Protein acetylation in the critical biological processes in protozoan parasites

Suellen Rodrigues Maran[1](ORCID: 0000-0003-0687-9186); Krista Fleck[2](ORCID: 0000-0001-6863-8631); Natália Melquie Monteiro-Teles[3](ORCID:0000-0002-7123-378X); Tony Isebe[4](ORCID: 0000-0003-3094-1832); Pegine Walrad[3](ORCID: 0000-0002-2302-0720); Victoria Jeffers[2](ORCID: 0000-0003-1233-1088); Igor Cestari[4,6] (ORCID: 0000-0003-3845-7535); Elton J. R. Vasconcelos[5] (ORCID: 0000-0001-5130-6622); Nilmar Silvio Moretti[1*](ORCID: 0000-0003-0455-2497)

[1] Laboratório de Biologia Molecular de Patógenos (LBMP) – Departamento Microbiologia, Imunologia e Parasitologia – Escola Paulista de Medicina – Universidade Federal de São Paulo (UNIFESP), Brazil.
[2] Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH, United States.
[3] York Biomedical Research Institute, Department of Biology, University of York, York, UK.
[4] Institute of Parasitology, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada,
[5] Leeds Omics, University of Leeds, Leeds, UK.
[6] Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada.
[*] **Correspondence:** nilmar.moretti@unifesp.br (N. S. Moretti)

Keywords: acetylation; acetylome; KATs; KDACs; bromodomain; glycolysis.

Abstract

Protein lysine acetylation has emerged as a major regulatory post-translational modification in different organisms, present not only on histone proteins affecting chromatin structure and gene expression, but also on non-histone proteins involved in several cellular processes. The same scenario was observed in protozoan parasites after the description of their acetylomes, indicating that acetylation might regulate crucial biological processes in these parasites. The demonstration that glycolytic enzymes are regulated by acetylation in protozoans shows that this modification might regulate several other processes implicated in parasite survival and adaptation during the life cycle, opening the chance to explore the regulatory acetylation machinery of these parasites as drug targets for new treatment development.

Protein acetylation

Apicomplexan and Trypanosomatid parasites are a group of protists with a broad range of species that cause various public health-impacting diseases worldwide. Among these species, *Toxoplasma gondii*, *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Trypanosoma evansi* - etiological agents of toxoplasmosis, malaria, African trypanosomiasis/Nagana, Chagas disease and surra, respectively, - are listed in the rankings of the most relevant protozoan parasites [1-4]. These microorganisms have complex life cycles shifting between different hosts and facing varied environmental conditions, which require alterations in several biological processes aimed at their survival and infection success [5-7].

Reversible **post-translational modifications** (PTMs) (see **Glossary**) represent a fast and economical way for cells to respond to physiological and environmental conditions. PTMs such as phosphorylation, methylation, ubiquitination and acetylation are found on several proteins in the cell [8-11]. Acetylation is one of the most common PTMs and is characterized by the addition of an acetyl group on the ε-amino group of lysine residues [8, 12]. This PTM neutralizes lysine positive charges and can confer novel properties to the modified proteins, comprising changes in enzymatic activity, subcellular localization and DNA binding [8, 12]. Moreover, acetylation is also found in the N-terminal regions of proteins and plays an important role in the synthesis, stability and cellular localization of proteins (32912665; 30054468).

Protein acetylation was first described on histones [13], and for many years the main interest was in understanding the impact of this modification on chromatin structure and gene expression regulation. However, the advent of new proteomic technologies allowed the identification of thousands of acetylated lysine sites (K-ac) in both prokaryotes and eukaryotes and described numerous non-histone acetylated proteins. Hence, the initial focus on chromatin-associated protein acetylation has now shifted to a

broader scope and highlights acetylation's regulatory functions in any subcellular location [8, 12].

Acetylomes of many organisms, including apicomplexan and trypanosomatids, have been described [14-19]. The widespread presence of lysine acetylation in these organisms indicates that its regulatory functions are diverse. Thus, in this review, we will consider the advances in the study of protein lysine acetylation in protozoan parasites of medical and veterinary importance that had their acetylomes published/described up to the date we wrote this article. We also discuss what these recent studies have taught us about the functionality of this particular PTM on essential biological processes in these organisms. Finally, we review the parasitic protein acetylation machinery and the potential to explore them as drug targets for the development of new therapeutic strategies against these parasites.

Regulatory machinery of protein acetylation in protozoan parasites

The addition, removal and recognition of acetyl groups on lysines are coordinated by **lysine acetyltransferases (KATs), lysine deacetylases (KDACs)** and **bromodomain-containing proteins (BDPs)**, respectively. KATs add acetyl groups to lysines [20], while KDACs remove the acetyl groups [21]. Bromodomains bind acetylated lysines, and link acetylation marks with the proteins that perform downstream regulatory functions [22]. The acetylation regulatory machinery of apicomplexans and trypanosomatids has shown to be similar (BOX1), but compared to higher eukaryotes such as humans, it is reduced and contains both highly conserved and parasite-specific acetylation factors (**Figure 1A**). In the next sections we will give more details about the acetylation machinery of protozoan parasites that have acetylome datasets available.

Lysine acetyltransferases (KATs)

KATs are grouped based on the sequence similarity of their acetyltransferase domain to historically well-conserved KATs. The most common families include GNAT, MYST, Hat1 and p300/CBP. Their specific domains/substrates are reviewed in [20]. While humans have a large repertoire of KATs, protozoans have far fewer. *T. gondii* and *P. falciparum* each have four GNAT family KATs, while trypanosomes only have two (**Figure 1** and Supplementary **Table S1**). *T. gondii* is unique in possessing two GCN5 homologues, and *P. falciparum* has a GNAT protein (PF3D7_1020700) with no similarity to other identified KATs outside of the GNAT domain.

The MYST family of KATs are named for the first identified proteins of this group (MOZ, Ybf2/Sas3, Sas2, Tip60). While *T. gondii* and *P. falciparum* contain more GNAT family KATs, trypanosomes have many MYST KATs [23, 24] (**Figure 1** and Supplementary **Table S1**). The third group of KATs is similar to Hat1; the first identified histone acetyltransferase in yeast [25, 26]. The genomes of *T. gondii* and *P. falciparum* each encode a single Hat1 homologue, but neither has been investigated, and no homologues have been identified in trypanosomes (**Figure 1** and Supplementary **Table S1**). The fourth family of eukaryotic KATs, p300/CBP, have no known homologues in apicomplexans or trypanosomes.

Lysine deacetylases (KDACs)

Lysine deacetylases (KDACs) are subdivided into four classes (I, II, III/sirtuins, IV). Classes I, II and IV are categorized based on sequence similarity to yeast deacetylases Rpd3, Hda1 and HDAC11, respectively. Class III KDACs, also referred to as sirtuins, are homologous to yeast Sir2 and require nicotinamide adenine dinucleotide (NAD^+) as a cofactor for their catalytic activity [21].

Toxoplasma possesses four class I KDACs, but only TgHDAC3 has been characterized [27], while *P. falciparum* has only class I KDAC. Trypanosomes have twice as many class II KDACs as *Toxoplasma* and *P. falciparum,* and of all the protozoan class II KDACs, only *T. brucei* enzymes have been characterized [28] (**Figure 1** and Supplementary **Table S1**).

Apicomplexans each have two class III KDACs (sirtuins), and while the precise function of the *Toxoplasma* sirtuins has not yet been identified, the role of the *P. falciparum* sirtuins in regulating gene expression has been reported [29-33]. *T. brucei* has two mitochondrial (TbSir2rp2 and TbSir2rp3) and one nuclear sirtuin (TbSir2rp1) [34, 35]. *T. cruzi's* two sirtuins, cytoplasmic TcSir2rp1 and mitochondrial TcSir2rp3, have distinct functions in parasite epimastigote multiplication and differentiation to metacyclic forms [36, 37]. The three *T. evansi* sirtuins remain uncharacterized. (**Figure 1** and Supplementary **Table S1**). No class IV KDACs have been identified in apicomplexans nor trypanosomes.

