, an expanded family of apatela-related transcription factors, the AP2 family of proteins (ApiAP2), appear to be the predominant transcription factors in this phylum, including *Cryptosporidium* (Oberstaller et al., 2014; Jeninga et al., 2019). AP2 domains in *C. parvum* are reported to have reduced binding diversity relative to the malaria parasite *Plasmodium falciparum* and proposed to possess less dominancy in transcriptional regulation in *C. parvum* (Campbell et al., 2010; Yarlett et al., 2020). It has been proposed that *C. parvum* is less reliant on ApiAP2 regulators in part because it utilizes E2F/DP1 transcription factors, which are present in *Cryptosporidium* while absent in other studied apicomplexans (Templeton et al., 2004; Yarlett et al., 2020). Based on the similarity of gene expression profiles, it has been suggested that the number of co-expressed gene clusters in *C. parvum* is somewhere between 150 and 200, and putative ApiAP2 and E2F/DP1*cis*-regulatory elements were successfully detected in the upstream region of many co-expressed gene clusters (Oberstaller et al., 2013). Additionally, low levels of DNA methylation in oocysts has been reported in several *Cryptosporidium spp.* (Gong et al., 2017), suggesting the requirement of additional regulatory mechanisms. At the level of post-transcriptional regulation, the RNA interference (RNAi) pathway, which plays a crucial role in gene silencing in most eukaryotes, is considered to be missing in *Cryptosporidium* due to the lack of identifiable genes encoding the microRNA processing machinery or RNA-induced silencing complex (RISC) components (Keeling, 2004). There is much that remains to be discovered with respect to regulation of gene expression in *Cryptosporidium*.

Long non-coding RNAs (lncRNAs) are transcripts without significant protein-encoding capacity that are longer than 200 nt. In eukaryotes, lncRNAs play critical regulatory roles in gene regulation at multiple levels, including transcriptional, post-transcriptional, chromatin modification and nuclear architecture alterations (Marchese et al., 2017). In humans,

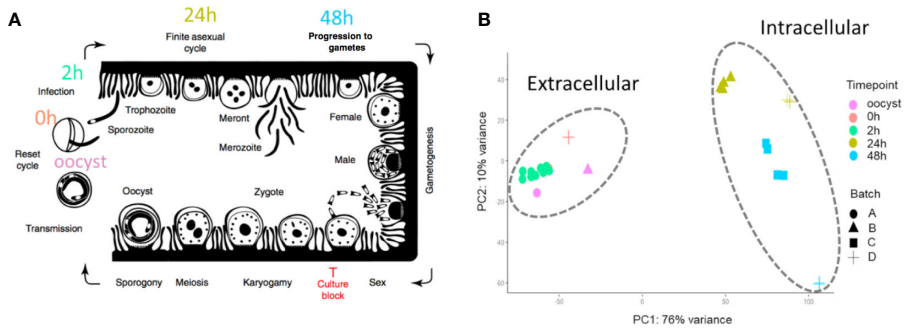


FIGURE 1 | The 33 RNA-Seq data sets used for expression analysis. **(A)** The time points indicate when RNA-Seq samples were collected and the associated *C. parvum* life cycle stage. The schematic model of the *C. parvum* life cycle is modified from (Tandel et al., 2019). "Culture block" refers to the developmental stage where parasites in traditional *in vitro* cultures fail to progress. **(B)** Principal component analysis of 33 *C. parvum* transcriptomes. The analysis is based on the normalized expression level (VST) of the *C. parvum* mRNA and predicted lncRNA genes. Samples collected from different time points are indicated by colors. Various projects/batches are represented by shapes. Batch A includes sample IDs 3–19 without host cells. Batch B includes sample IDs 1, 23–26 from IPEC host cells. Batch C includes sample IDs 27–30 from MDBK host cells. Batch D contains sample IDs 2, 20–22, 31–33 from HCT-8 host cells (**Table 1**).

3300 long intergenic ncRNAs (lincRNAs) were analyzed using chromatin state maps, and ~20% of these RNAs are bound to the polycomb repressive complex (PCR2, a complex with histone methyltransferase activity) (Khalil et al., 2009). Most lncRNAs share many characteristics of mRNAs, such as RNA polymerase II-mediated transcription, a 5′ 7-methylguanosine cap and a 3′ poly(A) tail [6]. The expression of lncRNAs is usually more tissue- or time-specific than mRNA expression (Necsulea et al., 2014; Tsoi et al., 2015). lncRNA sequences are not well conserved across species, but their structure could be conserved due to functional constraints (Ulitsky et al., 2011; Diederichs, 2014). By forming hybrid structural complexes such as RNA-DNA hybrid duplexes or RNA-DNA triplexes, lncRNAs can recruit or scaffold protein complexes to facilitate localization of protein machinery to specific genome target sites (Li et al., 2016). lncRNAs play important roles in regulating occurrence and progression of many diseases. After infected by *C. baileyi*, significant expression changes have been observed in the host (Ren et al., 2018). The mis-regulation of lncRNAs in multi-cellular eukaryotes has been shown to cause tumorigenesis (Chakravarty et al., 2014), cardiovascular diseases (Tang et al., 2019), and neurodegenerative dysfunction (Zhang et al., 2018) and thus can be used as diagnostic biomarkers.

Taking advantage of sequencing technologies, numerous lncRNA candidates have been detected in apicomplexans, some with proven regulatory potential during parasite invasion and proliferation processes. These discoveries have ushered in a new era in parasite transcriptomics research (Liao et al., 2014; Siegel et al., 2014; Broadbent et al., 2015; Ramaprasad et al., 2015; Filarsky et al., 2018). In *P. falciparum*, lncRNAs are critical regulators of virulence gene expression and associated with chromatin modifications (Vembar et al., 2014). Likewise, an antisense RNA of the gene *gdv1* was shown to be involved in regulating sexual conversion in *P. falciparum* (Filarsky et al., 2018). In *C. parvum*, putative parasite lncRNAs were found to be delivered into the host nucleus, some of which were experimentally proven to regulate host genes by hijacking the host histone modification system (Ming et al., 2017; Wang et al., 2017a; Wang et al., 2017b). The importance of lncRNA in *C. parvum* has been demonstrated, but no systematic annotation of lncRNA has been conducted. The systematic identification of lncRNAs will increase the pool of candidate regulatory molecules thus ultimately leading to increased knowledge of the developmental gene regulation in *C. parvum* and control of parasite interactions with its hosts.

In this study, we developed and applied a computational pipeline to systematically identify new lncRNAs in the *C. parvum* genome. We used a set of stranded RNA-seq data collected from multiple lifecycle stages that cover both asexual and sexual developmental stages. We conducted a systematic analysis of lncRNA that includes sequence characteristics, conservation, expression profiles and expression relative to neighboring genes. The results provide new insights into *C. parvum's* non-coding potential and suggest several areas for further research.

METHODS AND MATERIALS

RNA-Seq Data Pre-Processing/Cleaning

RNA-Seq data sets were downloaded from the NCBI Sequence Read Archive (SRA) and European Nucleotide Archive database (ENA). Detailed information on SRA accession numbers and Bioprojects are listed in **Table 1** and **Supplementary Table S1**. FastQC-v0.11.8 was used to perform quality control of the RNA-Seq reads. Remaining adapters and low-quality bases were trimmed by Trimmomatic-v-0.36 (Bolger et al., 2014) with parameters: Adapters:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:25. All reads were scanned with a four-base sliding window and cut when the average Phred quality dropped below 25. Bases from the start and end were removed when the quality score was below 20. The minimum read length was set at 25 bases. The processed reads are referred to as cleaned reads in this work.

TABLE 1 | RNA-Seq data sets used in this study.

ID*	Time point	Host cells	Condition
C. parvum			
1B	Oocyst	NA	Extracellular
2D	Oocyst	NA	Extracellular
3A	0 h	NA	Extracellular
4A	0 h	NA	Extracellular
5A	0 h	NA	Extracellular
6A	0 h	NA	Extracellular
7A	0 h	NA	Extracellular
8A	0 h	NA	Extracellular
9A	0 h	NA	Extracellular
10A	2 h	NA	Extracellular
11A	2 h	NA	Extracellular
12A	2 h	NA	Extracellular
13A	2 h	NA	Extracellular
14A	2 h	NA	Extracellular
15A	2 h	NA	Extracellular
16A	2 h	NA	Extracellular
17A	2 h	NA	Extracellular
18A	2 h	NA	Extracellular
19A	2 h	NA	Extracellular
20D	24 h	HCT-8	Intracellular
21D	24 h	HCT-8	Intracellular
22D	24 h	HCT-8	Intracellular
23B	24 h	IPEC	Intracellular
24B	24 h	IPEC	Intracellular
25B	24 h	IPEC	Intracellular
26B	24 h	IPEC	Intracellular
27C	48 h	MDBK	Intracellular
28C	48 h	MDBK	Intracellular
29C	48 h	MDBK	Intracellular
30C	48 h	MDBK	Intracellular
31D	48 h	HCT-8	Intracellular
32D	48 h	HCT-8	Intracellular
33D	48 h	HCT-8	Intracellular
C. hominis			
34E	Oocyst	NA	Extracellular
35E	Oocyst	NA	Extracellular
36F	Oocyst	NA	Extracellular
37F	Oocyst	NA	Extracellular
C. baileyi			
38G	Oocyst/sporozoite	NA	Extracellular

**Batch with various host cell types and parasites from different projects are indicated with the sample IDs. IDs designated with A, C and E, (Matos et al., 2019); B, (Mirhashemi et al., 2018); D, (Tandel et al., 2019); 36F: SRR1183950; 37F: SRR1183934; 38G: SRR1183952.*

Cell line synonyms/origin: HCT-8: Human intestine; IPEC: Intestinal Porcine Epithelial Cell line; MDBK: Bovine kidney.

Read Mapping and Transcript Assembly

Cleaned reads from each sample were mapped to the reference genome sequence for *Cryptosporidium parvum* IOWA-ATCC (BioProject ID:PRJNA573722, Baptista et al. *in prep*) downloaded from CryptoDB v46 (https://cryptodb.org/cryptodb/) using the mapping tool HISAT2-v2.1.0 (Kim et al., 2015) with maximum intron length (–max-intronlen) set at 3000, and remaining parameters as default. Uniquely mapped reads were selected for further study using SAMtools-v1.10 (view -q 10) (Li et al., 2009). StringTie-v2.0.6 (Pertea et al., 2015) was used to reconstruct transcripts using the reference annotation guided method (–rf -j 5 -c 10 -g 1). At least five reads were required to define a splice junction. A minimum read coverage of 10 was used for transcript prediction. Only overlapping transcript clusters were merged together. The stranded library types were all "fr-firststrand." Transcripts with FPKM lower than three were removed. The transcriptome assemblies from multiple samples were merged into one master transcript file using TACO-v0.7.3 with default settings (Niknafs et al., 2017).

lncRNA Prediction

Transcripts that overlapped with currently annotated mRNAs in the *C. parvum* IOWA-ATCC annotation in CryptoDB v46 with coverage >70% on the same strand were removed using BEDTools-v2.29.2 (Quinlan and Hall, 2010). The remaining transcripts were examined for coding potential using the online tool Coding Potential Calculator (CPC) v0.9 (Kong et al., 2007). Transcripts considered as "coding" by CPC were removed. Potential read-through transcripts resulting from transcription of neighboring mRNAs were removed using two criteria: 1) The transcript was <50 bp from the upstream coding region of another gene on the same strand. 2) The transcript was always transcribed together with the upstream mRNA on the same strand. Finally, the remaining transcripts which occurred in >2 RNA-Seq samples were kept as putative lncRNA candidates for further studies.

Transcriptome Data Normalization and Identification of Differentially Expressed Genes

The raw read counts for both mRNA genes and predicted lncRNAs were calculated using HTSeq-v0.12.4 (Anders et al., 2015). All genes were filtered to require > 50 reads in at least three samples. Variance stabilizing transformation (VST) from DESeq2-v1.28.1 (Love et al., 2014) was used to normalize the expression between samples. Principal components analysis (PCA) of the RNA-Seq samples was performed using the resulting VST values. VST values for each of the mRNA and lncRNA candidates were visualized using the R package pheatmap-v1.0.12 (https://github.com/raivokolde/pheatmap). The K-mean approach in pheatmap was applied to cluster genes based on the expression data.

Differentially expressed genes including mRNAs and lncRNAs were analyzed by EdgeR-v3.30.3 (Robinson et al., 2010) using the raw read count from HTSeq-v0.12.4. The expression time-points compared were between oocyst/sporozoites (oocysts, time point 0 h and sporozoites, 2 h), asexual stage (time point 24 h) and mixed asexual/sexual stage (only select samples from 48 h time point in which gametocyte marker genes are clearly expressed, i.e. batch D). The sex marker gene: cgd6_2090 encodes *Cryptosporidium* oocyst wall protein-1 (COWP1) produced in female gametes and cgd8_2220 encodes the homolog of hapless2 (HAP2) a marker of male gamonts (Tandel et al., 2019). A generalized linear model (GLM) approach was used for differential expression hypothesis testing. P-values were adjusted by the false discovery rate (FDR). Significant differentially expressed genes were declared at a log2-fold change ≥ 1.5 and an FDR < 0.05.

Expression Correlation

The expression correlation analysis between predicted lncRNAs and mRNAs was conducted with normalized expression data from all 33 samples (**Table 1**) of *C. parvum* using the Pearson test. P-values were adjusted by FDR.

Upstream Motif Analysis

MEME v5.0.0 (Bailey and Elkan, 1994) was used to discover motifs that may be present upstream of putative lncRNAs. For the promoter motif search, we extracted the 100 bp of (+) strand sequence upstream of the predicted lncRNAs and searched both strands using MEME for motifs with length six to 50 bp. The parameters were: -dna -mod anr -nmotifs 15 -minw 6 -maxw 50 -objfun classic -markov_order 0 -revcomp.

Evolutionary Conservation

RNA-Seq data sets from *C. hominis* and *C. baileyi* oocysts were mapped to reference genome sequences for *C. hominis* 30976 and *C. baileyi* TAMU-09Q1, respectively, downloaded from CryptoDB v46 (https://cryptodb.org/cryptodb/). Mapped reads were assembled into transcripts using the same methods mentioned above. tblastx from NCBI BLAST v 2.10.0 was used to search for conserved antisense candidates among *C. parvum*, *C. hominis* and *C. baileyi*, with parameter of E-value:1e-5 and best hit retained. To assess conservation among other apicomplexans, *P. falciparum*, antisense and associated sense mRNA data were retrieved from (Broadbent et al., 2015). *P. falciparum* orthologs of the sense mRNAs in *C. parvum* were retrieved from OrthoMCL DB v6.1 (Li et al., 2003). If antisense RNAs were detected between *P. falciparum* and *C. parvum* orthologs, the lncRNAs between *P. falciparum* and *C. parvum* were considered orthologous.

RT-qPCR Validation

We designed PCR primers using the PrimerQuest Tool from IDT (https://www.idtdna.com/pages/tools/primerquest) (**Supplementary Table S2**) to use for expression validation and exon structure confirmation of select lncRNA candidates using RT-PCR and qPCR. RNA was extracted from oocysts and provided by Boris Striepen. The cDNA for each sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) from 1μg of input RNA. The resulting cDNAs were used as templates for PCR amplification and qPCR detection. Strand-specific primers were designed to amplify antisense RNAs. The 18S rRNA gene of *C. parvum* was used as positive control and samples without RNA or primer were used as a negative control. Each RT-PCR reaction contained 1ul cDNA, 2 μl primer mix (10 μM), 2 μl water and 5 μl MyTaq™ HS Mix (Bioline). RT-PCR was performed in the following conditions: 35 cycles of 15 s at 95°C, 30 s at 64°C. Then the RT-PCR products were run on a 2% agarose gel. The cDNA was also subjected to qPCR with All-in-One qPCR Mix (QP001; GeneCopoeia, Rockville, MD, USA) using the Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA). All reactions, including no-template controls, were run in triplicate. Following amplification, the CT values were determined using fixed threshold settings. lncRNA expression was normalized to 18S rRNA expression.

Data Availability

GenBank accession records CP044415–CP044422 have been updated to include annotation of the lncRNAs identified in this study. These data have also been submitted to CryptoDB.org.

RESULTS

Analysis of the *Cryptosporidium* Stranded RNA-Seq Data Used in This Study

To identify and investigate the expression profile of lncRNAs in *C. parvum* during parasite development, we searched for stranded RNA-Seq data sets from *Cryptosporidium* available in public databases. Due to the small volume of *Cryptosporidium* relative to the host cells, RNA-Seq data usually suffer from high host contamination. In this study, we selected samples that had more than 100k *Cryptosporidium* reads generated from the Illumina platform mapped to the reference genome sequence to reduce bias mostly arising from sequencing platform and sequencing depth. In total, 38 stranded-RNA-seq data sets which originated from 33 *C. parvum* samples, four *C. hominis* samples and one *C. baileyi* sample were selected for further analysis. The details and mapping statistics of each sample are shown in **Table 1** and (**Supplementary Table S1**). The *C. parvum* samples represented five lifecycle time points: oocyst, 0 h (sporozoites immediately after oocyst excystation), 2 h (2-h incubation in the medium (Matos et al., 2019)), 24 h (24-h post host cell infection) and 48 h (48-h post host cell infection) (**Figure 1A**). The 24- and 48-h samples were derived from infections in different types of host cells (see details in **Table 1** and **Supplementary Table S1**). The *C. hominis* RNA-Seq and one *C. baileyi* RNA-seq sample were obtained from oocysts.

The transcriptomes of the 33 *C. parvum* RNA-Seq samples were compared by principal component analysis (PCA) based on the normalized mRNA gene expression level in each sample (**Figure 1B**). Extracellular stages, including oocyst and sporozoites from 0 h and 2 h, are differentiated from intracellular stages, including 24 h and 48 h. The transcriptomes of intracellular stages were demonstrated to be more heterogeneous, while extracellular samples formed a relatively compact cluster. This observation was consistent with a previous transcriptome study of *C. parvum* oocysts and intracellular stages (Matos et al., 2019). At time points 24 h and 48 h, different host cells and laboratory procedures were used, which could contribute to the distance observed between samples from the same time point.

To further explore whether sexual commitment was initiated in all 48 h samples, we profiled the transcriptome of marker genes cgd6_2090 and cgd8_2220 (**Supplementary Figure 1**). cgd6_2090 encodes the *Cryptosporidium* oocyst wall protein-1 (COWP1) which is produced in female gametes (Tandel et al., 2019); cgd8_2220 encodes the homolog of hapless2 (HAP2) which is a class of membrane fusion protein required for gamete fusion in a range of organisms including *Plasmodium falciparum* (Liu et al., 2008). HAP2 labeled protein was exclusively found in male gamonts in *C. parvum* (Tandel et al., 2019). In another study, the *C. parvum* transcriptome was elucidated over a 72 h *in vitro* time-course infection with

HCT8 cells using semi-quantitative RT-PCR (Mauzy et al., 2012). The 48 h-specific genes from that study were also examined in the 33 RNA-Seq data sets analyzed here (**Supplementary Figure 2**). Both the sex marker genes and 48 h-specific genes show an expression peak at 48 hr. At 48 h, expression levels from batch D samples are much higher than batch C samples. cgd8_2220 is very low/not expressed at 48 h in batch C samples. These results indicate that both batch C and D samples showed the commitment of sexual development, but commitment was more pronounced in batch D. It is possible that sequencing depth could be influencing this difference as the normalization process (VST) between samples tends to reduce the variation of genes with low read support. Interestingly, batch C and D samples used different host cell types (**Table 1**). Batch D *C. parvum* parasites were cultured in HCT-8 (Human intestine cells) while batch C parasites were cultured in MDBK (Bovine kidney cells). Although sexual stages have been observed in MDBK cells (Villacorta et al., 1996), it is possible that the adaptation of *C. parvum* to MDBK cells is lower than HCT-8 cells as hosts. Thus, a slower or lower conversion rate was observed.

Identification and Characteristics of lncRNAs

We began assembly of the *C. parvum* transcriptome using mapped RNA-Seq reads of samples in NCBI BioProject PRJNA530692 with a high sample quality. However, *C. parvum* has an extremely compact genome sequence. As calculated from the *C. parvum* IOWA-ATCC annotation, the average intergenic distance between the stop and start codon boundaries of neighboring genes is only 504 bp and this distance must also include promoter and UTR regions. The average length of an annotated *C. parvum* ATCC mRNA coding sequence, CDS, is 1853 bp. The high gene density and the short distance between genes make it difficult to accurately define UTR boundaries using short-read sequencing data as transcripts overlap and become merged. Transcriptome assembly using RNA-Seq without genome and reference annotation guidance leads to a high chimerism rate. Thus, we used reference annotation to guide the assembly process and set parameters to minimize the number of artificially fused transcripts. We then used the program TACO v0.7.3 (Multi-sample transcriptome assembly), which employs change point detection to break apart complex loci to lower the number of fused transcripts to obtain a non-redundant master transcriptome from all samples, resulting in 5818 transcripts in total. Of these, 4846 transcripts overlapped with an mRNA on the same strand and thus, were removed. Transcripts which were <200 bp or only detected in a single sample were removed to improve the lncRNA prediction quality. Transcripts that were considered as "coding" by the Coding potential analysis tool CPC were filtered out. To identify and remove potential read-through transcripts, predicted transcripts that were located closer than 50 bp from the coding region of the upstream gene on the same strand and always transcribed together with the upstream mRNA were removed (**Figure 2A**).

In total, 396 transcripts were selected as lncRNA candidates for further analysis, 363 (91.7%) are antisense to mRNAs and only 33 are lincRNAs (**Figure 2B**). This high percentage of antisense RNA transcripts is a noteworthy feature of the compact *C. parvum* genome given that CDSs cover >70% of the genome sequence. Interestingly, the nucleotide composition varies between antisense RNAs and lincRNAs with many antisense RNAs being CT-rich and most of the lincRNAs being AT-rich (**Supplementary Figure 3**).

Most of the lncRNAs we detected consist of a single exon however five lncRNAs contain introns. This is consistent with the low-intron rate in *Cryptosporidium*. Additional introns are expected to emerge with deeper RNA-Seq data. The average length of lncRNAs and annotated mRNAs is 1267 bp and 1853 bp, respectively (**Figure 2C**). When compared to mRNAs, one of the most distinguishing features of lncRNAs is the low Open Reading Frame (ORF) coding potential relative to the transcript length.

We further investigated the relative location of the antisense transcripts relative to the annotated sense mRNA coding sequence (CDS), and we found that many of lncRNAs' initiation and termination sites are located close to the sense CDS boundaries. The start site of the antisense lncRNA transcript is highly correlated with the end of the sense strand CDS (**Figure 2D**). When looking at the coverage of the antisense transcript along the mRNA, the *C. parvum* lncRNA antisense expression has a bias towards the 3′ end of the mRNA transcript (**Figure 2E**). This property has also been reported in other organisms, including the malaria pathogen *Plasmodium falciparum* (Lopez-Barragan et al., 2011; Siegel et al., 2014; Broadbent et al., 2015).

To better understand their transcription, we searched for potential promoter motifs within 100 bp of the (+) strand upstream from all 396 lncRNAs. This analysis returned five significant motifs (E-value <0.001) for 190 lncRNAs (**Supplementary Figure 4** and **Supplementary Table S3**). The top two motifs are known *Cryptosporidium* transcriptional factor binding motifs E2F/DP1 (5′-[C/G]GCGC[G/C]-3′) and ApiAP2_1 (5′-BGCATGCAH-3′) motifs (Oberstaller et al., 2013), both of which are palindromic (**Figure 2F**). Of the 190 lncRNAs with significant upstream motifs, 142 lncRNAs had only a single type of motif (93 with E2F/DP1 and 19 with ApiAP2_1). 70 (75.3%) of the 93 lncRNAs with upstream E2F/DP1 motifs had only one motif, 21(22.6%) had two occurrences. Of the 19 lncRNAs with upstream ApiAP2_1 motifs, 12 (63.2%) had only one occurrence of the motif and 6 (31.6%) had two. Co-occurrence of the E2F/DP1 and ApiAP2_1 motif was only observed upstream of 17 lncRNAs. The spacing of the motifs relative to the transcription start site was assessed and no clear pattern was observed (**Supplemental Figure 5**). These combined results suggest that lncRNA transcripts have the potential for being regulated independently during parasite development.

lncRNAs Are Developmentally Regulated

We visualized gene expression profiles across the 33 RNA-Seq samples used in this study (**Table 1**) for both mRNA (**Figure 3A**)

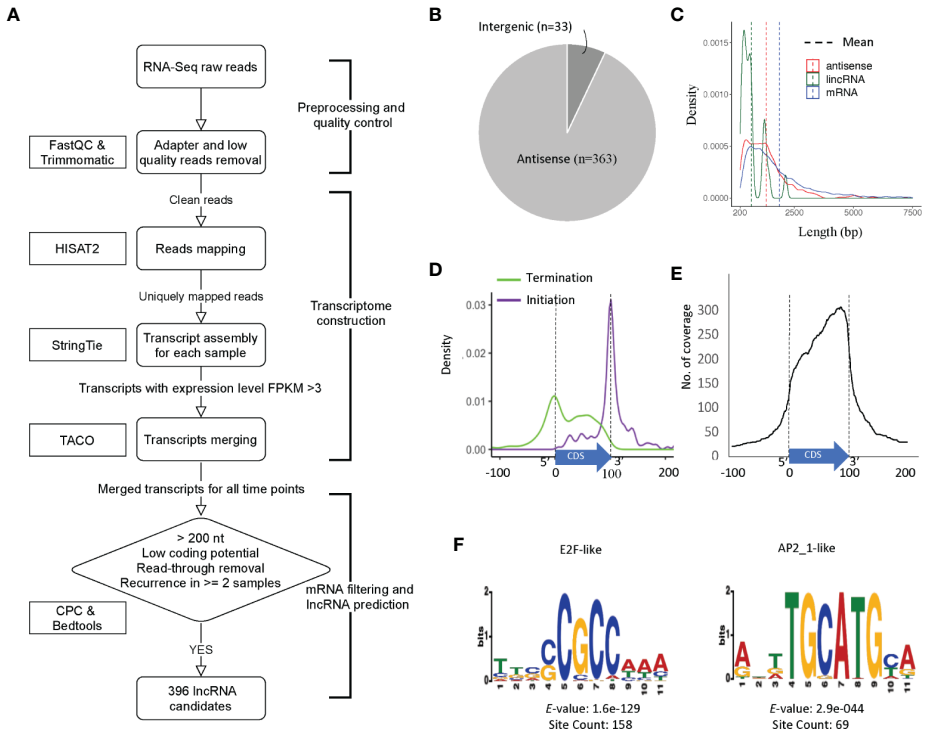


FIGURE 2 | Prediction and characterization of lncRNAs in *C. parvum*. **(A)** Pipeline of lncRNA prediction. **(B)** Genomic location of predicted lncRNAs. **(C)** The distribution of transcript lengths for mRNA genes, antisense RNAs and lincRNA candidates with mean lengths indicated. **(D)** Antisense transcription initiation and termination position relative to the sense CDS (normalized to 0–100). **(E)** Abundance and position of sense CDS (normalized to 0–100) covered by antisense transcription. **(F)** Top two enriched upstream motifs within 100 bp, the same strand (+) of lncRNA candidates.

and lncRNAs (**Figure 3B**). To identify genes with a similar expression profile, we used a k-means algorithm to cluster mRNAs and lncRNAs separately (**Supplementary Table S4** and **Supplementary Table S5**). The k value was selected as the smallest value that allowed the separation of genes from different time points while keeping genes from different samples of the same timepoint together despite the batch effects present within each time point. As a result, mRNAs and lncRNAs were clustered into seven and nine broad co-expression groups, respectively. Select highly expressed lncRNAs from clusters 2, 4 and 5 are visualized as examples (**Supplementary Figure 6**).

The expression of mRNAs in the extracellular stages (oocysts and sporozoites from 0h and 2h) showed a similar expression trend that is quite distinct from the latter two stages. For mRNAs, genes from cluster 1 and cluster 2 were more highly expressed in the extracellular stages but still show expression in the intracellular stages when the vast majority of mRNAs are active. This result is consistent with another transcriptome study of *C. parvum*, which used semi-quantitative RT-PCR over a 72 h time course during *in vitro* development (Mauzy et al., 2012). On the contrary, many lncRNAs showed enriched expression in extracellular stages (oocyst, 0 h, 2 h) and some had expression later at the intracellular sexual development stage (48 h) (**Supplementary Table S6**) while the asexual stage (24 h) showed the least lncRNA expression. When considering the correlation of antisense transcription to parasite development (according to the peak expression time point), 162 antisense transcripts were classified as extracellular stage enriched (oocyst, 0 h and sporozoite 2 h), 50 as merozoite enriched (24 h) and 151 as sexual stage enriched (48 h). It is noteworthy that both mRNAs and lncRNAs have gene sets that are specifically turned on at 48 h (mRNA cluster 5 and lncRNA cluster 4). The average expression level of lncRNAs suggests they are more abundant or were actively expressed in oocysts, 0 h, 2 h and gamont formation 48 h stages than the 24 h merozoite stage (p value <.0001, t-test) (**Figure 3C**). As was observed in the PCA to assess batch effect **(Figure 1B),** there is increased variation at the 48 h time point. Compared to mRNAs which show more upregulation when transitioning from oocyst/sporozoite to the asexual stage (1715 genes vs 1270 genes), lncRNAs have many more genes downregulated (218 vs 80) (**Figure 3D**). Comparing the asexual stage at 24 h to the sexually activated stage at 48 h, fewer mRNAs showed differential expression, with both having ~400 genes upregulated and downregulated. Very few lncRNAs were downregulated in the transition from the asexual to sexually activated stage but 85 lncRNAs were upregulated. The 85 upregulated lncRNAs did not significantly overlap with the lncRNAs that were downregulated between the extracellular to the asexual stage. Here we only used 48 h samples from batch D to analyze differential expression since this batch has clear sexual

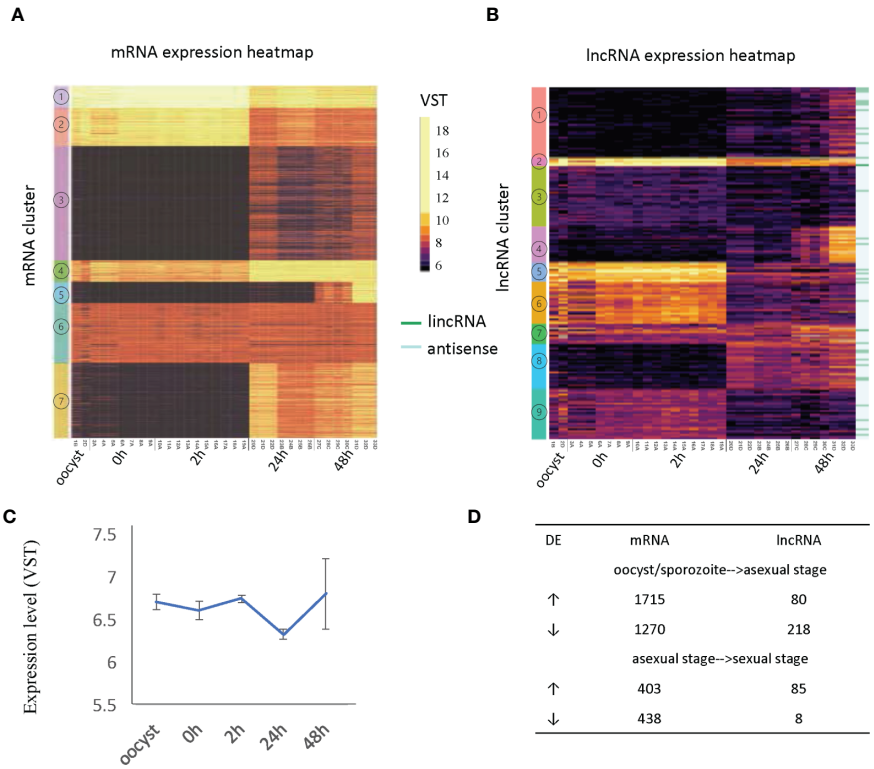


FIGURE 3 | Developmentally regulated lncRNAs. **(A)** Heatmap of mRNA expression across 33 RNA-Seq samples (**Table 1**). **(B)** Heatmap of lncRNA expression (396 transcripts) across 33 RNA-Seq samples (**Table 1**). Expression clusters generated by K-means are indicated by colored bars on the left-most edge. The 33 lincRNAs are identified by dark green lines on the righthand side of the figure. **(C)** The average expression level and standard deviation of lncRNAs at each time point. **(D)** Differentially expressed (DE) genes are compared between developmental transitions (oocyst/sporozoite stage, 24 h asexual stage and 48 h sexual stages. The arrows indicate the direction of change in gene expression. Normalized gene expression values are colored as indicated in the scale located between panels A and B with yellow indicating the highest levels.

stage marker gene expression, as discussed above. The developmentally regulated expression pattern of lncRNAs is indicative of their importance in extracellular and sexual stages. It is important to note that overall, the levels of lncRNA expression are lower than the expression levels observed for mRNAs (**Figure 3**).

Correlation of lncRNA Expression With Sense and Neighboring mRNA Expression

LncRNA mediated gene regulation can be achieved by various mechanisms (Li et al., 2020). One mechanism is transcriptional interference that usually results in repression of the target gene. LncRNAs can also activate target gene expression through epigenetic mechanisms. Additionally, translational regulation by lncRNA has also been reported. Therefore, to understand the potential roles of lncRNA transcription or transcripts, we studied the correlation between lncRNA and neighboring gene mRNA expression in *C. parvum* using the 33 RNA-Seq samples (**Supplementary Table S5**).

First, to estimate the background transcriptional correlation between genes, we calculated the expression correlation between 10,000 random gene pairs. 5748 pairs had a positive Pearson's correlation with an average r value of 0.56 while 4250 pairs had a negative Pearson's correlation with an average r value of −0.51. Thus, we consider r > 0.56 as an indicator of positive correlation and r >0.70 as significant correlation and r < −0.51 as an indicator of negative correlation with r < −0.7 as significant negative correlation and r values between −0.51 and 0.56 as indicative of no correlation.

