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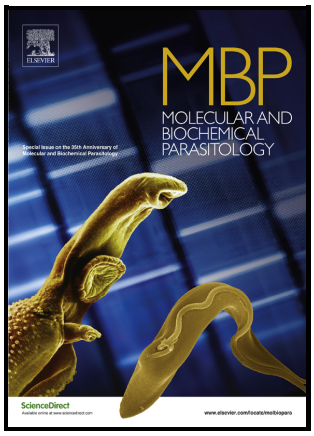

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PII: S0166-6851(24)00008-2

DOI: https://doi.org/10.1016/j.molbiopara.2024.111615

Reference: MOLBIO111615

To appear in: *Molecular & Biochemical Parasitology*

Received date: 15 September 2023
Revised date: 28 December 2023
Accepted date: 6 February 2024

Please cite this article as: Sergio O. Angel, Laura Vanagas and Andres M. Alonso, Mechanisms of adaptation and evolution in *Toxoplasma gondii*, *Molecular & Biochemical Parasitology,* (2024) doi:https://doi.org/10.1016/j.molbiopara.2024.111615

Mechanisms of adaptation and evolution in *Toxoplasma gondii*

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Abstract

Toxoplasma has high host flexibility, infecting all nucleated cells of mammals and birds. This implies that during its infective process the parasite must constantly adapt to different environmental situations, which in turn leads to modifications in its metabolism, regulation of gene transcription, translation of mRNAs and stage specific factors. There are conserved pathways that support these adaptations, which we aim to elucidate in this review. We begin by exploring the widespread epigenetic mechanisms and transcription regulators, continue with the supportive role of Heat Shock Proteins (Hsp), the translation regulation, stress granules, and finish with the emergence of contingency genes in highly variable genomic domains, such as subtelomeres. Within epigenetics, the discovery of a new histone variant of the H2B family (H2B.Z), contributing to *T. gondii* virulence and differentiation, but also gene expression regulation and its association with the metabolic state of the parasite, is highlighted. Associated with the regulation of gene expression are transcription factors (TFs). An overview of the main findings on TF and development is presented. We also emphasize the role of Hsp90 and Tgj1 in *T. gondii* metabolic fitness and the regulation of protein translation. Translation regulation is also highlighted as a mechanism for adaptation to conditions encountered by the parasite as well as stress granules

containing mRNA and proteins generated in the extracellular tachyzoite. Another important aspect in evolution and adaptability are the subtelomeres because of their high variability and gene duplication rate. *Toxoplasma* possess multigene families of membrane proteins and contingency genes that are associated with different metabolic stresses. Among them parasite differentiation and environmental stresses stand out, including those that lead tachyzoite to bradyzoite conversion. Finally, we are interested in positioning protozoa as valuable evolution models, focusing on research related to the Extended Evolutionary Synthesis, based on models recently generated, such as extracellular adaptation and ex vivo cyst recrudescence.

Key words: *Toxoplasma*; epigenetics; heat shock; subtelomeres; Extended evolutionary synthesis

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that belongs to the Phylum Apicomplexa. A distinctive aspect of this protozoan is the ability to infect all warm-blooded animals, and within the animal to potentially infect nearly all types of nucleated cells. It presents a complex cell cycle, with three main stages. One of these stages is the sporozoite, contained in oocysts, which results from sexual reproduction and occurs in the intestinal epithelium of felines. In the rest of the mammals or birds, *T. gondii* only reproduces asexually, going through the highly replicative tachyzoite stage in parasitophorous vacuoles, to the latent bradyzoite stage contained in tissue cysts. The ability of tachyzoites to infect different types of cells, including dendritic cells or macrophages, is crucial for dissemination to the whole body, including the central nervous system [1]. The differentiation from tachyzoite to bradyzoite occurs mainly in muscle tissue and Central Nervous System (CNS) [2], through different pre-bradyzoite stages, with the expression and sequential shutdown of some genes in a coordinated manner [3, 4]. In immunosuppressed individuals, reactivation of the bradyzoite to tachyzoite may occur, with catastrophic results for these patients [5]. In vitro, many stress situations can lead to the tachyzoite to bradyzoite differentiation in HFF cells [6]. In muscle cell culture, some *T. gondii* strains spontaneously

differentiate into bradyzoites [7]. There are *T. gondii* cystogenic and non-cystogenic strains. Cystogenic strains such as Prugniaud or Me49 are in turn non-virulent in mice and represent a model of chronic infection, while non-cystogenic strains, such as RH, are virulent in mice and represent a model of acute infection [8, 9]. In the feline intestinal epithelium, both the tachyzoite and the bradyzoite can differentiate into merozoite, initiating the sexual phase [10]. All these stage conversions indicate a high capacity of *T. gondii* to sense the different environments, either to multiply and propagate efficiently in the animal, or to initiate the differentiation process. The remarkable ability to transition from invading one cell, replicating, exiting into the extracellular space, and subsequently invading another cell, potentially in a vastly different environment, highlights its astonishing adaptive versatility (Fig 1 A). This implies that *T. gondii* can swiftly and effectively adapt to these variations, contributing to its success in infection rate, which spans from 30 to 90% of the global human population [11].

There are various strategies of the eukaryotic cell to be able to respond quickly to different environmental situations and reprogram gene expression at the RNA and protein level, which also exist in *T. gondii* (Fig. 1B). The mechanisms that allow these rapid adaptations and transcriptome reprogramming primarily relies on epigenetic mechanisms, which can sense the environment regulating gene expression. Although transcription factors (TF) have a gene-specific role, they are essential to activate gene transcription in different stress situations, including those that trigger the different stages of *T. gondii*. But they also include the involvement of heat shock proteins that regulate and safeguard the activity of essential proteins in face of diverse stress situations, as well as stress control of translation machinery that allows a selective protein biosynthesis. Furthermore, the existence of subtelomeric contingency genes, potentially with specific roles in subtelomeres (ST), adds to this complex process.

2. Epigenetics and transcriptional control

T. gondii possesses the 4 canonical histones that comprise the nucleosome, and also many variants. In addition to the well-known variants found in nature within the H2A (H2A.X and H2A.Z) and H3 (H3.1, H3.2, H3.3, and cenH3) families, the parasite showcases two canonical H2B

isoforms. Notably, a novel variant known as H2B.Z is also part of the natural repertoire and is shared across all apicomplexans [12]. Recently, a linker H1 histone was also described to be present in *T. gondii* [13]. Different from other eukaryotes, DNA methylation which is a well studied epigenetic regulator, appears to be lacking or underrepresented [14]. Histone post-translational modifications (PTMs), on the other hand, are essential to generate marks indicating active or silent chromatin. In turn, there is an orchestrated crosstalk between them, generating a complex picture in epigenetic regulation of gene expression. In *T. gondii* all the main PTMs present in other eukaryotes such as mammals are also present [15-17]. This includes the presence of "writers" and "erasers" for adequate control of these epigenetic marks, along with proteins that possess specific histone reader domains [18], constituting the so-called histone code [19]. However, *T. gondii* "readers" are not so many as those described in mammals. In this context, the novelty of a new histone variant, H2B.Z which is essential and highly acetylated in N-terminal lysines [20], is intriguing. Moreover, this histone dimerizes with H2A.Z [21], being both located in promoters of active genes and in the bodies of silent genes [22]. H2A.Z in turn, is also highly acetylated, conforming to a double variant nucleosome (DVN) that is likely being part of the epigenetic regulation. The epigenetic mechanism has been demonstrated to be involved in the fine regulation of transcription in *T. gondii*. As one example, an alteration of HDC3 histone deacetylase by an inhibitory drug, alters the tachyzoite program activating bradyzoite differentiation [23].

The significance of this DVN in epigenetic regulation under varying conditions becomes apparent through the observation of H2B.Z mutants with modifications to its five acetylatable lysine residues [24]. In this work, mutant lines were generated in a RH strain background. As mentioned above, RH is a highly virulent non-cystogenic strain with a lethal dose of just one tachyzoite to kill mice. Lysine is a positively charged residue that is neutralized with PTMs, such as acetylation. When these 5 lysines were replaced by alanines, which would mimic an acetylated lysine, no phenotypic changes were observed in the mutant parasites in vitro. Meanwhile, in the mouse infection model although still lethal, a small but significant delay in mice lethality was observed. On the contrary, substituting these 5 N-terminal lysines by arginines to mimic unacetylated lysines (creating a constitutive positive charge patch) yielded discernible phenotypic

alterations in vitro: growth reduction, enhanced differentiation, and increased parasite sensitivity to genotoxic agents. Despite these alterations, the parasite managed to replicate, albeit at a slower pace, successfully overcoming the challenges. The effects, however, were drastic within the in vivo mouse model. In that scenario, this mutant line ceased to be lethal, displaying a 100% survival rate in mice. These mice demonstrated a rapid control over the infection, revealing an immune response reminiscent of that observed in non-lethal strains during a typical infection progression, with the surplus of avoiding formation of brain cysts.