Bromodomain-containing proteins (BDPs)

Apicomplexans and trypanosomes have a limited repertoire of bromodomain proteins compared to humans. With twelve BDPs, *Toxoplasma* has the largest number of these reader proteins in its genome, almost twice the number found in *P. falciparum* or *Trypanosoma* species. Many human BDPs contain more than one bromodomain; however, this is only found for TgBDP3 in *Toxoplasma* and TcBDF5 in *T. cruzi,* each of which possesses two bromodomains. Importantly, apicomplexans and trypanosomes have many parasite-specific BDPs with no similarity to human BDPs, which have been seen as promising drug targets [38] (Supplementary **Table S1**).

Acetylated protein repertoire of protozoan parasites

General overview on published acetylomes

To date, acetylomes have been reported for five protozoan parasite species: *P. falciparum* [18, 19, 39], *T. gondii* [17, 38, Ref], *T. brucei* [14, 15], *T. evansi* [14] and *T. cruzi* [15]. For more details about the methods used to describe their acetylomes, see **Box 2**.

From the five parasite acetylomes addressed herein, *P. falciparum* and *T. brucei* were more comprehensively studied and combine several recent studies with revised and improved new methodologies, which have refined the acetylomes from each species [14, 19]. Twelve hundred and 2,756 acetylated proteins were identified in *P. falciparum* and *T. brucei*, corresponding to 21.6% and 24.6% of their total predicted proteome, respectively (**Figure 2A and B**). Similarly, 19.2% of *T. evansi* proteome contains K-ac-modified proteins, whereas 5.9% of *T. gondii* and 2.2% of *T. cruzi* proteomes have K-ac proteins (**Figure 2A and B**). These differences might reflect the use of distinct protocols and MS/MS technologies [17, 38]. Notably, the *T. cruzi* acetylome protocol did not include the K-ac immunoaffinity enrichment step [15]. Hence, the number of acetylated proteins identified in some of these parasites might be underestimated (as depicted in **Figure 2A to C**).

Comparative analyses of protozoan parasite acetylomes

Gene ontology (GO)-based enrichment analyses of several parasite acetylomes via the EuPathDB [40] revealed that chromatin and nucleosome GO cellular component (CC) terms are present in all three genera: *Trypanosoma, Toxoplasma, and Plasmodium* (**Figure 2D** and Supplementary **Table S2**); moreover, it showed that glucose metabolism is a GO biological process (BP) also common to both apicomplexan and trypanosomatid

159 species studied herein (**Figure 2D** and Supplementary **Table S2**). Furthermore, processes
160 related to nucleotide metabolism/biosynthesis were shared between *P. falciparum* and *T.*
161 *gondii*, whereas *T. evansi* and *T. cruzi* have "tRNA aminoacylation for protein translation"
162 (BP) and "proteasome complex" (CC) terms in common. Finally, “microtubule-based
163 transport” (BP)-associated Kac-containing proteins were enriched in *T. evansi* and *T.*
164 *brucei* acetylomes.

165 A survey using parasite acetylated proteins by Markov Clustering (MCL)
166 algorithm [41] indicated that those acetylome datasets form 20 distinct clusters that vary
167 in size from 5 to 45 proteins each, and they contain at least one protein from each species
168 (**Figure 2E** and Supplementary **Table S3**). Putative functional clusters of orthologous
169 groups (COGs) corroborate the GO-based enrichment analysis (**Figure 2D**), notably, the
170 chromatin, glucose metabolism, and protein synthesis/degradation-related COGs (**Figure**
171 **2E**). Chaperones, cell division, oxidative stress, and RNA degradation-associated COGs
172 also display functional groups of acetylated proteins in all analyzed species (**Figure 2E**).
173 Details on all 20 COGs are found in Supplementary **Table S3**. Hence, there are conserved
174 functional groups of acetylated proteins in protozoans, and they may play a role in specific
175 processes of each parasite biology.

176

177 **Physiological roles of protein acetylation in protozoan parasites**

178 *Regulation of chromatin structure and gene expression in apicomplexan parasites*

179 Protozoans possess the same basic components and assembly of chromatin as
180 higher eukaryotes. Acetylation alters the charge of lysine residues from positive to
181 neutral, thus reducing histone tails' affinity to DNA, allowing chromatin to relax and trans
182 factors to access DNA. *Toxoplasma* and *Plasmodium* both rely heavily on PTMs to
183 regulate gene expression (REF). They lack typical regulatory mechanisms present in

metazoans, such as a conserved TATA box in promoters, the linker histone H1, DNA methylation, and DNA-binding transcription factors. The only transcription factors identified in apicomplexans to-date are the plant-like transcription factors ApiAP2s (Apicomplexan APetala2) [42].

Analysis of the repertoire of PTMs in *Plasmodium* showed a prevalence of histone acetylation [19]. Histone H3 and H4 acetylation upstream of active genes has consistently been observed in *Toxoplasma* and *P. falciparum* [18, 19, 43-45]. The presence of histone acetylation in **euchromatin** and absence in **heterochromatin** is observed particularly well in *P. falciparum*, in which inactive var genes are devoid of acetyl marks and located in highly compacted chromatin at the nuclear periphery [46, 47]. Moreover, the acetyl marks H3K9ac, K3K14ac, H4K5ac and H4K12, and H3K4 trimethylation are present in intergenic regions of transcribed genes [43, 48, 49]. The correlation between H3K9ac and transcript levels is well established in *P. falciparum* asexual blood stages and **sporozoites** and also observed in male **gametocyte** ookinetes [50].

While most acetyl marks are associated with active transcription, other acetyl marks identified in *T. gondii* and *P. falciparum* show no such correlation. In the **oocyst** and **sporozoite** mosquito stages of *P. falciparum*, H3K27ac is enriched in intergenic euchromatin regions but is not associated with gene expression [51]. Whitmer et al. found the H3K9ac mark does not correlate with increased transcript levels in female gametocytes [50], and low passage strains of *T. gondii* have significant histone acetylation at inactive genes [52].

KATs and KDACs are typically associated with gene activation and repression, respectively. In apicomplexans, they appear to be multifunctional, present in various complexes and involved in regulating the expression of specific subsets of genes, including those involved in parasite growth and differentiation. *T. gondii* tachyzoite

growth during asexual replication requires the KAT TgGCN5B to regulate gene expression via histone acetylation [53]. The *P. falciparum* homologue PfGCN5 is also required for asexual replication. PfGCN5 increases erythrocyte invasion by hyperacetylation of histones, whereas histone hypoacetylation by the KDAC PfSir2A decreases invasion and delays trophozoite growth [54]. The *P. falciparum* KDACs PfSir2A, PfSir2B, and PfHda2 also play an essential role in heterochromatin formation and silencing var genes [29, 55, 56]. The differentiation between **tachyzoite** and **bradyzoite** stages of *T. gondii* is also affected by acetylation. TgHDAC3 is enriched at the stage-specific inactive genes in tachyzoites, and inhibition of TgHDAC3 with the compound FR235222 causes expression of bradyzoite-specific genes and differentiation [57]. In *P. falciparum*, PfHda2 is associated with gene regulation required to convert from the asexual to the sexual stage [55].

More recently, bromodomain-containing proteins have surfaced as critical players in protozoan gene regulation. Studies suggest that they might be essential for *T. gondii* growth [58] and verified to be critical for *P. falciparum* growth and invasion [59]. Hanquier et al. identified the bromodomain of TgGCN5B as essential for *T. gondii* viability [60]. It is likely that parasite GCN5 requires both the KAT and acetyl binding functions for gene regulation during parasite growth and differentiation. The bromodomain protein PfBDP1 associates with acetylated histones in actively transcribed genes, with the transcription factor PfAP2-I, and the bromodomain-containing protein PfBDP2 [59, 61]. Tang et al. showed the localization of the PfBDP1/PfBDP2/PfAP2-1 complex to nucleosomes containing PfH2A.Z and H3K18ac and H3K27ac marks at active transcription start sites [62].

In addition to histones, KATs, KDACs, and BDPs, transcriptional components are themselves acetylated. The effects of acetylation on the function of these proteins are

poorly understood. However, acetylation of ApiAP2s can alter their interactions with DNA and other proteins [39]. Moreover, the KAT inhibitor garcinol decreased acetylation of the KAT TgGCN5B itself, in addition to its substrate H3, resulting in disrupted tachyzoite growth [63]. It will be interesting to know how acetylation of these factors is regulated and how it contributes to regulating transcription.

