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Review

Protein acetylation in the critical biological processes in protozoan parasites

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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 nonhistone 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.

Highlights

Protein acetylation has emerged as a new regulatory post-translational modification in protozoan parasites.

Chromatin structure and gene expression are regulated by acetylation directly, impacting how protozoan parasites adapt during their life cycle.

Acetylation is present on hundreds of nonhistone proteins from different cellular compartments, which are involved in several biological processes in protozoan parasites.

Lysine acetylation is a new player in the regulation of glycolytic enzymes in *Trypanosoma brucei*.

The regulatory machinery of protein acetylation is present in protozoan parasites and can be explored for drug development.

Protein acetylation in protozoan parasites

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, requiring 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, and acetylation, are found on several proteins in the cell [8–10]. Acetylation is one of the most common PTMs and is characterized by the addition of an acetyl group to the ε-amino group of lysine residues [8,11]. 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 [8,11]. 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 [12]. This review focuses on the roles of lysine ε-amino acetylation.

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 the regulation of gene expression. 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 nonhistone 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 [8,11].

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Acetylomes of many organisms, including apicomplexans and trypanosomatids, have been described [14–20]. The widespread presence of K-ac in these organisms indicates that its regulatory functions are diverse. Thus, in this review, we consider the advances in the study of protein acetylation in protozoan parasites of medical and veterinary importance that had their acetylomes described so far. 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 [21], while KDACs remove the acetyl groups [22]. Bromodomains bind acetylated lysines, and link acetylation marks with the proteins that perform downstream regulatory functions [23]. The acetylation regulatory machinery of apicomplexans and trypanosomatids has been shown to be similar (Box 1) but compared to higher eukaryotes, such as humans, it is reduced and contains both highly conserved and parasite-specific acetylation factors (Figure 1). In the next sections we give more detail about the acetylation machinery of protozoans that have their acetylome available.

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 [21]. 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 have only two (Figure 1 and see Table S1 in the supplemental information

Box 1. Repertoire of proteins involved in lysine acetylation from protozoan parasites

Proteins involved in the regulation of lysine acetylation levels are present in organisms ranging from bacteria to humans, and the set of these proteins can vary depending on the complexity of each organism. For protozoan parasites, the number of genes coding for KDACs, KATs, and BDPs are similar, especially within species from the same group (Figure I). However, most of these proteins still need to be characterized.

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

Regarding the KATs, the smallest repertoire is found in the amoeba species (2 genes), and the biggest in *T. vaginalis* (13 genes). Apicomplexan species have genes coding for tree family of KATs [GNATs, MYST, and noncanonical (HAT1)], while the last group is not present in the other species analyzed. Trypanosomatids have two and four genes coding for GNATs and MYST, respectively; except *T. brucei* and *T. evansi* which have only tree MYST proteins.

The number of BDPs found in the groups of protozoan parasites varies from five in some trypanosomatids (*T. cruzi*, *Leishmania* spp., and *C. fasciculata*) to more than 100 in *T. vaginalis*. Amongst apicomplexans, the number of BDPs varies from seven in *P. falciparum* to 12 in *T. gondii* and *C. parvum*. It is important to mention that the bigger number of genes found in *T. vaginalis* for all groups 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 this parasite.

Glossary

Acetylome: set of lysine-acetylated proteins of a specific organism; the number of acetylated proteins can vary consistently depending on the organism.
Bloodstream form: a *T. brucei* parasite stage inhabiting the vertebrate host; it relies on glycolysis for energy production and has a degenerated mitochondrion.
Bradyzoite: the dormant stage of *Toxoplasma* in the intermediate host; it is responsible for chronic disease in humans.
Bromodomain-containing proteins (BDPs): proteins bearing BDP domains that bind to acetylated lysine; usually they are within protein complexes involved in downstream functions of acetylation.
Euchromatin: lightly packed chromatin, usually containing actively expressed genes.
Gametocyte: a *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): a family of enzymes responsible for the addition of acetyl groups to lysine residues; the number of members of this family varies depending on the organism.
Lysine deacetylase (KDAC): a group of enzymes involved in the removal of acetyl groups from lysine residues; they are divided mainly in two families: zinc- and NAD^+-dependent.
Oocyst: the form of *Plasmodium* in the mosquito that releases sporozoites.
Ookinete: the motile form of *Plasmodium* in the mosquito that forms oocysts.
Oxidative phosphorylation: the 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 (PTMs): covalent modifications of proteins following protein synthesis; they are frequently performed by enzymes.
Procyclic form: a *T. brucei* parasite stage present in the tsetse invertebrate host; this stage has an elaborated