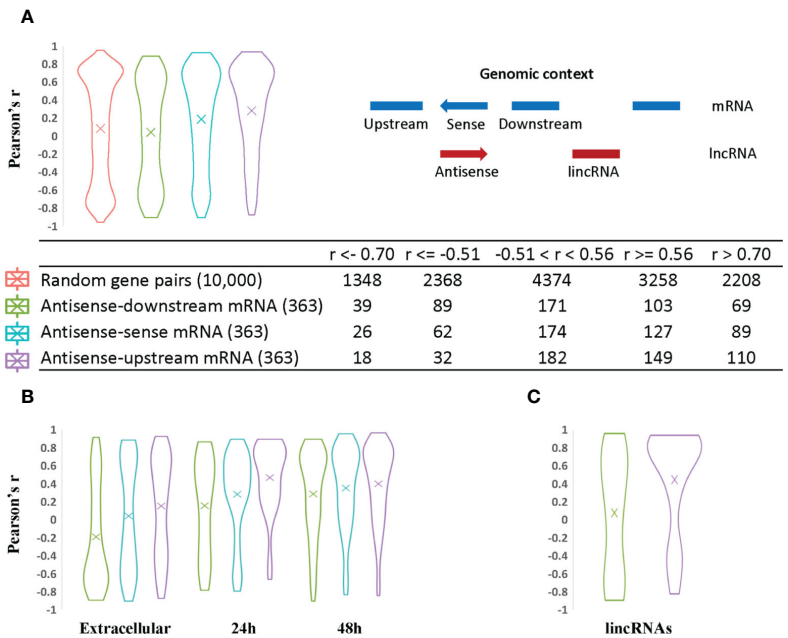
Next, transcriptional correlations between the sense mRNA gene and its antisense as well as between mRNAs located upstream and downstream (either DNA strand) of the antisense transcript were analyzed (**Figure 4A**). Most sense-antisense transcript pairs do not show a strong expression correlation but those that do, tend to have a positive correlation. We note that the levels of positive correlation vary by developmental stage (**Figure 4B**). For the antisense transcripts that have a negative correlation with the sense mRNA, we note that the antisense transcripts tend to have their expression peak in extracellular stages (**Figure 4B**). To explore the possibility of a repressive role for these negatively correlated anti-sense transcripts in oocysts, we examined GO enrichment for the corresponding sense mRNAs. No significant GO functions were enriched because most sense mRNAs are uncharacterized protein-encoding genes. However, one sense-antisense pair is noteworthy. Cp_lnc_7-RA is highly expressed in oocyst/

sporozoites and negatively correlated with its sense mRNA CPATCC_0034040 (Guanine nucleotide exchange factor/ Initiation factor 2 subunit family) with Pearson's r of −0.90. This protein facilitates and controls protein synthesis in eukaryotes by mediating guanine nucleotide exchange (Gordiyenko et al., 2014). We note that CPATCC_0034040 is silenced in oocyst/sporozoites and becomes active in later stages. Further experiments are needed to determine if Cp_lnc_7-RA plays any role in this regulation.

Compared to expression of random gene pairs, antisense transcripts and their upstream mRNAs have a higher positive correlation of expression level on average **(Figure 4A)**, despite the fact that potential read-through transcripts have already been removed (309 out of 363 antisense genes are arranged in a head-to-head orientation with the corresponding upstream mRNA). 110 antisense genes have high positive expression correlation with their upstream mRNAs (r > 0.7) while only 69 and 89 correlated with the downstream and sense mRNAs, respectively. LincRNAs also show a higher positive expression correlation with upstream mRNAs **(Figure 4C).** One possible explanation for the higher positive transcription correlation between lncRNAs and upstream mRNAs in *C. parvum* is the potential existence of bidirectional promoters. Bidirectional promoters have been reported in many organisms especially species with compact genome sequences. In *Giardia lamblia*, bidirectional transcription is considered to be an inherent feature of promoters and contributes to an abundance of antisense transcripts (Teodorovic et al., 2007).

When antisense expression is compared to downstream mRNA expression, more negative correlations are observed relative to antisense-sense mRNA and antisense-upstream mRNA pairs. This may suggest the presence of transcriptional interference such as transcriptional machinery collision between antisense and its downstream mRNA (Hobson et al., 2012; Kindgren et al., 2018) as one possible explanation.

Finally, we note that a large proportion of antisense transcripts (102 out of 363) and their corresponding sense, upstream and downstream neighboring mRNAs do not show significant expression correlations, i.e. their r values are between −0.51 and 0.56 (**Figure 4A; Supplementary Table S7**). Seven of these uncorrelated transcripts have been reported to be delivered into the host cell nucleus (Wang et al., 2017b) (**Supplementary Table S7**). Another reason for a lack of correlation may stem from possible roles in post-transcriptional and translational processes rather than transcriptional regulation.



	r <- 0.70	r <= -0.51	-0.51 < r < 0.56	r >= 0.56	r > 0.70
Random gene pairs (10,000)	1348	2368	4374	3258	2208
Antisense-downstream mRNA (363)	39	89	171	103	69
Antisense-sense mRNA (363)	26	62	174	127	89
Antisense-upstream mRNA (363)	18	32	182	149	110

FIGURE 4 | Analysis of lncRNA transcript expression correlation with sense and neighboring mRNA genes. The Pearson correlation coefficient was used to measure the expression correlation of different types of gene pair relationships using VST expression levels from the 33 RNA-Seq samples. The width of the plot is indicative of the number of genes at a given Pearson correlation value. **(A)** Antisense expression relationships with sense and neighboring mRNAs. Random gene pairs were selected from all genes including lncRNAs to assess their expression correlation as the background control. The relative genomic position of lncRNAs relative to other neighboring genes is indicated on the righthand side. For example, a given antisense RNA (red arrow) is shown relative to its sense gene mRNA on the opposite DNA strand and upstream and downstream mRNA encoding genes (shown in blue). The coding strand of the upstream and downstream mRNAs may be positive or negative for the purpose of the calculations. This context was used to evaluate the statistics for each category of lncRNA/mRNA relationship pair. The results are listed in the table at the bottom of panel **(A)**. **(B)** Antisense expression correlation with sense mRNA and neighboring gene mRNAs are distributed according to parasite developmental stage. Antisense transcripts were assigned to different developmental stages based on their expression peak. The mean value is indicated by an "X" in each plot. **(C)** LincRNA expression correlation with neighboring gene mRNAs.

Many lncRNAs Are Conserved Between *Cryptosporidium parvum*, *Cryptosporidium hominis*, and *Cryptosporidium baileyi*

Evolutionary conservation of a lncRNA can imply functional importance. lncRNAs can be conserved in different dimensions: the sequence, structure, function, and expression from syntenic loci (Diederichs, 2014). lncRNAs are considered to be poorly conserved at the primary sequence level between genera as reported in many higher eukaryotes. Here, we looked for expression conservation of *C. parvum* lncRNA in two other *Cryptosporidium* species from syntenic loci (observed conserved antisense expression from orthologous genes) with available stranded RNA-Seq data that include *C. hominis*, a very close relative of *C. parvum* and *C. baileyi*, a distant relative that infects birds (Slapeta, 2013). First, we assembled the oocyst/sporozoite transcriptome (the only stranded samples that exist) of *C. hominis* 30976 and *C. baileyi* TAMU-09Q1 by the same methods as used previously except we did not use reference genome annotation guidance (-G). A total of 167 *C. parvum* antisense lncRNAs were detected in both *C. hominis* and *C. baileyi* (**Supplementary Table S8**). Of these, 10 are putatively conserved in *P. falciparum* (**Supplementary Table S9**) based on the presence of antisense lncRNA expression of the orthologus sense gene in *P. falciparum* (Broadbent et al., 2015). The sense mRNAs were related to functions that included a methyltransferase protein, a palmitoyltransferase and a copper transporter. No significant sequence similarities were detected among the antisense lncRNAs of these orthologs in *C. parvum* and *P. falciparum*. This is not surprising given the evolutionary distance and AT bias of *P. falciparum*.

Since the samples of *C. hominis* 30976 and *C. baileyi* TAMU-09Q1 were from oocysts/sporozoites, we focused on 48 of the 167 conserved *C. parvum* lncRNAs that showed a higher expression level in the extracellular stages (**Figure 5**). The corresponding sense mRNAs were involved in various biological processes. Translation related functions, including translational initiation (cgd7_2430, translational initiation factor eIF-5) and protein folding (cgd2_1800, DnaJ domain-containing protein), were seen in the sense mRNAs. A positive correlation of 0.78 and a negative correlation of −0.78 was calculated for cgd7_2430 sense-antisense pair and cgd2_1800 sense-antisense pair, respectively. In addition, a few mRNAs that encode putative secreted proteins (cgd5_10 and cgd4_3550) also showed a high positive correlation of expression with the corresponding antisense.

lncRNA Prediction Validation

In the RNA-Seq data, Cp_lnc_51 was expressed in oocysts/sporozoites while the associated sense mRNA cgd1_380 (Ubiquinone biosynthesis protein COQ4) was seen to be silenced the RNA-Seq data. The expression levels for the sense and antisense transcripts were validated by stranded RT-qPCR (**Figure 6A**). To validate lncRNA by strand-specific RT-PCR (Ho et al., 2010), a specific RT primer was designed for each gene to generate the appropriate stranded cDNA (**Supplementary Table S2**). Cp_lnc_51 contains an intron as does its sense mRNA. Splicing of Cp_lnc_51 was confirmed by RT-PCR and agarose gel electrophoresis to assess the transcript size (**Figure 6B**), however, the splicing is observed in all RT-PCR products. The existence of splicing was also observed in the sense transcript (**Figure 6B**). We randomly selected five additional lncRNAs that were expressed in RNA-Seq data for oocyst/sporozoites for validation. Two out of the five, Cp_lnc_82 (**Figure 6C**) and Cp_lnc_93 (**Figure 6D**) were validated by qPCR. The relative expression of sense-antisense is consistent with the RNA-Seq data.

DISCUSSION

In this study, we utilized stranded RNA-Seq data from multiple time points during parasite development to systematically identify and characterize lncRNAs in *C. parvum*. 396 high-confidence lncRNAs were identified, 363 (91.7%) occur as antisense transcripts to mRNAs and 33 are encoded in intergenic locations. Nearly 10% of predicted mRNAs are covered by an antisense lncRNA. This level of antisense transcription suggests an important function for lncRNA in *C. parvum*. The lncRNAs were analyzed to determine expression profiles, promoter motifs for coordinately expressed transcripts, transcriptional relationships with upstream and downstream mRNAs and conservation among three *Cryptosporidium* species with stranded RNA-Seq data available.

To investigate the expression relationship of lncRNAs and their neighboring mRNA encoding genes, we calculated the expression correlation of different types of gene pairs by Pearson coefficient and noticed a higher positive correlation of expression between lncRNAs and their upstream mRNAs (primarily from the opposite strand) compared to random gene pairs. Notably, spurious correlations of gene expression can happen if the biological variation among samples is too large. Due to the challenge of *in vitro* culture for *C. parvum* and very low number of the parasite transcripts compared to their host cells, samples from the early intracellular stages are rare and usually contain very low levels of parasite transcripts. In this study, transcriptome data from early (< 24 h) intracellular timepoints are absent. We detected a bimodal shape for the distribution of expression correlation with random gene pairs showing trends at both high positive and negative values, probably due to spurious correlation. However, antisense transcripts showed much less negative correlation of expression with upstream than random gene pairs. One possible explanation for the higher positive correlation between lncRNA and their neighboring mRNAs would be the presence of bidirectional promoters in *C. parvum*. Another possibility is that lncRNAs function as positive regulators of neighboring mRNA expression. In *P. falciparum*, ncRNAs derived from GC-rich elements that are interspersed among the internal chromosomal *var* gene clusters are hypothesized to play a role in *var* gene activation while the mechanism is unclear (Guizetti et al., 2016; Barcons-Simon et al., 2020). lncRNAs have also been associated with chromatin remodeling to achieve transcriptional regulation in many studies (Li et al., 2020). One example is a lncRNA named HOTTIP which is transcribed from the 5′ tip of the HOXA locus that coordinates the activation of HOXA genes by maintaining active chromatin (Wang et al., 2011).

lncRNA ID	Pearson's r	Sense mRNA description
Cp_lnc_201	0.89	uncharacterized Secreted Protein
Cp_lnc_204	-0.37	CDC50/LEM3 protein
Cp_lnc_242	-0.09	DnaJ domain containing protein
Cp_lnc_316	-0.79	Uncharacterized protein
Cp_lnc_357	-0.62	unspecified product
Cp_lnc_362	-0.26	Signal Peptide Uncharacterized transmembrane Protein
Cp_lnc_45	NA	NA
Cp_lnc_88	0.56	Uncharacterized protein
Cp_lnc_149	0.55	Uncharacterized protein
Cp_lnc_19	0.78	Secreted Kazal domain-containing protein
Cp_lnc_193	-0.56	Uncharacterized protein
Cp_lnc_212	0.30	Actin depolymerizing factor
Cp_lnc_213	0.77	Zinc finger, RING/FYVE/PHD-type domain containg protein
Cp_lnc_226	-0.22	Zinc finger RanBP2-type domain containing protein
Cp_lnc_23	-0.01	SPAC694.02. SKI family SFII helicase
Cp_lnc_286	0.80	Uncharacterized protein
Cp_lnc_297	0.72	L-lactate/malate dehydrogenase
Cp_lnc_31	0.87	Uncharacterized protein with Tetratricopeptide-like helical
Cp_lnc_321	0.77	RRM/Zinc finger, RING/FYVE/PHD-type domain containg protein
Cp_lnc_322	0.41	Uncharacterized protein
Cp_lnc_327	0.51	Aspartate--tRNA ligase
Cp_lnc_337	-0.71	26S Proteasome non-ATPase regulatory subunit 7/8
Cp_lnc_360	-0.06	Uncharacterized protein
Cp_lnc_393	0.46	Nucleolar protein 14
Cp_lnc_64	-0.55	RNA recognition motif domain containing protein
Cp_lnc_87	-0.36	Proteasome regulatory subunit Rpn7/26S proteasome subunit 6
Cp_lnc_13	-0.37	O-acyltransferase WSD1 C-terminal domain containin
Cp_lnc_15	0.47	tRNA N6-adenosine threonylcarbamoyltransferase Kae
Cp_lnc_167	-0.27	60S ribosomal protein L27A
Cp_lnc_173	-0.67	Uncharacterized protein
Cp_lnc_174	0.55	NifS-like protein cysteine desulfurase/Pyridoxal phosphate-dependent transferase
Cp_lnc_257	0.83	DEAD/DEAH box helicase with GUCT domain
Cp_lnc_276	-0.67	Ferredoxin-like protein
Cp_lnc_320	-0.13	Amino acid transporter
Cp_lnc_326	0.79	Uncharacterized protein
Cp_lnc_33	0.78	Translation initiation factor eIF-5 Tif5p/ZnR+W2 domain-containing protein
Cp_lnc_373	-0.66	Asparaginyl-tRNA synthetase (NOB+tRNA synthase)
Cp_lnc_384	-0.74	HAS/SNF2/Helicase/SANT-associated domain containing protein
Cp_lnc_395	NA	NA
Cp_lnc_40	0.81	IMP4-like U3 small nucleolar ribonucleoprotein (SnoRNP)
Cp_lnc_44	0.76	Signal peptide region containing protein
Cp_lnc_50	-0.73	carboxypeptidase A with signal peptide region
Cp_lnc_6	0.63	Uncharacterized protein
Cp_lnc_7	-0.26	Guanine nucleotide exchange factor/Initiation factor 2 subunit family
Cp_lnc_75	0.84	Sas10/Utp3/C1D-like protein
Cp_lnc_82	0.88	ABC transporter
Cp_lnc_85	-0.78	DnaJ domain containing protein
Cp_lnc_95	-0.83	Uncharacterized protein

FIGURE 5 | Highly expressed *C. parvum* lncRNAs with conservation and expression in *C. hominis* and *C. baileyi* oocysts. The heatmap visualizes the lncRNA expression level of 48 conserved *C. parvum* genes across 33 RNA-Seq samples, grouped as extracellular (oocyst/sporozoite, 0 h and 2 h) and intracellular (24 h and 48 h) stages. The lncRNA name and the corresponding sense mRNA description are listed. An mRNA description of "NA" indicates the lncRNA is intergenic. The sense-antisense expression correlation coefficient is shown in the bracket. The color scale is shown on the left. Yellow indicates high levels of expression.

Antisense transcripts have a strong bias towards covering the 3′ end of the sense mRNA. This property has also been reported in other organisms, including the malaria pathogen *P. falciparum* (Siegel et al., 2014; Broadbent et al., 2015). As these authors suggest, one possibility is that antisense RNAs arise from promiscuous transcription initiation from nucleosome depleted regions (Siegel et al., 2014). It is also known that the 3′ UTR of mRNAs can contain elements that are important for transcript cleavage, stability, translation and mRNA localization. The 3′ UTR serves as a binding site for numerous regulatory elements including proteins and microRNAs (Jia et al., 2013; Tushev et al., 2018). In humans, the antisense *KAT5* gene has been reported to promoted the usage of distal polyA (pA) site in the sense gene *RNASEH2C*, which generated a longer 3′ untranslated region (3′ UTR) and produced less protein, accompanied by slowed cell growth (Shen et al., 2018). Whether the 3′ end bias of antisense expression related to its function and translation repressor need further investigation.

The enriched expression of lncRNAs in extracellular and late intracellular stages, the time point when the parasite expresses sexual commitment and produces gametes, suggests potential critical roles that lncRNAs may play during these life cycle stages. We specifically analyzed the transcription correlation between sense-antisense pairs in extracellular stages and late intracellular stages. Both significant positive and negative correlations between mRNA sense-antisense transcript pairs are observed in *C. parvum* which indicates that diverse RNA regulation strategies exist. Antisense transcripts/transcription as a negative regulator of sense mRNAs has been reported in many organisms including *P. falciparum* (Filarsky et al., 2018), which could also hold true in *C. parvum* for the negatively correlated sense-antisense pairs. It has been also reported that lncRNAs can regulate translation by stabilizing mRNAs, and in some cases triggering the translation process by interactions with the associated machinery (Li et al., 2020). For transcriptionally

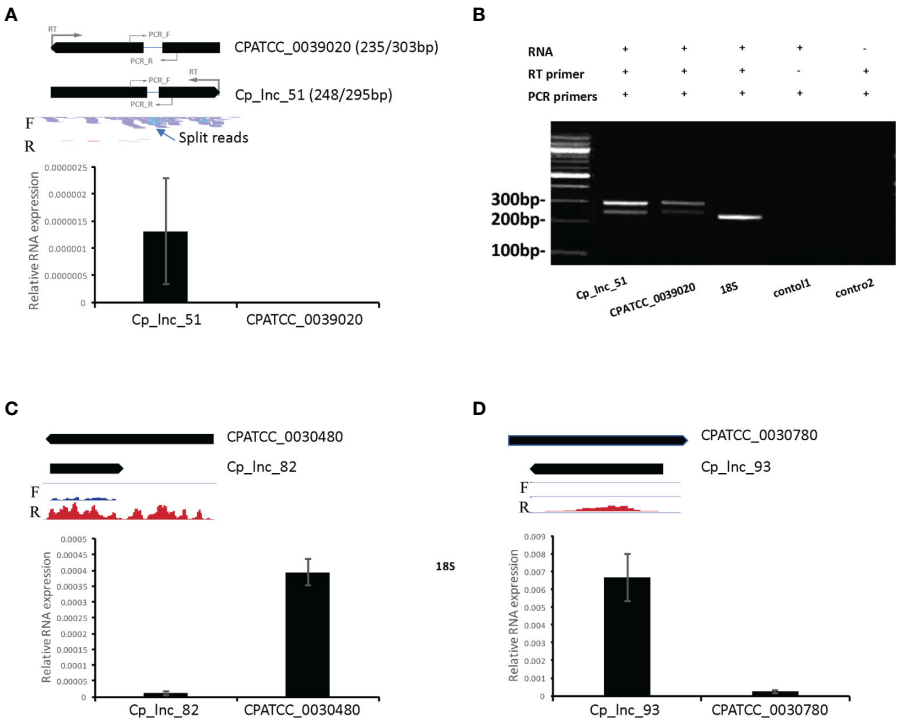


FIGURE 6 | lncRNA candidate expression and intron structure validation. **(A)** The expression levels of lncRNA Cp_lnc_51 and its corresponding sense mRNA CPATCC_0039020 were validated by RT-qPCR of RNA from oocyst/sporozoite the expression was normalized to 18S expression levels. The annotated gene model is shown on the top with RNA-Seq reads mapped to the genomic region. Location of RT primers and PCR primers for each gene are shown with the gene models. Reads are separated by the mapped strand: forward strand (F) and reverse strand (R). The intron structure of Cp_lnc_51 is indicated by the presence of split RNA-Seq reads. **(B)** Agarose gel electrophoresis of RT-PCR products for the sense and antisense transcripts. Splicing of the Cp_lnc_51 transcript and its sense mRNA CPATCC_0039020 are supported by detection of both spliced and unspliced forms for each of the transcripts of the expected sizes. The expected size of the products with/without their intron is indicated next to the gene name in panel A. 18S is used as positive control with expected size of 239bp. Control 1 is a negative control without the RT primer for CPATCC_0039020. Control 2 is a negative control for CPATCC_0039020 with both RT and PCR primers but no RNA template added. **(C)** The expression levels of lncRNA Cp_lnc_82 and its corresponding sense mRNA CPATCC_0030480 were validated by RT-qPCR. The expression was normalized to 18S expression levels. **(D)** The expression levels of lncRNA Cp_lnc_93 and its corresponding sense mRNA CPATCC_0030780 validated by RT-qPCR. The expression was normalized to 18S expression levels. The RNA-Seq coverage in C and D is with range 0–100 CPM (counts per million reads mapped).

positively correlated sense-antisense transcript pairs, we raise the testable hypothesis that lncRNAs may be involved in stabilizing transcripts that may be preloaded in macrogamonts that will eventually become oocysts. To test this hypothesis, one future direction is to take advantage of single-cell sequencing approaches and look at the transcriptomic details of male and female gametocytes. It will be interesting to see if lncRNAs are specifically expressed in male and female gametocytes and whether or not some lncRNAs are restricted specifically to these gamonts or if they are also detected elsewhere, e.g., in oocysts where they could, perhaps, have a role in transcriptional or post-transcriptional gene regulation, or mRNA stability. The amount of active transcription as opposed to RNA pre-loading in oocysts is not known.

Twenty-two *C. parvum* lncRNAs have been detected in the host cell nucleus (Wang et al., 2017a). Of these, 18 were detected in this study. Motif analysis was conducted on the exported lncRNA transcript sequences, but no significant similarity or motif was detected relative to the larger pool of lncRNA candidates identified in this study. This raises the question of what signal is responsible for lncRNA export. Further studies are needed.

A significant roadblock in lncRNA research is the determination of their function. Genetics studies are particularly tricky with antisense transcripts of the sequences overlap coding sequences closely, as they do in *C. parvum* because genetic alterations of the sequence affect the sense and antisense transcripts. lncRNAs with similar functions often lack sequence similarity (Kirk et al., 2018). Many known lncRNAs function by interacting with proteins. Proteins often bind RNA through short motifs (three to eight bp in length) (Ray et al., 2013). It was hypothesized that lncRNAs with shared functions should harbor motif composition similarities (Kirk et al., 2018). In this study, the difference in nucleotide composition between antisense RNA and lincRNA transcripts gives rise to the speculation that the machineries interacting with antisense and intergenic lncRNAs may be different.

lncRNA prediction using short reads in organisms with compact genome sequences, including *C. parvum* is limited due to the difficulty of separating independent lncRNA transcription from neighboring transcriptional read-through noise. In this study, we used a customized pipeline with strict criteria designed to minimize false positives from background noise such as transcriptional read-through. Many antisense transcriptions

overlap all or most of the sense mRNA transcript. To further improve the discovery of full-length lncRNA and any isoforms, long-read approaches such as Iso-Seq (Pacific Biosystems) and single molecule pore-sequencing approaches [Oxford Nanopore Technologies (ONT)] are needed. Although obtaining sufficient high-quality RNA from intracellular stages is still challenging, hybrid capture approaches (Gnirke et al., 2009; Amorim-Vaz et al., 2015) can be utilized to obtain *Cryptosporidium* RNA to be used for direct RNA sequencing on the ONT platform providing additional insights into the RNA biology of *Cryptosporidium*. Long-read sequencing also enables better annotation of mRNA UTR boundaries (Chappell et al., 2020) which can be used to further investigate the 3′ bias (relative to the sense transcript) of antisense transcription observed in this study.

The RNA-Seq samples from intracellular stages used in this study suffered from host contamination though we set thresholds for sample selection. For extracellular samples (sample ID 1-19 for *C. parvum*), mapping rate ranges from 76%–97% with the majority >95%. The low mapping rate dropped below 4% in intracellular. Low numbers of parasite-specific reads can lead to an underestimated number of lncRNAs, with lowly expressed lncRNAs possibly being undetectable. On the flip side, a lower false positive prediction rate is expected since the goal is to primarily characterize high-quality lncRNAs with good expression level by setting thresholds to filter out low-quality candidates.

Another limitation of this study results from the fact that the lncRNAs discussed here are exclusively generated from conventional poly(A)-enriched libraries. Thus, only polyadenylated transcripts will be identified. While many lncRNAs in the literature share characteristics of mRNAs, such as RNA polymerase II-mediated transcription, a 5′ 7-methylguanosine cap and a 3′ polyadenylated tail (Sun et al., 2018), many non-polyadenylated lncRNAs have been reported including enhancer-derived lncRNAs (Li et al., 2016) and RNA Polymerase III transcribed lncRNAs (Sun et al., 2018). Thus, determination of the full repertoire of lncRNAs requires an analysis of total RNA. The contributions of polyA+ and polyA- lncRNAs to the complete repertoire of lncRNA remains unclear including in model organism species. This is in part due to the instability of polyA- RNAs (Li et al., 2016) and the caveats of Illumina short read sequencing coupled with transcriptome assembly algorithms that predict transcripts (small RNAs excluded) which suffer from low 3′ and 5′ coverage (Uszczynska-Ratajczak et al., 2018). In *C. parvum*, the non-poly(A) selected library suffered from high levels of rRNA contamination (data not shown) since no commercial kits specific for apicomplexan or other protist parasite rRNA removal are available. In an rRNA depleted non-poly(A)+ selected total RNA library from *C. parvum* oocysts/sporozoites generated by our lab (data not shown), we were able to detect 134 (82.7%) out of the 162 antisense transcripts with peak expression in extracellular stages found in this study suggesting that we have not missed a huge population of non-poly(A) transcripts by focusing on the available poly(A)+ libraries (Li et al., in prep). Only ~20 new potential non-polyadenylated lncRNAs were detected in the rRNA depleted library. This finding suggests the possibility that lncRNAs in *C. parvum* are primarily polyadenylated.

It is important to understand how species evolve and adapt to their environment. Due to the poor conservation of lncRNA reported in higher eukaryotes (Johnsson et al., 2014) and the large phylogenetic distance among *Cryptosporidium* species (Slapeta, 2013), it is noteworthy that many lncRNAs detected in *C. parvum* were also seen expressed in *C. baileyi*. It indicates that RNA regulation could be a common and critical strategy for *Cryptosporidium* gene regulation or interactions with their hosts. The conserved antisense RNA expression between *C. parvum* and *P. falciparum* orthologs did not share detectable sequence similarity. However, the structure of lncRNAs could be under stronger selection than their sequence. Since most lncRNAs are antisense in *C. parvum*, to separate conservation of lncRNA from the conservation of mRNA sequence could provide further insights into lncRNA evolution. Selection pressures that independently act to maintain sequence and secondary structure features can lead to incongruent conservation of sequence and structure. As a consequence, it is possible that analogous base pairs no longer correspond to homologous sequence positions. Thus, possible selection pressures independently acting on sequence and structure should be taken into account (Nowick et al., 2019). Despite the increasing acknowledgment that ncRNAs are functional, tests for ncRNAs under either positive or negative selection did not exist until recently (Walter Costa et al., 2019). This type of analysis will assist in identifying candidates to prioritize for further functional lncRNA investigations.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

YL, RB, and JK conceptualized the work. BS and AS provided the *Cryptosporidium parvum* RNA and access to stranded libraries pre-publication. YL and JK wrote the manuscript, and all authors edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.608298/full#supplementary-material

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High-Resolution Mapping of Transcription Initiation in the Asexual Stages of *Toxoplasma gondii*

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Toxoplasma gondii is a common parasite of humans and animals, causing life-threatening disease in the immunocompromized, fetal abnormalities when contracted during gestation, and recurrent ocular lesions in some patients. Central to the prevalence and pathogenicity of this protozoan is its ability to adapt to a broad range of environments, and to differentiate between acute and chronic stages. These processes are underpinned by a major rewiring of gene expression, yet the mechanisms that regulate transcription in this parasite are only partially characterized. Deciphering these mechanisms requires a precise and comprehensive map of transcription start sites (TSSs); however, *Toxoplasma* TSSs have remained incompletely defined. To address this challenge, we used 5′-end RNA sequencing to genomically assess transcription initiation in both acute and chronic stages of *Toxoplasma*. Here, we report an in-depth analysis of transcription initiation at promoters, and provide empirically-defined TSSs for 7603 (91%) protein-coding genes, of which only 1840 concur with existing gene models. Comparing data from acute and chronic stages, we identified instances of stage-specific alternative TSSs that putatively generate mRNA isoforms with distinct 5′ termini. Analysis of the nucleotide content and nucleosome occupancy around TSSs allowed us to examine the determinants of TSS choice, and outline features of *Toxoplasma* promoter architecture. We also found pervasive divergent transcription at *Toxoplasma* promoters, clustered within the nucleosomes of highly-symmetrical phased arrays, underscoring chromatin contributions to transcription initiation. Corroborating previous observations, we asserted that *Toxoplasma* 5′ leaders are among the longest of any eukaryote studied thus far, displaying a median length of approximately 800 nucleotides. Further highlighting the utility of a precise TSS map, we pinpointed motifs associated with transcription initiation, including the binding sites of the master regulator of chronic-stage differentiation, BFD1, and a novel motif with a similar positional arrangement present at 44% of *Toxoplasma* promoters. This work provides a critical resource for functional genomics in *Toxoplasma*, and lays down a foundation to study the interactions between genomic sequences and the regulatory factors that control transcription in this parasite.

Keywords: Apicomplexa, transcription start site mapping, 5′-end RNA sequencing, 5′ untranslated regions, uORF, nucleosome positioning, transcriptional regulation, core-promoter elements

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INTRODUCTION

A precise map of transcription start sites (TSSs) is indispensable for identifying promoters and other regulatory factors that mediate gene expression in an organism. Quantitative maps of transcription initiation have revealed the complex and dynamic nature of transcription initiation landscapes in a range of model eukaryotes, including yeast, flies, worms, zebrafish, and mammals (e.g., Carninci et al., 2006; Hoskins et al., 2011; Chen et al., 2013; FANTOM Consortium and the RIKEN PMI and CLST (DGT) et al., 2014; Haberle et al., 2014; Lu and Lin, 2019). These studies have elucidated the architecture of prototypical eukaryotic promoters (Lenhard et al., 2012; Haberle and Stark, 2018). However, many unicellular eukaryotic organisms, like those belonging to the phylum Apicomplexa, differ extensively from model systems (Baldauf, 2003) and therefore demand tailored analyses of transcription initiation and regulation.

Apicomplexa include the causative agents of widespread human diseases, such as *Toxoplasma gondii*, *Plasmodium* spp., and *Cryptosporidium* spp. These parasites have complex life cycles that typically involve several developmental stages underpinned by distinct transcriptional programs. This complexity is illustrated by *Toxoplasma*, which can infect and replicate within any nucleated avian or mammalian cell, establishing both acute and chronic stages in intermediate hosts and undergoing sexual recombination within the definitive feline hosts. Asexual replication in intermediate hosts, like humans, is characterized by rapidly-dividing tachyzoites (Tz), which convert into the slow-replicating bradyzoites (Bz) that establish chronic infections lasting for the life of the host (Dubey, 2016). Transcriptome analyses using microarray and RNA sequencing (RNA-seq) methods have highlighted coordinate changes in the expression levels of numerous mRNAs as *Toxoplasma* transitions between intra- and extracellular environments (Hassan et al., 2017), converts between sexual and asexual stages (Fritz et al., 2012; Behnke et al., 2014; Farhat et al., 2020), and between acute (Tz) and chronic (Bz) stages (reviewed in Jeffers et al., 2018, also Garfoot et al., 2019; Ramakrishnan et al., 2019; Waldman et al., 2020; Xue et al., 2020).

Despite major progress in identifying the transcription factors that govern these transitions (e.g., Balaji et al., 2005; Farhat et al., 2020; Waldman et al., 2020), the lack of a comprehensive TSS map has limited advances in decrypting the interactions between *cis*- and *trans*-regulatory factors that converge at the promoters of regulated genes. The models of 8322 protein-coding genes in the *Toxoplasma* genome (ME49, ToxoDB v.45) were largely generated *via* computational prediction of coding sequences (CDSs) on the basis of RNA-seq and pre-RNA-seq expression data. However, 5′ and 3′ untranslated regions (UTRs), including TSSs, have remained incompletely defined due to the lack of data specifically addressing transcript boundaries. While standard RNA-seq is a powerful approach for quantifying gene expression, identifying splice isoforms, and discovering novel transcripts, it fails to accurately capture the 5′ end of transcripts, where information on TSSs and 5′ UTRs (also called 5′ leaders) is contained. This is because RNA-seq coverage is seldomly uniform, and typically diminishes toward the 5′ end of transcripts, owing to biases introduced during common sample preparation procedures (Wang et al., 2009; Levin et al., 2010). In particular, RNA-seq protocols do not select for 5′-intact mRNA, and instead often enrich mRNA *via* the polyadenylated tail, which favors 3′-end representation (Chao et al., 2019). In addition, the random fragmentation of mRNA reduces the efficiency of cDNA synthesis at transcript flanks, which contributes to the lack of sequencing coverage at 5′ ends (Wang et al., 2009). Indeed, available RNA-seq datasets in *Toxoplasma* are largely 3′-biased, and lack coverage of intact 5′ ends. This precludes the identification of 5′ termini at the resolution required to (i) correlate TSSs with sequence or chromatin elements that might influence gene expression, and to (ii) discern between and to accurately quantify distinct 5′ termini arising from alternative TSSs which can contribute to the functional or regulatory complexity of genes (Davuluri et al., 2008; de Klerk and 't Hoen, 2015).

Numerous assays have been developed for profiling transcription initiation genome-wide, all based on the capture and preferential sequencing of intact 5′ mRNA ends, called 5′ tags. In contrast to conventional RNA-seq, these 5′-end RNA-seq protocols provide high local coverage for precise TSS prediction, and enable the profiling of distinct TSSs that are linked to the same gene. A pioneering study published in 2010 provided the first such systematic assessment of transcription initiation in *Toxoplasma*, and suggested an absence of canonical eukaryotic core-promoter elements (Yamagishi et al., 2010). In *Plasmodium falciparum*, recent genomic mapping and profiling of TSSs throughout its intra-erythrocytic life cycle revealed a highly-complex and dynamic landscape of transcription initiation (Adjalley et al., 2016), suggesting a previously unknown diversity of transcripts with alternative 5′ termini. In *Toxoplasma*, the Tz-to-Bz stage conversion is accompanied by drastic changes in the mRNA expression levels of more than 2000 genes (Ramakrishnan et al., 2019; Waldman et al., 2020), but alternative stage-specific TSS usage has remained unexplored.

Here, we report an in-depth analysis of transcription initiation at *Toxoplasma* promoters in both acute (Tz) and chronic stages (Bz). Using state-of-the-art approaches for systematically characterizing mRNA 5′ ends (Batut and Gingeras, 2013; Adiconis et al., 2018), we generated a genome-wide map of transcription initiation at single-nucleotide resolution. We empirically defined dominant TSSs for the majority of *Toxoplasma* genes, revising most current gene models and providing an avenue for improved genome annotation through a comprehensive definition of 5′ leaders. Comparing TSS usage between Tz and Bz stages, we identified genes with alternative TSSs, some of which are regulated stage-dependently. Analysis of the nucleotide content and nucleosome occupancy around TSSs allowed us to examine the determinants for TSS choice, and outline features of *Toxoplasma* promoter architecture. Given the high complexity and pliability of *Toxoplasma* transcription, this report constitutes a highly-valuable resource for further

investigations into the mechanisms directing TSS selection. Our study also provides a framework for functional genomics studies, specifically for targeted promoter manipulations using forward genetic approaches.