With these results, a potential impact on a precise response to environmental fluctuations becomes evident. Perhaps, under in vitro culture conditions, with a single host cell type and non-fluctuating environmental conditions, the mutant is capable of adjusting its transcriptional program and replicating, although more slowly. However, within the in vivo context, it must adapt to different host cell types as well as different cellular environments of cells of the same lineage. Furthermore, transitioning from one host cell to another would require a quick recognition and adaptive reaction to the differing environment, as illustrated in Figure 1. For instance, moving from an intestinal epithelial cell to a macrophage, which generates NO or O_2 radicals capable of inducing DNA damage, demands a rapid adjustment. Similarly, transitioning from a macrophage or dendritic cell to an active muscle cell might entail significant metabolic changes. The regulation of the N-terminal positive charge patch of H2B.Z clearly contributes to the fine regulation of chromatin, although the precise mechanism of action remains to be fully elucidated.

2.1 H2A.Z/H2B.Z DVN as a sensor of metabolic changes?

Parasitic protozoa have evolved to have a small genome size, but sufficient to allow an efficient propagation. In the case of intracellular parasites as *T. gondii*, especially concerning the diversity of cell environments it encounters, it requires host cell nutrients, and adapting to their availability becomes an important task. However, this successful parasite presents diversified metabolic pathways that could be alternatively chosen in response to the signals in different cells invaded. In such a manner, the parasite becomes capable of generating enough energy and metabolites for propagation. Alternatively, it can trigger changes in its stage and/or pathogenicity. The

epigenetic regulatory mechanism emerges as a potential orchestrator of these adaptive responses, allowing the parasite to finely tune its genetic and phenotypic traits in accordance with the dynamic host environments (Fig. 2 A). As is known, the acetyl donor in the histone acetylation process is nuclear cytosolic acetyl-CoA [25]. Alternative pathways to glycolysis/tricarboxylic acid (TCA) could affect nuclear acetyl-CoA levels and thus affect the degree of histone acetylation. *T. gondii* presents great flexibility to use different carbon sources in energy generation [26-28], although when intracellular, the main route to obtain energy is glycolysis, starting from glucose until reaching the TCA cycle. This is the main source of cytosolic/nuclear acetyl-CoA, although acetate is also able to generate cytosolic acetyl-coA [29]. In a study where acetyl-CoA synthetase was knocked-down (iΔACS) in *T. gondii* parasites with deleted ATP citrate lyase gene (ΔACL), reduced levels of cytosolic/nuclear acetyl-CoA were observed [29]. As a consequence, protein acetylation levels were reduced not only in those associated with metabolism, but also histones (mainly H2B.Z and H2A.Z), histone writers and transcription factors, among others. These results likely indicate a possible effect on the regulation of gene expression due to the decrease of nuclear cytosolic acetyl-CoA. However, of 453 differentially regulated transcripts, only 76 were downregulated while 377 were upregulated. Many of the up-regulated genes are hypothetical, and some encoding lipid or amino acid metabolism genes [29]. Therefore, the lack of histones and/or other proteins associated with the epigenetic mechanism may be a signal for a change in the transcriptional program. As mentioned before, *T. gondii* H2A.Z/H2B.Z DVN is highly acetylated (in 10 and 5 N-terminal lysines, respectively), whereas their canonical counterparts are not [20]. The remarkable elongation of N-terminal lysines within DVN that needs acetylation regulation, could potentially indicate a novel avenue in the realm of epigenetic mechanism.

Histone methylations are driven by methyl transferases using S-adenosylmethionine as a donor, linked to the methionine pathway [25]. In the same line of thought as acetylation regulation, it is premature to propose a sensing of this metabolite by DVN, taking into account that only one of ten N-terminal lysines in *T. gondii* H2A.Z was shown be methylated (Fig. 2 B). But other methylated histone marks (e.g. H3K4me3 and H3K9me3) are also important modulators of *T. gondii* chromatin compaction [22]. In *Plasmodium falciparum*, S-adenosylmethionine is used

both for DNA methylation and for de novo synthesis pathway of phosphatidyl-choline [30]. The different parasite sexual forms present in the insect vector were observed to be sensitive to phospholipid precursors. Harris et al [30] observed that competition for the de novo synthesis of phosphatidyl-choline generates a reduction in the levels of silent mark H3K9me3 in the promoter of transcription factor PfAP2-G gene, involved in activating the sexual phase. In this case, lipid metabolism was linked to the epigenetic mechanism. In *T. gondii*, sexual differentiation occurs in the feline intestinal epithelium. The feline intestine lacks the enzyme D-6-desaturase, which leads to the accumulation of linoleic acid, becoming a signal to sexual differentiation [10]. However, the relationship between the presence of linoleic acid seems to be more related to its oxidation by *T. gondii* lipoxygenases that are secreted into the cytoplasm of leukocytes in vivo [31].

2.2 H2A.Z/H2B.Z DVN as a modulator of general gene expression

Exploring the intricate landscape of gene expression regulation unveils a captivating realm where epigenetic modifications play a pivotal role. Among these, the exchange of canonical histones with their variant counterparts has emerged as a dynamic mechanism that orchestrates the modulation of gene activity. By this mechanism, histones shape the architecture of DNA, being important to understanding how cells finely tune their genetic responses to environmental cues. Within Apicomplexa, the existence of a distinctive H2A.Z/H2B.Z DVN has been described to be important in gene expression regulation. In *Plasmodium falciparum* and *T. gondii*, this DVN has been observed as a component in the promoters of euchromatic genes, suggesting it may be involved in gene regulation [22, 32-34]. Furthermore, the co-localization of H2A.Z and H2B.Z within euchromatic intergenic regions has been established in *P. falciparum*, leading authors to hypothesize that this DVN serves as a defining feature of the genome's regulatory domains. This, in turn, is believed to exert a significant influence over the transcriptional dynamics of the pathogen in question [34]. Similarly, *T. gondii* has exhibited a dual presence, appearing both at gene promoters associated with active transcription and within the bodies of genes that remain transcriptionally silent across the genome [22]. In this work it was proposed that variant histones likely wield control over stage-specific gene expression by demarcating functionally distinct

chromatin regions. The DVN, therefore, emerges as a pivotal factor responsible for orchestrating not only gene activation but also gene silencing processes [22]. Likewise, acetylated H4K31 mark was observed in promoter of active genes whereas H4K31me3 was detected in body gene and heterochromatin [35]. Once again, histone PTMs have been proposed to regulate this disparity in genomic location. In this context, the involvement of N-terminal acetylation of H2B.Z has been demonstrated to play a significant role in various biological mechanisms within *T. gondii*, including differentiation, virulence, growth, and DNA damage repair [24]. This study also examined the patterns of gene expression following in vitro differentiation, revealing differences among several key genes.

Another insight concerning the role of DVN in gene expression modulation pertains to Plasmodial *var* genes. Even before describing the DVN, a significant enrichment of PfH2A.Z within active var genes, accompanied by euchromatin-associated factors H3K4me3 and H3K9ac was observed [32]. Subsequently, it was demonstrated that PfH2B.Z is also present in var introns and active var promoters along with PfH2A.Z [33]. Once the var gene is no longer expressed, i.e. in a poised state, this DVN is lost from the promoter [32, 33]. However, PfH2A.Z/PfH2B.Z DVNs remain present at inactive promoters of heterochromatic invasion gene clusters that display comparable variegated expression patterns. This would mean that the DVN has a distinctive role in modulating virulence genes within the malaria parasite. Once again, in this work it was suggested that their acetylations are critical for their function [33].

While H2B.Z is a distinct feature of Apicomplexa, other parasitic protozoa such as *Trypanosoma sp.* also exhibit an H2B.V, which is also associated with H2A.Z. As for Apicomplexa, the TSS-nucleosomes in *T. brucei*, comprising H2A.Z/H2B.V, were found to be highly acetylated. Notably, the depletion of HAT1, one of the acetyl-transferases active in this parasite, resulted in diminished levels of acetylation for both H2A.Z and H2B.V, consequently leading to a substantial decrease in transcript levels [36]. The authors suggested that the absence of acetylation on these variant histones potentially could disrupt the recruitment of RNA Pol II causing a reduction in transcriptional activity. Similar to what was proposed for Apicomplexa, there would be regions

demarcated by the presence of H2A.Z and H2B.V with acetyl and methyl marks specific to TSS that might be fundamental on the initiation of transcription [36].