Regulation of Chromatin Structure and Gene Expression by Acetylation in Trypanosomatids

The chromatin structure of Trypanosomatids is organized into 10 nm nucleosomal filaments [64]. Micrococcal nuclease digestion of chromatin followed by histone analysis revealed that the trypanosome chromatin's basic structure and organization is similar but not identical to others eukaryotes [65]. Proteomic analysis has identified over 170 PTMs in trypanosome histones [15, 66]. Acetylation was found in *T. cruzi* at H2A C-terminal tails and H2A.Z at the N and C-terminal tails. Moreover, H2B.V, H3 and H4 were predominantly acetylated at the N-terminal tails [15, 66, 67]. Notably, acetylation was also detected in the globular domains of several histones. In contrast, no acetylation was detected in H3.V and H4.V of *T. brucei* [68].

Much less is known about histone acetylation in *Leishmania ssp.* but H4 is acetylated at K4 and K10, whereas H3 is acetylated at the N-terminal tails [69]. KATs and KDACs are also encoded in the trypanosomatid parasite genomes (**Supplementary Table S1**). The *T. brucei* TbSir2rp1 acetylates histones H2A and H2B in vitro [34]. *T. brucei* HAT1 can acetylate the N-terminal tails of H2A.Z and H2B.V; whereas HAT2 can acetylate H4K10, and HAT3 can acetylate H4K4 [70, 71].

Dissecting the role of specific histone acetylation is technically challenging. A typical approach is the knockdown of genes encoding acetylase or deacetylase enzymes

combined with chromatin immunoprecipitation and sequencing or gene expression analysis. Kraus et al. identified H4 and H2A.Z acetylation associated with transcription start sites (TSSs) [70]. These modifications were mediated by the histone acetyltransferases, HAT2 and HAT1, respectively. The knockdown of HAT2 decreased H2A.Z deposition and resulted in changes in transcription initiation sites. In contrast, the knockdown of HAT1 decreased total mRNA levels by half, implying that these histones' acetylation plays a role in RNA polymerase II transcription [70]. The knockdown of Sir2rp1 in *T. brucei* bloodstream forms also affected transcription of a reporter gene near telomeres but did not affect transcription of variant surface glycoprotein (VSG) genes [35]. Respuela et al. found an enrichment of acetylated H3 and H4 at strand switch regions of divergent polycistronic genes in *T. cruzi*, which indicates a role for these modifications on transcription [72].

Little is known about the role of histone acetylation in *T. cruzi* and *Leishmania spp.* *L. donovani* HAT4 acetylates histone H4 at residues K2 and K14, but the function of these acetylations is unknown [73]. The expression of mutant non-acetylated forms of H4, which prevents acetylation at positions K10 or K14, affected DNA replication and repair and indicated a role in chromatin assembly/remodelling required for gene expression or DNA replication in *T. cruzi* [74]. Hence, many histone acetylation sites have been identified in trypanosomatids; however, their function is, in most cases, elusive. Nevertheless, it is clear that they play a role in transcription, DNA repair and recombination in these organisms.

Acetylation as a regulatory mechanism of RNA-binding proteins

RNA binding proteins (RBPs) are modular regulatory proteins that are characteristically rich in positively charged amino acids. These versatile proteins are

essential components of ribonucleoprotein (RNP) complexes that drive RNA metabolism to control gene expression regulation networks [75, 76]. Conventionally, RNP associations are mediated by RNA binding domains (RBDs) [77, 78]. Of great interest, many RNA-associated proteins lack traditional RBD motifs [79]. In such instances, RNA binding capacity can be accomplished through intrinsically disordered regions, protein-protein interaction interfaces, enzymatic cores, and through as yet undefined molecular affinities [80].

Beyond the regulatory functions of RBPs, these proteins also serve as regulatory targets for multiple enzymatic pathways. PTMs such as phosphorylation, methylation and acetylation can provide a post-transcriptional epigenetic layer of gene expression control [81, 82]. Arginine monomethylation (MMA) impacts both RBP protein stability and RNA binding capacity [81]. Modifying enzymes have a wide-reaching impact upon associated RNP complexes, expanding the 'Regulon" network paradigms. Such modifiers can alter RBP binding affinities to target transcripts in a very tailored manner, enabling cell type-specific selection of distinct RNA pools [79, 81].

Acetylation regulates several steps of post-transcriptional RNA processing, such as pre-mRNA splicing and polyadenylation, and polyadenylated mRNA degradation. Acetylation can modify RBPs and most commonly targets lysine residues of RNA interaction sites and can negatively or positively impact the RNA affinity of acetylated-modified RBPs in a manner similar to MMA [83, 84]. Several RBPs have been identified as acetylated in *T. gondii*, *P. falciparum* and *Trypanosomes* that are predominantly associated with RNA processing, splicing and ribosome biogenesis [14, 15, 17-19, 38]. For example, Pumilio homology domain family member 8 (PUF8) in *T. brucei* (Tb927.3.2470) [14]; RNA binding protein 42 (TcCLB.509167.140) in *T. cruzi* [15]; RNA binding protein (TGME49_105850) in *T. gondii* [16] and RNA binding protein

NOVA1 (Pf7G8_140020700) in *P. falciparum* [18]. Although there are many known acetylations of RBPs in protozoans, the regulatory implications of this modification are still poorly explored.

In summary, the impact of acetylation on the function, stability and binding properties of RNA binding proteins likely represents a global regulatory mechanism in need of further exploration. The trypanosomatids parasites present an excellent eukaryotic system to accomplish such investigation due to the relatively high abundance of RBPs and emphasis upon post-transcriptional gene regulation as mediated by RNP complexes [79, 85-88].

Glycolytic metabolism regulation by differential acetylation

During their life cycle protozoan parasites must adapt their metabolism in response to nutrient sources available in the different hosts [5, 6]. Metabolic enzymes are among the most prevalent acetylated proteins detected in the acetylomes of both prokaryotes and eukaryotes, indicating the critical regulatory function of this modification on specific metabolic pathways [89, 90]. In protozoan parasites, this is most evident in the glycolytic pathway. Most of the glycolytic enzymes were detected acetylated at different lysine sites in all protozoan acetylomes (see **Box 2** for more details), except for glucose phosphate isomerase (PGI), which is highly acetylated in mammals [89]. The number of lysine acetylated sites identified varies between protein homologues in each species [14-19] (see **Box 2**), suggesting that acetylation in glycolytic enzymes might have different purposes and outcomes for each parasite.

Functional studies to investigate the role of acetylation on glycolytic enzymes were recently published in *T. brucei* and *T. gondii* [91; REF]. *T. brucei* **procyclic form** that develops in the insect gut relies on amino acids as the primary carbon source and

obtains adenosine triphosphate (ATP) by **oxidative phosphorylation** in the mitochondrion. In contrast, the **bloodstream form** that replicates in the blood faces high glucose levels and generates ATP mainly by glycolysis in the **glycosomes** [5; REF]. Comparing the acetylation profile of both parasite forms, Moretti et al., found higher levels of acetylation on procyclic glycolytic enzymes compared to bloodstream forms [15] (**Figure 3A**), which suggested that acetylation might act as a regulator for glycolytic activity in *T. brucei*, as observed for aldolase and glycerol-3-phosphate dehydrogenase in mammals or enolase in bacteria [89, 90].

Interestingly, fructose 1,6-biphosphate aldolase, which converts fructose 1,6-biphosphate (F-1,6-P) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA-3-P), acetylation levels are higher in procyclic forms cultivated in the absence of glucose, compared to those cultivated in the presence of glucose. This observation is associated with lower aldolase activity in procyclics grown in the absence of glucose, a phenotype that is reverted after in vitro deacetylation of these enzymes [91] (**Figure 3B**).