MATERIALS AND METHODS

Parasites and Host Cells

Toxoplasma parasites were cultured at 37 °C in human foreskin fibroblasts (HFFs, ATCC SCRC-1041). Tz from the strains RH and ME49 were maintained at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 3% or 10% heat-inactivated fetal bovine serum (IFS) and 10 μg/ml gentamicin (Thermo Fisher Scientific), referred to as standard medium. Bz from the strain ME49 were maintained at ambient CO_2 in alkaline-stress medium, consisting of RPMI 1640 (Sigma), supplemented with 1% IFS and 10 μg/ml gentamicin, and buffered with 50 mM HEPES, adjusted to pH 8.1 with 10 N NaOH. HFFs that were destined for use in Bz experiments were maintained exclusively in DMEM supplemented with 10% IFS and 10 μg/ml gentamicin prior to infection.

Bradyzoite Conversion and Harvest

ME49 parasites were grown in HFFs and standard medium, before changing to alkaline-stress medium at 24 h post-infection. At this time point, to remove residual standard medium, the infected HFF monolayer was washed once with alkaline-stress medium. Parasites were grown for an additional 48 h at 37°C at ambient CO_2 to allow for stage conversion. The medium was then aspirated and HFF monolayers rinsed with PBS. Following addition of 3 ml PBS, the host-cell monolayer was detached by scraping, followed by the mechanical release of parasites by serially passing once through 27- and twice through 30-gauge needles before filtering through a polycarbonate filter with a 3-μm pore size (Whatman). The efficiency of stage conversion was estimated at 98%, as assessed by immunofluorescence staining and microscopy at the time of the Bz harvest. Guinea-pig anti-CDPK1, diluted 1:1000, provided a general parasite stain, while fluorescein-labeled dolichos biflorus agglutinin (DBA; Vector Laboratories), diluted 1:150, served as an early-Bz marker. DBA is a lectin that recognizes *N*-acetylgalactosamine on the Bz-specific cyst-wall protein CST1 (Tomita et al., 2013).

Tachyzoite Harvest

RH Tz were allowed to egress naturally, while ME49 Tz were mechanically released. First, the medium was aspirated and HFF monolayers rinsed with PBS. Following the addition of 3 ml PBS, the host-cell monolayer was detached by scraping, followed by the mechanical release of parasites by passing through a 27-gauge needle. Egressed RH, and mechanically-released ME49 Tz were passed through a polycarbonate filter with a 3-μm pore size (Whatman).

RNA Extraction and DNaseI Digest

Parasite suspensions were centrifuged for 5 min at 1000 × *g*, and washed once in PBS. Pellets were resuspended in TRIzol (Ambion) at about 1 ml per 5 × 10^6 parasites, followed by vortexing, and incubating at room temperature for 5 min. Homogenized samples were flash-frozen in liquid N_2 and intermittently stored at -80°C. Upon complete thawing at room temperature, samples were loaded onto MaXtract High Density tubes (Qiagen), and total RNA was extracted as per manufacturer's instructions. RNA pellets were resuspended in nuclease-free water and concentration was determined using the Qubit RNA HS Assay Kit (Thermo Fisher). Genomic DNA contamination was removed from total RNA extracts using the TURBO DNA-free Kit (Invitrogen), following the manufacturer's protocol (routine DNase treatment). Digest of DNA contaminants was confirmed by PCR on extracts before and after this procedure.

Construction of RAMPAGE Libraries

RAMPAGE libraries were constructed from 5 μg of DNaseI-digested total RNA per biological replicate, largely following a previously published protocol (Batut and Gingeras, 2013; Batut et al., 2013) with the following modifications. (1) We extended the digest of 5′-monophosphate RNAs with 5′-phosphate-dependent exonuclease (Terminator Exonuclease; Lucigen) from 90 to 120 min since we observed high-level (> 50%) rRNA contamination in a pilot preparation using the shorter incubation time. (2) We used a universal template-switching oligo (TSO) instead of the barcoded ones, as published elsewhere (Adiconis et al., 2018). (3) We used a random 15-mer (RTP) with a modified tag sequence (universal TruSeq adapter; Illumina) for reverse transcription to allow for a separate index read, and a Read 2 using standard Illumina sequencing primers, as previously published (Adiconis et al., 2018). (4) In the final PCR, we used an extended forward primer (Fwd) with an optimized melting temperature, and (5) we used reverse primers (Rev) with 6-base indices for barcoding of individual libraries for multiplexed sequencing. (6) We used Q5 DNA polymerase (NEB) in the final PCR, and we first determined the optimal cycle number in small-scale reactions to prevent over-amplification in the subsequent bulk PCR reaction. (7) We used a different custom sequencing primer for Read 1 (R1), as previously published (Adiconis et al., 2018).

Designation	Sequence (5′ – 3′)
TSO	TAGTCGAACTGAAGGTCTCCAGCArGrGrG
RTP	TAGTCGAACGAAGGTCTCCCGTGTGCTCTTCCGATCT(N)15
Fwd	AATGATACGGCGACCACCGAGATCTACACTAGTC GAACTGAAGGTCTCCAG
Rev	CAAGCAGAAGACGGCATACGAGAT[index] GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT [index] = CTTGTA, GCCAAT, AGTTCC, TAGCTT, TTAGGC, ATCACG
R1	TACACTAGTCGAACTGAAGGTCTCCAGCAGGG

Sequencing of RAMPAGE Libraries

Paired-end sequencing was performed with 75-nt reads, and a 6-nt index read. In a pilot, RH replicate libraries were sequenced on a MiSeq (Illumina), which revealed an ~85% G-nucleotide bias at the first position of Read 1, complicating the use of a NextSeq platform (Illumina) for sequencing of the full set of six RAMPAGE libraries. Read mapping to the ME49 genome assembly (v.45) revealed that ~85% of reads started with one non-encoded G, and ~10% of reads started with two non-encoded Gs, which were introduced in the non-templated addition of C nucleotides during reverse transcription. We therefore chose to employ an initial five chemistry-only (dark) cycles to enable sequencing on a NextSeq. 5′-tag positions were then shifted 5′ by 4 nt, which should match the position of the 5′-most nucleotide for ~85% of the original mRNA molecules, assuming all six libraries were structured similarly. This is expected to result in a minor loss of positional resolution. Analyzing the nucleotide composition around predicted TSSs revealed the canonical pyrimidine-purine dinucleotide at the -1 and 0 positions, which further validates the positional correction.

Processing RAMPAGE Sequencing Data

Low-quality reads, and reads aligning to rRNA were filtered using TagDust2 v.2.33 (Lassmann, 2015) and a curated reference FASTA file containing all rRNA sequences annotated in the ME49 genome assembly (v.45), and the following settings:

-t 4 -dust 97 -fe 3 -1 R:N

Filtered reads were mapped onto the ME49 genome assembly (v.45) using STAR v.2.7.1a (Dobin et al., 2013) with the following settings:

–runMode alignReads –sjdbFileChrStartEnd […] –alignIntronMax 6000 –alignSJoverhangMin 8 –alignSJDBoverhangMin 1 –readMatesLengthsIn NotEqual –alignMatesGapMax 1000 –outFilterMultimapNmax 1 –outFilterMismatchNoverLmax 0.04 –outSAMprimaryFlag AllBestScore –clip5pNbases 0 15 –outSAMtype BAM SortedByCoordinate –outSAMorder Paired

PCR duplicates were flagged within the BAM file using STAR v.2.7.1a with the following settings:

–inputBAMfile […] –bamRemoveDuplicatesType UniqueIdentical –runMode inputAlignmentsFromBAM –bamRemoveDuplicatesMate2basesN 15

Flagged duplicates were then removed with SAMtools v.1.10 (Li et al., 2009) using:

samtools view -b -F 0x400

BedGraph files of 5′ tags were then generated from BAM files using STAR v.2.7.1a:

–inputBAMfile […] –runMode inputAlignmentsFromBAM –outWigType bedGraph read1_5p –outWigNorm None

Positions of 5′ tags in the BedGraph files were shifted 5′ by 4 nt, and absolute values were normalized by dividing counts by the total number of counts per sample, and multiplying by 1000000 to generate counts per million (CPM).

Data Analysis

Analyses were performed in Python or R, and plots generated in R (http://www.R-project.org) unless otherwise noted.

Defining Gene-Association Windows

Strand-specific 5′-tag-count-to-gene–association windows were defined from -3500 nt to +200 nt relative to the start codons of the 8322 protein-coding genes (ME49 v.45). Upstream window sizes were shortened by the stop codon of an upstream gene on the same strand, or by contig boundaries.

Density of 5′ Tags Across Gene-Association Windows

This analysis was restricted to (i) genes with unshortened gene-association windows, and (ii) genes with cumulative 5′-tag counts above the 1st percentile of the respective dataset, arriving at the sample sizes indicated in the graphs. The 5′-tag count at each position within individual gene-association windows was divided by the cumulative 5′-tag count for the respective window. Density values from biological replicates were then averaged for each position, followed by averaging densities for each position across all gene-association windows to generate 5′-tag counts as percent of cumulative 5′-tag counts. A scatter plot with a trendline and 95% confidence interval was generated in R using the ggplot2 package with the geom_smooth argument set to: method = "gam", formula = y ~ s(x, bs = "cs"). The empirical cumulative density plot was also generated with these values.

Defining *Toxoplasma* TSSs

TSSs were identified separately for each biological replicate as the position with the highest 5′-tag count within a gene-association window. Replicate TSSs were flagged as reproducible if they were within 40 nt, or as non-reproducible if they were farther than 40 nt apart. For reproducible TSSs, the rounded geometric center between the two TSSs was used for plotting and subsequent analyses, and are also available in a table compilation (**Data S1**). Genes with a TSS determined in only one of the replicates were discarded. Scatterplots of reproducible and non-reproducible TSSs were generated in R, as were violin and box plots of reproducible TSSs.

Comparing ME49 Bz and Tz TSSs

This comparison was restricted to TSSs that were reproducible at the nucleotide level between biological replicates of ME49 Bz and Tz. TSSs between both stages were then compared by their distance to annotated start codons, whereas TSSs farther than 40 nt apart were flagged as putative stage-specific alternative TSSs. Genes for which a TSS was only identified in one stage were flagged as unique. The set of unique Bz genes (n = 1017) was defined as "inactive" genes in Tz for the analyses pertaining to the nucleosome occupancy around inactive Bz TSSs.

Comparison of TSS Predictions to Current Models

A Tz-biased approach was chosen to curate a list of TSSs from both Bz and Tz stages. TSS predictions were successively added, only if not already present in the list, starting with TSSs that were reproducible to the nucleotide position between all ME49 replicates (category I), followed by reproducible to the nucleotide position between ME49 Tz replicates (category II),

reproducible to the nucleotide position between ME49 Bz replicates (category III), reproducible within 40 nt between ME49 Tz replicates (category IV), reproducible within 40 nt between ME49 Bz replicates (category V), and reproducible at the nucleotide position between two ME49 replicates (category VI). For categories IV and V, the geometric center between TSSs from biological replicates was used to define the position of the TSS prediction. Predictions are available in a table compilation (**Data S1**) and BED format (**Data S2**) for display in genome browsers. Predictions from this study and TSS annotations in the ME49 reference annotation (v.45) were evaluated by their distance to annotated start codons of their associated genes.

Generation of DNA and RNA Sequence Logos

Sequence logos were generated using the WebLogo v.3.7.5 command line tool (Crooks et al., 2004).

RNA-Seq and Differential Expression Datasets

Data from conventional RNA-seq were previously published and used here as follows. For correlation analyses between RNA-seq TPM and RAMPAGE cumulative 5′-tag CPM, unstranded RNA-seq data was used from ME49 Tz and alkaline-stress–induced Bz (Waldman et al., 2020). ME49 Bz to Tz differential expression data was derived from these two ME49 datasets as previously published (Waldman et al., 2020); briefly, differential expression in Bz compared to Tz ($\log_2$ scale) was determined using the DESeq2 R package v.1.21.16 (Love et al., 2014), with an adjusted *p*-value cutoff of 0.001. For all visualizations of RNA-seq coverage, stranded RNA-seq data from an engineered ME49 Shield-1–inducible BFD1-overexpression strain was used, with data corresponding to populations maintained in the presence (Bz) or absence (Tz) of Shield-1 as described and analyzed previously (Waldman et al., 2020).

MNase-Seq Data and Analysis

Previously published MNase-seq data from *Toxoplasma* Pru Tz were retrieved (Farhat et al., 2020). Raw sequencing reads were adapter- and quality-trimmed using Trim Galore v.0.4.1 (github.com/FelixKrueger/TrimGalore) (-quality 20 –illumina -stringency 3 –paired –length 10). Processed reads were then mapped onto the ME49 reference assembly v.45 using bowtie2 v.2.4.1 (Langmead and Salzberg, 2012; Langmead et al., 2019) using an inter-mate distance of 100–200 bp to avoid dinucleosomes and other artifacts (–local -D 20 -R 3 -N 1 -L 20 -i S,1,0.50 –no-unal –no-mixed –no-discordant –phred33 -I 100 -X 200). SAM files were converted into BAM files, sorted and then used to generate a BedGraph coverage track using bamCoverage v.3.2.0 from the deepTools2 package (Ramírez et al., 2016), with a bin size of 1 bp, filtering for fragment sizes from 130 to 200 bp, and normalizing coverage to the size of the ME49 reference assembly (–binSize 1 –normalizeTo1x 65669794 –ignoreDuplicates –minFragmentLength 130 –maxFragmentLength 200). All MNase-seq visualizations represent mean coverages around indicated TSS subsets. Geometries of phased nucleosomal arrays were defined around the TSSs that matched at the nucleotide position between ME49 Tz replicates. For this, local maxima in nucleosome density were called in R to define the average center positions of nucleosomes relative to TSSs. A length of nucleosomal DNA of 147 bp was assumed to calculate the upstream and downstream edges of each nucleosome.

Frequency of Poly(dA:dT) Tracts

Tracts of at least four consecutive deoxyadenosines or deoxythymidines were detected using the Fuzznuc command line tool from EMBOSS v.6.6.0.0 (Rice et al., 2000) (-pattern AAAA -complement Yes). The frequency of poly(dA:dT) tracts was plotted within ±1000-bp windows centered around the TSSs that matched at the nucleotide level between ME49 Tz replicates.

Sense and Antisense 5′-Tag Densities at TSSs

ME49 Tz sense and antisense 5′-tag counts were normalized to the sense maximum within ±1000-bp windows around TSSs that replicated at the nucleotide position between biological replicates of ME49 Tz. These sense and antisense 5′-tag densities were then averaged across all sense or antisense windows. The mean densities from both biological replicates were then averaged, and plotted as a graph with no binning. The values for heatmaps were scaled differently to aid visualization. Here, replicate-averaged sense and antisense 5′-tag counts were normalized separately to the respective window maximum, averaged over 20-nt bins diverging from a 21-nt bin centered around TSSs. Local minima and maxima in 5′-tag density were determined on data that was smoothed using a 20-nt rolling average. Minima and maxima were superimposed on the schematic representation of average nucleosome positions, and used to derive a cutoff distance for bidirectionally-paired TSSs.

Detection of Bidirectionally-Paired Genes

Genes were sorted by the position of their start codons along chromosomes and contigs, followed by the pairing of adjacent gene pairs with opposite strandedness. Inter-TSS distances were calculated relative to the TSS of the upstream counterpart of each pair—upstream, as defined by the position of its start codon. Therefore, negative distances correspond to diverging TSSs, while positive values correspond to converging TSSs, which could result in overlapping transcripts. Distances were used for a density plot (bandwidth = 20) and a histogram (binwidth = 20 bp). A local minimum in antisense 5′-tag density demarcating the upstream border of the antisense cluster on the opposite pole of the NDR relative to the sense TSS was determined at -293 bp as described above. Diverging TSSs separated by 293 bp or less were considered to be bidirectionally-paired.

Identification of a Novel Promoter Motif

Enrichment for the putative binding motif of BFD1 was previously determined (Waldman et al., 2020). A composite list of TSSs from Bz and Tz stages was curated based on differential expression analysis, evaluated on the basis of previously published stage-specific RNA-seq data (Waldman et al., 2020): (i) for genes upregulated in Bz (≥ $\log_2$-fold change

of 2, *p*adj ≤ 0.001; *n* = 556), TSSs were selected from nucleotide-matching Bz replicates as determined above, and (ii) for genes downregulated in Bz or unregulated (*n* = 5412), TSSs were selected from nucleotide-matching Tz replicates as determined above. Motif analysis using MEME v.5.1.1 (Bailey and Elkan, 1994) was performed using sequences from -200 to 0 bp relative to TSSs (TSS windows; -dna -mod anr -nmotifs 3 -minw 5 -maxw 14 -objfun classic -revcomp -markov_order 0). MEME motif occurrences in the ME49 reference assembly (v.45) were identified using FIMO v.5.1.1 (Grant et al., 2011) with no strand-selectivity, and a *p*-value threshold of < 0.0001. A 40-bp rolling average of the frequencies of respective motifs and previously determined BFD1 CUT&RUN peaks (Waldman et al., 2020) in percent was calculated and plotted as a function of the distance to the TSSs of the subsets defined above.

Interspecies 5′-Leader Length Comparison

Data for *Homo sapiens*, *Drosophila melanogaster*, *Danio rerio*, and *Arabidopsis thaliana* were compiled from a previously published meta-analysis of RefSeq data on 5′-leader lengths (Leppek et al., 2018). For plotting and calculating sample sizes and median lengths, all 5′ leaders shorter than two nucleotides were discarded (*Homo sapiens*, *n* = 44100; *Drosophila melanogaster*, *n* = 29862; *Danio rerio*, *n* = 14364; and *Arabidopsis thaliana, n* = 42704). *Toxoplasma* ME49 Bz and Tz 5′ leaders were derived from the midpoint of TSSs that replicated within 40 nt between biological replicates as described above. 5′-leader lengths were then calculated from the distance between TSSs and start codons—ignoring the possibility for introns that may occur within the defined 5′-untranslated regions. For plotting and calculating the sample sizes (Bz: 5837; Tz: 5612) and median lengths (Bz: 837 nt; Tz: 792 nt), all 5′ leaders shorter than two nucleotides were discarded. For defining TSSs and 5′ leaders in *P. falciparum* 3D7, we retrieved previously published CAGE data from the Gene Expression Omnibus data repository [GSE68982 (Adjalley et al., 2016)]. We processed BedGraph files containing 5′-tag counts for the 3D7 reference genome (version 11) for both replicates of all six investigated intra-erythrocytic time points. Gene-association windows were defined from -3500 to +200 nt around the start codons of all protein-coding genes in the 3D7 annotation. Upstream window sizes were shortened by the stop codon of an upstream gene on the same strand, or by contig boundaries. 5′-tag counts were associated with specific positions in gene-association windows for each sample. 5′-tag-count maxima (TSSs) were then detected in each of the 12 samples, discarding genes that had no maximum, or multiple maxima with identical 5′-tag counts. For each time point, replicate data was used to define TSSs that matched within 40 nt, and the midpoint between these TSSs was defined as the consensus TSS. A consolidated list of 3D7 TSSs was then generated *via* random selection of one TSS per gene from all samples. 5′-leader lengths were calculated from the distance between TSSs and start codons —ignoring the possibility for introns that may occur within the defined 5′-untranslated regions. For plotting and calculating the sample size (3763) and median length (431 nt), all 5′ leaders shorter than two nucleotides were discarded.

Calculating Sizes of Exons, Introns, and Intergenic Spacers

A previously published python script [gtfstats.py (Francis and Wörheide, 2017)] was used to determine the length of each exonic, intronic, and intergenic feature in the genome annotation (GFF) files for *Toxoplasma* (TgME49 v.45) and *Homo sapiens* (GRCh38). This resulted in the following sample sizes for *Toxoplasma* vs. *Homo sapiens*: introns (40103 vs. 1716073), exons (49023 vs. 1716073), and intergenic spaces (7646 vs. 33985).

uAUG and uORF Analysis

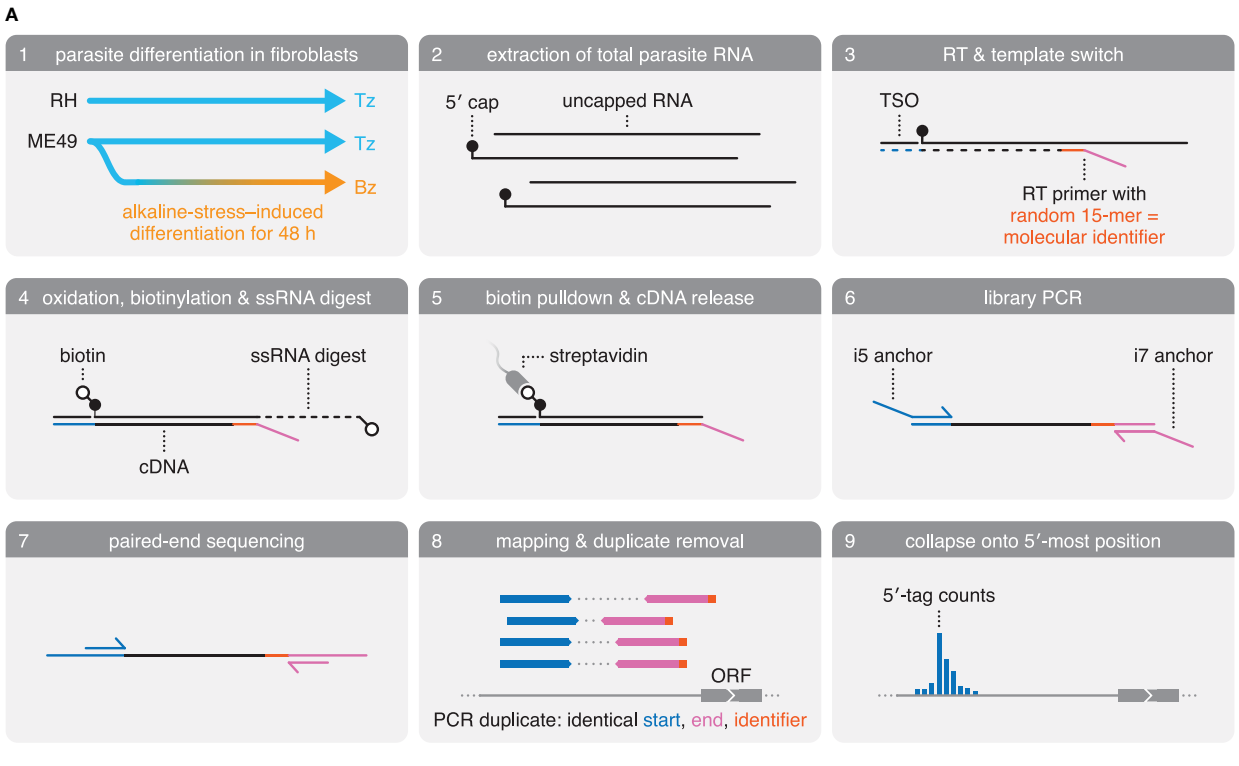
This analysis was performed on unspliced full-length 5′-leader predictions as defined above. We defined uAUGs as any AUG triplet upstream of the start codon of the CDS, and uORFs as any open reading frame with a minimal length of nine nucleotides, whose initiating codon lies within the 5′ leader. In the event of multiple initiation sites for a given stop codon, we selected the most distal in-frame uAUG as the uORF start. Out-of-frame overlapping uORFs (category B) were only detected down to the first codon that overlapped with the main ORF. Expected values were determined from reshuffled datasets, and are the average of 15 different simulations. These reshuffled datasets were generated using the Python module ushuffle (Jiang et al., 2008), which reshuffled the sequence of each 5′ leader, maintaining the same length and dinucleotide composition.

RESULTS

Generation and Sequencing of 5′-Intact cDNA Libraries From *Toxoplasma*

We adapted the RAMPAGE protocol (Batut and Gingeras, 2013) to systematically characterize mRNA 5′ ends in *Toxoplasma*, and to generate a genome-wide map of transcription initiation at single-nucleotide resolution. To capture strain- and life-cycle–dependent differences in transcription initiation, we constructed libraries from total RNA extracts of two canonical strains: the type I RH strain commonly used in cell culture and the type II ME49 strain frequently used in animal studies (**Figure 1A**, panels 1–2). Both strains were examined as acute-stage Tz, and ME49 was additionally analyzed as chronic-stage Bz, induced by culture under alkaline-pH stress.

The RAMPAGE protocol preserves the mRNA polarity in the resulting cDNA library by the directed introduction of Illumina-sequencing adapters for Read 1 at the 5′ end *via* a template switching oligo (TSO), and for Read 2 toward the 3′ end of the mRNA transcript *via* a randomly-priming reverse-transcription (RT) primer (**Figure 1A**, panel 3). RAMPAGE employs two approaches to enrich for 5′-intact mRNA based on the presence of the 5′-m7G cap structure (cap): by (1) introducing the 5′-sequencing adapter *via* template switching, which preferentially occurs at the cap (Wulf et al., 2019), and by (2) biotinylation of the cap, followed by capture on streptavidin-coated beads (**Figure 1A**, panels 3–5). Upon library amplification and paired-end sequencing, reads from both replicates of all three



B

	strain	ME49				RH	
	stage	bradyzoite (**Bz**)		tachyzoite (**Tz**)			
	replicate	A	B	A	B	A	B
read filtering	total number of reads	27,506,783	23,729,215	28,959,898	28,023,306	24,793,795	19,670,240
	rRNA removal	21,246,541	18,462,115	19,616,117	18,944,753	11,413,682	10,313,347
	multi-mapper removal	14,641,861	12,353,429	14,980,955	14,602,945	9,825,781	9,030,607
	duplicate removal	13,702,942	11,588,043	14,020,082	13,729,422	9,289,311	8,583,442

FIGURE 1 | Generation and sequencing of 5′-intact cDNA libraries from total RNA extracts of multiple *Toxoplasma* strains and life-cycle stages. **(A)** Preparation and sequencing of 5′-intact cDNA (RAMPAGE) libraries. Total RNA was extracted from RH and ME49 Tz, as well as ME49 Bz that were differentiated by culture in alkaline-pH medium. Sequencing adapters were introduced during reverse transcription (RT). Here, an RT primer anneals randomly to RNA transcripts, introducing the Read 2 sequencing adapter (magenta), and a template-switching oligo (TSO) introduces the Read 1 sequencing adapter (blue) at the 5′ end of the RNA transcript. 5′-intact cDNA was enriched *via* a cap-trapping strategy, in which riboses with free 2′- and 3′-hydroxyl groups are oxidized and biotinylated, and single-stranded portions of RNA (ssRNA) are digested by RNase I. This retains biotin only at the cap-structure of 5′-intact RNA transcripts, which was used in a streptavidin pulldown to enrich for the 5′-intact cDNA within the RNA-cDNA heteroduplex. Libraries were amplified by PCR, introducing the i5 and i7 anchor sequences for Illumina flow cells. Following size selection, libraries were paired-end sequenced. Reads were mapped onto the ME49 genome assembly. Duplicate reads were identified by their identical alignment positions; however, over-collapsing was prevented by exploiting the fact that the RT primer often primes with mismatches, providing a pseudo-random molecular identifier. Deduplicated and filtered reads were then collapsed onto the first nucleotide of Read 1 to generate 5′-tag counts across the genome. ORF, open reading frame. **(B)** Summary of sequencing of RAMPAGE libraries and read filtering. rRNA, ribosomal RNA.

samples were mapped to the ME49 genome assembly (ToxoDB, version 45), and reads originating from ribosomal RNA (rRNA) contamination were removed (**Figure 1A**, panels 6–8; **Figure 1B**). Reads originating from PCR duplicates were removed to improve the accuracy of RNA quantification (**Figure 1A**, panel 8; **Figure 1B**). Upon removing rRNA and PCR-duplicated reads, libraries retained 8.5–14 million uniquely-mapping reads (**Figure 1B**). These reads were collapsed onto the first nucleotide of Read 1 to provide a frequency distribution of 5′ tags across the ME49 genome assembly—an approximation of transcription initiation activity (**Figure 1A**, panel 9; data available from GEO, accession number GSE159515).

5′-Tag Counts Are Correlated With Levels of Polyadenylation and Gene Expression

As a first assessment of the quality and shape of our data, we examined 5′-tag distributions at the well-studied promoter of the *SAG1* gene, and found that 5′ tags indeed peaked at the previously identified major TSS (Burg et al., 1988) (**Figure 2A**). To systematically associate 5′ tags with ME49 gene

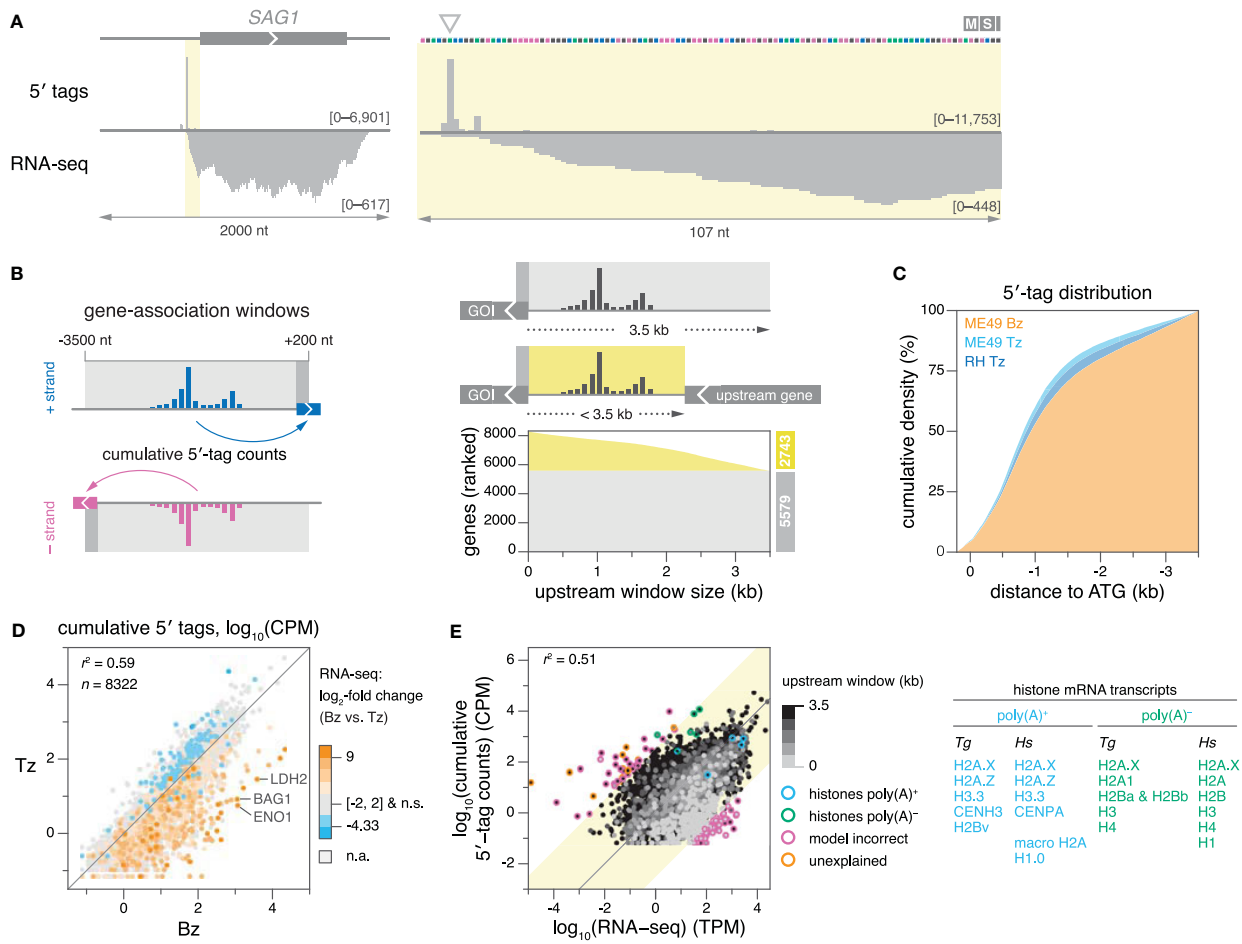


FIGURE 2 | 5′-tag counts correlate with polyadenylation and gene expression measured by RNA-seq. **(A)** 5′-tag and RNA-seq counts (ME49 Tz) on the (+) strand, upstream of the *SAG1* ORF (left), and zoomed in (right). Arrow indicates the major TSS previously determined (Burg et al., 1988). Data ranges indicated in counts per million (CPM) for 5′-tag data, and in transcripts per million (TPM) for RNA-seq data. **(B)** Strategy for associating 5′ tags to ME49 gene models. Strand-specific gene-association windows were defined around the start codons of 8322 protein-coding genes (left). The sum of all 5′-tag counts within a window constitutes a gene's cumulative 5′-tag count. Where applicable, upstream windows were shortened by the stop codon of an upstream gene on the same strand, or by contig boundaries (right). The graph shows the upstream window size of genes in ranked order. **(C)** Empirical cumulative density plots of gene-level–normalized 5′-tag counts from proximal to distal positions within windows. GOI, gene of interest. **(D)** Correlation of cumulative 5′-tag counts between ME49 Bz and Tz. Genes are colored according to differential expression in Bz compared to Tz (log_2 scale). Several canonical Bz genes are indicated. n.s., not significant. n.a., not applicable, i.e., genes below the coverage threshold required to calculate statistical significance. **(E)** Comparison of ME49 Tz cumulative 5′-tag counts (replicate-averaged) and Tz RNA-seq for relative quantification of gene expression (left). For clarity, genes with zero counts in either dataset are omitted. Genes are gray-shaded according to the size of their upstream gene-association window. RNA-seq libraries were generated using oligo(dT) priming for mRNA enrichment *via* poly(A) tails. Histone genes are highlighted according to the poly(A) status of their transcripts. Genes beyond three standard deviations (yellow background) are highlighted according to the accuracy of their model. Table of *Toxoplasma* (*Tg*) histone genes paired with their human (*Hs*) orthologues, grouped by the poly(A) state of their mRNA (right) (Dávila López and Samuelsson, 2008). The mRNA of H2A.X has both poly(A) and poly(A)-independent sequence motifs.

models, we defined strand-specific gene-association windows from -3500 to +200 nt relative to the start codons of all 8322 protein-coding genes in the reference annotation (version 45) (**Figure 2B**, left). Windows extended 200 nt into annotated open reading frames (ORFs) to enable the capture of 5′ tags for genes with erroneously-annotated start codons. The upstream distance of 3500 nt was chosen to maximize the number of genes for which the full distribution of 5′ tags could be captured, while minimizing the risk of erroneously capturing pervasive transcription from neighboring genes. As a point of reference, we used annotations from the *Toxoplasma* genome database (ToxoDB.org), and determined that the upstream window size of 3500 nt captures nearly all (98.7%) current TSS annotations (**Figure S1A**). Where applicable, upstream windows were shortened to terminate at the boundaries of chromosomes or contigs, or at stop codons of upstream ORFs with matching strandedness (**Figure 2B**, right). We found that 5′-tag density is normally-distributed across gene-association windows, with the mode of the distribution at ~700 nt upstream of the CDS and 50% of tags collected within ~900 nt proximal to the CDS

(**Figure 2C**; **S1B**). These metrics suggest that the selected gene-association windows capture the bulk of gene-associated transcriptional activity in these samples.