2.3 H2A.Z/H2B.Z DVN as a modulator of virulence gene expression

Genes specific for parasite virulence are present in *T. gondii*. Among them are the rhoptry proteins, specific of this Phylum. Two of these proteins (Rop5 and Rop18) were analyzed in lines of *T. gondii* expressing histone H2B.Z with mutations in the N-terminal acetylatable lysines (see above). Neither of the two genes showed expression at the protein level in the H2B.Z mutant featuring the constitutive positive charge patch. Conversely, while Rop18 protein was expressed, Rop5 had no protein-level expression, in the *T. gondii* mutant designed to emulate acetylated N-tail conditions [24]. As mentioned, the mutant was generated in the RH strain, highly virulent in mice (lethal dose of 1 parasite). In vivo experiments with these two mutant parasites showed revealed a striking disparity: the mutant carrying a constitutive positive charge patch, entirely forfeited its ability to induce mouse mortality, whereas the mutant with neutralized charges retained its virulent potential, albeit with a delayed onset compared to the parental strain. Taking everything together, the lack of lethality in vivo may be due to the lack of expression of virulence genes. Alternatively, it may respond to an essential function of the modulation of the positive charge patch to quickly respond to different cellular environments that occur in vivo but not in vitro as discussed above. More studies are needed to shed light on this subject. This includes analyzing, as an option, the regulation in the expression of non-coding RNAs (ncRNA) capable of blocking the specific translation of the target mRNA, in this case those encoding virulence proteins [37].

2.4 Transcription factors and development

Although epigenetic modulation of chromatin can be proposed as a rapid response mechanism to environmental changes, certainly the existence of transcription factors (TFs) is key to the correct expression of genes at the appropriate time and quantity. As shown in Figure 2 A, the regulatory TFs bind to an enhancer or activator element on DNA recruiting co-activators. Among

co-activators, it may find histone acetyltransferases (HAT) to acetylate and open chromatin at the transcription start site (TSS) flanking region. Another co-activator is the mediator complex, which recruits basal TFs and RNA polymerase II, facilitating their binding to the basal promoter [38]. In addition to activating TFs, there are also repressive TFs that have the opposite effect. *T. gondii* presents a larger group of regulatory TFs from the Apetala AP2 family. Although there are 67 AP genes, it is considered that only 50 could have the ability to bind DNA. These AP2 TFs seem to be associated with different functions, most of which are still unknown. Many of them present an expression associated with the cell cycle of the tachyzoite or in the extracellular tachyzoite [39]. The other recently described TF family belongs to the group that possesses Myb-like domains with 15 detected members [40].

The differentiation to the bradyzoite stage could be considered an evolutionary advantage that allows *T. gondii* to maintain an alternative cycle of infection and dissemination in addition to its sexual stage. This important process is regulated by a master gene that encodes a Myb family TF called bradyzoite formation deficient 1 (BFD1). The expression of this TF is enough to induce the bradyzoite stage as well as to bind to different bradyzoite-associated promoters [41]. An interesting aspect is that BFD1 is expressed as mRNA in the tachyzoite stage but is translated into protein only in bradyzoite-induction conditions. In part, the regulation of its translation is dependent on a protein with an RNA binding domain called BFD2/ROCY1 [42, 43]. Accompanying BFD1 in the fine gene expression regulation in the passage from tachyzoite to bradyzoite are at least 6 AP2 genes. AP2IV-3, AP21b-1, and AP2XI-4 were shown to be activators of bradyzoite induction and expression of specific bradyzoite genes while AP2IX-9, AP2IV-4, and AP2IX-4 were classified as repressors [16, 44-46]. AP2IV-3 and AP2IX-9 seem to bind to the same genes but with opposite effects [4]. To note, the lack of expression of AP2IV-4 generates an incorrect timing of expression of bradyzoite genes during experimental murine infection that leads to an inflammatory response that ends up eliminating the parasite [46].

Few works address the regulation of gene expression in the sexual phase of *T. gondii*. The TF AP2XII-1 and AP2XII-2 were recently identified as repressors of genes associated with the sexual phase [47, 48]. In vitro, it was observed that AP2XII-1 forms heterodimers with the TF

AP2XI-2, recruiting the epigenetic regulators HDAC3 and Microrchidia (MORC) proteins to silence the chromatin of the genes associated with the merozoite; a developmental stage critical for subsequent sexual commitment [49]. The conditional silencing of both genes results in the in vitro formation of merozoites, a stage previously achievable solely within the cat intestine.

3. Heat shock proteins and adaptability

There are several families of heat shock protein (Hsp) defined according to their molecular weight (small Hsp, which are smaller than 40 kDa, Hsp40, Hsp60, Hsp70, Hsp90, Hsp110, and so on). Unlike the other Hsps, eukaryotic Hsp90 has highly selective activity in stressed and non-stressed cells, where this chaperone is responsible for processes that control cell growth and differentiation [50]. Along with other Hsps (for example, the Hsp70 and Hsp40 chaperones), and other co-chaperones, Hsp90 helps unfolded proteins achieve their proper conformation for their biological activity [51]. Although there is an increase in the expression of Hsp90 under stress conditions, this is one of the most abundant proteins in eukaryotes [52]. This basal reservoir of Hsp90 activity under non-stress conditions allows cells to respond rapidly to modest levels of environmental fluctuations. The Hsp90 protein network includes hundreds of proteins, mainly transcription factors and kinases, but also proteins associated with chromatin, metabolism, translation, and DNA damage [53-56]. These client proteins are key in almost all developmental pathways, eukaryotic signaling, and environmental sensing, providing a plausible explanation for the impact of the Hsp90 chaperone on the genotype-phenotype relationship. The Hsp90 chaperone has an important role in evolution and fitness, evidenced by the fact that Hsp90 client proteins present higher evolution rates than non-client proteins [57]. Furthermore, it has been observed that this ability of Hsp90 to “protect” mutant proteins actually allows client proteins to acquire more and potentially more damaging mutations than non-clients, facilitating divergence between gene duplicates [58]. In addition, Hsp90 binds to several chromatin regulators, including the INO80 complex and histone-modifying enzymes [55].

The studies performed on *T. gondii* chaperones have been descriptive, being able to establish a possible biological role based on their interactomes that require further analysis for

confirmation. However, if we analyze these interactomes in the context of the protein-protein network interaction (PPI) and/or roles of chaperones in other species, we can verify that there is a good correlation with those observed in *T. gondii* (see [51, 59, 60]. Therefore, based on the *T. gondii* PPI of Tgj1 and Hsp90 we can infer a possible role of these chaperones in different biological processes of the parasite.

3.1 Hsps and metabolic fitness

Being a unicellular organism and an obligate intracellular parasite, each *T. gondii* cell needs to effectively react to shifts in its environment and the signals it encounters in its three-dimensional surroundings to be successful in its infective process. The possibility of parasite-parasite communication via extracellular vesicles should not be ruled out, but in this section, we are focusing on the individual response in a population of intracellular parasites. Each one of them is probably exposed at different times or to different stimuli according to the type or cellular state of the host cell. In this context, metabolic pathways play a crucial role in the evolution of this protozoan parasite. This is primarily due to the fact that the decision to undergo differentiation is frequently influenced by reaching a critical concentration of a low molecular weight metabolite [61]. When the parasite transitions from one host cell to another, encountering shifts in nutrients, it needs to adapt and counteract these changes (Fig. 1). This adaptation is carried out by adjusting its metabolic pathways. This process encompasses not only epigenetic mechanisms, as mentioned earlier, but also relies on the assistance of chaperones such as Hsp70 or Hsp90 [62].

For energy metabolism, *T. gondii* can use glucose, glutamine, alanine and lactate as carbon sources to generate energy via glycolysis, TCA and oxidative phosphorylation. However, in optimal conditions glucose is the preferred source [63-65]. To date, only a few studies have provided insights into bradyzoite metabolism, revealing increased reliance on glycolysis for energy production [66-68]. Also, a crucial role for the degradation of amylopectin [69, 70] and proteolysis within the vacuolar compartment have been described. Weilhammer et al. have proposed that low lactate production is a hallmark of host cells that trigger tachyzoite to bradyzoite conversion [71].

T. gondii tachyzoite is a highly replicative stage, with a cell cycle lasting between 5 and 12 hours depending on the genotype [72]. This rapid division cycle is likely to be important for the effective dispersion throughout the organism of the infected individual before the immune response stimulates its differentiation into a bradyzoite. It is important to note that this high rate of replication would only be possible with high energy expenditure. Despite the main carbon source of the tachyzoite is glucose, in its absence there are other ways to retake the glycolysis and TCA pathway. One of these alternatives is the use of glutamine that relies on fructose bisphosphatase 2, a constitutively expressed gluconeogenic enzyme which is essential for growth [63, 73].