Human aldolase is negatively regulated by the acetylation of lysine 147 (K147) present in the catalytic site [89]. We compared *T. brucei* and mammalian aldolase protein structures and observed a high degree of similarity between the structures with the conservation of the residues that compose the catalytic pocket, including the regulatory K147 residue, which corresponds to K157 in *T. brucei* [91] (**Figure 3C**). *T. brucei* aldolase recombinant proteins mimicking an acetylated state of K157 (lysine is replaced by glutamine) abolishes the enzyme activity compared to the native form [91], similarly to mammalian aldolase [89]. Indeed, *in silico* analyses suggested that acetylation of parasite aldolase at K157 affects its electrostatic potential, alters the substrate binding to the catalytic pocket, and reduces the catalytic site volume considerably compared to

native unacetylated protein [91]. These findings identify lysine acetylation as a new regulatory mechanism of *T. brucei* aldolase enzymes and the conservation of key lysine residues for the enzyme activity among other species (**Figure 3C**) might indicates that this regulatory mechanism could be conserved.

Recently, Kloehn et al., demonstrated hypoacetylation of glycolytic enzymes in *T. gondii* mutant parasites lacking cytosolic acetyl-CoA, but no alterations in glycolytic flux was observed by metabolome analyses, except a reduction in the levels of F-1,6P, the substrate of aldolase (32546260). Interesting, K216, the residue corresponding to K157 in *T. brucei* (Figure 3C), was not detected acetylated in *T. gondii* aldolase, which could lead to hyperactivation of the enzyme and explain the lower levels of F-1,6P. Also, K301, present in the catalytic site of *T. gondii* aldolase and conserved among other species (Figure 3C), was detected hyperacetylated, suggesting that acetylation of this residue could activates the enzyme (32546260). On the other hand, the authors also investigated the impact of acetylation in the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK-1) enzyme, but found no clear impact (32546260).

Further experiments are necessary to better understand and validate the regulatory mechanism of protein acetylation in the glucose metabolism of protozoan parasites and to identify the enzymes responsible for regulating glycolytic enzymes' acetylation level. Also, new proteomic analyses comparing parasite stages with different metabolic profiles will be important to increase our understanding about this process..

Exploring protein acetylation regulatory machinery as drug targets in protozoan parasites

Regulators of lysine acetylation have proven to be critical for parasite survival and development, and their potential as therapeutic targets for parasitic diseases has been

realized. The first indication that inhibiting acetylation modifiers could have antiprotozoal activity occurred in 1996 when Darkin-Rattray et al. found that apicidin, a fungal metabolite, was cytotoxic to several protozoan species by disrupting histone acetylation [92]. Since then, inhibitors of KATs, KDACs, and more recently, BDPs have been investigated for their potential as anti-protozoan therapeutic targets [38, 93, 94].

Protozoan KATs, KDACs and BDPs make excellent targets for chemical inhibitors. They are generally divergent from human proteins despite maintaining conserved domain structures responsive to small compound inhibitors. A shining example of these unique characteristics is the KDAC inhibitor FR235222. This compound was first identified as a human KDAC inhibitor, but apicomplexans are more susceptible to the drug due to two divergent amino acids located in the catalytic domain [57].

Several strategies have been employed to identify and develop anti-protozoan drugs that target the parasites' lysine acetylation network. Multiple groups have performed parasite growth assay screens with known synthesized and natural compounds to identify those with cytotoxic effects [95-97]. These have uncovered several promising compounds, and additional studies have used such hits to design derivatives with higher specificity. A recent approach to developing more effective drugs that may also help combat drug resistance is creating hybrid compounds that merge chemical structures of two or more compounds with confirmed antiparasitic properties. This has been used to develop SAHAquines consisting of the standard anti-malarial primaquine and the KDAC inhibitor SAHA [98]. Another successful strategy has been rational drug design, using in silico molecular modelling and docking to identify inhibitors with a high likelihood of binding parasite-specific KAT, KDAC and BDP domains [97, 99, 100]. The difficulty with this approach is the lack of structural data for protozoan proteins.

KDAC inhibitors have been the most studied and found to be the most effective against parasites thus far. This is in part due to a large number of KDAC inhibitors available from human and other model organism drug repositories. A couple of recent comparative studies of multiple epigenetic inhibitors against several stages of *P. falciparum* found that KDAC inhibitors consistently displayed the highest efficacy [95]. In trypanosomatids, such as *T. cruzi*, the inhibitors of parasite sirtuins seem to be the most effective drugs for control of the infection as observed from in vitro and in vivo infection assays with sirtinol, a known SIRT inhibitor [37], and from further screenings of 33 chemically different modulators of human SIRTs [101]. For more information about the potential of KDAC inhibitors against protozoan see [94, 102].

KAT and BDP inhibitors have been studied far less but have also proven to be effective at killing parasites. The natural products curcumin and anacardic acid have potent anti-malarial and anti-trypanosomal activity [103-105]. These compounds, while non-specific, have been identified as binding and inhibiting KATs. Garcinol, a polyisoprenylated benzophenone derivative, is another non-specific KAT inhibitor identified as targeting GCN5 homologues and disrupting parasite growth [63]. With recent studies identifying BDPs as essential to parasites and their amenability to drug design, BDP inhibitors are being investigated for their anti-protozoan activity. Jeffers et al. showed that the human BDP inhibitor I-BET151 is cytotoxic to *Toxoplasma* at concentrations that do not affect host cells [106]. The compound L-Moses has been reported to inhibit the bromodomain of the GCN5 homologues in both *P. falciparum* and *Toxoplasma*, revealing a second potential route for drug inhibition of the critical GCN5 homologues in the apicomplexans [60, 107]. The bromodomain of PfGCN5 was also recently reported to be a target of the bromodomain inhibitor SGC-CBP30, which was identified in a screen of 42

compounds for binding to the recombinant PfGCN5 BRD [97]. Recently, GSK2801 was demonstrated to bind to *T. brucei* TbBDF2 and reduce parasite growth [108].

Tremendous progress has been made in the last two decades at unveiling KATs, KDACs and BDPs as promising therapeutic targets and discovering many compounds that warrant further investigation. The repertoire of drug candidates will continue to expand and improve as a combination of approaches is employed, and the knowledge of these essential factors grows, helping in the development of new treatments for the diseases caused by these parasites.

Concluding remarks

The repertoire of acetylated proteins has increased substantially. It has revealed the diversity of targets for this modification, which has allowed researchers to propose that "acetylation is the phosphorylation rival", a well-known modification implicated in several regulatory pathways [109]. This prediction is proving to be true year by year and is not different regarding protozoan parasites. Still, our understanding of acetylation's real impact on non-histone proteins is only at the beginning. We expect that years ahead will precisely show how different acetylation sites can impact on protein function in these organisms (see Outstanding questions). One opportunity is to use protozoan parasites, early-branching organisms in the eukaryotic evolution, to investigate how acetylation has evolved to regulate specific biological processes.

The demonstration that glycolytic enzymes are directly regulated by acetylation in *T. brucei* opens the opportunity to investigative the role of this modification on other essential processes in protozoan, such as oxidative stress response, protein synthesis/degradation and amino acid metabolism, all processes with several components identified as acetylated in the parasites studied herein. Understanding how acetylation

457 regulatory machinery acts within each specific process will support efforts to explore
458 these enzymes as drug targets. Finally, it will be crucial to uncover the acetylome of other
459 protozoan species, either human parasites or free-living organisms, as they might provide
460 insights into how acetylation impacts parasitism development.
461
462 **Declaration of Interests**
463 The authors declare no competing interests.
464
465 **Acknowledgments**
466 We would like to thanks Jessica Diaz for helping with the initial analyses of regulatory
467 protein acetylation machinery in protozoan parasites. This work was supported by
468 Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant 2018/09948-
469 0 to N.S.M. and 2019/13765-1 to S.R.M]; Conselho Nacional de Desenvolvimento
470 Científico e Tecnológico (CNPq) [grant 424729/2018-0 to N.S.M.]; UNH's Center for
471 Integrated Biomedical and Bioengineering Research (CIBBR) through a grant from
472 NIGMS (NIH P20GM113131 to VJ); Natural Sciences and Engineering Research
473 Council of Canada [grants DGECR-2019-00081 and RGPIN-2019-04658 to IC]; Fonds
474 de Recherche du Québec – Nature et technologies [FRQ-NT NC-288072 to IC]. UK
475 Medical Research Council [GCRF MR/P027989/1 to PW and NMMT] and MRC
476 Newton/FAPESP Partnership [MR/S019472/1 for PW].
477
478 **Resources**
479 [i]PlasmoDB (www.plasmodb.org)
480 [ii]ToxoDB (www.toxodb.org)
481 [iii]TritrypDB (www.tritrypdb.org)
482 [iv]VEuPathDB (www.veupathdb.org)