As an approximation for transcript abundance, we integrated all 5′ tags within individual gene-association windows to generate cumulative 5′-tag counts for each gene (**Figure 2B**, left). These values were highly-reproducible between the two biological replicates of each sample (**Figure S1C**). We then compared our measurements to expression data derived from conventional RNA-seq (Waldman et al., 2020), which served as an orthogonally-sourced metric for transcript abundance. Indeed, cumulative 5′-tag counts of replicate-averaged ME49 Bz and Tz samples recapitulated differential expression as evaluated by RNA-seq (**Figure 2D**). Transcript abundances assessed *via* conventional and 5′-end RNA sequencing (RAMPAGE) were positively correlated (r^2 = 0.51–0.55; **Figure 2E**; **S1D**), within the range reported by previous studies (Batut et al., 2013; Kawaji et al., 2014; Bhardwaj et al., 2019). Using the ME49 Tz datasets as an example, we investigated the discrepancy between gene expression measurements from RAMPAGE and RNA-seq. We analyzed genes known to differ in their polyadenylation to determine whether technical differences in transcript enrichment might underlie the discrepancy between the two methods, i.e., selecting mRNA *via* the 3′-polyadenylated (poly[A]) tail or the 5′-cap structure in RNA-seq or RAMPAGE, respectively. Indeed, we found that histone variants lacking poly (A) tails were underrepresented by RNA-seq, while those with poly(A) tails (López and Samuelsson, 2008; Marzluff et al., 2008) were quantified comparably, consistent with the generation of these RNA-seq libraries *via* oligo(dT) priming (**Figure 2E**). Furthermore, we observed that transcripts with shorter gene-association windows were more likely to have their gene expression underestimated by RAMPAGE compared to RNA-seq, which upon manual inspection of some examples we attributed to one or more of the following reasons: (i) a lack of sequence assembly upstream of the CDS, (ii) erroneous gene models of upstream genes, or (iii) an overall diminished capture of background pervasive transcription. We used both strand-specific RNA-seq (Waldman et al., 2020) and 5′-tag data to manually inspect outliers further than three standard deviations from the mean of the distribution. We found that the majority (91 of 102) is explained by inaccurate gene models or incomplete genome assembly [e.g., erroneously-annotated start codons; entirely spurious hypothetical gene models; transcripts split into multiple genes; erroneous annotation of introns, 5′ leader, and 3′ trailer sequences (**Figures S2A–H** for examples)], which cause erroneous transcript quantification by RNA-seq and/or cumulative 5′-tag counts (**Figure 2E**; **Table S1** for gene IDs). Finally, we investigated genes that had no coverage by conventional RNA-seq (n = 144), RAMPAGE (n = 224), or either technique (n = 27; **Table S2**). Manual inspection of some of these loci suggested that many genes with no or low coverage by either method may not be expressed in ME49 Tz, or their expression levels may be below the limit of detection. In addition, we noticed that genes not found by conventional RNA-seq were enriched for hypothetical proteins (72% vs. 51% among all genes) with generally shorter transcript lengths (973 nt vs. 3308 nt among all hypothetical transcripts), which could be indicative of spurious gene models that were predicted exclusively on the basis of short ORFs. Among the genes that were well-represented by conventional RNA-seq but lacked coverage by RAMPAGE, we frequently found inaccurate gene models, such as erroneous start codons or unresolved complex loci that prevented TSS capture, similar to the examples provided (**Figures S2A–H**).

In summary, our definition of gene-association windows provides a robust approach for the association of 5′-tag counts with gene models by demonstrating (i) the ORF-proximal accumulation of 5′-tag counts within gene-association windows, (ii) the reproducibility of cumulative 5′-tag counts, and (iii) the correlation of cumulative 5′-tag counts with conventional RNA-seq–derived transcript counts.

Defining *Toxoplasma* Transcription Start Sites and 5′ Leaders

When assigning TSSs, we chose not to assess the spread of 5′ tags at individual promoters, and instead defined the TSS as the nucleotide position with the maximal 5′-tag count within a gene-association window (**Figure 3A**). As a measure of confidence for our predictions, we classified TSSs into three groups based on their reproducibility across biological replicates: (i) reproducible at the nucleotide position, (ii) reproducible within 40 nt, or (iii) not reproducible within 40 nt. The 40-nt cutoff was chosen semi-arbitrarily to allow for positional variability of TSSs within clusters while discarding TSSs that likely originate from transcriptional noise or spurious read mapping. Remarkably, most TSSs (67%–75%) were reproducible at the exact nucleotide position: 6127 (out of 8176) in ME49 Bz, 5868 (out of 8071) in ME49 Tz, and 5214 (out of 7775) in RH Tz (**Figure 3B**; **S3A**). An additional 9%–11% of TSSs were reproducible within 40 nt (713 in ME49 Bz, 729 in ME49 Tz, and 866 in RH Tz), half of which clustered within a 10-nt distance. The remaining 15%–18% of TSSs were not reproducible within 40 nt between biological replicates and were excluded from further analysis. Using TSSs replicable at the nucleotide position or within 40 nt, we determined that the distribution of TSS distances to annotated start codons was similar between ME49 Tz and Bz, as well as RH Tz (**Figure S3B**). For a number of genes (366 in ME49 Bz, 318 in ME49 Tz, 293 in RH Tz) TSSs were predicted downstream of annotated start codons; evaluation of these loci found these predictions to be indicative of erroneous gene models (**Data S1** for a comprehensive table).

Integrating TSSs from both ME49 Bz and Tz, we also curated a comprehensive set of predictions to serve as a resource for further studies and to amend current gene models. For the purpose of generating this set of predictions, we assumed that TSS usage does not change significantly between life-cycle stages (see analysis below), and thereby accumulated 7603 TSSs successively from six categories that may reflect the confidence of the prediction (**Figure S4A**). We categorized TSSs by their perfect agreement among all four ME49 samples (56%), the two Tz samples only (21%), or the two Bz samples only (13%). The remaining predictions used TSSs that agreed within a 40-nt window between

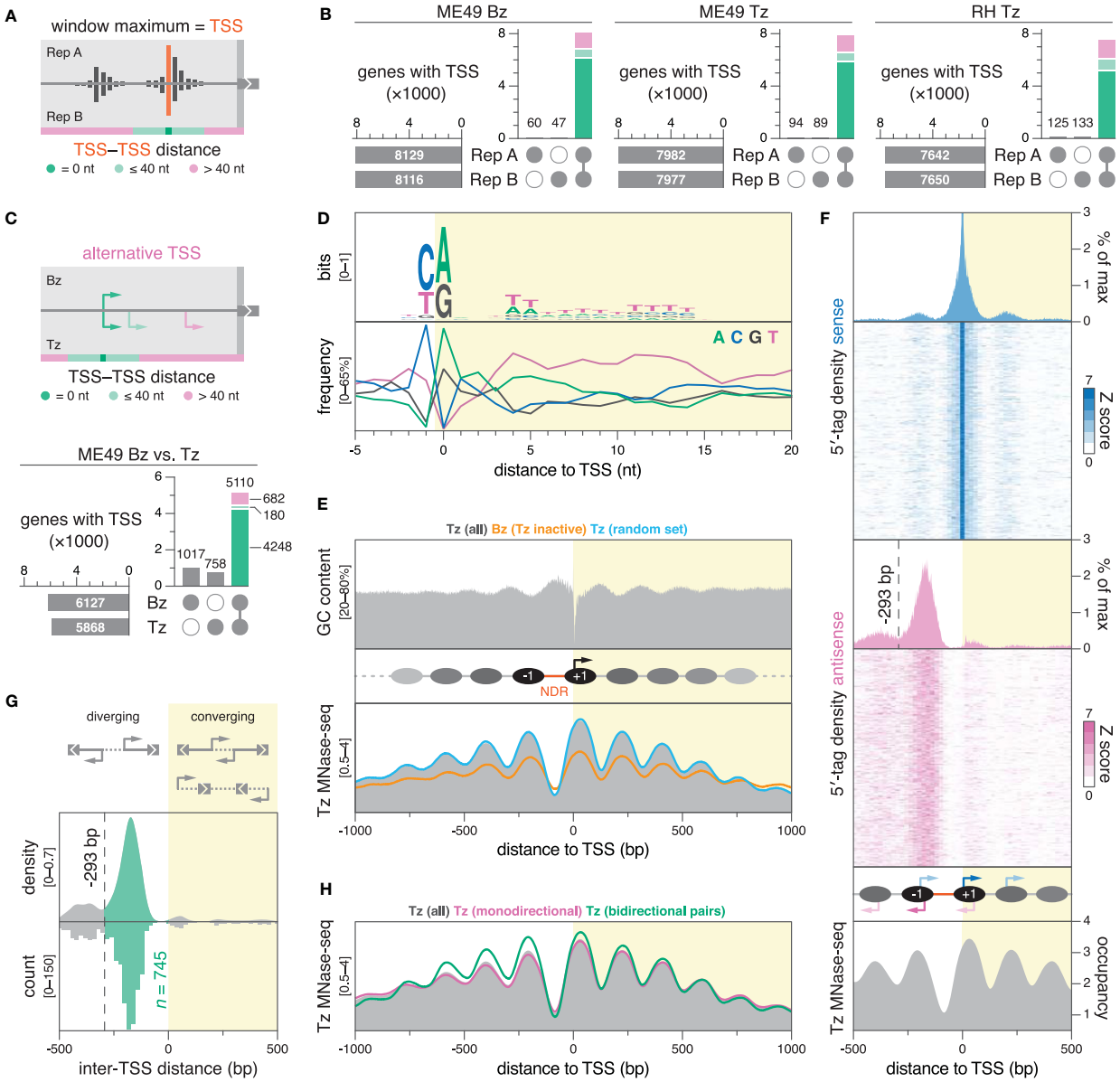


FIGURE 3 | DNA sequence and nucleosome positioning are strong determinants of transcription initiation in *Toxoplasma*. **(A)** Strategy for TSS mapping. We defined the TSS as the nucleotide position with the maximal 5′-tag count within a gene-association window. High-confidence TSSs were selected by assessing their reproducibility across biological replicates. TSSs separated by 40 nt or less were considered to be within the same cluster. **(B)** UpSet plots showing the intersection of TSSs between biological replicates, colored by inter-TSS distance. Only TSSs that replicate at the exact nucleotide position are considered for subsequent plots. **(C)** Differential TSS usage between ME49 Bz and Tz was similarly evaluated, clustering TSSs separated by less than 40 nt. UpSet plot shows the intersection of nucleotide-matching Bz and Tz TSSs, colored by inter-TSS distance. **(D)** Nucleotide composition around ME49 Tz TSSs; *n* = 5866. **(E)** GC content around ME49 Tz TSSs (top) and nucleosome occupancy around different subsets of ME49 TSSs (bottom). Nucleosome occupancy was determined by MNase-seq on Tz of the *Toxoplasma* Pru strain (Farhat et al., 2020). Nucleosome occupancy in Tz around the set of unique Bz TSSs (orange; *n* = 1017) or a randomly-sampled subset of Tz TSSs of equal size (blue). **(F)** Periodicity of secondary and antisense transcription initiation corresponds to nucleosome positioning. Sense (blue) and antisense (magenta) 5′-tag counts displayed as densities or stacked values, above nucleosome occupancy (gray). 5′-tag counts were normalized to the sense maximum before collapsing into density plots. Stacked values represent 5′-tag counts normalized strand-specifically to the maximum intensity of each locus along 20-nt bins. As the proportion of sense 5′-tag counts amounts to ~95% at position 0, the histogram is cropped to visualize minor density patterns. The dotted line marks a local minimum in antisense 5′-tag density at -293 bp, which subsequently served as a distance threshold for defining diverging gene pairs as bidirectional. **(G)** Density plot (bandwidth = 20) and histogram (binwidth = 20 bp) of the distance between TSSs of adjacent gene pairs in ME49 Tz. Negative distances correspond to diverging (head-to-head) TSSs, while positive values correspond to converging TSSs, which could result in overlapping transcripts. Distances from -293 to 0 bp indicate putative bidirectional gene pairs (green). **(H)** Nucleosome occupancy around indicated subsets of ME49 Tz TSSs.

Tz samples (5%) or Bz samples (3%), or at the same nucleotide between single Tz and Bz samples (2%). Collectively, we curated TSSs for 7603 genes, of which 5398 had predictions on ToxoDB (**Figure S4B**). Comparing predictions for these genes, we determined that 66% of our empirical predictions differed by more than 40 nt from those currently available on ToxoDB (**Figures S4B, C**), and we provide examples for how this data can improve current gene models (**Figure S4D**). Overall, our dataset provides empirically-determined TSSs for ~91% of *Toxoplasma* genes (**Figure S4E**; **Data S2** for BED file).

Evidence for the Stage-Specific Use of Alternative TSSs in *Toxoplasma*

Alternative TSSs increase protein and regulatory diversity by giving rise to transcripts encoding protein isoforms with alternative N termini, or transcripts with distinct 5′ leaders that can entail differential translational regulation (Davuluri et al., 2008; de Klerk and 't Hoen, 2015). A recent study in *P. falciparum* identified numerous alternative TSSs, and profiled their differential activity during the parasite's intraerythrocytic developmental cycle (Adjalley et al., 2016). In *Arabidopsis thaliana*, recent studies illustrated the systematic use of alternative TSSs as an adaptive mechanism in response to environmental stimuli (Ushijima et al., 2017; Kurihara et al., 2018). We wondered whether similar mechanisms may be present in *Toxoplasma*, and therefore explored the possibility of stage-specific alternative TSSs by comparing the high-confidence (nucleotide-matching) TSSs of ME49 Bz and ME49 Tz.

Analogously to our inter-replicate comparisons, we chose to employ a 40-nt distance cutoff for identifying TSS discrepancies between Bz and Tz (**Figure 3C**, top). To increase specificity, we only considered TSSs that replicated at the exact nucleotide position in both replicates from a given stage. Comparing the resulting TSSs from ME49 Bz (6127) to Tz (5868), we identified 5110 genes for which TSS predictions exist in both stages (**Figure 3C**, bottom). Identification of TSSs unique to ME49 Bz (1017) or Tz (758) correlated with their stage-dependent expression as measured by RNA-seq. Of the 5110 genes with predictions in both stages, 4428 genes had matching TSSs in both life-cycle stages, whereas 682 genes appeared to have alternative TSSs that were used in a stage-dependent manner (**Figure S5A**; **Data S3**). To evaluate the validity of this assignment, we manually inspected stage-specific stranded RNA-seq and 5′-tag data for 50 randomly-sampled loci from the 682 candidate genes (**Figure S5B**; **Table S3**). 24 of the sampled loci likely represent false positives for alternative TSS usage, with 15 explained by the TSS capture of pervasive transcription from neighboring differentially-expressed genes. However, we found 26 genes that clearly exhibited alternative TSSs, 16 of which showed a marked shift in stage-dependent usage, while 10 others had alternative TSSs that were used at similar levels stage-independently (**Figure S5C** for an example). Alternative TSS usage resulted in the gain or loss of upstream initiation triplets (uAUGs) and upstream ORFs (uORFs) in at least 17 of the 26 genes, which could conceivably affect translational regulation (**Table S3**). *Bona fide* stage-dependent alternative TSS usage was illustrated by *TGME49_200250*, where such a shift resulted in a 649-nt extension of the 5′ leader in Bz (**Figure S5D**). We note that while we have not systematically assessed differential activity of minor TSSs, we have observed such instances anecdotally, exemplified by *TGME49_262620*, where a minor TSS 317 nt upstream of the dominant TSS is specifically upregulated in Bz (**Figure S5E**). Collectively, we identified examples for alternative TSS usage, some of which are stage-dependent. Additional studies are needed to functionally characterize the different transcript isoforms that arise from these TSSs.

DNA Sequence and Nucleosome Positioning Govern Transcription Initiation in *Toxoplasma*

Hallmarks of eukaryotic TSSs include distinct nucleotide motifs, so-called core promoter elements, as well as a nucleosome-depleted region (NDR) upstream of a phased nucleosomal array. Generating a genome-wide map of transcription initiation in *Toxoplasma* enabled us to assess the impact of these structural features on TSS choice. Centering individual genomic sequences only on TSSs that were reproducible at the nucleotide level between biological replicates, we found no remarkable difference in nucleotide composition around subsets specific to ME49 Bz, ME49 Tz, or RH Tz, and chose to present results using the set of TSSs from ME49 Tz in all subsequent plots (**Figure 3D**; **S6A**). We found a strong preference for a pyrimidine-purine dinucleotide at the -1 to 0 position relative to the TSS—a universal core promoter motif in both eukaryotic and prokaryotic organisms. A preference for thymidine at positions +3 to +25 corroborated the findings of a previous study that suggested a downstream thymidine cluster at positions +2 to +14 in *Toxoplasma* (Yamagishi et al., 2010). On a broader scale, we noticed a consistent bias against adenosine downstream of the TSS, congruent with a relative adenosine depletion in 5′ leaders, ORFs, as well as CDSs specifically (**Figure S6B**).

We also observed a sinusoidal oscillation in GC content, reminiscent of phased nucleosomal arrays (**Figure 3E**, top). To relate this observation to data on nucleosome positioning, we reanalyzed publicly available MNase-sequencing (MNase-seq) data generated from Tz of the *Toxoplasma* Pru strain (Farhat et al., 2020). Surprisingly, we found that GC content anti-correlates with nucleosome density (**Figure 3E**, bottom), which is in contrast to genome-wide studies in yeast and *in vitro* studies, where GC content is a strong predictor for nucleosome positioning (Lee et al., 2007; Peckham et al., 2007). GC-rich sequences inherently facilitate nucleosome formation while AT-rich sequences, specifically homopolymeric (dA:dT) tracts, disfavor the process (reviewed in Jansen and Verstrepen, 2011). We found that the frequency of poly (dA:dT) tracts indeed correlates with AT content and nucleosome density, suggesting that *Toxoplasma*-specific nucleosome-positioning factors may override purely biophysical DNA sequence propensities (**Figure S7**).

Just upstream of active *Toxoplasma* TSSs, we found a prominent NDR of ~91 bp, flanked by remarkably symmetric phased nucleosomal arrays with highly-positioned -1 and +1 nucleosomes (**Figure 3E**, bottom; **S8A**). The size of the average

Toxoplasma NDR at active promoters is within the range described in yeast and metazoans (80–300 bp) (Raisner et al., 2005; Albert et al., 2007; Venters and Pugh, 2009). In yeast, the location of the TSS is just inside (~13 bp) the +1 nucleosome (Albert et al., 2007), whereas in metazoans, this nucleosome is positioned further downstream (~60 bp), leaving the TSS accessible within the NDR (Barski et al., 2007; Mavrich et al., 2008; Valouev et al., 2008). In *Toxoplasma*, we found that the TSS is located unusually deep into the +1 nucleosome, at ~41 bp from its upstream edge (**Figure S8A**). A nucleosome-internal TSS is compatible with a model described for yeast that suggests a role for the +1 nucleosome in facilitating transcription initiation (Albert et al., 2007).

Stage-specific data on transcription initiation also enabled us to compare nucleosome positioning at active and inactive *Toxoplasma* promoters. Studies in yeast and metazoans showed that nucleosome phasing and transcriptional activity are correlated and co-dependent (Tirosh and Barkai, 2008; Weiner et al., 2010; Oruba et al., 2020). Accordingly, inactive promoters are often characterized by minimal nucleosome phasing and the lack of an NDR (Mellor, 2005; Li et al., 2007; Lin et al., 2007; Jiang and Pugh, 2009). However, assessing Tz nucleosome positioning around the set of inactive Bz-specific TSSs (**Figure 3C**, bottom: unique set), we found that phasing, while reduced, was still detectable, and that the NDR was nearly as prominent as NDRs at randomly-sampled active promoters (**Figure 3E**, bottom). The maintenance of nucleosome phasing, and the presence of a constitutive NDR in particular, could facilitate inducible activation by rendering Bz-specific promoters accessible for the binding of transcription factors during the Tz stage.

Patterns of Pervasive Bidirectional Transcription at *Toxoplasma* TSSs

Although productive elongation may be unidirectional, generally, promoters are capable of initiating transcription bidirectionally (Neil et al., 2009; Xu et al., 2009). To assess the dynamics of bidirectional transcription within the nucleosomal context at *Toxoplasma* promoters, we projected averaged 5′-tag densities of both sense and antisense strands onto nucleosome density within ±1000-bp windows around ME49 Tz TSSs (**Figure 3F**; **S8A**). At these promoters, we found symmetrical patterns of bidirectional transcription initiation, clustered within nucleosomes. On the sense strand, the TSS accounts for the vast majority (~95%) of 5′ tags, yet secondary clusters peak at upstream (-198 nt) and downstream (+197 nt) locations, roughly corresponding to ±1 nucleosomal periodicity. Antisense 5′-tag density largely recapitulates these patterns: relative to the TSS, the major antisense cluster peaks at the opposite pole of the NDR (-169 nt), and is flanked by secondary clusters (-380 and +58 nt), again overlapping nucleosomes. We complemented averaged 5′-tag densities with heatmaps of 5′-tag counts for all ±1000-bp TSS regions, which showed that the observed patterns are indeed representative (**Figure 3F**). These patterns show that *Toxoplasma* promoters are inherently bidirectional, and that transcription initiates at regularly-spaced discrete loci within the nucleosomal array.

We wondered whether the symmetry in nucleosomal positioning, and incidence in antisense transcription at TSSs could also be indicative of bidirectionally-paired genes, i.e., non-overlapping (head-to-head) protein-coding genes that diverge from a common promoter. Bidirectional transcription of protein-coding genes is common in yeast, metazoans, and *P. falciparum* (Trinklein et al., 2004; Hermsen et al., 2008; Adjalley et al., 2016), and frequently allows for the co-regulation of functionally-related genes (Adachi and Lieber, 2002; Trinklein et al., 2004). To determine the proportion of the *Toxoplasma* genome that is organized into bidirectional gene pairs, we first defined such pairs as two adjacent genes whose coding sequences are located on opposite DNA strands, and with TSSs diverging from a shared NDR—a similarly strict definition was previously employed by genome-wide studies in yeast (Xu et al., 2009). We reasoned that antisense TSSs originating from the antisense 5′-tag cluster at the -1 nucleosome satisfy this definition. Consequently, we empirically derived a distance threshold between diverging TSSs to be considered as bidirectionally-paired by determining the upstream edge of the antisense 5′-tag cluster at the opposite pole of the NDR *via* a local minimum (-293 bp; **Figure 3F**). Using this threshold, we found 745 bidirectionally-arranged TSS pairs, corresponding to 1490 bidirectionally-paired genes, which represent a significant proportion (25%) of the genes considered in this analysis (n = 5868) (**Figure 3G**; **Data S4** for Gene IDs). In contrast to observations in *P. falciparum* (Adjalley et al., 2016), we found no evidence for an abundance of gene pairs with overlapping 5′ ends (**Figure 3G**, right). Conversely, the striking enrichment among diverging gene pairs for bidirectionality is consistent with findings in *P. falciparum*, where ~23% of genes were arranged head-to-head, with TSS blocks separated by 400 bp or less (Adjalley et al., 2016). As expected, nucleosome phasing was more symmetrical at TSSs of bidirectionally-paired genes, given that both sides of the NDR lie downstream of a TSS (**Figure 3H**). Notably, correlating RNA-seq–derived data on expression and stage-dependent changes in expression between bidirectional gene pairs, we found no evidence for co-regulation (**Figure S8B**). Manual evaluation of a subset of putative bidirectional gene pairs on the basis of our stranded RNA-seq data, showed that the majority (~85%) of predictions indeed represent *bona fide* bidirectional pairs; however, our analysis also captured artefacts, caused by (i) the erroneous association of antisense transcription from highly-active upstream promoters of neighbouring genes, or (ii) by the pairing with spurious hypothetical gene models that appear to have been annotated on the basis of antisense transcription from the same promoter (**Figure S8C** for examples). We therefore note that the presented number of bidirectional gene pairs may be overestimated.

To summarize, we found pervasive bidirectional transcription at *Toxoplasma* promoters, that initiates within discrete clusters, localized deep into nucleosomes. This is consistent with a model described for yeast, in which the transcriptional machinery assembles in bidirectional configuration at internucleosomal spaces, and transcription initiation is facilitated by the respective +1 nucleosome (Albert et al., 2007). Similar to

P. falciparum, we found that *Toxoplasma* organizes about a quarter of its protein-coding genome in bidirectional pairs. The lack of evidence for co-regulation of adjacent genes suggests mechanisms for the directional control of transcription.

Discovery of a New Sequence Motif Using Nucleotide-Resolution TSS Data

Transcriptional regulation is mediated by the collective binding of transcription factors to *cis*-regulatory elements in promoter and enhancer DNA. At promoters, transcription-factor–binding sites are typically positioned at defined distances relative to the TSS (Lim et al., 2004; Vardhanabhuti et al., 2007). Therefore, nucleotide-resolution data on TSSs is key for both the prediction of regulatory motifs, as well as the association of experimental data on transcription factor binding and motifs with annotated genes. Recently, we identified the transcription factor BFD1, a master regulator of Bz differentiation, and determined its genomic binding sites and putative binding motif *via* CUT&RUN (Waldman et al., 2020) (**Figure 4A**). Here, we assessed the frequency of both the putative BFD1 binding motif and CUT&RUN peaks within 5-kb windows centered on the TSSs of genes upregulated in Bz, compared to those that were downregulated in Bz or expressed similarly across the two life-cycle stages (**Figure 4B**). The aggregate signals for both CUT&RUN peaks and binding-motif frequency were enriched within a narrow interval, peaking at ~98 bp upstream of the TSSs of genes upregulated in Bz. Therefore, like most *cis*-regulatory sequences, the BFD1 binding site resides in the NDR, where the binding of transcription factors is not obstructed by the presence of nucleosomes (Lee et al., 2004). To discover novel motifs, we defined an interval at -200 to 0 bp relative to the TSS, reasoning that additional motifs may be positioned similarly to those bound by BFD1. Using the MEME program for motif discovery (Bailey and Elkan, 1994), we identified significant enrichment of the gCATGCa motif within the TSS windows of all genes (**Figure 4C**). Specifically, this motif was present in 44% of TSS windows (2637 out of 6053), and most concentrated at ~82 nt upstream of TSSs, independent of gene regulation in Bz (**Figure 4D**). The gCATGCa sequence resembles two known motifs: the sporozoite-specific regulatory motif PfM24.1 (CATGCA) identified in *P. falciparum* (Young et al., 2008)—the putative binding motif of the *P. falciparum* ApiAP2

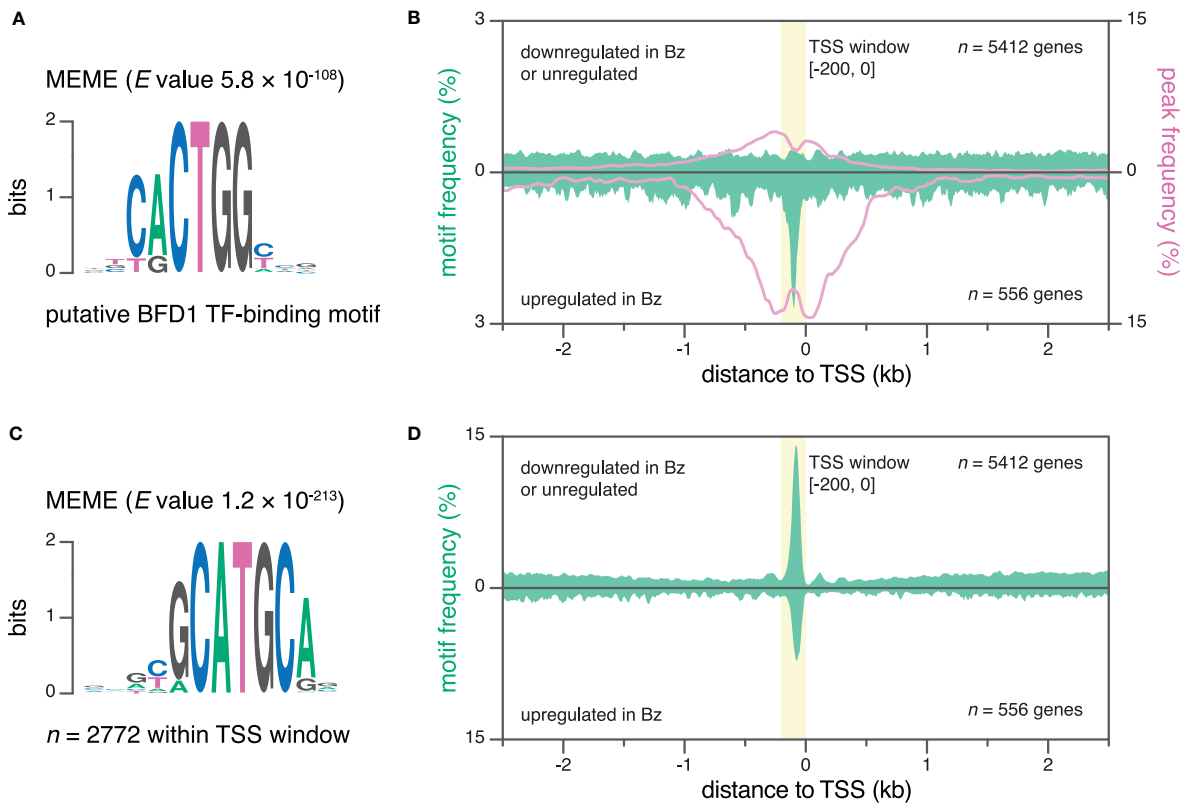


FIGURE 4 | Discovery of new sequence motifs using base-pair-resolution transcription start site (TSS) data. **(A)** Putative binding motif of the transcription factor BFD1 (Waldman et al., 2020). **(B)** Frequencies for the putative BFD1 motif (green, left y-axis) and CUT&RUN peaks (magenta, right y-axis) around different TSS subsets. Top, ME49 Tz TSSs of genes that are either downregulated (≥ 2-fold change on a $\log_2$ scale) in ME49 Bz or not regulated, as evaluated by RNA-seq. Bottom, ME49 Bz TSSs of genes that are upregulated in ME49 Bz (≥ 2-fold change on a $\log_2$ scale). A TSS window (-200 to 0 bp; yellow) captures the peak in motif frequency at TSSs of genes upregulated in ME49 Bz. Data was smoothed using a 40-bp rolling average. **(C)** The MEME motif (gCATGCa) was significantly enriched within TSS windows combined from both ME49 Bz and Tz. **(D)** Mean gCATGCa-motif frequency around different TSS subsets, as in **(B)**.

transcription factor PF14_0633 (YGCATGCP) (De Silva et al., 2008)—and the consensus binding sequence of plant-specific B3-domain–containing proteins (CATGCA; also known as RY element) (Suzuki et al., 1997). Collectively, our nucleotide-resolution data on TSSs enabled (i) the association of transcription-factor–binding data and a known binding motif to regulated genes, and (ii) the identification of a new putative *cis*-regulatory element. Similarly, future studies may use this data as a framework for motif discovery, and for assessing their regulatory potential.

Toxoplasma 5′ Leaders Are Unusually Long and Lack Suppression of uAUGs and uORFs

5′ leaders serve as the entry point for the ribosome during cap-dependent translation, and can regulate gene expression at the post-transcriptional level by affecting translational efficiency and mRNA localization. Between individual genes, 5′-leader lengths can vary dramatically, ranging from a few, to thousands of nucleotides (Leppek et al., 2018). The median length of 5′ leaders in non-apicomplexan eukaryotes varies between approximately 53 nt in yeast and 218 nt in humans (Leppek et al., 2018); however, previous data have suggested that 5′ leaders are significantly longer in apicomplexans, including *Toxoplasma*, *P. falciparum*, and *Neospora caninum* (Yamagishi et al., 2012; Russell et al., 2013; Caro et al., 2014; Ramaprasad et al., 2015).

Here, we comprehensively compared the length distribution of 5′ leaders between apicomplexans, and other model eukaryotes. First, we compiled data from a meta-analysis of RefSeq data on 5′-leader lengths from several model eukaryotes, including *Homo sapiens*, *Drosophila melanogaster*, *Danio rerio*, and *Arabidopsis thaliana* (Leppek et al., 2018). Secondly, we added 5′-leader data from (i) *Toxoplasma* as defined by TSSs that were predicted upstream of CDSs, and replicated within 40 nt in both biological replicates of ME49 Bz or Tz, and (ii) *P. falciparum* 3D7 as defined strictly analogously, using publicly-available 5′-end RNA-seq data (Adjalley et al., 2016) (see materials and methods for details). Indeed, the median 5′-leader lengths of both *Toxoplasma* (792–837 nt) and *P. falciparum* (431 nt) were substantially longer than those of the other analyzed eukaryotes (114–220 nt) (**Figure 5A**). These results suggest that extended 5′ leaders may be a conserved feature among diverse apicomplexan species. Remarkably, the expansion of 5′ leaders in *Toxoplasma* relative to other eukaryotes such as humans (median lengths of 792–837 vs. 220 nt) contrasts with its otherwise compact genome reflected in the reduced median lengths of introns (468 vs. 1747 bp) and intergenic sequences (1198 vs. 15396 bp) (**Figure 5B**).

A long region between the point of transcription initiation and the translation-initiation site in the mature mRNA provides a substrate for the evolution of mechanisms for post-transcriptional regulation. Various 5′-leader–localized linear and structural elements can modulate translation initiation (Wilkie et al., 2003; Jackson et al., 2010; Leppek et al., 2018). We therefore wondered whether the expansion of 5′ leaders may have been accompanied by a proliferation of mechanisms for post-transcriptional regulation. Specifically, we chose to analyze the occurrence of uAUGs and uORFs, which are common *cis*-regulatory elements in mammalian transcriptomes, often modulating the translational activity of downstream protein-coding ORFs by sequestering ribosomes (Morris and Geballe, 2000; Iacono et al., 2005). Here, we defined uAUGs as any AUG triplet upstream of the start codon of the CDS (main AUG, or mAUG), and uORFs as any open reading frame with a minimal length of nine nucleotides, whose initiating codon lies within the 5′ leader (**Figure 5C**). In our curated set of ME49 Bz and Tz 5′ leaders, we detected putative uORFs in ~90% of transcripts, surpassing the frequency observed in mammals (~50%) (**Figure 5D**; **Data S5** and **S6** for BED files) (Iacono et al., 2005).