T. gondii Hsp90 is an essential chaperone, important for the parasite lytic cycle as well as for the differentiation processes [51, 74]. This chaperone is part of a Hsp70/Hsp90 cycle in *T. gondii* with many co-chaperones that were also identified [60, 75]. The first step for the selection of the client proteins is determined by chaperones from the Hsp40 family, primarily DNAja1 and DNAja2. In *T. gondii* these chaperones are named Tgj1 and Sis1, respectively [59, 76]. The study of the PPI network of Tgj1 and cytosolic/nuclear Hsp90, showed a clear and strong association with the translation system and energy metabolism [59]. Among them, Tgj1 and Hsp90 would interact with fructose bisphosphatase 2 and pyruvate kinase 1 (PYK1), while PYK2 was only detected in the Tgj1 interactome [59], indicating a specific function for this chaperone in *T. gondii*. Fructose 2,6-bisphosphate is the substrate to generate Fructose 6_phospahate, thus regulating glycolysis. TgFBP2 isoform is essential for parasite replication and virulence in mice, and is required for growth even when parasites have access to host glucose [77]. PYK has a key role in *T. gondii* metabolism. The maintenance of a constant pyruvate supply is critical to parasite growth [65, 78]. *T. gondii* PYK1 is expressed in the cytosol whereas PYK2 is expressed in the apicoplast, a parasite-specific non-photosynthetic plastid [65]. Genetic deletion of PYK2 did not noticeably affect parasite growth and virulence, which contrasts with the current model of carbon metabolism in the apicoplast. Conditional depletion of PYK1 resulted in global alteration of carbon metabolism, amylopectin accumulation, and reduced cellular ATP, leading to severe growth impairment. All these results place Tgj1 and Hsp90 in a putative key role to modulate enzymes of the glycolytic

process and TCA. Considering their potential significance in promptly reacting to shifts in the environment and *T. gondii´*s adaptable nature in utilizing diverse carbon sources for energy production, we should consider this group of chaperones as potential contributors to such metabolic adjustments.

3.2 Hsps and translation

Transition from the tachyzoite to the bradyzoite stage may be induced by numerous types of stress in vitro [6, 79]. This in vitro stress model is accompanied by increased expression of Hsp70 and Hsp90 [74, 80]. In the case of Hsp90, it also shifts from being localized within the cytosol (tachyzoite) to both the cytosol and nucleus (bradyzoite). While the transition to the bradyzoite phase is evidently driven by epigenetic mechanisms and transcription factors [23, 24, 41, 45, 81, 82], the precise role of *T. gondii* Hsps in facilitating these processes remains elusive. Recently, the study of the *T. gondii* single cell transcriptome from PTG parasites (cystogenic strain) infecting bone marrow dendritic cells showed a certain association in the co-expression of ribosomal proteins with Hsp70 and Hsp90, as well as proteins of the inner membrane complex, important for host cell invasion [83]. This is largely due to the fact that studies of the PPI network of the Hsps have thus far been conducted solely in the tachyzoite stage. Nevertheless, based on this PPI, certain associations can be inferred, allowing for speculative insights into potential roles these Hsps might play in translation.

Experiments in stress conditions conducted to study the differentiation process in *T. gondii*, have also rendered the identification of many translation machinery components [84]. As occurs in other organisms, during stress the translation machinery is affected in different aspects, reacting, among other things, on the dynamics of eukaryotic initiation factor-2 (eIF-2). In stress situations, the alpha subunit of eIF2 (eIF2α) is phosphorylated by an eIF2a kinase (eIF2aK). Phosphorylated eIF2α is disabled to continue general peptide synthesis, but focusing on the translation of specific mRNAs [85]. In *T. gondii* it was observed that under stress conditions eIF2α is highly phosphorylated by eIF2K-A and eIF2K-B [86, 87]. Similarly, eIF2K-B was shown to

phosphorylate eIF2α under oxidative stress conditions [88]. This links the regulation of translation with an environmental response, in this case in a process that induces differentiation. Both *T. gondii* Hsp90 and Tgj1 have been shown to pull down the translation machinery, which includes almost all ribosomal subunits, aminoacyl tRNA synthetases, and initiation and elongation factors [84]. Among them, eIF2α was detected in the Tgj1 and Hsp90 proteomes. The eIF4-A and two subunits of eIF3 were also detected, which allow the assembly of the smaller ribosomal subunit with the mRNA.

There are trans-acting regulators of translation from proteins that bind to mRNA. One of these is the Acetylation lower binding affinity (Alba) family protein, which can bind to DNA or RNA. Several members of the Alba family were detected in *P. falciparum and T. gondii* [89, 90]. *Plasmodium* Alba1 was shown to bind to a subset of mRNAs linked to the host cell invasion machinery. This is likely indicative of a regulatory activity on its association with the ribosome, and therefore of its translation rate and timing [91]. Similarly, *T. gondii* Alba1 and Alba2 were shown to be associated with the regulation of the translation of some mRNAs [90]. In fact, the pull down of *T. gondii* Alba1 retrieved 30 mRNAs. Both Alba1 and Alba2 were pulled down by Tgj1 [59] revealing a potential specific role in regulating the translation of a group of mRNAs. Something similar had been already observed in yeast, where Brodsky et al. [92] observed that that Ydj1 altered the translation a subset of proteins was affected, especially of poorly translated mRNAs.

4. Translation control in *T. gondii*

A process highly linked to modifying the protein expression profile in response to different environmental situations lies in the control of the selective translation of mRNAs. The translation mechanism is highly conserved and comprises three major stages: initiation, elongation, and termination. In eukaryotes, translation regulation is associated with cis-elements of the mRNA that include: the CAP, secondary structures in the 5'UTR and 3'UTR regions, which may or may not be associated with interacting proteins, the presence of multiple start and stop codons, which can be chosen during scanning and translation elongation in different environmental situations,

and the presence of microRNAs [93]. Regulation can also occur as a result of the diversity of ribosomal subunits and/or PTMs that influence their activity, potential variations in subcellular localization, and interactions with other proteins, among other factors [94-96].

As mentioned above (section 3.2), stress situations and induction of the bradyzoite stage produced an increase in the phosphorylation of eIF2α, with a decline in global translation in *T. gondii* [87]. Since there is a set of mRNAs selectively translated in different environmental and stress situations, Joyce et al (2013) were able to determine the existence of 499 mRNAs that upregulated their translation in presence of tunicamycin, endoplasmic reticulum stressor [97]. In this way, the importance of translation control as an environmental response in *T. gondii* could be highlighted. A relevant aspect in the control of translation lies in the initiation of the process, which in eukaryotes relies both on CAP-dependent and CAP-independent pathways. Briefly, in the CAP-dependent pathway, the eukaryotic translation initiation factor eIF4E recognizes CAP. This in turn binds to eIF4G and eIF4A, to form the complex called eIF4F. The presence of the eIF4F complex in the CAP recruits the 40s ribosomal subunit to the mRNA. In stress situations, this process is inhibited by inducing CAP-independent translation [93]. There are different mechanisms of inhibition of the CAP-dependent translation pathway [98-100]. Combining both concepts, the selective control of translation and the possible role of the CAP binding complex, Sullivan's Lab delved into the analysis of the factor associated with the CAP-binding complex eIF4E mRNA in *T. gondii* [101]. Three eIF4E were identified, of which only eIF4E1 was associated with its binding to CAP. As expected, the knock down of eIF4E reduced the expression at mRNA level of 223 genes, while 153 presented alterations in translation efficiency sensitive to eIF4E. Interestingly, 54 genes were upregulated in their translation. More striking was that the depletion of eIF4E induced the formation of bradyzoites. Among the mRNAs that upregulated their translation are those that code for the transcription factors AP2IX-9 and AP2IV-3, both related to bradyzoite induction, as mentioned above. An important aspect in the regulation of the CAP-dependent translation pathway lies in the function of the mTORC1 kinases [93, 102]. mTORC1 phosphorylates the eIF4E binding factor (4B-BP), inhibiting the binding of eIF4AE to the CAP. This process is relevant, even more so when the function of mTORC1 is associated with different

types of stress such as nutrient starvation, energy status, and the presence of oxygen. As mentioned by the authors [101], in the future some association of this pathway in environmental stress conditions such as those just mentioned could be interesting for further analysis.