References

1. Robert-Gangneux, F. and Darde, M.L. (2012) Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 25 (2), 264-96.
2. Buscher, P. et al. (2019) Equine trypanosomosis: enigmas and diagnostic challenges. Parasit Vectors 12 (1), 234.
3. Altamura, F. et al. (2020) The current drug discovery landscape for trypanosomiasis and leishmaniasis: Challenges and strategies to identify drug targets. Drug Dev Res.
4. Phillips, M.A. et al. (2017) Malaria. Nat Rev Dis Primers 3, 17050.
5. Smith, T.K. et al. (2017) Metabolic reprogramming during the Trypanosoma brucei life cycle. F1000Res 6.
6. Srivastava, A. et al. (2016) Stage-Specific Changes in *Plasmodium* Metabolism Required for Differentiation and Adaptation to Different Host and Vector Environments. PLoS Pathog 12 (12), e1006094.
7. Moretti, N.S. and Schenkman, S. (2013) Chromatin modifications in trypanosomes due to stress. Cell Microbiol 15 (5), 709-17.
8. Narita, T. et al. (2019) Functions and mechanisms of non-histone protein acetylation. Nat Rev Mol Cell Biol 20 (3), 156-174.
9. Humphrey, S.J. et al. (2015) Protein Phosphorylation: A Major Switch Mechanism for Metabolic Regulation. Trends Endocrinol Metab 26 (12), 676-687.
10. Wu, Z. et al. (2017) Beyond histones - the expanding roles of protein lysine methylation. FEBS J 284 (17), 2732-2744.
11. Swatek, K.N. and Komander, D. (2016) Ubiquitin modifications. Cell Res 26 (4), 399-422.
12. Choudhary, C. et al. (2014) The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol 15 (8), 536-50.
13. Phillips, D.M. (1963) The presence of acetyl groups of histones. Biochem J 87, 258-63.
14. Zhang, N. et al. (2020) Landscapes of Protein Posttranslational Modifications of African Trypanosoma Parasites. iScience 23 (5), 101074.
15. Moretti, N.S. et al. (2018) Comparative Proteomic Analysis of Lysine Acetylation in Trypanosomes. J Proteome Res 17 (1), 374-385.
16. Jeffers, V. and Sullivan, W.J., Jr. (2012) Lysine acetylation is widespread on proteins of diverse function and localization in the protozoan parasite *Toxoplasma gondii*. Eukaryot Cell 11 (6), 735-42.
17. Xue, B. et al. (2013) Protein intrinsic disorder in the acetylome of intracellular and extracellular *Toxoplasma gondii*. Mol Biosyst 9 (4), 645-57.
18. Miao, J. et al. (2013) Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during *Plasmodium falciparum* intraerythrocytic development. Mol Microbiol 89 (4), 660-75.
19. Wang, J. et al. (2020) Protein modification characteristics of the malaria parasite *Plasmodium falciparum* and the infected erythrocytes. Mol Cell Proteomics.
20. Marmorstein, R. and Zhou, M.M. (2014) Writers and readers of histone acetylation: structure, mechanism, and inhibition. Cold Spring Harb Perspect Biol 6 (7), a018762.
21. Seto, E. and Yoshida, M. (2014) Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol 6 (4), a018713.

22. Fujisawa, T. and Filippakopoulos, P. (2017) Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. Nat Rev Mol Cell Biol 18 (4), 246-262.
23. Alonso, V.L. and Serra, E.C. (2012) Lysine acetylation: elucidating the components of an emerging global signaling pathway in trypanosomes. J Biomed Biotechnol 2012, 452934.
24. Saha, S. (2020) Histone Modifications and Other Facets of Epigenetic Regulation in Trypanosomatids: Leaving Their Mark. mBio 11 (5).
25. Parthun, M.R. (2007) Hat1: the emerging cellular roles of a type B histone acetyltransferase. Oncogene 26 (37), 5319-28.
26. Parthun, M.R. (2013) Histone acetyltransferase 1: more than just an enzyme? Biochim Biophys Acta 1819 (3-4), 256-63.
27. Saksouk, N. et al. (2005) Histone-modifying complexes regulate gene expression pertinent to the differentiation of the protozoan parasite *Toxoplasma gondii*. Mol Cell Biol 25 (23), 10301-14.
28. Ingram, A.K. and Horn, D. (2002) Histone deacetylases in *Trypanosoma brucei*: two are essential and another is required for normal cell cycle progression. Mol Microbiol 45 (1), 89-97.
29. Tonkin, C.J. et al. (2009) Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in *Plasmodium falciparum*. PLoS Biol 7 (4), e84.
30. Chakrabarty, S.P. et al. (2008) Biochemical characterization of *Plasmodium falciparum* Sir2, a NAD+-dependent deacetylase. Mol Biochem Parasitol 158 (2), 139-51.
31. Mancio-Silva, L. et al. (2013) Sir2a regulates rDNA transcription and multiplication rate in the human malaria parasite *Plasmodium falciparum*. Nat Commun 4, 1530.
32. Merrick, C.J. et al. (2015) Functional analysis of sirtuin genes in multiple *Plasmodium falciparum* strains. PLoS One 10 (3), e0118865.
33. Merrick, C.J. and Duraisingh, M.T. (2007) *Plasmodium falciparum* Sir2: an unusual sirtuin with dual histone deacetylase and ADP-ribosyltransferase activity. Eukaryot Cell 6 (11), 2081-91.
34. Garcia-Salcedo, J.A. et al. (2003) A chromosomal SIR2 homologue with both histone NAD-dependent ADP-ribosyltransferase and deacetylase activities is involved in DNA repair in *Trypanosoma brucei.* EMBO J 22 (21), 5851-62.
35. Alsford, S. et al. (2007) A sirtuin in the African trypanosome is involved in both DNA repair and telomeric gene silencing but is not required for antigenic variation. Mol Microbiol 63 (3), 724-36.
36. Ritagliati, C. et al. (2015) Overexpression of cytoplasmic TcSIR2RP1 and mitochondrial TcSIR2RP3 impacts on *Trypanosoma cruzi* growth and cell invasion. PLoS Negl Trop Dis 9 (4), e0003725.
37. Moretti, N.S. et al. (2015) Characterization of *Trypanosoma cruzi* Sirtuins as Possible Drug Targets for Chagas Disease. Antimicrob Agents Chemother 59 (8), 4669-79.
38. Jeffers, V. et al. (2017) Bromodomains in Protozoan Parasites: Evolution, Function, and Opportunities for Drug Development. Microbiol Mol Biol Rev 81 (1).
39. Cobbold, S.A. et al. (2016) Proteome-wide analysis reveals widespread lysine acetylation of major protein complexes in the malaria parasite. Sci Rep 6, 19722.
40. Warrenfeltz, S. et al. (2018) EuPathDB: The Eukaryotic Pathogen Genomics Database Resource. Methods Mol Biol 1757, 69-113.
41. Enright, A.J. et al. (2002) An efficient algorithm for large-scale detection of protein families. Nucleic Acids Res 30 (7), 1575-84.