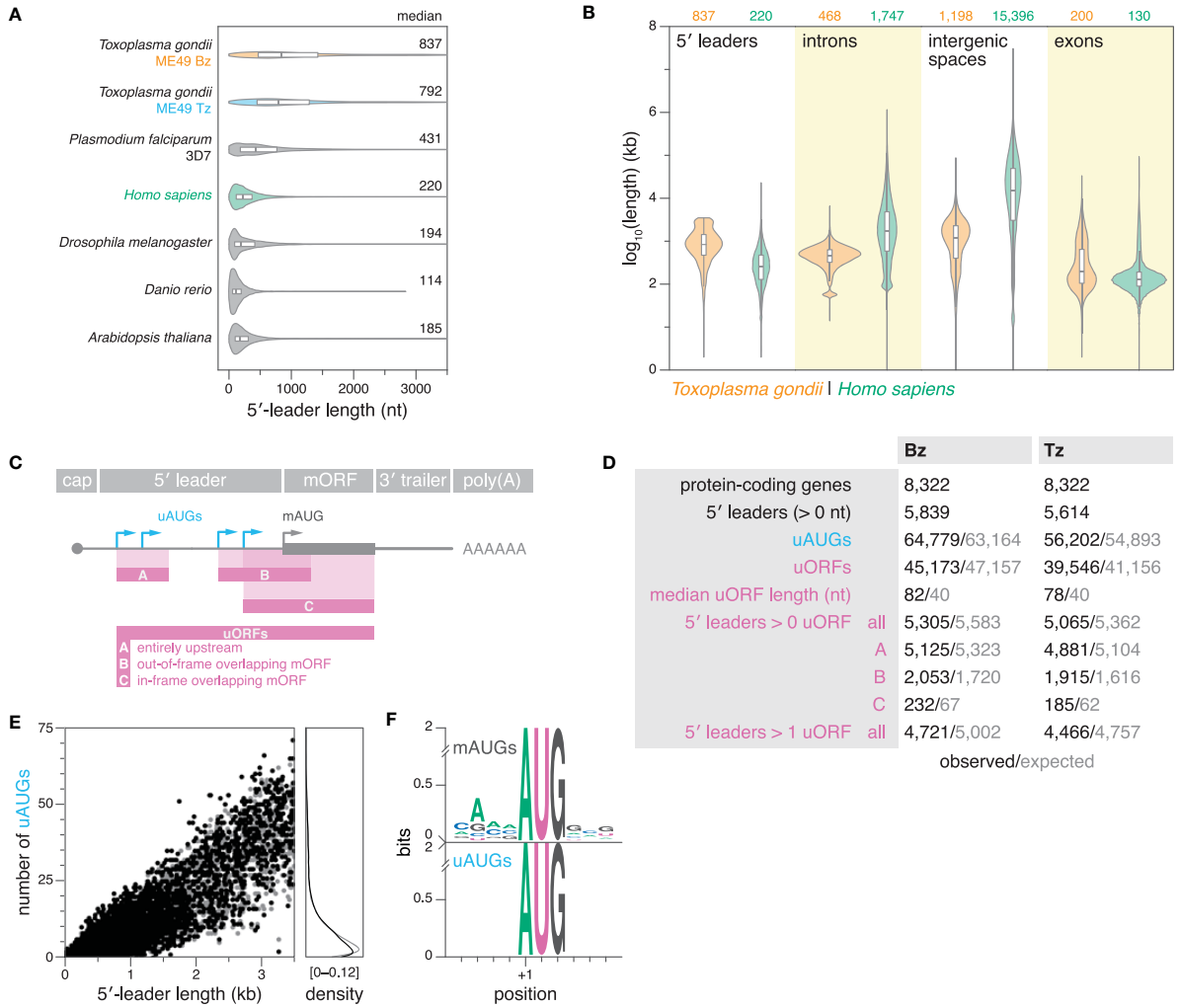
As expected, the number of uAUGs and uORFs correlated with 5′-leader length (**Figure 5E**; **S9**). In a range of eukaryotes, genome-wide studies have previously demonstrated that there are significantly fewer AUG triplets in 5′ leaders than expected by chance, which suggested that uORFs tend to be depleted from 5′ leaders due to their deleterious effects (Rogozin et al., 2001; Lynch et al., 2005; Neafsey and Galagan, 2007). To assess whether the occurrence of uAUGs and uORFs in *Toxoplasma* differed from what would be expected by chance, we compared observations with expectations calculated from reshuffled sequences of identical length and dinucleotide composition (see materials and methods for details). 5′ leaders with 1–6 uAUGs or 1–5 uORFs were slightly less prevalent than expected; however, there was no consistent deviation between observed and expected occurrences of these features, suggesting reduced selection against them in *Toxoplasma* (**Figures 5D, E**; **S9**).

Sequences immediately surrounding an AUG triplet can dramatically affect the frequency of translation initiation (Kozak, 1978; Kozak, 1986). Favourable consensus sequences for translation initiation have been defined in various species including *Toxoplasma* (Seeber, 1997). Importantly, in contrast to the conserved sequence context around mAUGs, we found that the sequences surrounding uAUGs were random, suggesting that uAUGs typically represent weak start codons (**Figure 5F**). This is consistent with the findings of a recent study that assessed the translational potential of some sequence-based uORF predictions in *Toxoplasma via* ribosome profiling, suggesting that uORF translation is typically inefficient, and that both mRNA sequence and secondary structure context may regulate translation efficiency (Hassan et al., 2017).

Collectively, we found that *Toxoplasma* 5′ leaders are on average among the longest of any eukaryote studied thus far. The occurrence of uAUGs and uORFs is generally not suppressed, and we find that sequence context may provide one mechanism to privilege a particular AUG for translation initiation by the *Toxoplasma* ribosome.

DISCUSSION

The lack of defined *Toxoplasma* TSSs has impeded genomic efforts in this divergent eukaryote. Using 5′-end RNA-seq, we



		Bz	Tz
protein-coding genes		8,322	8,322
5′ leaders (> 0 nt)		5,839	5,614
uAUGs		64,779/63,164	56,202/54,893
uORFs		45,173/47,157	39,546/41,156
median uORF length (nt)		82/40	78/40
5′ leaders > 0 uORF	all	5,305/5,583	5,065/5,362
	A	5,125/5,323	4,881/5,104
	B	2,053/1,720	1,915/1,616
	C	232/67	185/62
5′ leaders > 1 uORF	all	4,721/5,002	4,466/4,757

observed/expected

FIGURE 5 | *Toxoplasma* 5′ leaders are unusually long and lack suppression of upstream initiation triplets (uAUGs) and upstream ORFs (uORFs). **(A)** Violin and box plots showing the distribution of 5′-leader lengths in various species. Outliers were removed for clarity. **(B)** Violin and box plots showing the length distributions of 5′ leaders (this study), introns, intergenic spaces, and exons in ME49 Bz and humans. Outliers are omitted for clarity. **(C)** Definitions of uAUGs and uORFs. uORFs have a minimum length requirement of nine nucleotides. In the case of multiple in-frame overlapping uORFs that share the same stop codon, only the longest was reported. **(D)** Summary statistics of uAUGs and uORFs in ME49 Bz and Tz. Simulated values represent the average of 15 different simulations, in which 5′-leader sequences were individually shuffled while maintaining dinucleotide frequency. Refer to previous panel for uORF categories. **(E)** Left, scatter plot showing the number of uAUGs per gene as a function of 5′-leader length in ME49 Tz. Right, corresponding density plot. **(F)** Sequence logos of weighted position-specific scoring matrices showing nucleotide conservation around mAUGs (top; n = 5614) and uAUGs of uORFs (bottom; n = 39546) in ME49 Tz.

mapped transcription initiation activity at single-nucleotide resolution in acute and chronic stages of *Toxoplasma*, providing a much-needed resource for functional genomics studies. We empirically defined TSSs for ~91% of *Toxoplasma* genes, effectively revising 68% of current models and providing an avenue for improved genome annotation. We identify genes that putatively exhibit alternative TSSs, some of which appear to be differentially regulated in a stage-specific manner suggesting an additional transcript diversity that has not previously been appreciated. The canonical pyrimidine-purine dinucleotide motif at *Toxoplasma* TSSs is accompanied by sequence patterns such as a downstream thymidine cluster and a large-scale sinusoidal oscillation in GC content that appears anticorrelated with nucleosome occupancy. The *Toxoplasma* TSS lies unusually deep within nucleosomes, and is preceded by a prominent NDR that is at the center of a highly-symmetric phased nucleosomal array. Pervasive bidirectional transcription initiates with a defined pattern along the nucleosomal array. Demonstrating the utility of this data, we found that the putative binding motif of BFD1, a master regulator of Bz differentiation, resides at a specific distance from the TSSs of targeted genes, and we identified a novel motif with a similar positional arrangement at 44% of *Toxoplasma* promoters. Corroborating previous observations, we asserted that *Toxoplasma* 5′ leaders are among the longest of any eukaryote studied thus far. We found that 5′ leaders lack suppression of uAUGs and uORFs,

and we determined that sequence context may privilege mAUGs for translation initiation by the *Toxoplasma* ribosome. Collectively, our work provides a framework to investigate the interactions between genomic sequences and regulatory factors governing the complex transcriptional program of this parasite.

The *Toxoplasma* reference annotation (ToxoDB v.45) was largely generated *via* computational CDS prediction on the basis of RNA-seq and pre-RNA-seq expression data. However, untranslated regions, including TSSs, have remained incompletely defined due to the lack of integration with specific data on transcript boundaries, which has complicated investigations into the molecular mechanisms of transcriptional control. A pioneering study from 2010 used oligo-capping (Suzuki and Sugano, 2003) for the enrichment of 5′-intact cDNA and provided the first systematic assessment of transcription initiation in *Toxoplasma* (Yamagishi et al., 2010); however, these data never informed transcript annotations, and substantially more accurate and specific approaches have since been developed (Adiconis et al., 2018). With RAMPAGE (Batut and Gingeras, 2013), we used state-of-the-art approaches for systematically characterizing mRNA 5′ ends, thereby generating a genome-wide map of transcription initiation at single-nucleotide resolution. We empirically define dominant TSSs for 91% of protein-coding genes, compared to 68% of reference annotations, of which we extensively revised 66%. Apart from missing or erroneous TSS annotations, we identified various other gene-model inaccuracies, and putatively spurious models of hypothetical genes, highlighted by discrepancies between conventional and 5′-end RNA-seq. Future integration of the two modalities of strand-specific RNA-seq data—conventional and 5′-end—may facilitate the automated generation of high-quality empirical genome annotations (Boley et al., 2014; Wang et al., 2019). The integration of these datasets will likely be key in the *de novo* annotation of non-coding RNAs in *Toxoplasma*. Generating similar datasets from other life-cycle stages of *Toxoplasma* will be important in assessing stage-specifically–expressed genes that could not be captured in the present study. In addition, integrating our stage-specific 5′-end data with emerging long-read RNA-seq data from published (Lee et al., 2020) and future studies will likely improve the association of (alternative) TSSs to annotated genes.

We found evidence for the stage-specific use of alternative TSSs in *Toxoplasma*. Alternative TSSs are common in eukaryotic genomes where they increase protein and regulatory diversity by giving rise to transcripts encoding protein isoforms with alternative N termini, or transcripts with distinct 5′ leaders that can impart differential translational regulation (Trinklein et al., 2003; Zhang et al., 2004; Davuluri et al., 2008; FANTOM Consortium and the RIKEN PMI and CLST (DGT) et al., 2014; Haberle et al., 2014; Ushijima et al., 2017; Kurihara et al., 2018). Numerous alternative TSSs are differentially used during progression of *P. falciparum* through its intra-erythrocytic life-cycle stages (Adjalley et al., 2016). We note that our analysis focused on the identification of stage-dependent shifts between dominant TSSs in *Toxoplasma*; however, we have not comprehensively identified minor TSSs, or assessed their differential activity. Such an example is illustrated by the gene *TGME49_262620* where appearance of a minor TSS 317 nt upstream of the dominant TSS suggests the existence of a transcript isoform with an expanded 5′ leader specifically in Bz. Shifts in dominant TSSs were observed at some loci, such as *TGME49_200250*, where alternative TSS usage results in a 649-nt extension of its 5′ leader in Bz. Experimental validation will be needed to assess the functional impact that these 5′-leader extensions may have. Since our RAMPAGE data reflects transcription initiation activity within a population of parasites at various stages of their cell cycle, it will also be interesting to investigate synchronized cultures of *Toxoplasma* to resolve any cell-cycle–dependent shifts between alternative TSSs.

Canonical eukaryotic core promoter elements are hardly used in *Toxoplasma* and related *P. falciparum* (Yamagishi et al., 2010; Adjalley et al., 2016). Transcription initiates at the universal pyrimidine-purine (YR) dinucleotide (-1, 0), which is thought to facilitate the transition of RNA polymerase II from transcription initiation to mRNA elongation (Winkelman et al., 2016). While the YR dinucleotide may be required for efficient transcription initiation at a given site, it lacks the selectivity to explain the tight distribution of *Toxoplasma* TSSs. We corroborated the presence of a *Toxoplasma*-specific downstream thymidine cluster (+3–25) (Yamagishi et al., 2010), which may facilitate positioning of the +1 nucleosome and the preinitiation complex. Similar to other eukaryotes, the *Toxoplasma* TSS and the +1 nucleosome have maintained a fixed distance, suggesting that their positions have not arisen independently. The metazoan TSS resides within the NDR, ~60 nt upstream of the +1 nucleosome (Barski et al., 2007; Mavrich et al., 2008; Valouev et al., 2008), whereas *S. cerevisiae* initiates transcription ~13 nt inside the +1 nucleosome (Albert et al., 2007). In *Toxoplasma*, transcription initiates even deeper into the +1 nucleosome, clustered at around ~41 nt inside the nucleosome's upstream edge. Histone acetylation marks at the +1 nucleosome are thought to recruit and position the preinitiation complex at the TSS of *S. cerevisiae* (Jacobson et al., 2000; Matangkasombut et al., 2000; Hassan et al., 2002), and similar mechanisms may focalize transcription initiation in *Toxoplasma*.

At *Toxoplasma* promoters, we found that transcription initiates bidirectionally within discrete clusters that co-localize with the nucleosomes of a highly-symmetric phased nucleosomal array. Such patterns of pervasive divergent transcription at eukaryotic promoters are common (Jensen et al., 2013). However, the unusually-pronounced nucleosome phasing upstream of promoter-associated NDRs in *Toxoplasma* may structurally enhance the incidence of antisense transcription. Consistent with the presence of bidirectional activity and upstream nucleosome phasing, we found that *Toxoplasma* may organize up to a quarter of its protein-coding genome as bidirectional pairs that diverge from a common NDR. This configuration is similarly prevalent in *P. falciparum* (Adjalley et al., 2016). Manually evaluating a subset of putative bidirectional gene pairs on the basis of our stranded RNA-seq data, we found that ~15% were artefactual, caused by the

erroneous TSS capture of antisense transcription from highly-active upstream promoters of neighbouring genes, or by the pairing with spurious hypothetical gene models from the ME49 reference annotation that may have been predicted based on antisense promoter transcription. We therefore note that the presented number of bidirectional gene pairs may be overestimated. Such loci are likely to be resolved with additional analyses, improved genome annotations, and particularly with the integration of data from stranded long-read 5′-end and conventional RNA-seq. In metazoans, bidirectional gene pairs are also frequent (Trinklein et al., 2004; Hermsen et al., 2008), and often indicative of co-regulated adjacent genes with shared *cis*-regulatory elements (Adachi and Lieber, 2002; Trinklein et al., 2004); however, we could not establish a co-regulatory relationship between bidirectionally-paired genes in *Toxoplasma*, suggesting the presence of separate regulatory elements that allow for the directional control of promoter activity (Bagchi and Iyer, 2016). Even in the absence of shared *cis*-regulatory elements, the proximity between TSSs of bidirectionally-paired genes must be considered in forward genetic approaches that hinge on targeted promoter manipulations, such as CRISPR interference and activation (Rosenbluh et al., 2017).

Nucleosome positioning in *Toxoplasma* does not correlate with canonical sequence determinants. In yeast and metazoans, GC content is a strong predictor of nucleosome positioning (Lee et al., 2007; Peckham et al., 2007), and NDRs are often enriched in poly(dA:dT) tracts, which inherently disfavor nucleosomes (Yuan et al., 2005). By contrast, we found that nucleosome occupancy at *Toxoplasma* promoters strictly follows oscillations in AT content and poly(dA:dT) frequency, suggesting that *Toxoplasma*-specific nucleosome positioning factors may override intrinsically unfavorable DNA sequences.

We found that nucleosome phasing at inactive Bz promoters is maintained during the Tz stage. The maintenance of nucleosome phasing, and the presence of a constitutive NDR in particular, is consistent with a model in which Bz promoters are structurally poised for activation already prior to stage conversion. This would be similar to observations at stimulus-inducible cell-type–specific promoters in mammals (Oruba et al., 2020), where the magnitude of transcription after activation has been shown to correlate with promoter nucleosome depletion (Scruggs et al., 2015). The transcription factors bound at these NDRs and the chromatin remodelling factors involved in maintaining nucleosome patterning at Bz promoters remain to be identified. However, we note the following caveats to this analysis, in that (i) we cannot exclude potential Bz contamination in the MNase-seq data, and (ii) by assessing the gross average of nucleosome positioning across all Bz-specific TSSs, we ignored the potential presence of heterogeneous promoter patterning between individual genes. With promoters mapped in the asexual stages, and with the advent of an inducible system for *Toxoplasma* stage conversion (Waldman et al., 2020), future work may study the dynamics of nucleosome positioning at stage-dependent *Toxoplasma* promoters in greater detail.

We found that the putative BFD1 transcription-factor-binding motif is positioned at a defined distance relative to the TSSs of genes upregulated in Bz, consistent with its role as a master regulator for Bz differentiation (Waldman et al., 2020). We also identified a novel gCATGCa motif present at similarly defined positions upstream of 44% of *Toxoplasma* TSSs. The remarkable prevalence of this motif suggests an eminent role in regulating transcription, but its cognate transcription factor remains unidentified. The positioning of the BFD1 and gCATGCa motifs is typical for *cis*-regulatory elements in that they are at defined distances relative to the TSS (Lim et al., 2004; Vardhanabhuti et al., 2007), and reside roughly at the center of promoter-associated NDRs, where they are accessible for the binding of transcription factors (Lee et al., 2004). The herein-defined high-resolution TSSs for most *Toxoplasma* genes will likely facilitate similar studies into the association between transcription-factor-binding data, motifs, and annotated genes.

Our data corroborates that 5′ leaders of both *Toxoplasma* and *P. falciparum* are on average among the longest of any eukaryote studied thus far, suggesting that extended 5′ leaders may be a conserved feature among diverse apicomplexan species. Since 5′ leaders are associated with mechanisms of post-transcriptional regulation (Wilkie et al., 2003; Sonenberg and Hinnebusch, 2009; Jackson et al., 2010), the elongation of these non-coding regions may have contributed to increased regulatory complexity. However, 5′ leaders also provide a substrate for the mutational origin of potentially deleterious uAUGs and uORFs. These features are prevalent and regulatory in a variety of eukaryotes, including *P. falciparum* (Amulic et al., 2009; Caro et al., 2014), and typically occur significantly less frequently than would be expected by chance (Rogozin et al., 2001; Lynch et al., 2005; Neafsey and Galagan, 2007). In *Toxoplasma*, however, we found an abundance of putative uAUGs and uORFs, and no evidence for purifying selection against these features. The overall lack of statistical deviation between observed and expected frequencies of uAUGs and uORFs suggests that these elements are often irrelevant. This conclusion is consistent with ribosome-profiling data which found that putative *Toxoplasma* uORFs are typically not translated (Hassan et al., 2017); however, a more comprehensive functional evaluation of the herein-defined uORFs on the basis of both ribosome-profiling and proteomics data is ultimately needed. Mechanistically, the weak sequence context for translation initiation that we and others found at uAUGs may enable the *Toxoplasma* ribosome to bypass (through 'leaky scanning') these elements, and instead initiate translation at the downstream mAUG (Kozak, 1986; Hassan et al., 2017), and mRNA secondary structure around mAUGs has also been proposed to define the sites of translation initiation in *Toxoplasma* (Hassan et al., 2017).

Our precise genome-wide mapping of TSSs constitutes a major advance toward deciphering the molecular basis of both transcriptional and translational control in *Toxoplasma*. These predictions will both benefit from and also inform future revisions of reference assemblies and annotations. This resource opens new avenues into the characterization of the mechanisms that direct TSS choice and regulate promoter activity, and will be essential for the development of functional

genomics tools like CRISPR activation and interference in *Toxoplasma*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study are accessible through online repositories. Raw RAMPAGE sequencing data, and processed BedGraph files of strand-specific 5′-collapsed read coverage have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE159515 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159515). BedGraph files are also accessible through the genome browser at ToxoDB.org.

AUTHOR CONTRIBUTIONS

BM and SL conceptualized and designed the study. BM conducted experiments and analyzed the data. BW supplied the conventional RNA-seq datasets and provided key technical advice. BM wrote the first draft of the manuscript. HL and SL acquired funding. SL supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.617998/full#supplementary-material

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REVIEW
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Preparing for Transmission: Gene Regulation in *Plasmodium* Sporozoites

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Plasmodium sporozoites are transmitted to mammals by anopheline mosquitoes and first infect the liver, where they transform into replicative exoerythrocytic forms, which subsequently release thousands of merozoites that invade erythrocytes and initiate the malaria disease. In some species, sporozoites can transform into dormant hypnozoites in the liver, which cause malaria relapses upon reactivation. Transmission from the insect vector to a mammalian host is a critical step of the parasite life cycle, and requires tightly regulated gene expression. Sporozoites are formed inside oocysts in the mosquito midgut and become fully infectious after colonization of the insect salivary glands, where they remain quiescent until transmission. Parasite maturation into infectious sporozoites is associated with reprogramming of the sporozoite transcriptome and proteome, which depends on multiple layers of transcriptional and post-transcriptional regulatory mechanisms. An emerging scheme is that gene expression in *Plasmodium* sporozoites is controlled by alternating waves of transcription activity and translational repression, which shape the parasite RNA and protein repertoires for successful transition from the mosquito vector to the mammalian host.

Keywords: *Plasmodium*, malaria, sporozoite, gene regulation, quiescence, transcription, translational repression

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INTRODUCTION

Malaria is caused by protozoan parasites of the *Plasmodium* genus, and remains a major global health problem in endemic countries. *P. falciparum* is the deadliest species infecting humans, causing more than 200 million cases and 400,000 deaths every year, mainly in sub-Saharan Africa (World Health Organization, 2019). The absence of an available efficacious vaccine and the threat of parasite resistance to anti-malarial drugs emphasize the need for novel anti-malarial intervention strategies. To this end, a better understanding of the biology of the parasite is needed.

Plasmodium spp. have a complex life cycle that alternates between a mosquito vector and a vertebrate host (**Figure 1**). They are transmitted to mammals by the bite of infected female *Anopheles* mosquitoes, which deposit *Plasmodium* sporozoites in the host dermis (Sinnis and Zavala, 2012). The motile sporozoites actively migrate to the liver, where they invade hepatocytes for an initial and obligatory replication phase. Inside infected hepatocytes, *Plasmodium* resides in a specialized membrane-bound compartment, the parasitophorous vacuole (PV), where sporozoites transform into replicative exoerythrocytic forms (EEFs) (Frischknecht and Matuschewski, 2017). EEFs undergo a dramatic parasite multiplication, lasting 2 to 15 days depending on the parasite

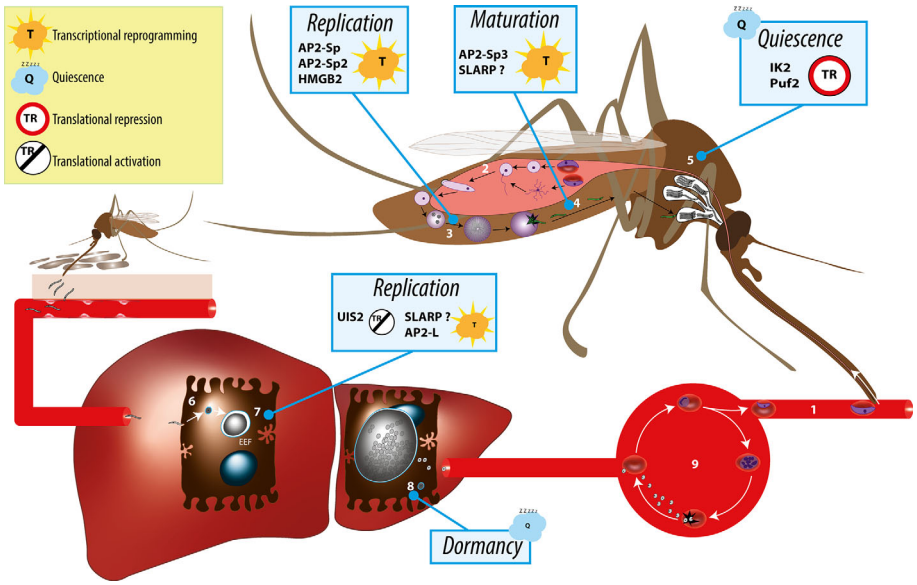

FIGURE 1 | Overview of the *Plasmodium* life cycle and the gene regulation mechanisms controlling parasite transmission from the mosquito vector to the mammalian host. During blood feeding on an infected host, female anopheline mosquito ingest male and female gametocytes (1), which transform into male and female gametes in the insect midgut lumen, followed by fertilization (2) and formation of motile ookinetes that cross the midgut epithelium and transform into extracellular oocysts. Oocysts undergo intense parasite replication (3) until releasing thousands of immature sporozoites that traffic to the salivary glands and mature into infectious sporozoites (4). In the salivary glands, sporozoites persist in a poised state for several days or weeks (5), awaiting transmission, which occurs when the mosquito feeds for blood on a new host. Infectious sporozoites are injected in the host skin and migrate to the liver, where they invade hepatocytes (6) and transform into replicative exoerythrocytic forms (EEFs) (7). In species such as *P. vivax*, some of the parasites do not replicate immediately after invasion but, instead, transform into dormant stages called hypnozoites (8). Parasite replication in the liver culminates with the release of thousands of merozoites into the circulation, which invade erythrocytes and initiate the asexual blood stage cycle (9). Individual factors with functional evidence for a role in transcriptional or post-transcriptional gene regulation during sporozoite development, maturation, or establishment of infection in the liver are indicated.

species and leading to the release of thousands of merozoites, which invade erythrocytes and initiate the pathogenic blood stage cycle. In some species such as *P. vivax*, a fraction of the parasites inside hepatocytes do not replicate immediately after invasion but, instead, transform into dormant stages called hypnozoites (Adams and Mueller, 2017). Activation of hypnozoites weeks or months after the infectious mosquito bite results in malaria relapses. During blood stage replication, a fraction of the parasites differentiates into sexual stage gametocytes, which upon ingestion by a blood-feeding anopheline mosquito can initiate sporogony in the insect.

Sporozoites develop within oocysts underneath the basal lamina of the mosquito midgut. Upon egress, they are released into the hemolymph and invade the insect salivary glands, where they persist in a poised state for several days or weeks, awaiting transmission, which occurs when the mosquito feeds for blood on a new host. The mosquito-mammal transition is a major bottleneck in the life of the parasite, as only a minor proportion (<20%) of sporozoites successfully invade the salivary glands (Hillyer et al., 2007), from which only a few dozen are inoculated during a mosquito bite (Frischknecht et al., 2004). Sporozoites released from oocysts are weakly infectious for the mammalian host, and gradually acquire infectivity while trafficking in the hemolymph, to become highly infectious after colonization of the insect salivary glands (Touray et al., 1992; Sato et al., 2014).

Comprehensive transcriptome and proteome surveys have shown that phenotypic maturation from immature oocyst-derived sporozoites in the mosquito midgut to mature infectious sporozoites in the insect salivary glands is associated with global changes in the parasite transcriptomes and proteomes (Matuschewski et al., 2002; Lasonder et al., 2008; Mikolajczak et al., 2008; Lindner et al., 2019). Studies based on RNAseq and LC-MS/MS performed with human-infecting species *P. falciparum* and rodent-infecting parasites *P. yoelii* revealed that around 3,500–4,200 genes (representing 60-70% of the genome) have detectable RNAs in sporozoites, while 1,500–2,000 proteins can be detected by mass spectrometry (about one third of the parasite proteins encoded by the genome) (Lindner et al., 2019). Comparison of the midgut and salivary gland sporozoite populations identified up-regulated in oocyst sporozoites (UOS) and up-regulated in infectious sporozoites (UIS) mRNAs and proteins (Matuschewski et al., 2002; Lasonder et al., 2008; Mikolajczak et al., 2008; Lindner et al., 2019). While these studies showed a correlation between RNA and protein abundance for many essential and conserved gene products, they also revealed extensive temporal dissociation between transcript and protein steady state abundance, with two overlapping and independent programs of translational repression that temporally regulate protein expression during sporozoite maturation and following transmission to the mammalian host (Lindner et al., 2019).

Plasmodium alternates between active proliferation, in the mosquito midgut and in mammalian hepatocytes and erythrocytes, and differentiation into transmission stages, the gametocytes and sporozoites (**Figure 1**). During progression through this complex life cycle, the parasite switches transcriptional programs. Like in other eukaryotes, gene expression is regulated at both transcriptional and post-transcriptional levels in *Plasmodium*. While gene regulation has been mostly investigated in the parasite asexual and sexual blood stages, fewer studies have looked into the sporozoite stage. In this review, we summarize the current understanding of the gene regulation mechanisms underpinning *Plasmodium* maturation into highly infectious sporozoites capable of invading the mammalian host hepatocytes, establish parasite reservoirs and transmit the disease.

CHALLENGES IN STUDYING GENE REGULATION IN *PLASMODIUM* SPOROZOITES

Studies of gene regulation in the malaria parasite have been greatly facilitated by the availability of genome sequence data, as illustrated by the first identification of a specific transcription factor (Myb1) in *P. falciparum* (Boschet et al., 2004; Gissot et al., 2005), rapidly followed by the discovery of the plant-like Apetala-2 (AP2) domain family of DNA-binding proteins (Balaji et al., 2005). However, the divergence of protein sequences compared to other eukaryotes, with nearly 30% of the parasite proteins still lacking an annotated function, has hindered the identification of key regulators. Genome-wide mutagenesis studies revealed that 60% of genes without a functional annotation in *P. berghei* show phenotypic defects during the erythrocytic stages, and 36% during the mosquito or liver stages (Bushell et al., 2017; Stanway et al., 2019), while in *P. falciparum* around 1,000 genes of unknown function were predicted to be essential in blood stages (Zhang et al., 2018). Prediction of domain architecture has been used to improve domain recognition methods in *Plasmodium* genomes, expanding new predicted domains up to 30% in *P. falciparum* (platform Plasmobase) (Bernardes et al., 2017; Briquet et al., 2018). While many transcription or translation associated proteins have been predicted in *Plasmodium* through bioinformatics (Roos et al., 2001; Bischoff and Vaquero, 2010; Bennink and Pradel, 2019), only a few of them have been biologically investigated, and it is plausible that other yet unidentified proteins play key roles in the gene regulation networks that concur to the progression of *Plasmodium* through its developmental stages. In addition, the genome extreme AT richness in some species such as *P. falciparum*, with more than 80% AT especially in intergenic regions, is a major obstacle for the identification of *cis*-regulatory elements.

Gene regulation has been mostly investigated in the context of asexual erythrocytic cycle and sexual conversion of *Plasmodium* parasites. In contrast, studies of the mosquito stages, including the sporozoite, are more scarse. Sporozoites must be isolated from anopheline mosquitoes, through hand dissection of the salivary glands of infected females. As a result, only limited numbers of sporozoites can be obtained (typically 10^4 to 10^5 per mosquito), resulting in limited sample amounts for downstream analysis of mRNA and/or proteins, with the additional issue of contamination with mosquito cellular content. Despite these limitations, highly sensitive next generation sequencing and mass spectrometry approaches, combined with methods to purify salivary gland sporozoites (Kennedy et al., 2012), have facilitated the deciphering of transcriptomes, epigenomes and proteomes of *Plasmodium* sporozoites (Lasonder et al., 2008; Lindner et al., 2013b; Gómez-Díaz et al., 2017; Swearingen et al., 2017; Zanghì et al., 2018; Lindner et al., 2019; Muller et al., 2019; Hamada et al., 2020).

Nevertheless, steady state mRNA levels result from a balance between mRNA synthesis and mRNA decay, which remain difficult to address in *Plasmodium* sporozoites. Importantly, detection of transcripts and proteins depends on their concentration and on the sensitivity of the analysis. A given messenger might be present in absence of its cognate protein because of translational repression or because the protein is expressed at low level even though it might be functionally competent. Reciprocally, mRNAs present below the detection limit may still be translated into functionally active proteins. Limited sample size is a major hindrance for proteomic detection of transcription factors, which are typically present at low concentrations in eukaryotic cells, allowing to switch on and off rapidly gene expression. Finally, while most studies have looked at bulk salivary gland sporozoites, single cell RNAseq approaches recently revealed some degree of transcriptome heterogeneity in sporozoite populations within the salivary glands (Howick et al., 2019), which could reflect continuing parasite maturation after invasion of the glands and until transmission. Liver stages, especially early exoerythrocytic forms, are typically present in extremely small numbers in experimental culture systems, and pose additional challenges as they cannot be easily isolated from the infected cell. For this reason, the mechanisms underlying stage conversion between quiescent sporozoites to proliferative liver stages remain poorly understood.

TRANSCRIPTIONAL REGULATION IN *PLASMODIUM* SPOROZOITES

The steady state transcriptome of sporozoites is remarkably stable during residence of the parasite in the mosquito salivary glands (Gomes-Santos et al., 2011). This suggests that mature sporozoites are transcriptionally quiescent, a notion further supported by the observation that sporozoites retain infectivity after exposure to the transcription inhibitor Actinomycin D (Silvie et al., 2014). However, exposure of *P. falciparum* and *P. vivax* sporozoites to conditions that mimic the host environment result in profound transcriptome reprogramming, with up-regulation of genes associated with liver stage development (Siau et al., 2008; Roth et al., 2018), illustrating the plasticity of sporozoite transcriptional control.

Plasmodium shares with other eukaryotic organisms common mechanisms for transcriptional gene regulation, including remodeling of the chromatin, post-translational modifications of histones, recruitment of the basal transcription machinery and the concerted action of transcription factors (TFs) that recognize *cis*-regulatory elements in gene promoter regions (Bischoff and Vaquero, 2010; Toenhake and Bártfai, 2019). Many transcription associated proteins (TAPs) have been annotated in *Plasmodium* (Roos et al., 2001; Bischoff and Vaquero, 2010), although few have been biologically investigated in the parasite, especially in sporozoites. They can be classified in three main groups according to their putative function in transcription: general transcription factors (GTF), chromatin-related transcription factors (CTF) and specific transcription factors (STF) (Bischoff and Vaquero, 2010). The three groups are represented in the proteome of sporozoites, albeit at variable levels (**Figure 2A**). **Table S1** lists annotated TAPs and protein detection by mass spectrometry in sporozoites from human and rodent *Plasmodium* species. The lower representation for some of the TAP subcategories could be explained by the absence of expression in sporozoites or by their low abundance in the minute biological material, below the detection limit of mass spectrometry. In eukaryotes, gene expression is largely governed at the level of transcription by stochastic molecular interactions between DNA and TAPs, linked to the fact that most of the proteins exist in cells in very small quantities, which allows switching on and off rapidly gene transcription (Kupiec, 1997). The constant association and dissociation events of DNA with interacting proteins, including histones, contributes to instability, while post-translational modifications of transcriptional regulators can stabilize stochastic gene expression (Kupiec, 1997; Nicolas et al., 2017).