In a mutagenesis study and analysis of mutants defective in bradyzoite formation, Carruthers's Lab identified that the F97L mutation of the eIF1.2 factor was responsible for the defect [103]. In eukaryotes, the 40s ribosomal subunit, bound to eIF1, eIF2+tRNAmet and eIF3, is recruited to the mRNA to begin scanning in search of the start codon. *T. gondii* ΔeIF1.2 mutant showed a reduction in the transcription and translation of bradyzoite genes and upregulation of acute infection (tachyzoite) genes. Furthermore, the lack of eIF1.2 reduces the translation of the master transcription factor BFD1 and the transcript and translation of BFD2, suggesting that eIF1.2 would be necessary for its correct expression in stressful situations.

5. Stress granules and adaptation to extracellular environment

Cells contain membrane-less organelles, called processing bodies (P-bodies) and stress granules that are involved in regulating gene expression. P-bodies are cytosolic granules of ribonucleoproteins, associated with mRNA decay and silencing [104]. Stress granules are transiently induced in response to stress situations associated with a global silencing of translation [105, 106]. Stress granules contain mRNA in the translation initiation phase to stall its translation and allow the translation of mRNAs associated with stress. In fact, in the presence of arsenite, an inhibitor of the citric acid cycle and an inducer of oxidative stress, eIF4E together with eIF4G1 and eIF4G2 were located in stress granules in *T. gondii* [101]. The first studies on stress granules in *T. gondii* showed that these were formed in the extracellular tachyzoite [107]. Noteworthy, the formation of stress granules in the extracellular form improved the invasion capacity, replication and resistance to apoptosis. Subsequently, the formation of stress granules in extracellular tachyzoites was detected in the presence of other stressors such as the aforementioned arsenite and salubrinal, the latter an inhibitor of eIF2α-P dephosphorylation [108]. More recently, it was observed that salubrinal does not induce the formation of stress granules in bradyzoites, rather stress granules are formed before the egress of the replicative tachyzoite present in the

parasitophorous vacuoles [109]. In addition, stress granules were not detected under alkaline or nutrient starvation stress, both processes generating repression in the parasite translation. Therefore, the authors inferred that stress granules in *T. gondii* would be formed by a rapid increase in untranslated mRNAs due to a sudden change in translational state. The proteomic study of stress granules in *T. gondii* carried out 10 and 30 minutes after its formation shows differences in its composition [110]. Although the proteins detected at 10 minutes also appear in the proteome of the 30-minute stress granules, in the latter 43 additional proteins appear. However, at 10 minutes there is a greater proportion of cytoskeletal proteins while at 30 minutes there are more proteins associated with translation. Many of the proteins that were detected are associated with invasion, so their role may not be relevant according to the authors. Up to now, is intriguing how the stress granules improve the tachyzoite invasion capacity in *T. gondii*.

6. Subtelomeres and contingency

Subtelomeres (ST), regions close to or contiguous with telomeres, are transition regions between the telomere and the chromosome-specific DNA sequence, euchromatin. ST are difficult to define, ranging in length from 20 kbp in some yeast strains to 200 kbp in several higher eukaryotes [111, 112]. The significance of subtelomeric regions has been acknowledged due to the connection established with various biological functions [113]. They are involved in (1) telomere maintenance and regulation of telomere length, (2) proper chromosome segregation, (3) chromosome recognition and pairing during meiosis (4) replicative senescence, (5) the nuclear positioning of telomeres, (6) the control of the spread of telomeric heterochromatin, (7) the phenotypic polymorphism and plasticity of the genome and, finally, in (8) the regulation of the transcription of TERRA, a family long non-coding RNA (lncRNA). These lncRNAs contain telomeric repeats transcribed in telomeric tracts from promoters embedded in ST.

However, an extremely important aspect of ST is that they are variable loci that contain rapidly evolving gene families involved in adaptive processes [112, 114]. One possible reason for this rapid evolution may be that ST are more tolerant to deletions or duplications [115]. Although STs are known as gene-poor regions, the presence of some groups of genes whose functions

may be relevant to them has been observed in many organisms. In yeast, an enrichment in genes related to the stress response and metabolism has been observed in ST. These include metabolite transporters and genes related to nutrients uptake, that are essential under non-optimal growth conditions [116, 117]. In pathogenic fungal species such as *Candida albicans* and *Candida glabrata* there are families of subtelomeric genes (ALS genes and EPA genes, respectively), which code for proteins responsible for adherence to mammalian tissues [118-120]. In *Saccharomyces cerevisiae*, subtelomeric genes encode proteins that confer adherence to agar, solid surfaces, and other yeast cells [121, 122]. In these examples, the protein encoded by each member of the mentioned families may alter the properties of the cell surface, generating phenotypic variation in response to the host environment [123]. In the protozoan parasites *Trypanosoma* sp. and *Plasmodium* sp., the presence of subtelomeric multigenic families that code for membrane proteins whose function is associated with evasion of the immune response and pathogenicity was observed [124, 125]. Barry et al. [126] suggest that subtelomeric positioning of pathogen contingency systems serves as a platform for reversible gene silencing, enabling gene switching, mutually exclusive expression, and gene recombination, ultimately driving the diversification of gene families. In *T. cruzi* as well, genes involved in pathogenesis like trans-sialidase superfamily, and other surface glycoprotein, are localized to the subtelomeric regions [127].

6.1. T. gondii *ST and integral membrane multigenic families*

Toxoplasma presents 94 subtelomeric genes distributed in 13 functional annotated genes. The function of the remaining 81 subtelomeric genes is unknown, but among them two multigenic families of highly variable integral membrane proteins (FamB and FamC) have been described [128]. The deduced protein structure of FamB and FamC resembles the *Plasmodium* variable antigen genes, although they are not phylogenetically related. Orthologues were also detected in *Neospora caninum* and *Hamondia hammondi*, but it seems that there was a gene expansion in *T. gondii* (Fig. 3 A). Interestingly, a clear expression of FamB and FamC is observed at the mRNA level (data from toxodb and our own, unpublished), but there is no evidence of their translation

[128]. Therefore, the role of these genes remains unclear. It may be hypothesized that their translation depends on environmental signals or, alternatively, that they are part of lncRNAs. The existence of a 15-kbp fragment conserved in all ST that contain FamC genes is striking (Fig. 3 B). This fragment extends beyond the annotated gene, with evidence of RNA transcription in gene depleted regions (Fig. 3 C). An interesting aspect of this 15-kbp fragment, associated with FamC or FamC-like sequences, is that it is also associated with two satellite DNAs: the 350-bp family [129] and a new 240-bp family [128]. This situation may be explained by inter-ST recombination and gene duplication, which in addition can explain FamC expansion (Fig. 3 B).

Recently, an ex vivo model of *T. gondii* recrudescence was generated, which allows the analysis of the bradyzoite-to-tachyzoite reactivation process, using a Type II ME49EW strain unadapted to cell culture [130]. The authors were able to detect HFF-adapted *T. gondii* line that spontaneously emerged, designated ME49EW1, which presented a dramatic reduction in producing tissue cysts in murine brain tissue. In this way, it was observed that *T. gondii* adaptation to HFF cells led to a loss of developmental competence, similar as occurring in other cystogenic lines. Transcriptome analysis of ME49EW cysts and HFF-adapted tachyzoites showed that 2,916 mRNAs were downregulated in cyst (up-regulated in tachyzoite) while 1,585 were upregulated in cyst. Of the genes that were upregulated in cyst, 28 were located in the ST, while in tachyzoite only 6 ST genes were upregulated (Table S1). Interestingly, in the cyst transcriptome 8 FamB and 4 FamC genes were observed, and only one annotated, while the rest belong to uncharacterized genes. To note, in some cases most of ST genes from one subtelomere were simultaneously upregulated (Table S1). In the case of tachyzoites, none of the FamB and FamC genes were detected, but there were at least 3 annotated genes. This difference shows that ST could provide more genes associated to bradyzoite development, and that FamB and FamC could play a relevant role in this conversion. Despite almost all ST genes upregulated either in cyst or tachyzoite present high synteny along *T. gondii* strain [128], it would be interesting to study the role of this variability for ST genes and the differences at *T. gondii* strains with greater or lesser cystogenic capacity. Taking all results together, the understudied FamB and FamC multigene families, with highly variable genes in sequence, should be given more attention in the future. The

appearance of FamB and FamC and their expansion, rather than their disappearance, could be indicative of an important role for this group of genes, at least in some situations faced by *T. gondii* in its life cycle.