42. Painter, H.J. et al. (2011) The Apicomplexan AP2 family: integral factors regulating *Plasmodium* development. Mol Biochem Parasitol 176 (1), 1-7.
43. Gissot, M. et al. (2007) Epigenomic modifications predict active promoters and gene structure in *Toxoplasma gondii*. PLoS Pathog 3 (6), e77.
44. Nardelli, S.C. et al. (2013) The histone code of *Toxoplasma gondii* comprises conserved and unique post-translational modifications. mBio 4 (6), e00922-13.
45. Trelle, M.B. et al. (2009) Global histone analysis by mass spectrometry reveals a high content of acetylated lysine residues in the malaria parasite *Plasmodium falciparum*. J Proteome Res 8 (7), 3439-50.
46. Ay, F. et al. (2015) Multiple dimensions of epigenetic gene regulation in the malaria parasite Plasmodium falciparum: gene regulation via histone modifications, nucleosome positioning and nuclear architecture in *P. falciparum*. Bioessays 37 (2), 182-94.
47. Salcedo-Amaya, A.M. et al. (2009) Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of *Plasmodium falciparum*. Proc Natl Acad Sci U S A 106 (24), 9655-60.
48. Batugedara, G. et al. (2017) The Role of Chromatin Structure in Gene Regulation of the Human Malaria Parasite. Trends Parasitol 33 (5), 364-377.
49. Bougdour, A. et al. (2010) Chromatin modifications: implications in the regulation of gene expression in *Toxoplasma gondii*. Cell Microbiol 12 (4), 413-23.
50. Witmer, K. et al. (2020) An epigenetic map of malaria parasite development from host to vector. Sci Rep 10 (1), 6354.
51. Gomez-Diaz, E. et al. (2017) Epigenetic regulation of *Plasmodium falciparum* clonally variant gene expression during development in Anopheles gambiae. Sci Rep 7, 40655.
52. Behnke, M.S. et al. (2008) The transcription of bradyzoite genes in *Toxoplasma gondii* is controlled by autonomous promoter elements. Mol Microbiol 68 (6), 1502-18.
53. Wang, J. et al. (2014) Lysine acetyltransferase GCN5b interacts with AP2 factors and is required for *Toxoplasma gondii* proliferation. PLoS Pathog 10 (1), e1003830.
54. Xiao, B. et al. (2019) Epigenetic editing by CRISPR/dCas9 in *Plasmodium falciparum*. Proc Natl Acad Sci U S A 116 (1), 255-260.
55. Coleman, B.I. et al. (2014) A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. Cell Host Microbe 16 (2), 177-186.
56. Duraisingh, M.T. et al. (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in *Plasmodium falciparum*. Cell 121 (1), 13-24.
57. Bougdour, A. et al. (2009) Drug inhibition of HDAC3 and epigenetic control of differentiation in Apicomplexa parasites. J Exp Med 206 (4), 953-66.
58. Sidik, S.M. et al. (2016) A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential Apicomplexan Genes. Cell 166 (6), 1423-1435 e12.
59. Josling, G.A. et al. (2015) A *Plasmodium Falciparum* Bromodomain Protein Regulates Invasion Gene Expression. Cell Host Microbe 17 (6), 741-51.
60. Hanquier, J. et al. (2020) Evaluating the GCN5b bromodomain as a novel therapeutic target against the parasite *Toxoplasma gondii*. Exp Parasitol 211, 107868.
61. Santos, J.M. et al. (2017) Red Blood Cell Invasion by the Malaria Parasite Is Coordinated by the PfAP2-I Transcription Factor. Cell Host Microbe 21 (6), 731-741 e10.
62. Tang, J. et al. (2020) Histone modifications associated with gene expression and genome accessibility are dynamically enriched at *Plasmodium falciparum* regulatory sequences. Epigenetics Chromatin 13 (1), 50.
63. Jeffers, V. et al. (2016) Garcinol Inhibits GCN5-Mediated Lysine Acetyltransferase Activity and Prevents Replication of the Parasite *Toxoplasma gondii*. Antimicrob Agents Chemother 60 (4), 2164-70.

64. Toro, G.C. and Galanti, N.J.E.c.r. (1988) H 1 histone and histone variants in *Trypanosoma cruzi*. 174 (1), 16-24.
65. Hecker, H. et al. (1994) The chromatin of trypanosomes. 24 (6), 809-819.
66. de Lima, L.P. et al. (2020) Improvements on the quantitative analysis of *Trypanosoma cruzi* histone post translational modifications: Study of changes in epigenetic marks through the parasite's metacyclogenesis and life cycle. J Proteomics 225, 103847.
67. de Jesus, T.C. et al. (2016) Chromatin Proteomics Reveals Variable Histone Modifications during the Life Cycle of *Trypanosoma cruzi*. J Proteome Res 15 (6), 2039-51.
68. Picchi, G.F. et al. (2017) Post-translational Modifications of *Trypanosoma cruzi* Canonical and Variant Histones. J Proteome Res 16 (3), 1167-1179.
69. Chandra, U. et al. (2017) Cell cycle stage-specific transcriptional activation of cyclins mediated by HAT2-dependent H4K10 acetylation of promoters in *Leishmania donovani*. PLoS Pathog 13 (9), e1006615.
70. Kraus, A.J. et al. (2020) Distinct roles for H4 and H2A.Z acetylation in RNA transcription in African trypanosomes. Nat Commun 11 (1), 1498.
71. Kawahara, T. et al. (2008) Two essential MYST-family proteins display distinct roles in histone H4K10 acetylation and telomeric silencing in trypanosomes. Mol Microbiol 69 (4), 1054-68.
72. Respuela, P. et al. (2008) Histone acetylation and methylation at sites initiating divergent polycistronic transcription in *Trypanosoma cruzi*. J Biol Chem 283 (23), 15884-92.
73. Kumar, D. et al. (2012) Histone H4 lysine 14 acetylation in *Leishmania donovani* is mediated by the MYST-family protein HAT4. Microbiology (Reading) 158 (Pt 2), 328-337.
74. Ramos, T.C. et al. (2015) Expression of non-acetylatable lysines 10 and 14 of histone H4 impairs transcription and replication in *Trypanosoma cruzi*. Mol Biochem Parasitol 204 (1), 1-10.
75. Ottoz, D.S.M. and Berchowitz, L.E. (2020) The role of disorder in RNA binding affinity and specificity. Open Biol 10 (12), 200328.
76. Kumar, S. and Maiti, S. (2013) The effect of N-acetylation and N-methylation of lysine residue of Tat peptide on its interaction with HIV-1 TAR RNA. PLoS One 8 (10), e77595.
77. Van Nostrand, E.L. et al. (2020) A large-scale binding and functional map of human RNA-binding proteins. Nature 583 (7818), 711-719.
78. Karamysheva, Z.N. et al. (2020) Regulation of Translation in the Protozoan Parasite *Leishmania*. Int J Mol Sci 21 (8).
79. de Pablos, L.M. et al. (2019) The mRNA-bound Proteome of *Leishmania mexicana*: Novel Genetic Insight into an Ancient Parasite. Mol Cell Proteomics 18 (7), 1271-1284.
80. Hentze, M.W. et al. (2018) A brave new world of RNA-binding proteins. Nat Rev Mol Cell Biol 19 (5), 327-341.
81. Ferreira, T.R. et al. (2020) PRMT7 regulates RNA-binding capacity and protein stability in *Leishmania* parasites. Nucleic Acids Res 48 (10), 5511-5526.
82. Thangima Zannat, M. et al. (2011) In the absence of cellular poly (A) binding protein, the glycolytic enzyme GAPDH translocated to the cell nucleus and activated the GAPDH mediated apoptotic pathway by enhancing acetylation and serine 46 phosphorylation of p53. Biochem Biophys Res Commun 409 (2), 171-6.
83. Gal, J. et al. (2019) The Acetylation of Lysine-376 of G3BP1 Regulates RNA Binding and Stress Granule Dynamics. Mol Cell Biol 39 (22).