Chromatin-Associated Regulation in *Plasmodium* Sporozoites

While the regulation of gene expression *via* the binding of specific TFs on their cognate regulatory elements is a central mechanism that controls complex processes of development and differentiation, transcription is for a large part influenced by chromatin organization, in *Plasmodium* as in other eukaryotes (Toenhake and Bártfai, 2019). Chromatin remodeling relies on a variety of readers, writers and erasers, involved in post-translational modifications of histones and driving the highly dynamic structure of nucleosomes underlying gene reprogramming (Tyagi et al., 2016). Architectural remodeling provides plasticity by bringing DNA loops and bound TFs in close proximity to scaffold functional multi-protein complexes. Transcription and genome activity can also be modulated by incorporation of long non-coding RNAs (lncRNA). This well-organized environment, which depends on the number and affinity of regulatory elements, defines transcriptional ecosystems of connected genes that allow the fine tuning of transcription programs for a steady-state level of gene expression (Silveira and Bilodeau, 2018). Disturbance of these ecosystems, including through the action of environmental factors, can lead to reprogramming of gene expression.

Epigenetic regulation is a key control mechanism of *Plasmodium* sexual conversion. In *P. falciparum*, the heterochromatin protein 1 (HP1) plays a central role in epigenetic silencing of the master regulator of gametocytogenesis AP2-G (Brancucci et al., 2014). Derepression of *ap2-g* gene expression depends on gametocyte development 1 (GDV1), itself controlled by an antisense RNA (Filarsky et al., 2018). Furthermore, histone post-translational modifications (hPTMs) act as primary epigenetic regulators of monoallelic expression of variant gene families and phenotypic plasticity in *P. falciparum* erythrocytic stages (Lopez-Rubio et al., 2007; Lopez-Rubio et al., 2009). Recent studies in *P. falciparum* mosquito stages revealed that similar chromatin regulation mechanisms also operate in sporozoites. Analysis by ChIP-Seq of the genome-wide distribution of HP1 and the repressive histone H3 lysine 9 trimethylation (H3K9me3) mark showed that *P. falciparum* (NF54 strain) sporozoites expand heterochromatin at subtelomeric regions to silence blood-stage-specific genes, which correlates with the transcription status of clonally variant gene members (Zanghì et al., 2018). One single *var* gene remained euchromatic, associated with expression of the cognate PfEMP1 protein at the sporozoite surface. Similar results were obtained with *P. falciparum* sporozoites from field isolates, where transcription of a single (but distinct) *var* gene was observed, correlating with the presence of low levels of the repressive H3K9me3 mark in its promoter and with the expression of an antisense lncRNA (Gómez-Díaz et al., 2017). Consistent with observations in *P. falciparum*, the H3K9me3 heterochromatin marks primarily clustered in telomeric and subtelomeric regions in *P. vivax* sporozoites, while marks associated with active transcription, H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 9 acetylation (H3K9ac), were distributed broadly across the chromosomes outside of the telomeric regions, with no overlap with regions under H3K9me3 suppression (Muller et al., 2019).

Sporozoites express several chromatin modifying enzymes, as shown by proteomic analysis, including chromatin writers (SET7 histone methyltransferases, GCN5 histone acetyl transferase) and erasers (HDAC1, Sir2A, Sir2B) (**Figure 2A** and **Table S1**). However, their biological function has not been investigated in sporozoites. One study showed that *P. falciparum* SET7 is distributed throughout the cytoplasm in salivary gland sporozoites, and localizes to distinct cytoplasmic foci adjacent to the nucleus in erythrocytic and liver stage parasites (Chen et al., 2016). Sporozoite proteomes also include chromatin readers, which participate in chromatin structuring and play an important role in transcriptional programming in eukaryotic cells. In *P. falciparum*, the bromodomain protein BDP1 has been reported to bind acetylated histone H3 and plays a role in regulating expression of genes encoding invasion factors in *P. falciparum* blood stages (Josling et al., 2015). BDP1 has been detected in sporozoites by mass spectrometry, as well as the SNF2 helicase and CHD1 chromodomain helicase (**Table S1**). Among TAP partners, the pleckstrin homology domain finger protein PHD1 is expressed in *P. falciparum* and *P. berghei* sporozoites, and could play a role in nodes of connectivity involving epigenetic complexes with bromodomain readers

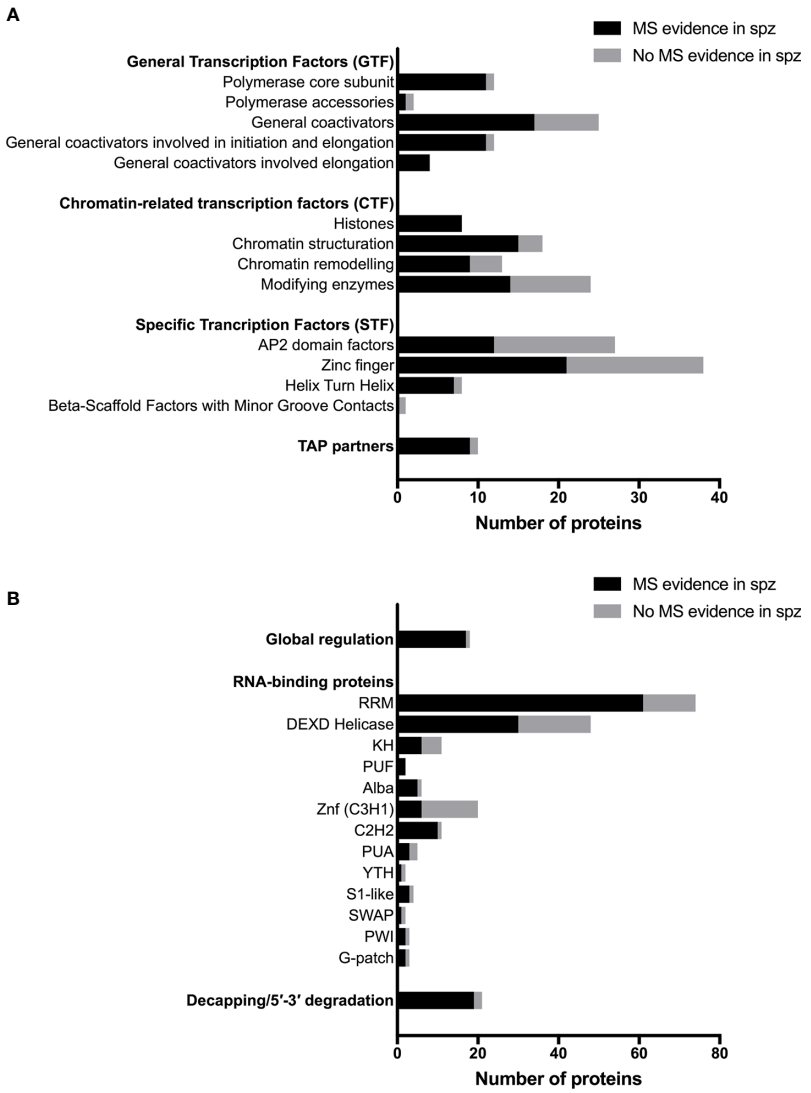

FIGURE 2 | Transcriptional, post-transcriptional and translational regulators in *Plasmodium* sporozoites. **(A)** Number of transcriptional-associated proteins (TAPs), based on the classification of Bischoff and Vaquero (2010). Black bars represent proteins with mass spectrometry evidence in *Plasmodium* spp. sporozoites. Gray bars represent proteins not detected in sporozoites by mass spectrometry. For details on protein identity and mass spectrometry results, see **Table S1**. **(B)** Number of post-transcriptional and translational regulators, based on the classification of Reddy et al. (2015) and Bennink and Pradel (2019). Black bars represent proteins with mass spectrometry evidence in *Plasmodium* spp. sporozoites. Grey bars represent proteins not detected in sporozoites by mass spectrometry. For details on protein identity and mass spectrometry results, see **Table S2**.

(Hoeijmakers et al., 2019). Again, none of these factors has been biologically investigated so far in sporozoites.

The final step leading to the recruitment of RNA polymerase II and the onset of transcription is chromatin remodeling by increasing nucleosome sliding and accessibility of the chromatin (Agresti and Bianchi, 2003). High-mobility group B (HMGB) proteins are typical members of this category, with architectural properties to recognize distorted DNA and induce stabilization of DNA bending. HMGB proteins appear to be associated with active chromatin (Németh and Längst, 2004; Briquet et al., 2006). HMGB and other chromatin remodeling factors are well represented in the sporozoite proteome (**Figure 2A** and **Table S1**). Interestingly, HMGB2 appears to be a critical regulator of parasite transmission to mosquitoes, since disruption of *hmgb2* gene in *P. yoelii* caused a major reduction of oocyst numbers, associated with the down-regulation of a subset of gametocyte transcripts (Gissot et al., 2008). However, *hmgb2*(-) oocysts were still capable of producing viable sporozoites that were infectious to mice. Although mainly nuclear, HMGB2 was also detected in the cytoplasm of *P. falciparum* gametocytes (Briquet et al., 2006), consistent with the dual life of the protein both as a nuclear protein and as a secreted alarmin (Briquet et al., 2015).

Sequence-Specific Transcription Factors

Apicomplexan AP2 (ApiAP2) DNA-binding domain proteins form the largest known family of specific TFs in *Plasmodium*, with 27 members in *P. falciparum* (Balaji et al., 2005; Bischoff and Vaquero, 2010). This family includes sequence-specific TFs with homology to the plant apetala 2/ethylene response TF, and are characterized by the presence of one to three DNA-binding AP2 domains (Balaji et al., 2005; Painter et al., 2011). *Plasmodium* AP2 TFs are considered as the main specific TFs responsible for parasite progression through distinct developmental stages (Painter et al., 2011). Twelve AP2 factors have been detected by mass spectrometry in sporozoites from at least one species (**Figure 2A** and **Table S1**), including six that have been detected in all tested species. Reverse genetics studies in *P. berghei* and *P. yoelii* have identified several AP2 factors that play an important role for parasite development in mosquitoes. Four are essential for the formation of infectious ookinetes (AP2-O, AP2-O2, AP2-O3, and AP2-O4), and three are required for sporogony (AP2-Sp, AP2-Sp2, AP2-Sp3) (Yuda et al., 2009; Yuda et al., 2010; Modrzynska et al., 2017; Zhang et al., 2017). Among these, AP2-Sp (Apetala 2 in sporozoites, annotated as AP2-Exp in *P. falciparum*) was one of the first AP2 TFs to be functionally characterized *in vivo* in *P. berghei* with AP2-O. AP2-Sp is expressed from the late oocyst to the salivary gland sporozoite, where it regulates specific gene expression (Yuda et al., 2010). The single AP2 domain of *P. falciparum* and *P. berghei* AP2-Sp binds to the TGCATGCA motif, found in the promoter region of many sporozoite-specific genes (Campbell et al., 2010; Iwanaga et al., 2010; Westenberger et al., 2010), which acts as a *cis*-acting element specific for the sporozoite stage (Yuda et al., 2010; Klug et al., 2018). The AP2-Sp domain was shown to dimerize upon DNA binding (Lindner et al., 2010). *P. berghei* mutants lacking AP2-Sp form oocysts but fail to produce sporozoites (Yuda et al., 2010). Genes under control of AP2-Sp encode proteins associated with a wide range of functions, including sporozoite formation in the mosquito (CSP) (Ménard et al., 1997), sporozoite motility and invasion of the mosquito salivary glands (TRAP, MAEBL) (Sultan et al., 1997; Kariu et al., 2002), sporozoite cell traversal (SPECT, PLP1) (Ishino et al., 2004; Ishino et al., 2005a), invasion of mammalian host cells (P36, P52) (Ishino et al., 2005b) or liver stage development (UIS3, UIS4) (Mueller et al., 2005a; Mueller et al., 2005b). This strongly suggests that additional factors and possibly complex gene regulatory networks participate in the fine-tuning of gene expression during sporozoite development and maturation. Systematic genetic screens in *P. berghei* and *P. yoelii* identified two additional AP2 factors, AP2-Sp2, and AP2-Sp3, as essential for sporozoite production or maturation in oocysts, respectively (Modrzynska et al., 2017; Zhang et al., 2017). Another AP2 TF of the rodent malaria parasite *P. berghei* expressed in liver stages and designated AP2-L was reported to be necessary for parasite development in hepatocytes (Iwanaga et al., 2012). AP2-L disruption does not affect the ability of sporozoites to migrate through cells and to invade hepatocytes but induce an important delay in patency in mice. The AP2-L phenotype is associated with a decreased level of several transcripts specific to the early liver stages (UIS2, UIS3, UIS4, EXP1, and LISP1) but there was no evidence for a binding DNA motif shared within these co-regulated genes (Iwanaga et al., 2012). Finally, among *P. berghei* essential AP2 factors (Modrzynska et al., 2017), some are highly expressed in the sporozoite stage, including AP2-I, which regulates invasion-related genes in *P. falciparum* merozoites (Santos et al., 2017). In fact, among AP2 factors identified in the proteome of sporozoites, six were also identified in the first *P. falciparum* blood stage nuclear proteome, including AP2-I and AP2-Sp (Oehring et al., 2012), showing some overlap in regulatory components between distinct developmental stages of the parasite. AP2-I and other essential AP2 factors expressed in sporozoites possibly participate in the regulation of sporozoite development and/or maturation. As these AP2 factors are refractory to conventional knockout approaches, conditional genome editing will be required for functional characterization in mosquito stages.

Proteomic studies identified other putative specific TFs in sporozoites, i.e., zinc finger and helix turn helix (HTH) proteins (**Figure 2A** and **Table S1**), but their biological role remains unknown. This group includes HMGB3, the only HMGB factor which exhibits one Myb-binding domain (Plasmobase, Vaquero & Briquet personal communication). HMGB3 was detected in the proteome of all tested sporozoite species, as well as the HTH factor ADA2 (Alteration/Deficiency in Activation 2). The first specific TF identified in *Plasmodium*, Myb1 (Boschet et al., 2004; Gissot et al., 2005), is expressed in oocysts and in liver stages, at least at the mRNA level, but not in sporozoites (Howick et al., 2019). A Myb-like TF was recently identified as a master regulator of *Toxoplasma* differentiation, representing a counterpoint of the ApiAP2 factors that dominate the common view of *Plasmodium* gene regulation (Waldman et al., 2020).

In summary, *Plasmodium* sporozoites express many factors potentially involved in transcription regulation, very few of which have been functionally characterized. In addition, unconventional factors could also participate in gene regulation at the transcriptional level, as illustrated by SLARP, which is discussed in a specific section of this review, raising the possibility that non-canonical regulators remain to be discovered in the malaria parasite.

POST-TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL IN *PLASMODIUM* SPOROZOITES

Plasmodium sporozoites can persist and remain infectious within the salivary glands of the mosquito for several days until they are eventually transmitted to a mammalian host. Remarkably, the parasite mRNA repertoire remains stable during protracted sporozoite residency in the salivary glands (Gomes-Santos et al., 2011). Two control mechanisms are known to operate in sporozoites at the post-transcriptional level: global translation inhibition, at the level of the translation initiation complex,

and mRNA-specific post-transcriptional silencing, which typically involves *cis*-regulatory RNA elements and mRNA-binding proteins. Other mechanisms could also be involved, such as mRNA decay mediated by ribonuclease complexes which plays a role in antigenic variation in *P. falciparum* (Zhang et al., 2014), but have not been characterized so far in sporozoites.

Translational regulation plays an important role in quiescent *Plasmodium* transmission stages, i.e., sporozoites and gametocytes, allowing rapid adjustment of gene expression and protein synthesis in response to the new environment that the parasite encounters when transiting from the mosquito vector to the mammalian host, and reciprocally (Bennink and Pradel, 2019). The eukaryote translation machinery is conserved in *Plasmodium*, where bioinformatics predict the presence of initiation, elongation and termination proteins (Cui et al., 2015; Bennink and Pradel, 2019). Many of the factors involved in translation regulation are expressed in sporozoites, as shown by mass spectrometry (**Figure 2B** and **Table S2**). Malaria parasites also express distinct rRNA genes at different developmental stages (Waters et al., 1989), with transcription of S-type rRNA genes taking place as the parasite begins to proliferate in the mosquito (Thompson et al., 1999).

The transient developmental arrest of sporozoites inside mosquito salivary glands implies efficient control mechanisms to prevent premature transformation before transmission. Sporozoite latency in the mosquito is controlled, at least in part, by global inhibition of protein synthesis, mediated through the phosphorylation of the translation initiation factor eIF2α by the sporozoite protein kinase IK2 (Zhang et al., 2010), also termed UIS1 (Matuschewski et al., 2002). *P. berghei* lacking IK2 display a partial loss of infectivity associated with premature transformation of sporozoites in the mosquito salivary glands (Zhang et al., 2010). *In situ* hybridization with oligo-d(T) or labelling with antibodies to the polyA-binding protein showed that sporozoites harbor cytoplasmic RNA granules (Zhang et al., 2010). *Ik2*(-) sporozoites lack such granules and show a general increase in protein synthesis (Zhang et al., 2010). Since global translation inhibition *via* eIF2α phosphorylation is a conserved stress response in eukaryotes, the premature transformation of sporozoites in the absence of IK2 possibly reflects an increased susceptibility of the mutant parasites to stress encountered during residence in the mosquito salivary glands.

Phosphorylation of eIF2α is regulated by the essential phosphatase UIS2, which is expressed in sporozoites and blood stages (Zhang et al., 2016). UIS2 conditional knockout *P. berghei* mutants have a defect in liver stage development, associated with increased levels of phosphorylation of eIF2α. UIS2 may promote reactivation of translation once the sporozoite entered the mammalian host, to allow sporozoite transformation and liver stage development. Intriguingly, UIS2 was recently identified as a PVM protein in *P. falciparum* asexual erythrocytic stages, indicating that UIS2 may have alternative roles (Khosh-Naucke et al., 2018).

Comparison of transcriptome and proteome datasets from *P. yoelii* and *P. falciparum* sporozoites revealed that translational repression affects a large subset of genes, including 70%–80% of UIS (Lindner et al., 2019). Transcriptomic and proteomic data from *P. vivax* sporozoites indicate that translational repression occurs in this species as well (Swearingen et al., 2017; Muller et al., 2019). Post-transcriptional silencing plays a crucial role in female gametocytes, where a defined population of mRNAs encoding ookinete proteins such as P25 and P28 are not translated but instead stably stored in quiescent messenger ribonucleoprotein particles (mRNPs) (Mair et al., 2006; Mair et al., 2010). Transcript-specific translational repression also operates in salivary gland sporozoites, where translation of one of the most abundant mRNAs, encoding UIS4, is inhibited (Silvie et al., 2014; Silva et al., 2016; Lindner et al., 2019). Inside hepatocytes, *Plasmodium* develops within a parasitophorous vacuole (PV) and remodels the membrane of its vacuole by inserting early transcribed membrane proteins such as UIS4 (Mueller et al., 2005a). *uis4* transcripts accumulate in cytoplasmic granules and are translationally repressed in sporozoites, a process that depends on *cis*-regulatory elements contained in the open reading frame of *uis4* (Silvie et al., 2014). UIS4 protein synthesis is activated only after host cell invasion, allowing PVM remodeling. Storage of translationally silent RNA probably permits swift stage conversion yet avoids premature expression of liver stage-specific proteins.

Activation of the translational repression machinery is linked with sporozoite maturation, at least in the case of UIS4 (Silvie et al., 2014), but the effectors remain to be characterized. One possible candidate is the RNA-binding protein Puf2 (Pumilio and fem-3 binding factor homology 2). Puf are evolutionary conserved proteins that typically bind to the 3′ UTR of target mRNAs and repress their translation or induce their degradation (Wickens et al., 2002; Quenault et al., 2011). Silva et al. reported that Puf2 binds to UIS4 mRNA in RNA-immunoprecipitation experiments, and observed an up-regulation of UIS4 protein levels in *puf2(-)* sporozoites (Silva et al., 2016). However, Puf2 is probably not the only effector of UIS4 repression, and overexpression of Puf2 in liver stages has no impact on parasite development in the liver (Lindner et al., 2013a), indicating that Puf2 is not sufficient to repress UIS4 protein expression. Puf2 localizes to cytoplasmic granules in *P. berghei* and *P. yoelii* sporozoites (Gomes-Santos et al., 2011; Lindner et al., 2013a; Silva et al., 2016), and could stabilize sporozoite mRNAs directly or indirectly by regulating other factors controlling RNA metabolism. These storage granules disappear after sporozoite invasion of hepatocytes, possibly releasing specifically bound mRNAs for selective and immediate translation in early EEFs (Lindner et al., 2013a). RNAseq documented genome wide transcriptional alterations in *Puf2*(-) as compared to WT sporozoites, indicating that Puf2, beyond specific translational repression, contributes to mRNA homeostasis in sporozoites (Lindner et al., 2013a). *Puf2*(-) sporozoites can invade hepatocytes and differentiate into EEFs, indicating that Puf2 is not required for liver stage development (Müller et al., 2011; Lindner et al., 2013a). However, Puf2 is essential for maintaining sporozoite infectivity during prolonged parasite residence in the mosquito salivary glands (Gomes-Santos et al., 2011; Müller et al., 2011; Lindner et al., 2013a),

a function that relies on the protein RNA-binding domain (Lindner et al., 2013a). Over time, *Puf2*(-) sporozoites transform prematurely into round forms resembling EEFs and loose infectivity, a phenotype that is reminiscent of the behavior of *ik2*(-) mutants (Zhang et al., 2010). In fact, *IK2* mRNAs are down-regulated in *puf2*(-) sporozoites, suggesting that their phenotype could be due, to a large extent, to depletion of IK2 (Gomes-Santos et al., 2011; Müller et al., 2011; Lindner et al., 2013a).

Sporozoites express many other putative RNA-binding proteins in addition to Puf proteins (**Figure 2B** and **Table S2**) (Reddy et al., 2015), some of which likely contribute to global or specific control of RNA homeostasis. As an example, ALBA proteins are DNA/RNA binding proteins that have been detected in DOZI/CITH mRNPs in gametocytes (Mair et al., 2010). In *P. yoelii*, ALBA4 has a dual function, first in suppressing the activation of male gametocytes, then allowing semi-synchronous development of sporozoites. ALBA4 localizes in cytoplasmic granules and deletion of *alba4* gene modifies the steady state level of many genes in sporozoites, showing that ALBA4, like Puf2, contributes to mRNA homeostasis (Muñoz et al., 2017).

SLARP, A MASTER REGULATOR OF *PLASMODIUM* PRE-ERYTHROCYTIC DEVELOPMENT

Once transmitted to the mammalian host, *Plasmodium* sporozoites invade hepatocytes and rapidly transform into round forms, which then grow and undergo multiple nuclear divisions. The Sporozoite and Liver Stage Asparagine-Rich Protein (SLARP, also called SAP1), which is conserved among *Plasmodium* species and specific for the genus, has been identified as a master regulator of liver stage development (Aly et al., 2008; Silvie et al., 2008). Gene deletion of *slarp* in *P. berghei* and *P. yoelii* does not affect parasite blood stage growth, transmission to mosquitoes nor production of salivary gland sporozoites. However, *slarp(-)* sporozoites are non-infective to mice, due to an early developmental arrest in the liver. *slarp(-)* sporozoites invade hepatocytes and transform into small uninuclear EEFs, but fail to replicate and are rapidly eliminated (Aly et al., 2008; Silvie et al., 2008; Manzoni et al., 2014).

SLARP plays an essential role in shaping the steady state mRNA repertoire of sporozoites in the mosquito (Aly et al., 2008; Silvie et al., 2008; Aly et al., 2011). Transcriptome analysis with oligonucleotide microarrays revealed 38 down-regulated and 14 up-regulated genes in *P. yoelii slarp*-deficient sporozoites compared to WT parasites (Aly et al., 2011). Several *uis* genes, including *uis4*, were enriched among down-regulated genes in *P. berghei* and *P. yoelii slarp*(-) sporozoites (Aly et al., 2008; Silvie et al., 2008). The mechanism of SLARP-mediated regulation of gene expression is still unclear. SLARP is a large protein containing long stretches of low complexity, with an overrepresentation of asparagine residues, but lacks any known functional domain such as DNA- or RNA-binding domains. The C-terminal portion of SLARP is highly conserved across *Plasmodium* species and contains two putative nuclear localization signals (Silvie et al., 2008). The cellular localization of SLARP remains controversial, as different studies documented a cytoplasmic distribution in *P. yoelii* sporozoites (Aly et al., 2008; Aly et al., 2011) and a nuclear localization in *P. berghei* sporozoites (Silvie et al., 2008). Based on 3' and 5' RACE PCR experiments in *P. yoelii* sporozoites, Aly *et al.* concluded that SLARP depletion causes specific mRNA degradation, suggesting a role at the post-transcriptional level (Aly et al., 2011). However, experiments with reporter centromeric plasmids established that SLARP controls gene expression by acting on the promoter region of target genes (Briquet & Silvie personal communication). Combined with the nuclear localization of the protein in *P. berghei* sporozoites and EEFs (Silvie et al., 2008), we propose that SLARP functions as a master transcriptional regulator in sporozoites and liver stages (**Figure 1**). The absence of nucleic acid binding domain identified in SLARP sequence suggests an indirect role, perhaps in complex with AP2 domain transcription factors or chromatin-associated regulators.

HYPNOZOITES

P. vivax, unlike *P. falciparum*, is characterized by relapsing infections, caused by the reactivation of dormant hypnozoites in the liver. Relapses account for a major proportion of *P. vivax* malaria cases, and constitute a major obstacle for malaria eradication (White et al., 2014). This phenotype is shared with a few other primate malaria parasites, including the sister species *P. cynomolgi*, which infects Asian macaques (Krotoski et al., 1982a; Krotoski et al., 1982b). The mechanisms underlying hypnozoite formation, persistence and reactivation remain unknown. In contrast with *P. falciparum, in vitro* culturing of *P. vivax* blood stages is not yet feasible (Gunalan et al., 2020), limiting the access to sporozoites since mosquitoes must be infected by feeding on blood collected from individuals carrying circulating gametocytes. In this context, *P. cynomolgi* provides a relevant model to investigate the hypnozoite biology, and recent progress in genetic manipulation of *P. cynomolgi* has opened new perspectives for the study of hypnozoite formation and reactivation (Voorberg-van der Wel et al., 2013; Voorberg-van der Wel et al., 2020).

It has been hypothesized that, in relapsing *Plasmodium* species, cell fate could be predetermined in sporozoites, which may exist in two forms in the salivary glands of infected mosquitoes, tachysporozoites that develop directly into replicating EEFs, and bradysporozoites, which transform into dormant hypnozoites (Lysenko et al., 1977; White et al., 2017). However, transcriptome and proteome analysis of *P. vivax* sporozoites revealed overall similar steady state mRNA and protein compositions as observed in non-relapsing *P. falciparum* and *P. yoelii*, with stage-specific expression of the same genes needed for hepatocyte invasion and liver stage

development (Westenberger et al., 2010; Swearingen et al., 2017; Muller et al., 2019). Nevertheless, orthologs with significantly different expression levels between species were observed, as well as highly expressed species-specific *P. vivax* genes with no known ortholog (Westenberger et al., 2010; Swearingen et al., 2017). While the similar transcriptome and proteome profiles of *P. vivax* as compared to non-relapsing parasites does not favor the hypothesis of predetermined sporozoite fate, single cell approaches are needed to determine whether sporozoites form two distinct populations in relapsing species. Also, other mechanisms could differentially operate in sporozoite subpopulations, including chromatin regulation, which could contribute to stochastic generation of phenotypic diversity in sporozoites, as observed in *P. falciparum* blood stages with clonally variant gene expression (Cortés et al., 2012). Again, this hypothesis is difficult to explore without epigenome studies performed at the single cell level. Another possible model is that formation of hypnozoites takes place in the environment of infected hepatocytes, following sporozoite invasion. A phosphatidylinositol 4-kinase (PI4K) inhibitor reduced the number of hypnozoites in a prophylactic regimen but not when administered as a radical cure scheme (Zeeman et al., 2016), supporting the idea that the dormant phenotype is established post-invasion. Cell fate determination may be influenced by environmental factors in the host cell and/or stochastic regulatory events.

A still open question is whether hypnozoites represent a distinct stage or merely correspond to arrested early EEFs. To solve this question, comparison of hypnozoites and early non-arrested EEFs is still needed, yet would require a hypnozoite-specific marker to distinguish and sort dormant and non-dormant parasites. Unfortunately, there is to date no specific marker of hypnozoite identified. Candidate dormancy-related genes have been identified based on *in silico* studies (Carlton et al., 2008; Tachibana et al., 2012; de Souza Ribeiro et al., 2018), but none has been validated yet. The recent identification of LISP2 as an early marker of liver stage development should facilitate the differential analysis of hypnozoite versus developing early EEFs (Gupta et al., 2019). Indeed, transgenic *P. cynomolgi* expressing mCherry under control of *lisp2* promoter allowed for the first time live imaging of hypnozoite reactivation events (Voorberg-van der Wel et al., 2020).

To circumvent the lack of hypnozoite-specific marker, several recent studies have unraveled the transcriptome of *P. vivax* or *P. cynomolgi* cultured hypnozoites in comparison with hepatic schizonts, either after pharmacological enrichment (Gural et al., 2018) or after isolation of hypnozoites and schizonts by laser capture microdissection (Cubi et al., 2017) or sorting of fluorescent *P. cynomologi* by flow cytometry (Voorberg-van der Wel et al., 2013; Voorberg-van der Wel et al., 2017; Bertschi et al., 2018). These studies revealed that hypnozoites express a lower number of genes compared to schizonts, representing a limited number of pathways. Not surprisingly, the level of gene expression was higher in schizonts than in hypnozoites. When comparing transcriptomes across distinct stages, hypnozoites appear to be a transition point between sporozoites and replicating schizonts, supporting the idea that hypnozoites correspond to arrested early EEFs rather than a distinct stage (Muller et al., 2019). These transcriptional studies failed to identify a specific transcriptional marker for hypnozoites, but revealed that despite a general metabolic shutdown hypnozoites continue to express several specific pathways likely involved in the maintenance of dormancy, including ATP homeostasis and chromatin maintenance (Voorberg-van der Wel et al., 2017; Bertschi et al., 2018).

P. cynomolgi hypnozoites display the acetylated H4K8 mark of open chromatin, indicating that they are metabolically active with persistent transcriptional activity (Voorberg-van der Wel et al., 2017). One AP2 domain protein (coined AP2-Q) was proposed as a candidate marker of quiescence, together with two other AP2 factors (AP2-O2 and AP2-G2) (Cubi et al., 2017). However, up-regulation of this putative AP2-Q was not confirmed in other studies (Voorberg-van der Wel et al., 2017; Gural et al., 2018). AP2-L, which plays a role during liver stage development (Iwanaga et al., 2012), was equally expressed in hypnozoites and schizonts in both *P. vivax* and *P. cynomolgi*. Another AP2 domain protein (PVP01_0916300) was found to be differentially expressed in *P. vivax* hypnozoites (Gural et al., 2018), and its ortholog was also found to be expressed in *P. cynomolgi* hypnozoites (Voorberg-van der Wel et al., 2017; Bertschi et al., 2018). Although not specific for relapsing malaria parasites, this particular AP2 factor could be involved in the maintenance of dormant liver stage parasites, together with other AP2 factors identified in hypnozoites (Voorberg-van der Wel et al., 2017).

Cubi et al. also noted an up-regulation of the eIF2 kinase IK2 in *P. cynomolgi* hypnozoites, suggesting a possible role of translational repression in liver stage dormancy (Cubi et al., 2017). Analysis of the phosphoproteome of *P. vivax* sporozoites revealed enrichment in DNA and RNA-binding proteins among phosphorylated proteins, raising the possibility that post-translational control may also participate in regulation of dormancy (Swearingen et al., 2017).

Dormancy could be regulated at least in part by chromatin modifications, since inhibitors of histone methyltransferases stimulate the reactivation of *P. cynomolgi* hypnozoites in simian hepatocyte cultures (Dembélé et al., 2014). In agreement with this idea, hypnozoites were found to express genes involved in histone acetylation and methylation as well as chaperone-mediated modulation of nucleosome-histone interactions, in addition to members of the *alba* gene family, which have DNA and RNA binding activities (Bertschi et al., 2018). Finally, non-AP2 transcriptional regulators could also participate in parasite dormancy, including Myb proteins and SLARP. A Myb-like transcription factor was recently identified as a master regulator of bradyzoite differentiation in *Toxoplasma gondii* (Waldman et al., 2020). *Plasmodium* genome encodes Myb-like proteins, including Myb1 (Gissot et al., 2005), for which transcripts have been found in *P. berghei* liver stages in single cell RNAseq experiments (Howick et al., 2019). Intriguingly, SLARP was detected in *P. falciparum*, *P. berghei* and *P. yoelii* sporozoites (Lindner et al., 2013b; Lindner et al.,

2019; Hamada et al., 2020), but not in the *P. vivax* sporozoite proteome (Swearingen et al., 2017). Rodent parasites with targeted gene deletion of *slarp* show a complete developmental arrest in early liver stages, prior to replication, resulting in small hypnozoite-like forms in cell cultures (Aly et al., 2008; Silvie et al., 2008; Manzoni et al., 2014). SLARP is conserved across *Plasmodium* species, yet its contribution during liver stage dormancy is still unknown.

CONCLUSION AND PERSPECTIVES

Transmission of *Plasmodium* from the insect vector to a mammalian host is a critical step of the parasite life cycle, and requires coordinated gene expression for development and maturation into infectious sporozoites that can establish infection in the host liver. Although the knowledge of the gene regulatory mechanisms operating in sporozoites is still fragmented, master transcriptional regulators of sporozoite development and maturation have been identified, which probably participate in more complex regulatory circuits that still need to be characterized. In addition, translational repression plays a crucial role in mosquito stages, ensuring that quiescent sporozoites awaiting transmission in the mosquito salivary glands are already prepared for swift transition to the next replicative stage in the liver of the mammalian host. Many questions remain to be addressed, including the role of chromatin regulators, non-coding RNAs and specific TFs in sporozoite development and maturation. Also, the mechanisms underlying sporozoite transformation into dormant liver stages and hypnozoite reactivation in the medically important *P. vivax* still need to be elucidated. Deciphering gene regulation in *Plasmodium* sporozoites not only will help us to better understand development of this unicellular parasite but will also provide insights into many parasite-specific phenomena such as antigenic variation, alternative invasion pathways and dormancy, which may ultimately lead to the development of novel control strategies targeting host-parasite interactions. More importantly, the panel of the parasite regulation machinery distinct from those of its mammalian hosts makes it a promising target for novel antimalarial chemotherapies.