6.2. T. gondii *ST and contingency genes*

It is important to mention that a high percentage of *T. gondii* subtelomeric genes increase their expression in at least two cases of environmental stress mentioned in the previous sections: adaptation to in vitro passage (adaptation to extracellular environments), and the lack of cytoplasmic/nuclear acetyl-coA (metabolic stress) [128]. In both cases, the percentage of subtelomeric genes that present a differential expression is significantly higher than that of the euchromatin genes, suggesting a possible role of subtelomeric genes in adapting to these stress situations. Moreover, gene deletion of subtelomeric TGGT1_264240 (chromosome VII_b), produced parasites with reduced growth in a plaque size assay [131], highlighting the importance of these genes in *T. gondii* extracellular adaptation. As expected, some *T. gondii* ST genes, like TGGT1_264240, present a low conservation among *T. gondii* genotypes, with cases of gene loss as well as gene duplication [128]. All these findings suggest that the *T. gondii* ST could be enriched in contingency genes, being a relevant contribution to the adaptability and evolution of the different *T. gondii* genotypes.

T. gondii, like other organisms, presents a group of genes that code for Late Embryogenesis Abundant (LEA) proteins. Among the biological roles of these proteins are cellular dehydration tolerance, stabilizing membranes or sensitive enzymes during freezing or drying [132]. In *T. gondii*, the knock-out of LEA genes was associated with greater susceptibility of oocysts to high salinity, freezing, and desiccation [133]. It was also observed that the LEA proteins were cryopreservatives of the enzyme lactate dehydrogenase 1. LEA proteins are encoded by 4 genes which appear in tandem and have a chromosomal location compatible with the subtelomeric domain in *Neospora caninum* and *Besnoitia besnoiti*. In *T. gondii* these genes are located 200 kbp from the telomere of chromosome XII, right arm. A detailed analysis shows that these genes are immediately upstream of TGME49_276820, a gene proposed to limit the

subtelomere of that chromosome, at the right arm [128]. Furthermore, TGME49_276820 is precisely 191.5 kbp from the telomere. Taking in consideration that the longest ST defined in *T. gondii* is 232 kbp, we should consider whether the expression of LEA genes is affected by telomeric position effect [134]. Moreover, it is also possible that LEA genes should be considered subtelomeric, and it seems clear that they were the result of evolution associated with subtelomere plasticity.

7. *T. gondii* in the context of Extended Evolutionary Synthesis

The concepts of adaptation and evolution of organisms have quite changed since Darwin's theory of Evolution. Nowadays, science has incorporated the analysis of phenotypic variability as a result of the interaction between the genotype and the environment. Very important, only a small proportion of the observed variability and adaptation to environmental changes is explained by genetic variation, incorporating transgenerational epigenetic inheritance as a determining factor. Among others, this is a phenomenon that gives rise to the so-called extended evolutionary synthesis (EES) [135]. The EES theory incorporates the concept of epigenetic variation between individuals with the same or similar genotype. Epigenetic alterations have been specifically characterized as heritable changes in gene expression through mitotic and/or meiotic processes, which cannot be attributed to modifications in the underlying gene sequence [136]. Thus, within a few or even just a single generation, populations can display adaptive changes in response to a shifting environment, all without significant genetic modifications. This highlights how epigenetic variation serves as an evolutionary mechanism within natural populations [137]. Here we consider that other elements could be included to support EES, such as assistance by heat shock proteins and contingency genes.

Until now, the concept of EES has produced publications mainly from studies in animals and plants [137]. However, protozoan parasites can be an interesting EES model: they have a high rate of replication and, as mentioned above, a remarkable adaptive capacity to environmental stimuli. Recently, Primo et al [131], carried out an assay of evolution and adaptation to the extracellular life of *T. gondii* as part of an in vitro propagation experiment. *T. gondii* tachyzoites

have a high rate of replication which occurs only within the parasitophorous vacuole that is generated intracellularly after invasion. After several rounds of replication, tachyzoites egress and invade neighboring cells. Although this step is expected to be fast enough to escape from the extracellular environment, the tachyzoite is capable of surviving days outside any cell. In the context of in vitro tissue culture, the lytic cycle remains consistent, albeit with a prolonged interval from discharge to invasion by another cell. Primo's study focused on examining the phenotypic changes in the tachyzoites of a *T. gondii* GT1 strain isolate, following around 5,000 generations of in vitro passages. Given that the culture conditions remained constant throughout, the sole variation between successive passages and the original isolate pertained to extracellular adaptation and the lack of in vivo environmental pressure. In fact, tachyzoites "evolved" to last longer outside the cell, and to acquire a greater capacity for invasion and transmigration. Genetic studies of the new generations with respect to the original isolate showed only one mutated gene, *P4 flippase*, that was likely selected for the evolving population. Noteworthy, the phenotypic changes observed would be likely explained by changes at the transcriptome level. Among the transcriptomic changes, those related to fatty acid metabolism, transcription factors and epigenetics were highlighted. Among the epigenetic mechanisms, a histone demethylase, TgJmjC-put5/TgNO66 [138], and a nucleosome remodeler, SWI/SNF2-containing protein-b [139], stand out. The last one also plays a role in the incorporation of histone variant H2A.Z to the nucleosome. Consequently, the change in the gene expression profile was responsible for driving the adaptation of *T. gondii* tachyzoites to the extended extracellular conditions. It is expected that the mechanism of chromatin modulation could be involved in this transcriptional reprogramming.

As mentioned above, the development of an ex vivo model of bradyzoite recrudescence showed a clear adaptation of *T. gondii* ME49EW to passage in HFF host cells [130] In this passage, the tachyzoites showed a loss of competence to generate bradyzoites in the brain of the infected mice. Once again, all of these changes appear to be related to changes in the transcriptome. Transcriptome analysis showed that numerous transcription factors of the ApiAP2 family were detected at tachyzoite and cyst stages. Interestingly, during bradyzoite to tachyzoite transition, the enrichment of mRNAs coding for H2A.Z and H2B.Z (previously named H2Bv) was

detected. Furthermore, some of the H2B.Z interactors, acetyl-CoA carboxylase ACC1, H3.3 and RNA Recognition motif protein (TGME49_262620) [24] were also enriched in the tachyzoite. All these data suggest that the epigenetic mechanism based on the H2A.Z/H2B.Z DVN could be involved in the process of conversion to tachyzoite and perhaps also in the adaptive cell culture process.

The most interesting aspect is that in both cases the observed phenotypic changes seem to fit perfectly into the concept of EES. In this way, protozoan organisms could be extremely interesting models for evolutionary studies to be incorporated into this field that presents different points of view. An important aspect of the EES is the construction of the niche, where organisms select components of the environment for their use, but at the same time this modification of the niche arises as a selection pressure for the rest of the offspring [140]. Although this should be studied in more detail, *T. gondii* is capable of interacting with the host cell, strongly changing its transcriptome [141]. Furthermore, single cell transcriptome studies show heterogeneity in *T. gondii* population [130]. How these modify the host cell and then select the tachyzoite populations is the subject of future studies.

8. Conclusions

T. gondii is a versatile parasite with high adaptable capacity to varying environments, a trait that aligns with its unicellular nature and should fall on rapid response programs. Analogous to the observed patterns in other species, this adaptation process relies on evolutionarily conserved pathways; each species tailoring them to their specific needs. In the context of *T. gondii*, at least three fundamental mechanisms are participating: an epigenetic pathway with the addition of an unconventional histone variant (H2B.Z), heat shock proteins, which exhibit notable conservation in chaperone and co-chaperone pathways, and rapidly evolving contingency ST genes. Relevant for the evolution and adaptability of organisms, *T. gondii* presents TF with specific functions and determinants in the transitions to the different stages of its life cycle.

These mechanisms collectively underlie rapid reactions to shifts in metabolism, a trait fundamentally conserved across nature. In the case of *T. gondii*, this adaptability extends to

encompass significant flexibility in altering energy metabolic pathways, translation, and development. ST FamB and FamC also arise as an interesting family of genes that can have a relevant role in the adaptation to different stress or differentiation.

While divergences exist at the gene sequence level (e.g. virulent genes), many of the rapid changes involving stress response and differentiation stem from alterations in the transcriptome. This phenomenon hinges on the framework of epigenetic modulation. In this sense, *T. gondii*, along with other protozoa, emerges as an exemplary model for exploring the EES.

Acknowledgements

SO Angel (Researcher), L. Vanagas (Researcher), and A.M. Alonso (Researcher) are members of National Council of Research (CONICET). Funds were obtained from CONICET (Grant PIP 0572) and ANPCyT, (Grant BID-PICT 2021-0331 (SOA), BID-PICT 2019-0513 (AMA), BID-PICT 2018-2434 and BID-PICT 2021-0169 (LV)).

Declarations of interest

none

Author contribution

Writing - original draft SOA; Writing - review & editing LV and AMA; Funding acquisition, SOA, LV and AMA,

Legends

Fig. 1: Adaptation model of *Toxoplasma gondii* to environment and different host cells.