84. Babic, I. et al. (2004) The RNA binding protein Sam68 is acetylated in tumor cell lines, and its acetylation correlates with enhanced RNA binding activity. Oncogene 23 (21), 3781-9.
85. Dupe, A. et al. (2015) Differential Subcellular Localization of *Leishmania* Alba-Domain Proteins throughout the Parasite Development. PLoS One 10 (9), e0137243.
86. Rochette, A. et al. (2008) Genome-wide gene expression profiling analysis of *Leishmania major* and *Leishmania infantum* developmental stages reveals substantial differences between the two species. BMC Genomics 9, 255.
87. Nandan, D. et al. (2017) Comprehensive Identification of mRNA-Binding Proteins of *Leishmania donovani* by Interactome Capture. PLoS One 12 (1), e0170068.
88. Terrao, M.C. et al. (2017) Disclosing 3' UTR cis-elements and putative partners involved in gene expression regulation in *Leishmania* spp. PLoS One 12 (8), e0183401.
89. Lundby, A. et al. (2012) Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. Cell Rep 2 (2), 419-31.
90. Nakayasu, E.S. et al. (2017) Ancient Regulatory Role of Lysine Acetylation in Central Metabolism. mBio 8 (6).
91. Barbosa Leite, A. et al. (2020) Effect of lysine acetylation on the regulation of *Trypanosoma brucei* glycosomal aldolase activity. Biochem J 477 (9), 1733-1744.
92. Darkin-Rattray, S.J. et al. (1996) Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. Proc Natl Acad Sci U S A 93 (23), 13143-7.
93. Alonso, V.L. et al. (2019) Aim for the Readers! Bromodomains As New Targets Against Chagas' Disease. Curr Med Chem 26 (36), 6544-6563.
94. Hailu, G.S. et al. (2017) Lysine Deacetylase Inhibitors in Parasites: Past, Present, and Future Perspectives. J Med Chem 60 (12), 4780-4804.
95. Coetzee, N. et al. (2020) Epigenetic inhibitors target multiple stages of Plasmodium falciparum parasites. Sci Rep 10 (1), 2355.
96. Wang, Q. et al. (2015) Targeting Lysine Deacetylases (KDACs) in Parasites. PLoS Negl Trop Dis 9 (9), e0004026.
97. Chua, M.J. et al. (2018) Activity of bromodomain protein inhibitors/binders against asexual-stage *Plasmodium falciparum* parasites. Int J Parasitol Drugs Drug Resist 8 (2), 189-193.
98. Beus, M. et al. (2018) SAHAquines, Novel Hybrids Based on SAHA and Primaquine Motifs, as Potential Cytostatic and Antiplasmodial Agents. ChemistryOpen 7 (8), 624-638.
99. Kumar, A. et al. (2018) In silico identification of inhibitors against *Plasmodium falciparum* histone deacetylase 1 (PfHDAC-1). J Mol Model 24 (9), 232.
100. Elbadawi, M.A. et al. (2015) Valproic acid as a potential inhibitor of *Plasmodium falciparum* histone deacetylase 1 (PfHDAC1): an in silico approach. Int J Mol Sci 16 (2), 3915-31.
101. Matutino Bastos, T. et al. (2020) Identification of Inhibitors to *Trypanosoma cruzi* Sirtuins Based on Compounds Developed to Human Enzymes. Int J Mol Sci 21 (10).
102. Fioravanti, R. et al. (2020) Targeting histone acetylation/deacetylation in parasites: an update (2017-2020). Curr Opin Chem Biol 57, 65-74.
103. Cui, L. et al. (2007) Cytotoxic effect of curcumin on malaria parasite *Plasmodium falciparum:* inhibition of histone acetylation and generation of reactive oxygen species. Antimicrob Agents Chemother 51 (2), 488-94.
104. Cui, L. et al. (2008) Histone acetyltransferase inhibitor anacardic acid causes changes in global gene expression during in vitro *Plasmodium falciparum* development. Eukaryot Cell 7 (7), 1200-10.

105. Matutino Bastos, T. et al. (2019) Chemical Constituents of Anacardium occidentale as Inhibitors of *Trypanosoma cruzi* Sirtuins. Molecules 24 (7).
106. Jeffers, V. et al. (2017) TgPRELID, a Mitochondrial Protein Linked to Multidrug Resistance in the Parasite *Toxoplasma gondii*. mSphere 2 (1).
107. Moustakim, M. et al. (2017) Discovery of a PCAF Bromodomain Chemical Probe. Angew Chem Int Ed Engl 56 (3), 827-831.
108. Yang, X. et al. (2017) Recognition of hyperacetylated N-terminus of H2AZ by TbBDF2 from *Trypanosoma brucei*. Biochem J 474 (22), 3817-3830.
109. Kouzarides, T. (2000) Acetylation: a regulatory modification to rival phosphorylation? EMBO J 19 (6), 1176-9.

Glossary

Acetylomes: set of lysine acetylated proteins of a specific organism; the number of acetylated proteins can vary consistently depending on the organism.

Bloodstream form: *T. brucei* parasite stage inhabiting the vertebrate host; relies on glycolysis for energy production and has a degenerated mitochondrion.

Bradyzoite: The dormant stage of *Toxoplasma* in the intermediate host, responsible for chronic disease in humans.

Bromodomain containing protein (BDP): proteins bearing BDP domains that bind to acetylated lysine; usually are within protein complexes involved in downstream functions of acetylation.

Euchromatin: lightly packed chromatin, usually containing actively expressed genes

Gametocyte: *Plasmodium* sexual precursor cell transmitted from human to mosquito

Glycosomes: specialized enclosed-membrane organelles that contain glycolytic enzymes found in Trypanosomatids.

Heterochromatin: densely packed chromatin, usually containing repressed genes

Lysine acetyltransferase (KAT): family of enzymes responsible for the addition of acetyl groups on lysine residues; the number of members of this family varies depending on the organism.

Lysine deacetylase (KDAC): group of enzymes involved in the removal of acetyl groups of lysine residues; divided mainly in two families: zinc and NAD^+-dependent.

Ookinete: the motile form of *Plasmodium* in the mosquito that forms oocysts.

Oocyst: the form of *Plasmodium* in the mosquito that releases sporozoites

Oxidative phosphorylation: process in which ATP is formed as a result of the transfer of electrons from NADH or $FADH_2$ to O_2 by a series of electron carriers; this process occurs in the mitochondria.

Post-translational modifications: covalent modifications of proteins following protein synthesis; frequently performed by enzymes.

Procyclic form: *T. brucei* parasite stage present in the tsetse invertebrate host; this stage has an elaborated mitochondrion and ATP production relies mainly on oxidative phosphorylation.

RNA binding proteins (RBPs): enzymes that bind to single or double strand RNAs in cells and are important for gene expression regulation.

Sporozoite: the form of *Plasmodium* that is transmitted from mosquitoes to a new host during a blood meal.

Tachyzoite: The proliferative stage of *Toxoplasma*, found in intermediate hosts. Responsible for acute disease in humans.

BOX1. Repertoire of proteins involved in lysine acetylation from protozoan parasites

Proteins involved in the regulation of lysine acetylation levels are present from bacteria to human and the set of these proteins can varies depending of the complexity of each organism. For protozoan parasites the number of genes coding for lysine deacetylases (KDACs), lysine acetyltransferases (KATs) and bromodomain proteins

780 (BDPs) are similar, especially within species from the same group. However, most of
781 these proteins still need to be characterized.

782 In general, the number of lysine deacetylases (Zn-dependent and sirtuins) varies
783 from 3 to 20 among the species analyzed, with *Eimeria brunetti* with the smallest and
784 *Trichomonas vaginalis* with the biggest set. The sirtuins are present in similar numbers
785 amid the species. For example, most of the trypanosomatids (blue circles), *T. brucei*, *T.*
786 *evansi*, *Leishmania* spp., *Crithidia fasciculata* and *Leptomonas seymouri*, have three
787 genes for sirtuins, while *T. cruzi* and *T. rangeli* have two and *Leptomonas pyrrhocoris*
788 four. Differences are also observed for the apicomplexan species, where *P. falciparum*
789 and *Neospora caninum* has two sirtuins, while *Cryptosporidium parvum* and *E. brunetti*
790 only one. The intestinal parasites, amoebas and *Giardia lamblia*, have four sirtuins. In
791 contrast, the set of Zn-dependent enzymes detected among the species is more similar,
792 except for amoebas and *E. brunetti* that have only two genes and *T. vaginalis* that has
793 nine, compared to four genes found in the other species.

794 Regarding the KATs, the smallest repertoire is found in the amoeba species (2
795 genes), and the biggest in *T. vaginalis* (13 genes). Apicomplexan species have genes
796 coding for tree family of KATs, GNATs, MYST and non-canonical (HAT1), while the
797 last group is not present in the other species analyzed. Trypanosomatids have two and
798 four genes coding for GNATs and MYST, respectively; except *T. brucei* and *T. evansi*
799 that have only tree MYST proteins.