AUTHOR CONTRIBUTIONS

SB, OS, and CV drafted the manuscript. CM and SB computed the proteome data. CM drew **Figure 1**. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Pleiotropic Roles for the *Plasmodium berghei* RNA Binding Protein UIS12 in Transmission and Oocyst Maturation

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Colonization of the mosquito host by *Plasmodium* parasites is achieved by sexually differentiated gametocytes. Gametocytogenesis, gamete formation and fertilization are tightly regulated processes, and translational repression is a major regulatory mechanism for stage conversion. Here, we present a characterization of a *Plasmodium berghei* RNA binding protein, UIS12, that contains two conserved eukaryotic RNA recognition motifs (RRM). Targeted gene deletion resulted in viable parasites that replicate normally during blood infection, but form fewer gametocytes. Upon transmission to *Anopheles stephensi* mosquitoes, both numbers and size of midgut-associated oocysts were reduced and their development stopped at an early time point. As a consequence, no salivary gland sporozoites were formed indicative of a complete life cycle arrest in the mosquito vector. Comparative transcript profiling in mutant and wild-type infected red blood cells revealed a decrease in transcript abundance of mRNAs coding for signature gamete-, ookinete-, and oocyst-specific proteins in *uis12(-)* parasites. Together, our findings indicate multiple roles for UIS12 in regulation of gene expression after blood infection in good agreement with the pleiotropic defects that terminate successful sporogony and onward transmission to a new vertebrate host.

Keywords: malaria, *Plasmodium*, RNA binding protein (RBP), translational regulation, RNA, gametocyte, oocyst

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INTRODUCTION

During the complex life cycle of *Plasmodium*, malarial parasites switch between different host cells, extra- and intracellular life cycle forms, and an invertebrate and a vertebrate host. As the parasites progress in their developmental program, and particularly after switching host, adaptation to new environmental conditions, including temperature and immune defense, is central. For instance, *Plasmodium* gametocytes must remain infectious in the blood of the vertebrate host until they are eventually transmitted to the vector during a blood meal. The gametocytes need to quickly adapt to new environmental factors, leave the digestive environment of the blood meal, and colonize the

mosquito midgut (Billker et al., 1998; Billker et al., 2004). To swiftly accomplish a host switch, transcription and translation are tightly coordinated.

Transcriptional reprogramming is controlled by the apicomplexan Apetala-2 (Api-AP2) transcription factors (Balaji et al., 2005; Painter et al., 2011). Many genes needed for gametocytogenesis and early events of mosquito colonization are transcribed by the interplay of the *Plasmodium* transcription factors AP2-G and AP2-I, while asexual gene expression is repressed by AP2-G2 and AP2-FG controls female-specific gene regulation (Sinha et al., 2014; Yuda et al., 2015; Josling et al., 2020; Yuda et al., 2020). This transcriptional switch leads to the expression of mRNAs which are required for the multiple steps from gametocytogenesis to zygote formation until the motile ookinete stage is reached and the next checkpoint in transcription control is initiated by the AP2-O 1-4 family (Kaneko et al., 2015; Modrzynska et al., 2017). Therefore, a tight fine-tuning is essential to synthesize tailored proteins at the right time.

Translational repression occurs during host switch to keep gametocytes and sporozoites in a latent state. Accordingly, translation of pre-synthesized mRNAs, which are required for mosquito colonization, occurs only after transmission and constitutes a mechanism to be prepared for the new host environment. In gametocytes a large number of genes have been described to be translationally repressed (Le Roch et al., 2004; Mair et al., 2006; Lasonder et al., 2008; Mair et al., 2010; Zhang et al., 2010; Lasonder et al., 2016). Translational repression is particularly dominant in female gametocytes and affects a wide range of mRNAs, including those that encode surface proteins, e.g., P25, P28, and CCP2, transcriptional regulators, e.g., AP2-O, and proteases, e.g., plasmepsin IV. A conserved U-rich 47 nucleotide-long cis-acting consensus motif in the 5' and 3' UTRs of a subset of translationally repressed transcripts in *P. berghei* gametocytes was shown to be important in translational regulation in gametocytes (Hall et al., 2005; Braks et al., 2008). In the female gametocyte, the DDX6-class DEAD box RNA-helicase DOZI (development of zygote impaired) is bound to its target transcripts in a large storage mRNP complex and silences their translation (Mair et al., 2006; Mair et al., 2010; Guerreiro et al., 2014). The Pumilio-family RNA binding protein Puf2 increases proliferation and decreases stage differentiation. As a consequence, deletion of *P. berghei* and *P. falciparum Puf2* resulted in higher gametocyte rates and premature transformation of sporozoites in the mosquito salivary glands (Miao et al., 2010; Gomes-Santos et al., 2011; Muller et al., 2011; Lindner et al., 2013). In *pfpuf2(-)* parasites, an up-regulation of many gametocyte specific transcripts, mostly independent of those that are regulated in *dozi(-)/cith(-)*, was observed (Miao et al., 2010). Specific binding of *pfs25*, *pfs28*, and *Plasmepsin VI* mRNA by the *Pf*Puf2 protein was shown by RNA immunoprecipitation (Miao et al., 2013).

During host switch to the mosquito vector Puf2 negatively regulates gametocyte formation, and a post-transcriptional activator of gametocyte formation still remains elusive. In a complex with other proteins DOZI control of mRNA translation occurs much later, shortly after fertilization of female gametocytes inside the mosquito. Therefore, it is likely that additional factors participate in the regulation of post-transcriptional control of gene expression during mosquito transmission. In the present study we characterized an RNA binding protein, originally identified as up-regulated in infectious sporozoites gene 12 (*UIS12*), which is also highly expressed in gametocytes. A suppression subtractive hybridization screen aimed at discovering genes which are up-regulated in salivary gland sporozoites first described the *P. berghei* RNA binding protein UIS12 (PBANKA_0506200) (Matuschewski et al., 2002). As expected, protein prediction programs indicate an intracellular localization. Comparison of transcriptome and proteome data indicate that *P. berghei UIS12* is expressed in sporozoites, but the corresponding protein is not detectable, suggesting that UIS12 expression is regulated post-transcriptionally (Lasonder et al., 2008; Le Roch et al., 2004; Lindner et al., 2019). Due to the presence of the two RRM motifs, UIS12 is likely involved in post-transcriptional control of gene expression in gametocytes and pre-erythrocytic stages. Employing experimental genetics, the role of *UIS12* for parasite life cycle progression was analyzed with an emphasis on a potential function in post-transcriptional regulation.

RESULTS

The RNA Binding Protein *Pb*UIS12 Is Expressed at Multiple Life Cycle Stages

We initiated our analysis by an NCBI conserved domain database (CDD) search (Marchler-Bauer et al., 2015), which revealed two ~70 amino acid long RNA-recognition motifs (RRM), belonging to the class of RNA-binding domains (RBD) (Dreyfuss et al., 1988), in the *P. berghei* UIS12 protein sequence. The *UIS12* gene, and particularly the two RRM domains, are highly conserved between different *Plasmodium* UIS12 orthologs (**Figure 1A**), while in a candidate *Toxoplasma gondii* ortholog (TGME49_268380) the conservation is limited to the two RRM domains only. In general, the amino- and carboxy-terminal regions are less conserved, and all *Plasmodium* ortholog proteins have similar sizes of ~1,300 amino acid residues. The *PbUIS12* coding region has a total length of 4,070 base pairs and consists of two exons (**Figure 2A**). Of note, the two regions encoding the RRM domains are separated by the single intron, which also exhibits a degree of conservation.

We next measured *UIS12* transcript abundance throughout the *P. berghei* life cycle by quantitative RT-PCR (**Figure 1B**). In good agreement with the previous notion of up-regulation in salivary gland sporozoites, *UIS12* mRNA steady state levels are low in midgut oocysts and highest in salivary gland sporozoites. High *UIS12* transcript levels were also detected in early (24h) liver stages. In mixed asexual blood stages *UIS12* mRNA levels are low, whereas in schizonts, gametocytes and ookinetes, considerable expression was detected. Our observations mostly corroborate published data (Howick et al., 2019; Otto et al., 2014). However, we did not specifically analyze ring stages or

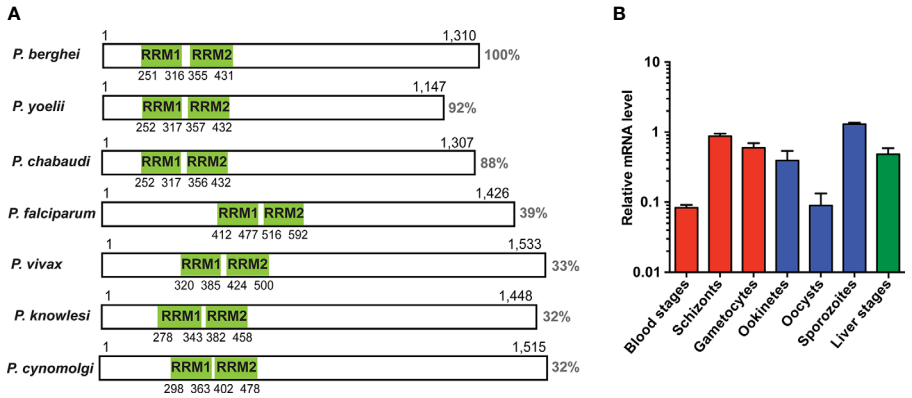

FIGURE 1 | Expression analysis of UIS12. **(A)** Schematic display of protein primary structure illustrating length, homology and position of the two predicted RRM domains (green) of the UIS12 orthologues of different *Plasmodium* species. Protein sequence identity is shown to the right. Gene identification and accession numbers are as follows: *P. berghei*, PBANKA_0506200; *P. yoelii*, PY17X_0507300; *P. chabaudi*, PCHAS_0506300; *P. falciparum*, PF3D7_0823200; *P. vivax*, PVX_001965; *P. knowlesi*, PKNH_0606300; *P. cynomolgi*, PCYB_061630. **(B)** Expression profile of *UIS12* in different *P. berghei* life cycle stages; mixed blood stages, schizonts, gametocytes (red), ookinetes, oocysts, sporozoites (blue), and 24 h liver stages (green). Expression levels were quantified by qRT-PCR, and data were normalized to GFP, constitutively expressed under the *PbEF1α* promoter (cl507) (Janse et al., 2006a). Mean values (± SD) from two independent experiments are shown.

distinguished between male and female gametocytes (Yeoh et al., 2017). Together, *UIS12* is expressed at multiple points of the *P. berghei* life cycle, indicative of multiple functions during life cycle progression and parasite stage conversion.

Targeted Gene Disruption of the RNA-Binding Protein *PbUIS12*

In order to study the gene function, we generated a *UIS12* loss-of-function mutant. *PbUIS12* deletion was generated by double homologous recombination employing a replacement strategy, as described previously (van Dijk et al., 1995) (**Figure 2A**).

The *UIS12* targeting construct (pΔPBANKA_UIS12) comprised ~500 bp each of the 5' and 3' non-coding sequences flanking a *TgDHFR/TS* pyrimethamine resistance cassette for positive selection with the anti-malarial drug pyrimethamine. Upon a double homologous recombination event, the *UIS12* gene is expected to be replaced by the selectable marker (**Figure 2A**). As recipient parasites we used *P. berghei* (strain: ANKA; clone: cl507), which expresses the fluorescent protein GFP under the control of the constitutive *eIF1α* promoter (Janse et al., 2006a). After transfection we recovered parental *uis12(-)* blood stage populations, from which clonal lines were generated by injection of limiting serial dilutions into mice. The desired recombination event was confirmed by diagnostic PCR, which confirmed the presence of 5' and 3' integration in the parental and the clonal *uis12(-)* populations, and by the absence of wild-type-specific PCR fragments in the clonal *uis12(-)* populations. During the course of our *UIS12* knock-out analysis and to further corroborate our findings we successfully generated an independent second *uis12(-)* knockout line (*uis12(-) clone 2*), in a different *P. berghei* ANKA recipient line, Bergreen, which constitutively expresses GFP under the control of the *HSP70* promoter (Kooij et al., 2012) (**Figure 2B**: clone 2; **Figure S1A**: clone 1).

At this stage, we could already conclude that the successful generation of clonal *uis12(-)*-knockout lines demonstrates that the gene is not essential for normal progression of asexual blood stages, although *UIS12* is expressed considerably in schizonts (**Figure 1B**).

Impaired Gametocyte Development of *uis12(-)* Parasites

After successful selection of *uis12(-)* parasites, we first examined asexual and sexual blood stage growth. To this end, C57BL/6 mice (n=5) were infected by intravenous injection of either 10,000 *uis12(-)* or WT-infected erythrocytes. Starting three days after infection the mice were monitored by daily microscopic examination of thin blood films, and parasitemia and gametocytemia were quantified (**Figures 2C, D**). Parasitemia increased daily in wild-type- and *uis12(-)*-infected mice to a similar extent. On day 7, wild-type and *uis12 (-)*-infected mice started developing symptoms indicative of onset of experimental cerebral malaria, supporting the notion that *uis12(-)* parasites display virulence typically seen in infections with *P. berghei* ANKA parasites.

The blood films were also analyzed for the presence of male and female gametocytes (**Figure 2D**). This analysis revealed that, in contrast to normal asexual blood infection, gametocytemia was significantly reduced in *uis12(-)* parasites as compared to the wild-type. *Uis12(-)* gametocyte numbers were already slightly reduced on day four after infection, when the first fully mature gametocytes became visible. The difference between WT and *uis12(-)* parasites increased further over time. Examination of size and morphology of male or female gametocytes and ookinetes revealed no difference between the two parasite lines (**Figure 2E**). The presence of ookinetes indicates that *uis12(-)* gametocytes are able to fertilize and subsequently form a zygote that develops into an ookinete.

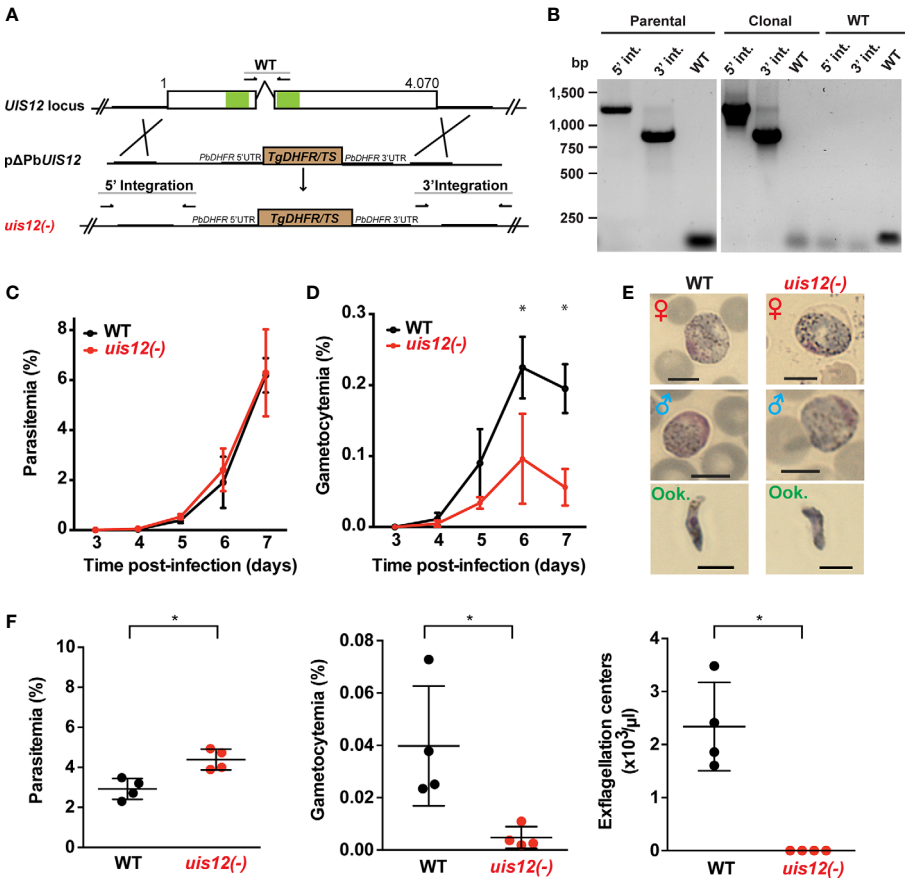

FIGURE 2 | Normal asexual blood stage growth in *uis12(-)* parasites, but reduced gametocyte development and exflagellation. **(A)** Graphical scheme showing the replacement strategy to generate *uis12(-)* parasites, and *UIS12* gene structure. The *UIS12* locus was replaced by double-homologous recombination with the linearized replacement plasmid pΔPBANKA_UIS12, which includes a resistance marker (*Toxoplasma gondii DHFR/TS*) and the 5' and 3' flanking regions of *UIS12*. Arrows and lines indicate positions of knockout- and wild-type-specific oligonucleotide positions and PCR fragments, respectively. The RNA recognition motif domains (RRM) are shown in green. **(B)** Confirmation of *UIS12* gene disruption by diagnostic PCR. Genomic DNA of parental *uis12(-)*, clonal *uis12(-)* clone 2, and wild-type parasites served as templates. 5'- and 3'-integration-specific primer combinations amplify the predicted fragment only in the recombinant locus. Wild-type-specific primer pairs that do not produce a PCR fragment in the recombinant locus amplified the wild-type locus (right panel). Absence of residual wild-type confirms a clonal *uis12(-)* line (central panel). Time courses of parasitemia **(C)** and gametocytemia **(D)** of *uis12(-)* (clone 1, red) and wild-type (WT) (black) parasites, starting three days after intravenous injection of 10,000 mixed blood stages into C57BL/6 mice (n=5). Daily microscopic analysis of Giemsa-stained blood films was used to determine parasitemia and gametocytemia. **(C)** Parasitemia, percentage of asexual parasites per total erythrocytes **(D)** gametocytemia, percentage of gametocytes per total erythrocytes. Means of parasitemia and gametocytemia (± SD) are shown. *$p < 0.05$ (multiple t-tests, one per row). **(E)** Gametocytes and ookinetes of *uis12(-)* (clone 1) are morphologically indistinguishable from wild-type (WT). Shown are microscopic images of Giemsa-stained female (♀) and male (♂) gametocytes and ookinetes (Ook., from ookinete cultures). Brightfield microscopy, magnification 1,000-fold. Scale bars, 5 µm. **(F)** Asexual and sexual blood stage development. Infected blood (C57BL/6 mice; n=4) was analyzed four days after injection of 10^7 *uis12(-)* (clone 1, red) or WT (black)-infected erythrocytes, respectively. Plots show parasitemia (left), gametocytemia (center), and numbers of exflagellation centers per microliter of mouse tail blood (right). Results are expressed as means (± SD). *$p < 0.05$ (Mann-Whitney test).

Both, male and female gametocytes were found in the expected female biased proportions. Together, the data indicate that UIS12 is dispensable for asexual growth, but plays an important role during gametocytogenesis. Analysis of the second *uis12(-)* clone corroborated these findings (**Figures S1B, C**).

Exflagellation Is Markedly Reduced in *uis12(-)* Parasites

The potential of male gametocytes to produce motile microgametes is an active process and termed exflagellation. In both *uis12(-)* clonal lines, exflagellation events were detected very rarely by microscopic examination of undiluted blood from *uis12(-)* infected mice at any given time point. Yet, this already showed that *uis12(-)* male gametocytes are able to produce motile male gametes, albeit at very low frequencies. To quantify the exflagellation centers, C57BL/6 mice (n=4) were injected intravenously with a high dose of 10^7 blood stages. Four days later, parasitemia, gametocytemia and the number of exflagellation centers were enumerated (**Figure 2F**, *uis12(-)* clone 1). Interestingly, in this experiment, the parasitemia was slightly, but significantly higher in *uis12(-)*-infected mice. In

agreement with our first experiment (**Figure 2D**), gametocytemia was 8.6-fold reduced in *uis12(-)* parasite-infected mice. Exflagellation was not visible under these conditions, i.e., 1:25 diluted blood, in *uis12(-)*-infected mice (**Figure 2F**). Even after an extended incubation or when examined the following days, exflagellation was not detectable. In conclusion, male *uis12(-)* gametocytes retain the ability to progress to functional microgametes, but at a very low rate. The defect in exflagellation is more severe than the reduction in gametocytemia indicating additive defects in absence of *UIS12* upon progression from blood infection to transmission to the insect vector.

Reduced Oocyst Formation By *uis12(-)* Gametocytes, But Failure to Produce Sporozoites

The severe defect in *in vitro* exflagellation implied a large impact in mosquito colonization. However, after transmission to *Anopheles stephensi* mosquitos, *uis12(-)* parasites were able to establish midgut infections and produce oocysts, showing that early events of midgut colonization are not abolished *in vivo* (**Figure 3**). The mean mosquito infection prevalences were 54% (± 18%) for wild-type (n=5) and 46% (± 23%) for *uis12(-)* (n=5).

This finding prompted us to quantify oocyst maturation. To this end, midguts were isolated daily starting on day 4 after mosquito infection up until day 14. Oocyst numbers and morphology as well as their ability to produce sporozoites were analyzed by fluorescence microscopy (**Figure 3**, *uis12(-)* clone 2). In *uis12(-)*-infected mosquitoes, the number of oocysts per midgut was more than 10-fold reduced as compared to wild-type, consistent with reduced gametocyte numbers and the very low exflagellation rate (**Figure 3B**). Strikingly, in the absence of *UIS12* oocysts were markedly smaller in size (**Figures 3A, C**). While up until day 4 the oocyst size was comparable between both groups, over time *uis12(-)* oocysts displayed an abnormal

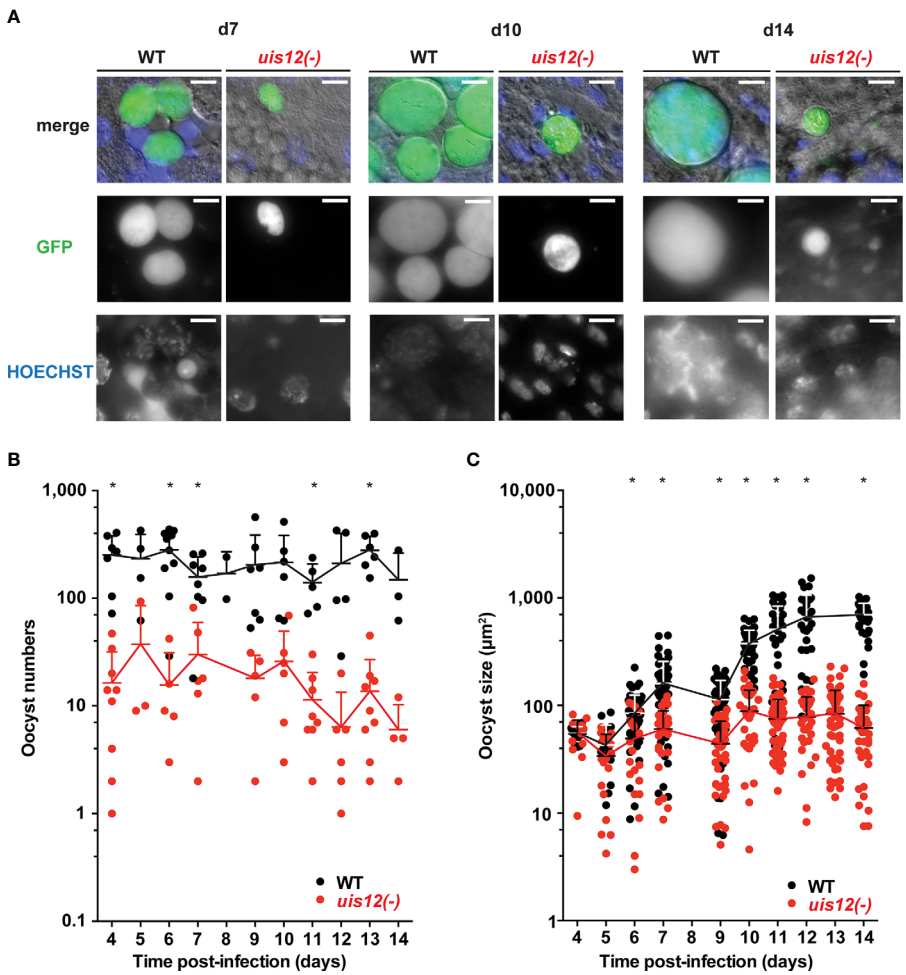

FIGURE 3 | Disruption of *UIS12* leads to a reduced oocyst size and a defect in sporozoite formation. **(A)** Shown are representative live micrographs of *uis12(-)* and wild-type oocysts 7, 10 and 14 days after an infectious blood meal. GFP (green) is constitutively expressed under the *HSP70* promoter (*P. berghei* ANKA Bergreen) (Kooij et al., 2012). *Uis12(-)* clone 2 was used for this experiment. Nuclei were stained with Hoechst (blue). Note the reduced density and size of *uis12(-)* oocysts, and absence of sporozoites in *uis12(-)* oocysts. Magnification, 630-fold; scale bars, 10 µm. **(B, C)** Time course of oocyst numbers per infected mosquito midgut **(B)** and oocyst size (µm^2) **(C)** at different time points after midgut infection (*uis12(-)* clone 2, red; WT, black). *p < 0.05 (multiple t-tests).

growth pattern. Their size remained unchanged, whereas the WT oocyst diameter increased ~10-fold over the following ten days. This finding indicates that the early events of oocyst development, including transformation of the motile ookinete to the spherical oocyst, and the initial increase in biomass (Carter et al., 2007) are reduced, but not completely abolished. An additional defect leads to arrested oocyst maturation. The analysis of the second *uis12(-)* clone corroborated these findings (**Figures S1D–F**).

Upon close examination at later time points, *uis12(-)* oocysts displayed little or no nuclear staining, harbored many large vacuoles, and frequently were surrounded by an apparently wrinkled oocyst wall (**Figure 3A**, **Figure S1F**). As a consequence, no sporozoites were seen inside *uis12(-)* oocysts. This lack of sporozoite formation was confirmed by analysis of extracted midguts; no midgut- associated sporozoites were detectable. In WT-infected midguts sporoblast formation was observed inside the oocysts starting around day 11 after infection, and from day 12 onwards oocysts started to fill with sporozoites (Matuschewski, 2006) (**Figure 3A**). In contrast, *uis12(-)* midguts remained free of sporozoites even until late time points of mosquito infection, excluding delayed sporozoite formation.

In good agreement, initial microscopic screening of salivary gland extracts of *uis12(-)*-infected mosquitoes for presence of sporozoites did not return a single sporozoite. To obtain independent support for the complete absence of sporozoites in *uis12(-)*-infected mosquitoes, we inoculated C57BL/6 mice with salivary gland extracts of 20 *uis12(-)*-infected mosquitoes. In addition, we exposed C57BL/6 mice to ~100 *uis12(-)*-infected mosquitoes (**Table 1**, both clones). In sharp contrast to control infections with wild-type-infected mosquitoes under similar conditions, none of the attempts to transmit *uis12(-)* parasites resulted in blood stage infections (**Table 1**). This defect in transmission confirms that *uis12(-)* parasites are unable to complete their development inside the mosquito.

Deregulation of Transcripts Important for Mosquito Transmission in *uis12(-)* Blood Stages

We hypothesized that due to the presence of the two RRM motifs, UIS12 is likely involved in post-transcriptional control of gene expression. Reduced gametocyte production, exflagellation, and oocyst development detected in *uis12(-)* parasites suggests that UIS12 might be regulating target mRNAs and alter expression of the respective proteins, which in turn play important roles in these stages.

To profile the transcriptome of *uis12(-)* parasites, we performed a comparative microarray analysis on *uis12(-)* and wild-type mixed blood stage RNAs (**Figure 4**, **Figure S2**). Since the first defect is detected during gametocyte formation, we limited our analysis to mixed blood stages, that include the asexual blood stages, from which gametocytes originate. Attempts to retrieve sufficient *uis12(-)* gametocytes for a microarray failed. The microarray analysis was performed with two biological replicates (R1 and R2), both with *uis12(-)* clone 1. For both replicates, parasitemia of the samples were comparable (R1: WT 6.2%; *uis12(-)* 6.3%; R2: WT 6.4%; *uis12(-)* 8.7%). As expected, gametocytemia was reduced in *uis12(-)* parasites (R1: WT 0.2%; *uis12(-)* 0.06%). Each microarray experiment was performed by dual-color hybridizations, and a color-swap dye-reversal was included in order to compensate for dye-specific effects. In this microarray analysis, the -fold change of the expression levels of 2,890 *P. berghei* genes could be successfully compared (**Figure 4A**, **Figures S2A, D**).

Overall, absence of *PbUIS12* correlated with a global perturbation of 30% of all mRNA expression levels (threshold, <-2 or >2). More transcripts were down-regulated (646, 23%) than up-regulated (199, 7%). The mean down-regulation was -6.4-fold, whereas the mean up-regulation was +2.5-fold (GEO Series accession number: GSE152686). As expected, the *UIS12*-knockout was independently confirmed by the microarray analysis; *UIS12* transcripts were down-regulated by -22.3-fold (**Table 2**). Within the 25 most down-regulated transcripts known proteins required for the switch to the mosquito vector, CCp1 (LAP2), LAP5, P48/45, and P28, as well as numerous previously unrecognized proteins are represented (**Table 2**). The list of the 25 most up-regulated genes contains, among others, PIR proteins (**Table S1**). Remarkably, many transcripts that were down-regulated more than 10-fold in *uis12(-)* blood stages, are reported to be expressed in gametocytes (Hall et al., 2005). We also analyzed the proportions of up-, down-, and non-regulated transcripts and found distinctions between stage-specific RNAs, as defined by RNA sequencing data (Otto et al., 2014). For instance, gametocyte transcripts were defined by >2-fold up-regulation in gametocytes as compared to asexual blood

TABLE 1 | No malaria transmission by *uis12(-)*-infected mosquitoes.

Parasites	Route of injection[a]	Infected/Injected	Pre-patency (days)[b]
WT 507cl	10,000 sporozoites i.v.	5/5	3
uis12(-) clone 1	mosquito bite (~100 mosquitoes)	0/5	N.A.
	salivary gland extract i.v. (20 mosquitoes)	0/4	N.A.
WT Bergreen	mosquito bite (~100 mosquitoes)	3/3	3
uis12(-) clone 2	mosquito bite (~100 mosquitoes)	0/3	N.A.
	salivary gland extract i.v. (20 mosquitoes)	0/3	N.A.

[a]*C57BL/6 mice were exposed to the bites of ~100 infected mosquitoes for 15–20 min or injected intravenously (i.v.) with salivary gland extract isolated from 20 mosquitoes (uis12(-)) and, as a control, 10,000 sporozoites (WT). Data are pooled from two separate experiments.*

[b]*the pre-patent period represents the days until detection of the first infected erythrocytes by microscopic thin blood film examination after sporozoite inoculation. N. A. not applicable. The colors were used to highlight different conditions.*

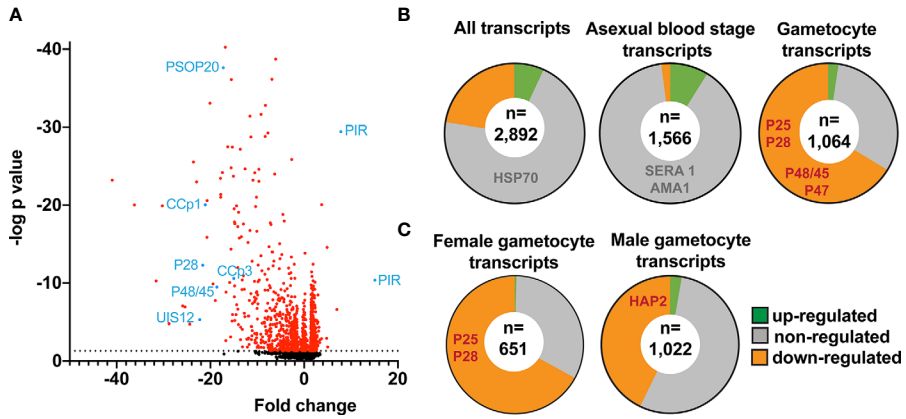

FIGURE 4 | Down-regulation of distinct mRNAs coding for signature gamete-, ookinete- and oocyst-specific proteins in *uis12(-)* blood stage parasites. Shown are mean values of microarray results of total RNA isolated from mixed infected erythrocytes from *uis12(-)* (clone 1)- and WT- infected mice from two biological replicates. **(A)** Shown is a volcano-plot illustrating the fold change of the expression levels and the negative log *p*-values of all analyzed 2,890 *P. berghei* genes. The dotted black line represents a *p*-value of 0.05 and all transcripts with a *p*-value <0.05 are shown in red. Exemplary transcripts are highlighted and labeled in blue. **(B)** Pie charts displaying the proportions of up- (green >2), non- (grey), and down- (orange <-2) regulated transcripts among all transcripts (upper left), blood stage-specific transcripts (center), and gametocyte-specific transcripts (upper right) (Otto et al., 2014). Exemplary transcripts are listed in the respective region of the chart. The number of transcripts analyzed is shown in a white circle inside the center. **(C)** Pie charts displaying the proportions of up- (green >2), non- (grey), and down- (orange <-2) regulated transcripts among female (left) and male (right) gametocyte-specific transcripts (Yeoh et al., 2017). The number of transcripts analyzed is shown in a white circle inside the center.

TABLE 2 | The top 25 down-regulated genes in blood stages of *uis12(-) vs.* wild-type.

Gene ID	Product description	Mean -fold change R1[a] R2[b]	-fold change R1[a]	-fold change R2[b]
PBANKA_0806800	unknown	-41.0	-37.5	-44.4
PBANKA_1449000	candidate microgamete surface protein MiGS	-36.2	-25.4	-47.0
PBANKA_1349400	candidate DNA replication complex GINS protein	-31.6	-29.0	-34.2
PBANKA_1340400	candidate lactate dehydrogenase	-30.2	-24.2	-36.3
PBANKA_1400400	fam-b-protein	-28.8	-14.3	-43.4
PBANKA_1361600	E1-E2 ATPase	-25.9	-25.9	-25.9
PBANKA_1040300	fam-b-protein	-25.4	-18.5	-32.3
PBANKA_1315300	LCCL domain-containing protein (LAP5)	-24.4	-27.4	-21.4
PBANKA_0413000	unknown	-23.6	-29.3	-18.0
PBANKA_1246600	cleft-like protein 1	-22.9	-9.7	-36.2
PBANKA_0506200	RNA binding protein (UIS12)	-22.3	-31.7	-12.8
PBANKA_0514900	ookinete surface protein P28	-21.6	-15.5	-27.8
PBANKA_1300700	LCCL domain-containing protein (CCp1)	-21.1	-20.7	-21.4
PBANKA_0719700	unknown	-20.7	-19.2	-22.2
PBANKA_0707100	inner membrane complex protein 1i	-20.7	-26.5	-15.0
PBANKA_1334900	unknown	-20.4	-20.1	-20.7
PBANKA_1353800	unknown	-20.1	-26.1	-14.1
PBANKA_1432400	perforin-like protein 2	-19.4	-16.9	-21.9
PBANKA_0608600	candidate EGF-domain protein	-19.0	-23.9	-14.0
PBANKA_1400900	unknown	-18.9	-20.3	-17.5
PBANKA_1359600	6-cysteine protein (P48/45)	-18.6	-21.0	-16.2
PBANKA_1106000	unknown	-17.9	-22.2	-13.5
PBANKA_1414800	unknown	-17.9	-19.6	-16.1
PBANKA_1120400	candidate inner membrane complex protein 1j	-17.8	-18.9	-16.7
PBANKA_1318600	unknown	-17.8	-15.9	-19.7

[a]*biological replicate 1.*
[b]*biological replicate 2.*
The colors were used to highlight different conditions.

stages (**Figure 4B**, **Table 3**, **Figures S2B, E**). In good agreement with normal replication rates, very few asexual blood stage-specific transcripts were down-regulated in *uis12(-)* parasites. Instead, the proportion of up-regulated transcripts was highest for asexual blood stage-specific mRNAs, which might reflect over-representation of these stages due to defects in gametocytogenesis. However, signature transcripts, like *AMA1* (-0.3), *HSP70* (-1.6), *MSP1* (1.7) and *MSP8* (1.5), were neither

TABLE 3 | Examples for deregulation of signature vector-stage transcripts in *uis12(-)* parasites.