A. *T. gondii* is capable of infecting nearly all types of nucleated cell of mammals and birds. At the initial stages of infection, the tachyzoite must spread rapidly throughout the organism, invading different cell types of the host. Upon invasion, it must sense the environment for cues such as nutrient availability and oxygen derivatives. Once established, it undergoes replication and subsequently egresses the host cell to infect another. This new host cell can either belong to a

different cell type or may be of the same type but in a distinct metabolic state, necessitating an adaptation to the altered environment. In the model, the yellow and green cells symbolize the same cell type undergoing different metabolic states, while the light blue cell represents a distinct cell type.

B. *T. gondii* is a unicellular parasite that must sense environmental changes and respond quickly in order to adapt to them and survive. For this, like every cell, it has different molecular mechanisms: 1. Transcription factors that regulate the expression of specific genes; 2. the epigenetic mechanism that presents high flexibility to modulate chromatin and regulate different biological processes such as replication, gene transcription, DNA repair; 3. the stress protein system, which assists affected proteins in their folding or, as in the case of Hsp90, client proteins that must engage in different cellular scenarios; 4. the global translation rate of mRNAs and the selective translation of mRNAs to express only proteins associated to environmental changes response; 5. related to translation, the stress granules that allow the rapid removal of mRNAs in the translation process in the event of a stress situation; 6. the presence of contingency genes that are expressed only in stressful situations. TF: transcription factor; E: enhancer; P: promoter; Me: methylation; Ac: acetylation; ST: subtelomere; TgJ1: *Toxoplasma gondii* DNAJ1; HSP70 Heat shock protein 70; HSP90: Heat shock protein 90; CP: client protein; eIF4E: eukaryotic translation initiation factor 4E.

Fig. 2: Importance of acetylations for the epigenetic mechanism.

A. Model of epigenetic regulation of gene expression based on acetyl-CoA metabolism proposed for *T. gondii*. In the upper section, we observe the interaction of a transcription factor (TF) with its enhancer (blue arrow), where it recruits the histone acetyltransferase (HAT) enzyme. This HAT uses acetyl-CoA as the source of acetyl groups. The maintenance of high cytoplasmic/nuclear acetyl-CoA levels is ensured by the glycolysis/TCA pathway. Consequently, this HAT acetylates the histones within the basal promoter region, leading to chromatin opening. Additionally, the TF recruits mediator complex proteins (green), which subsequently facilitate the recruitment of basal TFs. These basal TFs bind to the basal promoter and, in turn, recruit RNA polymerase II (RNAP)

for gene transcription. In the model below, a deficiency in glucose results in decreased levels of cytosolic/nuclear acetyl-CoA. Consequently, even if a TF can bind to its enhancer and recruit HAT, it lacks the necessary substrate to acetylate the chromatin. As a result, the basal promoter will remain in a closed chromatin state. Moreover, the mediator complex, if bound to the TF, cannot load the basal TFs onto the basal promoter, thereby silencing gene expression. **B.** The N-terminal and C-terminal tails of histone H2A.Z from various organisms and H2B.Z from *T. gondii* are represented. The PTMs are indicated in each case. Ac, acetylation, Me, methylation; Ubi, ubiquitination and Sum, sumoylation. DVN, Double variant nucleosome generated by histone variants H2A.Z and H2B.Z. The extensive positive charge patch (non-acetylated lysines) that would be generated in the *T. gondii* DVN if the histones are not acetylated is highlighted.

Fig. 3. Expansion of FAmB and FamC in *T. gondii*.

A. The phylogenetic tree was based on the publication of Hu et al [142]. The numbers indicate the FamB and FamC genes present in each species, showing the expansion of both integral membrane protein families in *T. gondii*. **B.** Expansion model of FamC family based on a DNA element named ST15KFamC. ST15KFamC, is a ST fragment of near 15 kpb which contains a member of the FamC at one end. The model is based on the ST15KFamC of subtelomere from chromosome Ia, left arm (ST_Ia_L), which *tgc* gene is 2,889-bp long. It is proposed that this element could have “jumped” to other STs due to recombination events. However, the possibility that it is a transposable element cannot be ruled out. **C.** RNA-seq analysis of tachyzoites (Tz) and bradyzoites (Bz) along the ST_Ia_L. Coverage plots for Tz and Bz stages at this subtelomere (chromosome Ia: 1-14kb; ME49 strain). RNA seq data was extracted from Waldman et al. [41]. On the right, the brown bar shows the location of the FamC gene. The light blue bar shows the region where RNA is detected without the identification of coding genes, suggesting the existence of a possible long non-coding RNA (lncRNA). The line above shows the scale in kilobase pairs (Kb). The Y axis indicates CPM-Normalized Coverage (CPM= count per million of mapped reads).

Table S1. Subtelomeric genes upregulated in in vivo cyst and in vitro tachyzoite during bradyzoite to tachyzoite transition.