800 The number of BDPs found in the groups of protozoan parasites varies from five
801 in some trypanosomatids (*T. cruzi*, *Leishmania* spp. and *C. fasciculata*) to more than
802 hundred in *T. vaginalis*. Amongst apicomplexans, the number of BDPs varies from seven
803 in *P. falciparum* to twelve in *T. gondii* and *C. parvum*. It is important to mention that the
804 bigger number of genes found in *T. vaginalis* for all group of proteins can be explained

by the fact that this parasite has a huge genome with more than 60,000 genes and further analyses are necessary to understand the functional importance of this for the parasite.

Figure I (in Box 1). Comparative analyses of the regulatory protein acetylation repertoire of several protozoan parasites' species.

Box 2. Proteome-wide analysis used for protozoan parasites acetylomes description.

Although there are differences in the approaches used to describe protozoan parasites' acetylomes, it generally follows similar methodologies applied to others prokaryote and eukaryote acetylomes. The methods are laborious, containing several steps schematically represented in **Figure I**, and are described in more detail here. 1) Sample preparation: protein extracts are obtained from the specific parasite stages (described in the figure) with lysis buffer and digested into peptides using proteases, usually trypsin. Whole-cell protein extracts were obtained for all parasites, except for the *T. cruzi* and *T. brucei* procyclic acetylomes, in which organelle fractionation was performed before lysate preparation. 2) Acetylated peptide enrichment: trypsin digestion of total protein extracts generates several peptides, but only a minor proportion is acetylated (indicated by a yellow circle). To decrease sample complexity and increase the detection capacity, acetylated peptides are enriched by immunoaffinity purification using pan-acetyl-lysine antibodies that bind to acetylated peptides. This step was not applied to *T. cruzi* acetylome, and total trypsin-digested peptides were used directly in mass spectrophotometry analysis. 3) Peptide fractionation: sample complexity can be further decreased using peptide fractionation steps and the methods vary. This step was employed on *Plasmodium* (new version) [42], *T. brucei* bloodstream stage and *T. evansi* acetylome descriptions. Strong cation exchange was the method used. 4) LC-MS/MS and

computational analysis: peptide samples are submitted to high resolution nano-UPLC-MS and MS/MS. MS/MS spectra are then computationally processed to define the peptide sequences and the presence/position of acetylated sites. The more recent versions of *T. brucei* and *Plasmodium* acetylomes considerably improved the number of acetylated sites identified, helping to increase our knowledge about the function of acetylation in these parasites (**Figure 2A and B**).

Figure I (in Box 1). Methodological steps used to describe protozoan parasites acetylomes.

Box 3. Acetylation of glycolytic enzymes from protozoan parasites

Glycolysis is an ancient and regulatory mechanism used by most organisms to breakdown glucose and generate energy. The pathway is a sequence of ten enzyme-catalyzed reactions that converts glucose into pyruvate and has a net of two molecules of ATP (**Figure I**). In most organisms, glycolysis takes place in the cytosol, but in trypanosomes, the first five or six steps of the pathway (depending on parasite stage) happens in specialized organelles, called glycosomes.

The first reaction of the pathway is catalyzed by hexokinase (HK) that phosphorylates glucose, producing glucose 6-phosphate; one molecule of ATP is consumed. Glucose 6-phosphate is then isomerized into fructose 6-phosphate by phosphoglucose isomerase (PGI), which is next phosphorylated by phosphofructokinase (PFK) to generate fructose 1,6-biphosphate. Fructose 1,6-biphosphate is split on dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate by fructose 1,6-biphosphate aldolase (ALD). Next, triose phosphate isomerase (TIM) converts DHAP into glyceraldehyde 3-phosphate, which is first dehydrogenated by glyceraldehyde 3-

855 phosphate dehydrogenase (GAPDH), releasing $NADH^{+}$, and then adds a phosphate to
856 generate 1,3-biphosphoglycerate. Phosphoglycerate kinase (PGK) transfers a phosphate
857 from 1,3-biphosphoglycerate to ADP forming ATP and two molecules of 3-
858 phosphoglycerate. These two molecules of 3-phosphoglycerate are converted to 2-
859 phosphoglycerate by phosphoglycerate mutase (PGM), which has a water molecule
860 removed by enolase (ENO) to obtain phosphoenolpyruvate. Finally, pyruvate kinase
861 transfers a phosphate group from phosphoenolpyruvate to ADP generating ATP and
862 pyruvate.

863 The description of protozoan parasite acetylomes identified most of the glycolytic
864 enzymes acetylated (left panel, each colored circle represents the presence of acetylation).
865 The only exception was PGI, in which acetylation was not detected in any species.
866 Moreover, HK, PFK, PGM, PGK, ENO and PK were not identified acetylated in *T. cruzi*,
867 while TIM was not identified as acetylated in *Toxoplasma*. One explanation for the low
868 number of acetylated enzymes in *T. cruzi* could be the low coverage of the acetylome.
869 Interestingly, the number of lysine acetylated sites for each enzyme varies depending on
870 the protozoan specie (right panel) and could reflect the distinct regulatory function of
871 acetylation or the parasite stage's metabolic state to perform the acetylome analysis.

872

873 **Figure I (in Box 3). Acetylation profile of glycolytic enzymes from protozoan**
874 **parasites.**

875

Figure 1. Regulatory lysine acetylation machinery of protozoan parasites. A. Overview of "writers", “erasers” and “readers” from protozoan parasites with described acetylomes compared to human. **B.** Diversity of lysine acetylation machinery components of protozoan parasites. Although the repertoire of protozoan machinery is smaller, the diversity of components is comparable to human, as observed by the families of enzymes from each species.

Figure 2. Protein lysine acetylation repertoire of protozoan parasites. **A.** Number of acetylated proteins detected from each species. **B.** Percentage of acetylated proteomes over their respective total predicted proteomes. **C.** Number of acetylated sites identified for each species. **D.** Cellular component (CC) and biological process (BP) distribution of acetylated proteins in each species. The three most fold enrichment-based prevalent CC and BP for each species are listed (adj. p-value < 0.01). Data from species with more than one available acetylome were combined to obtain the whole set of acetylated lysine sites and proteins. *Plasmodium falciparum* (Pf); *Toxoplasma gondii* (Tg); *Trypanosoma brucei* (Tb); *Trypanosoma cruzi* (Tc); *Trypanosoma evansi* (Tev). **E.** Putative clusters of orthologous groups (COGs) comprising acetylated proteins from all five species. An all-versus-all (acetylomes) BlastP alignment file (e-value < 0.001, >35% identity and >25% query coverage) was used as input for the Markov Clustering algorithm (MCL) with a 2.0 inflation value. Some COGs’ functions corroborated GO-based enrichment analysis results.

Figure 3. Regulatory function of acetylation on glycolytic enzymes from protozoan parasites. A. Changes in metabolism during the life cycle of *T. brucei*. The bloodstream of a mammalian host is a very rich environment, containing high levels of glucose, while

the nutrient sources found in the tsetse fly (insect vector) is glucose-poor but amino acid-rich. Thus, the ATP generation of *T. brucei* bloodstream (BSF) and procyclic (PCF) stages relies mainly on glycolysis and oxidative phosphorylation (OXPHOS), respectively. Comparative analysis demonstrates that PCF glycolytic enzymes have higher acetylation levels compared to BSF enzymes, suggesting a negative regulatory mechanism of this modification in *T. brucei*. **B.** *T. brucei* aldolase activity is regulated by acetylation. Fructose 1,6-biphosphate aldolase (aldolase) splits fructose 1,6-biphosphate (F-1,6-P) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA-3-P). PCF parasites cultivated in the presence of glucose have lower aldolase acetylation and higher enzyme activity compared to PCF cultivated in the absence of glucose, which have higher aldolase acetylation and lower enzymatic activity. **C.** Regulatory aldolase lysine acetylation site conservation within protozoan parasites. The K157 or K147 residue that negatively regulates *T. brucei* and human aldolase activity when acetylated, respectively, is conserved in *Toxoplasma*, *Plasmodium* and other Trypanosomes, suggesting a conserved regulatory mechanism. Other lysine residues important for aldolase activity, K52, K117 and K240 in *T. brucei* (red), are also conserved and acetylated in some of these parasites.