	Known blood stage transcripts		Known transmission stage transcripts	
Up-regulated	Pb235	2.8		
Unaffected	AMA1	-0.3	AP-2 G*	-0.9
	MSP8	1.5	p230p	-0.3
	HSP70	-1.6	NPT1*	-1.3
	SERA3	1.7	Puf2	-1.9
	MSP1	1.7		
Down-regulated (< 10-fold)			CITH*	-2.3
			Actin II	-2.8
			CDPK4	-3.1
			MAPK 2	-3.3
			GAP45	-5.1
			AP2-0	-5.7
			CDPK3	-5.8
			Plasmepsin VI	-6.3
			HAP2	-7.5
			MDV1	-10
Down-regulated (> 10-fold)			CCp5*	-11.2
			CCp4*	-12.2
			CCp3*	-15
			P47	-15
			CCp2*	-15.3
			P25	-17.1
			P48/45	-18.6
			CCp1*	-21.1
			P28	-21.6

*Shown are exemplary blood and transmission stage transcripts. The mean -fold change represents steady-state mRNA levels in uis12(-) parasites compared to the corresponding WT parasite stages. *genes with a knock-out phenotype similar to uis12(-).*

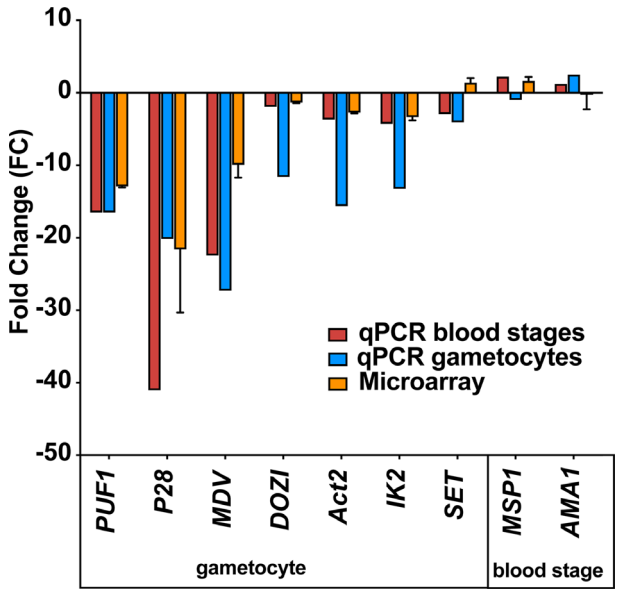

FIGURE 5 | Confirmation of down-regulation of selected mRNAs by quantitative RT-PCR. Quantitative RT-PCR on selected blood stage and gametocyte transcripts validate the results of the microarray analysis. Shown are -fold changes of steady state mRNA levels of *uis12(-)* clone 1 compared to wild-type mixed blood stages for selected genes (*Puf1*, *P28*, *MDV*, *DOZI*, *Actin II*, *UIS1/IK2*, *SET*, *MSP1*, and *AMA1*). qRT-PCR data were normalized to the steady state levels of *HSP70* mRNA. The microarray data represent mean values of biological replicate 1 and 2 (± SD).

up- nor down-regulated in the absence of *UIS12* (**Figure 4B**, **Table 3**). We also noted that the mRNA levels of many signature mosquito stage-specific proteins were down-regulated in *uis12(-)* mixed blood stages. In addition, we also compared the proportions of up-, down- and non-regulated transcripts and found distinctions between male- and female-specific transcripts, as defined by RNA sequencing data (Yeoh et al., 2017) (**Figure 4C**, **Figures S2C, F**). While 67% of the female gametocyte transcripts were down-regulated in absence of *UIS12,* only 43% of male-specific transcripts were down-regulated, indicating differential impact of UIS12 on male and female gametocyte-specific transcripts in mixed blood stages.

Next, we wanted to independently confirm the microarray results by quantitative RT-PCR of selected transcripts on blood stage and gametocyte mRNA from *uis12(-)* and WT samples (**Figure 5**). This analysis revealed a good agreement with the mean -fold changes determined in the microarray for gametocyte- and blood stage- specific transcripts. The gametocyte-expressed transcripts *Puf1*, *DOZI*, *UIS1/IK2*, *SET*, and *Actin II* (Mair et al., 2006; Pace et al., 2006; Deligianni et al., 2011; Gomes-Santos et al., 2011; Muller et al., 2011) showed similar -fold changes in both assays. Similarly, the blood stage-expressed transcripts *AMA1* and *MSP1* remained un-regulated. For some transcripts, e.g., male development factor (*MDV1*) (Lal et al., 2009), the reduction in *uis12(-)* parasites was more pronounced by quantitative RT-PCR in comparison to the microarray data. qRT-PCR on gametocyte mRNA samples were consistent with the data from asexual blood stages (**Figure 5**, *uis12(-)* clone 1). Overall, we could confirm the results of the microarray on these samples, indicating that the down-regulation of the gametocyte transcripts is a direct result of a specific UIS12-dependent regulation and not merely a consequence of the absence of gametocytes in *uis12(-)* mixed blood stages.

Gene Ontology Analysis of the Deregulated Transcripts in *uis12(-)* Parasites

We next performed a gene ontology (GO) enrichment analysis (Ashburner et al., 2000) (**Tables S2** and **S3**). According to this analysis the 199 up-regulated and the 646 down-regulated transcripts are involved in 21 and 42 different biological processes, as described by distinct GO terms, respectively. Examples for GO terms of the set of down-regulated genes include reproduction, cell communication and phosphorylation (**Table S2**), in good agreement with the known roles of kinases in gametogenesis (Invergo et al., 2017). Examples for GO terms of the set of up-regulated genes include RNA processing and histone acetylation (**Table S3**), further indicative of perturbations in gene expression and mRNA translation in absence of *UIS12*.

A 10-Nucleotide Motif Shared By Up-Regulated Transcripts in *uis12(-)* Parasites

Since UIS12 contains two RNA recognition motifs and the combination of at least two RRM domains allows the continuous recognition of a 8-10 nucleotide-long sequence

motif with high affinity (Maris et al., 2005), we screened all ORFs, and 1,000 base pairs of the flanking 5' UTR and 3' UTR sequences of genes up-regulated >2.5-fold in replicate 1 for common 8-10 nucleotide-long motifs using MEME (Bailey et al., 2009) using the respective portions of the non-regulated transcripts as reference. While this search did not return a motif in either the 5' or 3' UTR sequences, we found a 10 nucleotide-long motif (TTTYTTTTTC) in the ORF (**Figure 6A**). This signature was found 751 times in 174 of the 220 up-regulated sequences (e-value: $4.2x10^{-4}$). We restricted our search to up-regulated transcripts, since down-regulation can at least be partially attributed to fewer gametocytes in mixed *uis12(-)* blood stages.

DISCUSSION

In this study, deletion of the candidate RNA binding protein UIS12 led to the discovery of a potential post-transcriptional regulator of gametocytogenesis and mosquito transmission. Our results underscore the key roles of a fine-tuned transcriptional control and post-transcriptional regulation during transmission of the malaria parasite between hosts. When *UIS12* is absent, fewer gametocytes are produced and *in vitro* exflagellation is severely impaired, yet not abolished. The defects of *uis12(-)* parasites during host switching cannot be compensated for in subsequent steps in the *Plasmodium* life cycle, and *uis12(-)* infection leads to fewer oocysts, which fail to undergo oocyst maturation. We can correlate these defects with a set of signature gametocyte and mosquito-stage transcripts that are down-regulated in *uis12(-)* parasites already during blood infection. Parasites lacking *UIS12* produce ~80% fewer gametocytes. Whether this reduction is due to a defect in sexual conversion or later during the maturation and/or survival of the sexual stages cannot be distinguished in the *P. berghei in vivo* model, where gametocytes are constantly formed. Both male and female gametocytes account for this reduction, indicative of a role of *UIS12* in early events of gametocytogenesis, perhaps in conjunction with transcriptional control by AP2-G, AP2-I, AP2-FG, and AP2-G2. Gene deletion of *AP2-G* resulted in a complete loss of gametocytes and the deletion of *AP2-G2* in a major reduction of gametocytes (Sinha et al., 2014). While *AP2-G* expression was unaffected in *uis12(-)* parasites, *AP2-G2* was not included in our microarray screen.

Plasmodium falciparum possesses at least 189 candidate RNA-binding proteins (RBP), which comprise 3.5% of all annotated genes. In good agreement with the complex regulation network required for stage conversion, a third of all RBPs show elevated expression during the gametocyte stage (Reddy et al., 2015). In contrast to a broad role in gene regulation, such as translational inhibition by phosphorylation of eIF2α, RRM-containing RBPs are mainly known for their tight RNA-specific control of RNA processing, export, and stability. The presence of tandem RRM domains, a feature observed in UIS12, ensures high sequence specificity (Maris et al., 2005).

Whether UIS12 is a positive regulator of gametocytogenesis and how it influences this process awaits further molecular and biochemical analysis, including RNA-pull down studies to capture UIS12 target transcripts. The role of UIS12 in promoting gametocytogenesis shares similarities with another RNA-binding protein, the pumilio-familiy RNA-binding protein *Puf1* which was shown to function in *P. falciparum* gametocyte maturation, and especially of female gametocytes (Shrestha et al., 2016). Both proteins likely inhibit asexual propagation and, thereby, promote gametocyte formation. A related pumilio-familiy RNA-binding protein, *Puf2*, exerts the corresponding

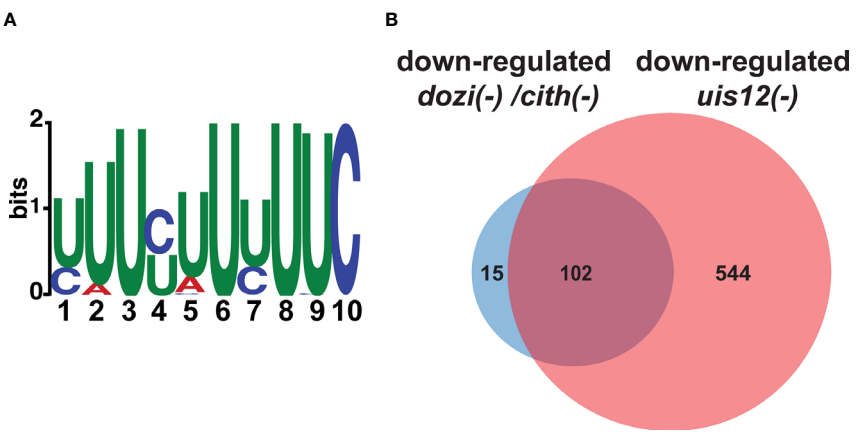

FIGURE 6 | A motif is shared by up-regulated transcripts in *uis12(-)* parasites, and the comparison of *dozi(-), cith(-)* and *uis12(-)* target transcripts reveals possible overlapping functions. **(A)** Shown is a graphic display of the 10-nucleotide U-rich motif found in the ORF of transcripts that are up-regulated in absence of *UIS12* in the microarray with biological replicate 1. The size of the depicted nucleotide represents its probability at the respective position. **(B)** Venn diagram comparing shared down-regulated transcripts of the gametocyte microarray analyses performed for *dozi(-)* and *cith(-)* with a -fold change lower than -1 (Mair et al., 2010) and down-regulated transcripts in mixed blood stages of *uis12(-)* with a mean -fold change lower than -2.

function in sporozoites and prepares them for transmission to the vertebrate host (Miao et al., 2010; Gomes-Santos et al., 2011; Muller et al., 2011). We note that UIS12 likely executes a second function, similar to the one analyzed in the present study, in pre-erythrocytic stages, the stage where it was originally identified (Matuschewski et al., 2002), but the complete block in sporogony prevented us from studying this by classical reverse genetics. It, thus, still remains unclear if *UIS12* also plays a role in the host switch back to the mammalian host. Of note, in sporozoites translational repression of *UIS12* was described, and the protein is only detectable in liver stages (Lindner et al., 2019). Accordingly, a stage-specific knockout, which depletes *UIS12* during sporogony is required to study its cellular roles for sporozoite transmission and liver infection.

The few remaining *uis12(-)* gametocytes were able to form male and female gametes, fertilize, and form ookinetes. Accordingly, numbers of oocysts are extremely low in *uis12 (-)*-infected *Anopheles* mosquitoes. Complete life cycle arrest in *uis12(-)* infections occurs at an early time-point after oocysts are formed, indicating that the events leading to oocyst formation are not impaired (Carter et al., 2007).

Our microarray analysis revealed a sharp drop in the steady state mRNA levels of numerous well-characterized *Plasmodium* genes. The *P. berghei* LCCL protein family members CCp1 and lectin adhesive-like protein 5 (LAP5) are expressed in female gametocytes and ookinetes. *Pb*LAP proteins are important for sporogenesis inside oocysts, yet known to be expressed already in gametocytes (Pradel et al., 2004; Trueman et al., 2004; Raine et al., 2007; Lavazec et al., 2009; Saeed et al., 2010). The surface proteins of the 6-cysteine repeat protein family P48/45 are essential for male gametocyte fertility (van Dijk et al., 2001). P25 and P28 are surface proteins of sexual stages, with distinct roles in ookinete to oocyst transformation (Siden-Kiamos et al., 2000; Tomas et al., 2001). Interestingly, some transcripts that are severely depleted, including P28, AP2-O, CCP2, and CCP4, have been reported to be translationally controlled (Vervenne et al., 1994; Saeed et al., 2013; Lasonder et al., 2016). Strikingly, the function of some of these repressed targets extends far beyond the ookinete stage, and the corresponding proteins are important for oocyst and sporozoite formation, illustrating the complexity of regulation of post-transcriptional control of gene expression in gametocytes (Lasonder et al., 2016). A deregulation of mRNAs already in mixed blood stages in absence of *UIS12* might partially explain the observed inability to complete oocyst development. However, the abundant *UIS12* mRNA levels in ookinetes suggest UIS12 presence at that stage and a potential additional checkpoint of post-transcriptional control by *UIS12* possibly after transcription of mosquito stage-specific target genes is orchestrated by a family of closely related Apetala-2 proteins, AP2-O 1-4, in ookinetes (Kaneko et al., 2015; Modrzynska et al., 2017). And this additional checkpoint in ookinetes might be the reason for the pleiotropic effects observed upon absence of *UIS12*, finally resulting in the aberrant maturation of oocysts. Due to the low *UIS12* transcript levels in oocysts a direct role at this point in the life cycle is less likely. We, thus, propose that UIS12 exerts multiple regulatory roles at consecutive life cycle stages, and, hence, is together with Puf2 one of the first known *Plasmodium* RNA binding proteins with pleiotropic functions for life cycle progression (Gomes-Santos et al., 2011; Lindner et al., 2013; Miao et al., 2013; Miao et al., 2010; Muller et al., 2011).

The GO-term enrichment of down-regulated transcripts in *uis12(-)* shows perturbation of many kinases and GO-term enrichment of up-regulated transcripts revealed perturbation of gene expression. A MEME search in the ORFs, 5' UTRs and 3' UTRs of the up-regulated transcripts identified in the microarray did reveal a 10-nucleotide U-rich motif in the ORFs unique to the analyzed subsets when compared to sequences that were not perturbated in absence of *UIS12*. This 10 nucleotide-long U-rich motif might represent a signature of UIS12 interaction with target mRNAs during blood infection, which might be required for proper preparation for host switching. We wish to point out that down-regulation of gametocyte signature transcripts in *uis12(-)* blood stages can be largely attributed to the at least 3.5-fold reduction in gametocyte numbers, yet the levels of down-regulation ranged from a low (-2, e.g., *Puf2*) to more than 15-fold (e.g., *CCP1* and *CCP2*). By qPCR we could confirm the down-regulation of some of the gametocyte specific transcripts such as P28 in a gametocyte enriched mRNA preparation of *uis12(-)* blood stage parasites, indicating that the down-regulation of gametocyte transcripts might not only be a consequence of the absence of gametocytes in *uis12(-)* mixed blood stages, but a direct result of a specific UIS12-dependent regulation.

Further analyses employing RNA-Seq or single cell transcriptomics on synchronized and purified stages, ranging from ring stages to ookinetes, are warranted to gain an in-depth understanding of the mRNA repertoire that is under UIS12 control. Since the shared signature motif was identified in up-regulated transcripts, this would argue against a role of UIS12 as an mRNA stabilizing factor. At least three alternative scenarios emerge (**Figure 7**). One potential, albeit unusual, mechanism of UIS12 could be binding and stabilizing of large set of target mRNAs that encode gametocyte- and mosquito stage-specific proteins. Alternatively, UIS12 acts on one or few mRNAs, which in turn drive expression control, for instance by stabilizing transcripts that encode checkpoint activators. A reverse, and perhaps more likely function would be an inhibitory role towards target mRNAs or a partner protein, which in turn modulate the half-lives of target mRNAs. According to such a scenario, up-regulation of transcripts in the absence of UIS12 might be a consequence of increased mRNA stability. We consider the identification of a nucleotide motif a good starting point for future work, and are hesitant to propose a molecular mechanism at this early stage of discovery.

In gametocytes, the conserved DOZI/CITH RNA storage complex (Mair et al., 2006; Mair et al., 2010; Guerreiro et al., 2014) is central to translational repression. For instance, the DOZI/CITH complex is involved in translationally repressing *P25*, *P28, PlasmepsinVI*, *AP2*-O, and *GAP45*. These transcripts are also contained on our list of down-regulated transcripts in *uis12(-)* blood stages. Thus, we were curious how many targets

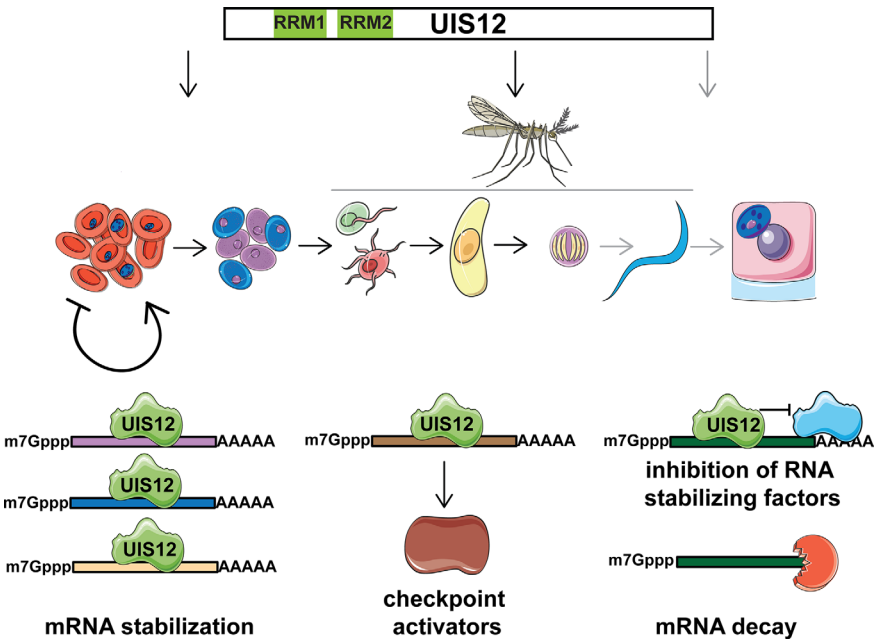

FIGURE 7 | Potential roles of *Plasmodium* UIS12 in expression control of gametocyte- and mosquito stage-specific mRNAs. UIS12 appears to influence parasite stage conversion at three different points in the complex *Plasmodium* life cycle (top). During blood infection commitment to onward transmission is initiated and *Plasmodium* parasites undergo a series of developmental transitions (center); female (blue) and male gametocytes (purple) are formed in the mammalian host; inside the mosquito midgut male gametes (red) fertilize female gametes (green), leading to formation of a motile zygote, the ookinete (yellow); oocyst development leads to formation of sporozoites (blue), which upon another mosquito bite invade hepatocytes (pink) and form liver stages (blue). Note that the last checkpoint (grey) could not be addressed experimentally because of complete life cycle arrest prior to sporozoite formation. The candidate RNA-binding protein UIS12 might modulate expression of gametocyte- and mosquito stage-specific transcripts by one of at least three alternative mechanisms (bottom); i) stabilization and/or translational repression of a large set of target mRNAs (left); ii) binding and stabilization of few mRNAs that in turn encode checkpoint activators (center); iii) inhibition of partner protein(s), which in turn stabilize target mRNAs (right). In the absence of *UIS12* post-transcriptional expression of a large set of gametocyte and mosquito stage -specific genes is impaired resulting in reduced differentiation and, ultimately, arrest of life cycle progression.

overlap when we compared the reported 117 genes down-regulated in both *dozi(-)* and *cith(-)* as determined by microarray on gametocytes (Mair et al., 2010) with the 646 down-regulated transcripts in *uis12(-)* blood stages (<-2-fold change), we found a surprisingly high overlap, of 102 of the 117 transcripts (**Figure 6B**). The RNA-helicase DOZI was previously shown to be a key regulator of *P25* and *P28* transcript stability, as well as of 731 other transcripts, and they all share a conserved U-rich nucleotide motif mainly in the 5' and 3' UTR (Hall et al., 2005; Braks et al., 2008; Guerreiro et al., 2014). The abundance of U residues in the DOZI and UIS12 motifs could hint at a related mechanism. The key differences are that the U-rich motif in our study is found in the ORFs of target genes, while the U-rich motif bound by the DOZI complex is located in 5' and 3' regions of its targets (Braks et al., 2008). Also, the U-rich motif identified for DOZI binding has a length of 47 nucleotides, whereas the motif identified herein is only 10 nucleotides long. Despite these clear distinctions a potential functional link of these two proteins remains an attractive possibility, since UIS12 and DOZI have >100 overlapping down-regulated targets (**Figure 6B**).

We note that, in marked contrast to *UIS12*, gene deletion of *DOZI* leads to a developmental arrest of the fertilized female gamete, a process that is not abolished in the absence of UIS12. In all three knockout lines, *uis12(-)*, *dozi(-)*, and *puf2(-)* parasites the mRNA levels of *P25* and *P28* were perturbated. While in *dozi(-)* and *uis12(-)* the transcripts were down-regulated, in *puf2(-)* an up-regulation was described, indicating involvement in distinct shared and opposing regulatory processes. On the other hand, PlasmepsinVI is translationally repressed by *Pf*Puf2 (Miao et al., 2013) and reduced in *uis12(-)* parasites, similar to the meiotic recombinase *DMC1* (Mlambo et al., 2012). Both knockouts as well as the *CCP* knockouts resemble the defects of *uis12(-)*. Clearly, further work is warranted to decipher the molecular mechanisms that orchestrate the sequential post-transcriptional control events and contributions of the regulatory molecules.

In conclusion, our experimental genetics analysis of the *Plasmodium berghei* candidate RNA binding protein *UIS12* uncovered broad perturbations of mRNA expression during blood infection, including up-regulation of several hundred transcripts, which share a U-rich 10 nucleotide-long motif in their open reading frames. The observed mRNA deregulation correlates with pleiotropic defects after blood stage propagation during sexual stage development, mosquito midgut colonization and oocyst maturation, ultimately leading to a complete block in the *Plasmodium* life cycle inside the *Anopheles* vector.

METHODS

Ethics Statement

All animal work conducted in this study was carried out in strict accordance with the German "Tierschutzgesetz in der Fassung vom 18. Mai 2006 (BGBl. I S. 1207)", which implements the directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocol was approved by the ethics committee of MPI-IB and the Berlin state authorities (LAGeSo Reg# G0469/09, G0294/15).

Parasites and Experimental Animals

P. berghei ANKA cl507 parasites that constitutively express GFP under the *Pb*EF1α promoter and *P. berghei* ANKA Bergreen parasites that express the green fluorescent protein GFP under the *Pb*HSP70 promoter throughout their life cycle have been used in our experiments (Franke-Fayard et al., 2004; Kooij et al., 2012).

All mouse experiments were performed with female NMRI or C57BL/6 mice purchased from Charles River Laboratories (Sulzfeld, Germany) or bred in house. NMRI mice were used for transfection experiments, blood stage infections, and transmission to *Anopheles stephensi* mosquitos. C57BL/6 mice were used for sporozoite and blood stage infections, exflagellation essays and ookinete cultures.

Gene Deletion of *P. berghei UIS12*

The *P. berghei UIS12* gene deletion was performed by double crossover homologous recombination of a replacement pB3D-knockout plasmid (van Dijk et al., 1995). The gene knockout construct targeting the endogenous *UIS12* locus comprised a 647 bp *UIS12* 5' UTR and a 524 bp *UIS12* 3' UTR sequence flanking a *T. gondii DHFR*/TS pyrimethamine-resistance cassette. **Table S4** lists the oligonucleotides used for generation of the knockout construct. Prior to transfection, plasmids were linearized by restriction digest. A digest with *Sac*II and *Kpn*I produced a fragment consisting of the pyrimethamine resistance cassette flanked by the *UIS12* 5' and 3' UTRs. Transfection by electroporation of cultured schizonts was performed as previously described (Janse et al., 2006b). Two independent parasite lines, one in *P. berghei* Bergreen and one in *P. berghei* cl507 recipient lines were generated and phenotypically characterized. For genotyping of recombinant parasites, knockout- and wild-type-specific PCR fragments were amplified from genomic DNA of parental and clonal lines with the oligonucleotides listed in **Table S4**.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR was used to quantify *UIS12* gene expression and for comparing gene expression in *uis12(-)* and wild-type (WT) mixed blood stages and gametocytes. For mixed blood stage analyses, C57BL/6 mice were infected i.v. with 10,000 iRBC, and 7 days after infection whole blood was isolated. The gametocyte samples were generated by infecting phenylhydrazine-treated NMRI mice with 10^6 iRBC. Three to four days later, when gametocytemia reached the highest levels, 12.5 mg sulfadiazine was supplemented to 1 liter of drinking water. Within 48 h the sulfadiazine treatment eliminated the asexual stages, which was confirmed by a Giemsa-stained thin blood film. Next, the infected blood was harvested. To isolate mixed blood stages or gametocytes, whole blood was cleaned from white blood cells and erythrocytes using cellulose-glass bead columns and saponin (0.3%; Sigma-Aldrich) lysis. Parasites were stored in 1 ml TRIzol (Invitrogen) or RLT-Buffer with β-mercaptoethanol (Qiagen). Parasite RNA was isolated with the RNeasy Kit (Qiagen) or TRIzol-Reagent (Sigma-Aldrich). DNase treatment with Quiagen RNase free DNase Set or Ambion Turbo DNA free Kit was performed to remove residual genomic DNA.

Reverse transcription to cDNA was done using the RETROscript Kit (Ambion). The qPCR was performed on parasite cDNA that was generated from total RNA of selected *P. berghei* life cycle stages. Quantitative real-time PCR was performed as previously described (Silvie et al., 2008) using Power SYBR Green PCR Master Mix and the StepOnePlus Real-Time PCR System (both Applied Biosystems). All procedures were performed according to the manufacturer's instructions. Life cycle expression data were normalized to GFP that is constitutively expressed under the *EF1α* promoter in *P. berghei* 507cl. Mixed blood stage and gametocyte expression data for comparison of knockout and WT parasites were normalized to *PbHSP70*. The relative transcript abundance was determined by the $2^{\Delta\Delta CT}$ method. For oligonucleotide sequences refer to **Table S4**.

Plasmodium Life Cycle: Parasite Asexual and Sexual Growth, Mosquito Infection, and Sporozoite Transmission

For asexual blood stage and gametocyte growth curves, C57BL/6 mice were injected intravenously with 10,000 infected red blood cells. From day 3 to 7, Giemsa (VWR)-stained thin blood films were microscopically examined to determine parasitemia and gametocytemia. Parasitemia and gametocytemia are defined as the percentage of red blood cells infected with asexual stages or gametocytes, respectively.

To analyze male exflagellation, C57BL/6 mice were intravenously injected with 10^7 infected red blood cells (iRBC). After four days, parasitemia and gametocytemia were determined. Next, five microliters of tail blood were diluted 1:25 in ookinete medium (RPMI 1640, 10% FCS and 50 mM xanthurenic acid, pH 8.0) and incubated for 12 min at room temperature. For the next 6 min exflagellation centers were counted with help of a Neubauer chamber.

Ookinete cultures were set up as previously described (Weiss et al., 1997). Four days after the intravenous injection of 10^6 iRBC into C57BL/6 mice pre-treated one day earlier with phenylhydrazine 97% (Sigma-Aldrich, 100µl of 6.18 µl/ml) 1 ml of fresh mouse blood was mixed with 10 ml of ookinete medium. After 20 h incubation at 20°C, at 80% humidity the culture was examined for development of ookinetes and ookinetes were purified with anti-P28-coupled magnetic beads.

Anopheles stephensi mosquitoes were kept at 28°C (non-infected) or 20°C (infected) under a 14 h light/10 h dark cycle

at 80% humidity. Four days after the transfer of 10^7 iRBC, when exflagellation reached a maximum, mosquitoes were infected as described (Vanderberg, 1975). After 4 to 14 days, midguts were isolated and oocyst development analyzed by fluorescent microscopy (Zeiss AxioObserver Z1). For visualization of nuclei, HOECHST 33342 (Invitrogen) was added at a 1:1,000 dilution. Images were analyzed using the FIJI processing (oocyst diameter) and/or Photoshop (oocyst numbers) software.

Midguts and salivary glands were analyzed at day 14–18 and day 18–30, respectively, for presence of sporozoites.

To test *Plasmodium* transmission, age-matched female C57BL/6 mice were either subjected to the bites of ~100 infected mosquitos or inoculated with 10,000 sporozoites, or for *uis12(-)* the salivary gland extract of 20 mosquitos, in RPMI intravenously. After three days Giemsa-stained thin blood films were analyzed for presence of infected red blood cells.

Microarray Expression Profiling of *P. berghei* Blood Stages

The microarray was performed on two biological replicate samples of *uis12(-)* and wild-type with comparable parasitemia. One replicate was generated by infecting one C57BL/6 mouse each with 10,000 *uis12(-)* or wild-type blood stages intravenously (R1, biological replicate 1). After 7 days of infection, mixed blood stages were isolated from whole blood. For both samples, the parasitemia was comparable (*uis12(-)* 6.3%, WT 6.2%). The other replicate (R2, biological replicate 2) was generated by injecting one NMRI mouse with 10^7 *uis12(-)* or wild-type blood stages and mixed blood stage parasites were harvested after 3 days (*uis12(-)* 8.7%, WT 6.4%).

Whole blood was isolated and cleaned from white blood cells and erythrocytes using cellulose-glass bead columns and saponin (0.3%; Sigma-Aldrich) lysis. About 10^8 parasites were stored in 1ml TRIzol (Invitrogen). Total RNA was isolated using glycogen as a carrier according to the 'TRIzol Reagent RNA preparation method' (Invitrogen). The amount of RNA was determined by OD 260/280 nm measurement using a 'NanoDrop 1000' (peQlab) spectrophotometer. The RNA size, integrity and the amount of total RNA were measured with a 'Bioanalyzer 2100' using an 'RNA Nano 6000 Microfluidics Kit' (both Agilent genomics). RNA labeling was performed with the two color 'Quick Amp Labeling Kit' (Agilent genomics) using a 1:5 mixture of 'FullSpectrum MultiStart Primer' (Systembio). mRNA was reverse transcribed and amplified using the primer mixture. The RNA was split and labeled with Cyanine 3-CTP and Cyanine 5-CTP, respectively. After precipitation, purification and quantification, labeled samples were hybridized to the Agilent '4x44K custom-commercial microarrays' according to the manufacturers protocol. Microarray experiments were performed as dual-color hybridizations. A color-swap dye-reversal was performed in order to compensate specific effects of the dyes and to ensure statistically relevant data analysis (Churchill, 2002; Wernersson et al., 2007). Scanning of the microarrays was performed with 5 μm resolution and the extended mode using a 'High Resolution Microarray Laser Scanner' (G2505, Agilent Technologies). Raw microarray image data were extracted and analyzed with the 'G2567AA Image Analysis/Feature Extraction software' (Version A.10.5.1.1, Agilent Technologies). On reporter and sequence level, the extracted MAGE-ML files were further analyzed with the 'Rosetta Resolver Biosoftware' (7.2.2.0 SP1.31). All subsequent data analysis was performed with the Microsoft Excel, Prism and the PlasmoDB (http://plasmodb.org/plasmo/) database. *P. berghei* microarrays were designed on the OligoWiz (http://www.cbs.dtu.dk/services/OligoWiz/) server that included annotated open reading frames (ORF) from the whole genome of *P. berghei*. ORF specific probes were designed as sense oligonucleotides of the coding strand (Wernersson et al., 2007). The aimed oligonucleotide length was between 50 and 60 bases. To avoid self-hit, the maximal homology was 97%, the cross hybridization maximum length was at 80% and random primed position preference scores within OligoWiz were chosen. Depending on these parameters and the sequence input length, each ORF was covered by different numbers of specific oligonucleotide probes. In total 41,133 specific probe sequences were uploaded to eArray (Agilent Technologies, https://earray.chem.agilent.com/earray/) as expression application with a customer specified feature layout.

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE152686.

Statistical Analysis and Online Bioinformatic Tools

Statistical significance was determined with the help of Graph Pad Prism software, using Mann-Whitney test for comparison of parasitemia, gametocytemia, and exflagellation dot-plots and multiple t-tests (one per row) for all growth curves.

The identification and positioning of the two RRM domains of the UIS12 orthologous proteins has been performed by NCBI WEB batch-CD Search Tool (Marchler-Bauer et al., 2015). Protein comparison was done by NCBI protein blast (blastp) with default settings, no compositional adjustment and without low complexity filter. Sequence coverage of all *Plasmodium* orthologues was higher than 90%.

Gene ontology (GO) enrichment analysis (Ashburner et al., 2000) was performed with help of the Gene Ontology enrichment analysis tool from PlasmoDB, with the default settings and the redundant GO terms were removed with help of the REVIGO software.

To screen for common 8-10 nucleotide-long motifs we used the MEME webtool (Bailey et al., 2009), screened all ORFs, 5'UTR sequences and 3'UTR sequences (1,000 nucleotides per gene for ORFs) of transcripts up-regulated >2.5-fold and compared them to the respective gene parts of the non-regulated (-1.5 to -1.5) transcripts from our microarray analysis of biological replicate 1.

Parts of the illustrations used in **Figure 7** were obtained from Servier Medical Art, (http://www.servier.fr/servier-medical-art) licensed under a Creative Commons Attribution 3.0 Unported License.

DATA AVAILABILITY STATEMENT

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE152686 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152686).

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of MPI-IB and the Berlin state authorities (LAGeSo Reg# G0469/09, G0294/15).

AUTHOR CONTRIBUTIONS

KMa, OS, and KMü designed the experiments. KMü performed experiments and analyzed data. H-JM performed and analyzed the microarray. KMa and KMü wrote the paper. All authors commented on and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.624945/full#supplementary-material

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