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Figure 1

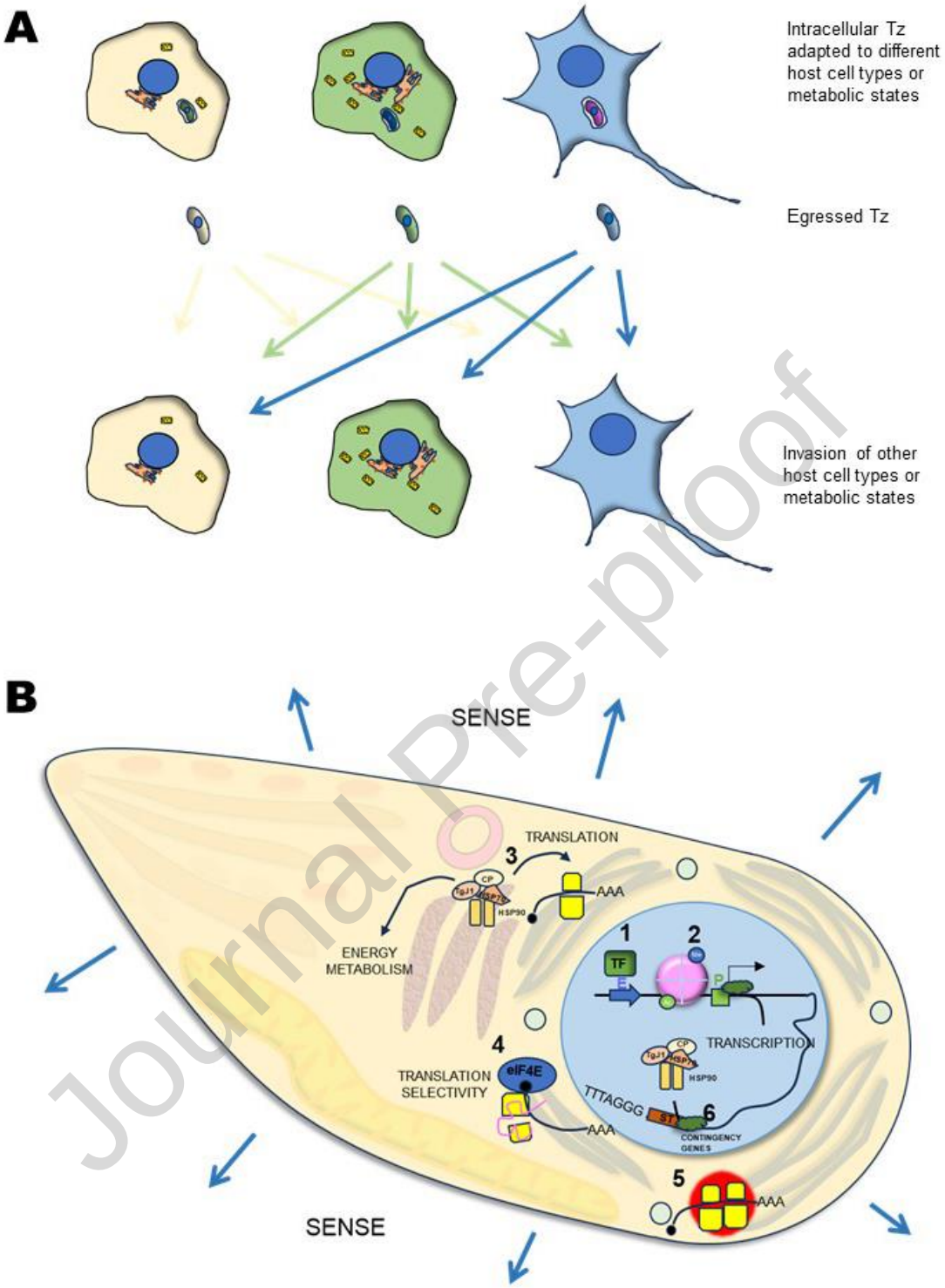
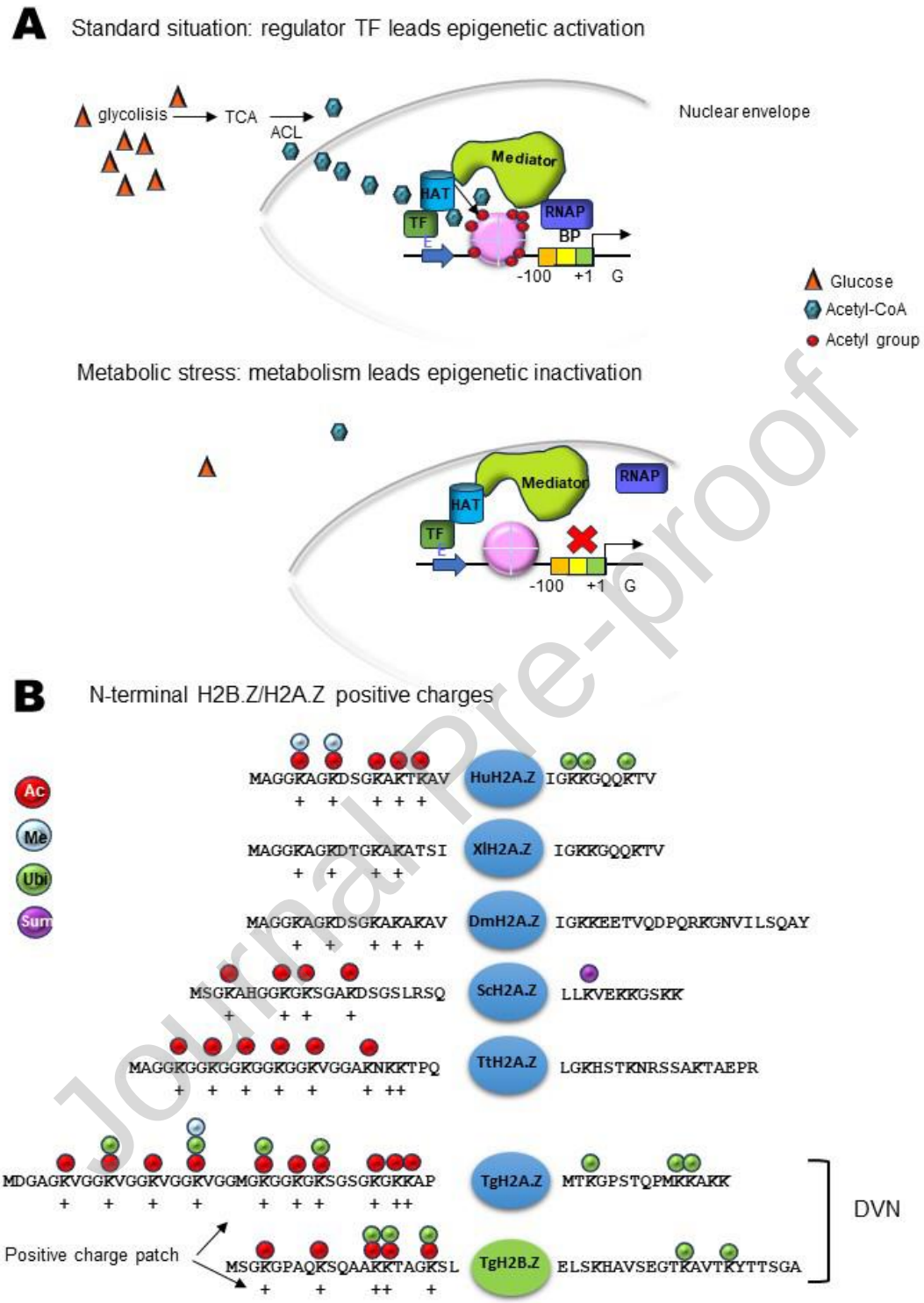


Figure 2


A
Standard situation: regulator TF leads epigenetic activation
glycolisis
TCA
ACL
Nuclear envelope
Mediator
HAT
TF
RNAP
BP
-100
+1
G
Glucose
Acetyl-CoA
Acetyl group
Metabolic stress: metabolism leads epigenetic inactivation
Mediator
RNAP
HAT
TF
-100
+1
G
B
N-terminal H2B.Z/H2A.Z positive charges
Ac
Me
Ubi
Sum
MAGGKAGKDSGKAKTKAV
HuH2A.Z
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TgH2A.Z
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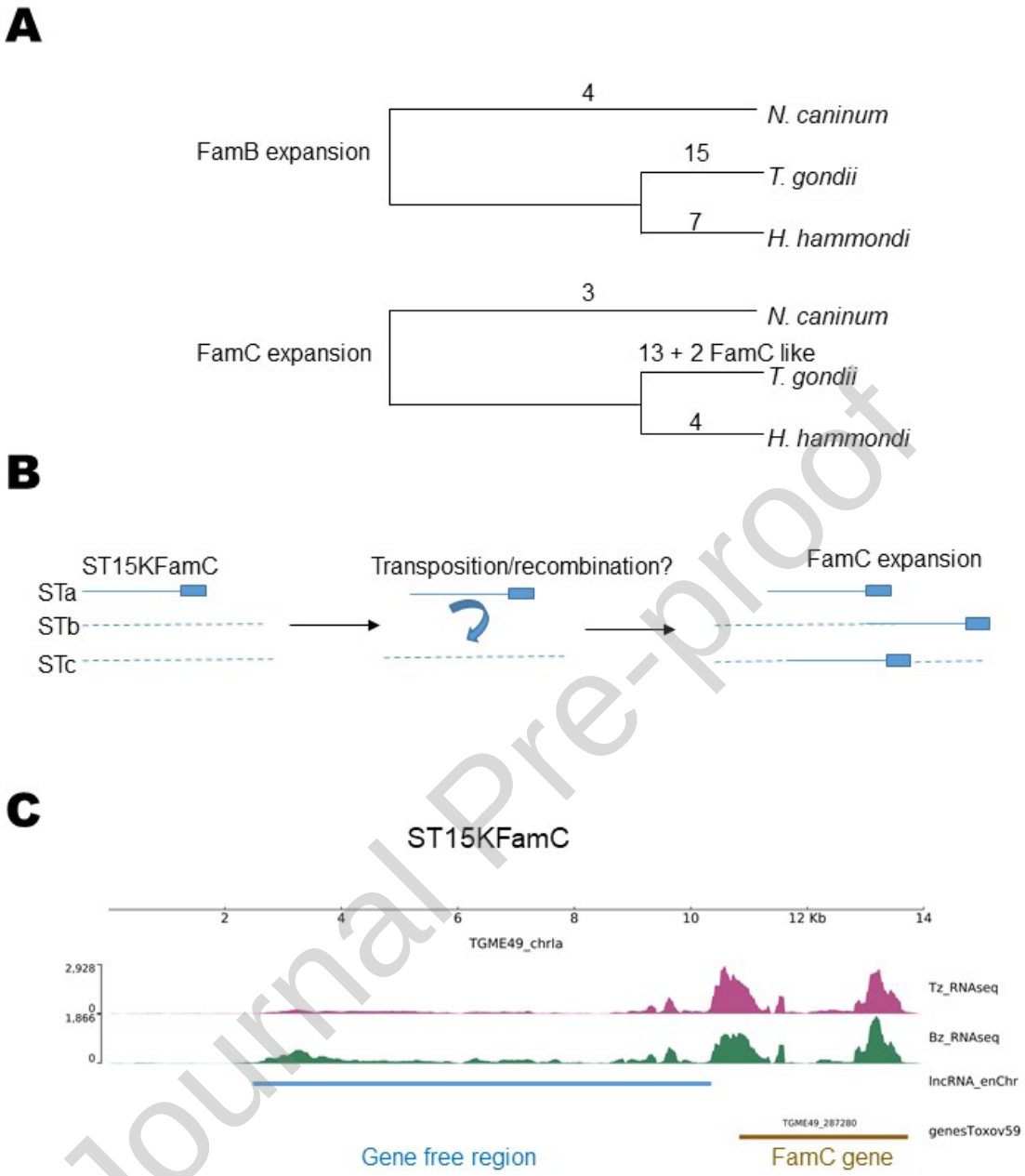
Figure 3



Declaration of Competing Interest

none

Highlights

- Toxoplasma must constantly adapt to different environmental situations
- Epigenetic modifications and heat shock proteins can provide support for adaptability
- Subtelomeric contingency genes can contribute to adaptation and evolution
- The presence of a high adaptability feature can drive the evolution of Toxoplasma