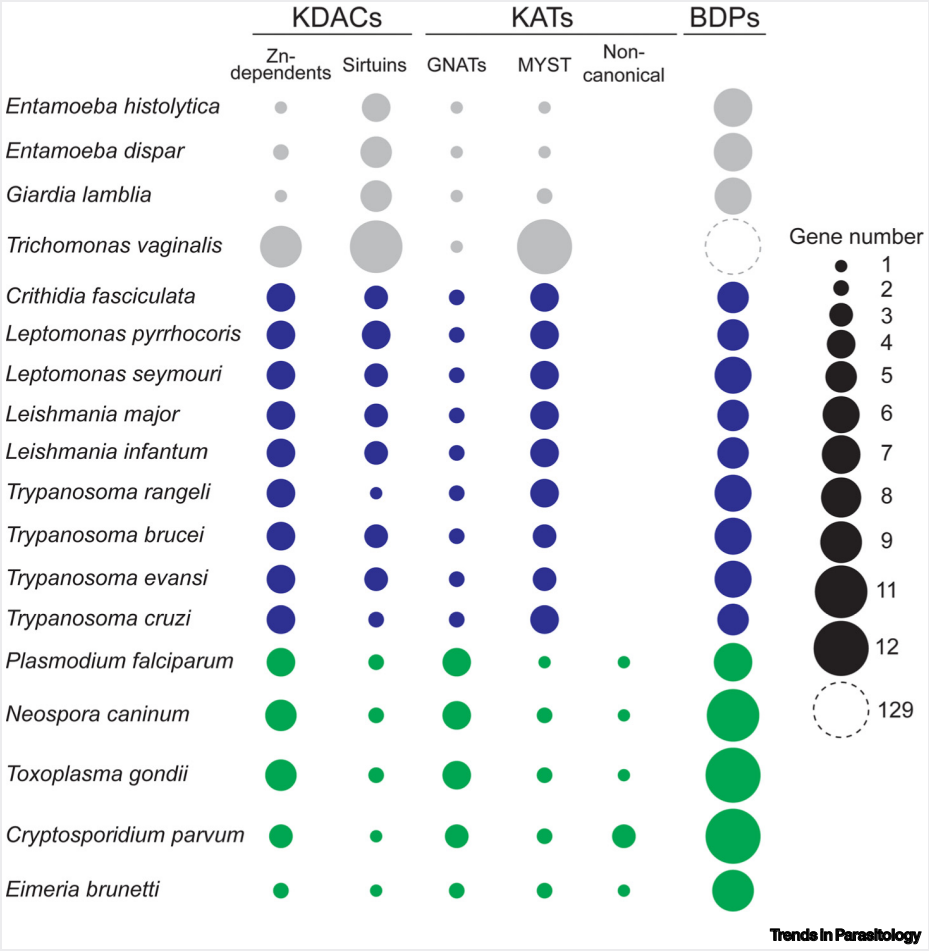


Figure I. Comparative analyses of the regulatory protein acetylation repertoire of several protozoan species.
Abbreviations: BDP, bromodomain-containing protein; KAT, lysine acetyltransferase; KDAC, lysine deacetylase.

mitochondrion, and ATP production relies mainly on oxidative phosphorylation.
RNA-binding proteins (RBPs): enzymes that bind to single- or double-stranded RNAs in cells and are important for regulating gene expression.
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. It is responsible for acute disease in humans.

online). *T. gondii* is unique in possessing two GCN5 homologs, 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 [24] (Figure 1 and Table S1). The third group of KATs is similar to Hat1, the first identified histone acetyltransferase in yeast [25]. The genomes of *T. gondii* and *P. falciparum* each encode a single Hat1 homolog, but neither has been investigated, and no homologs have been identified in trypanosomes (Figure 1 and Table S1). The fourth family of eukaryotic KATs, p300/CBP, have no known homologs in apicomplexans or trypanosomes.

KDACs

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.

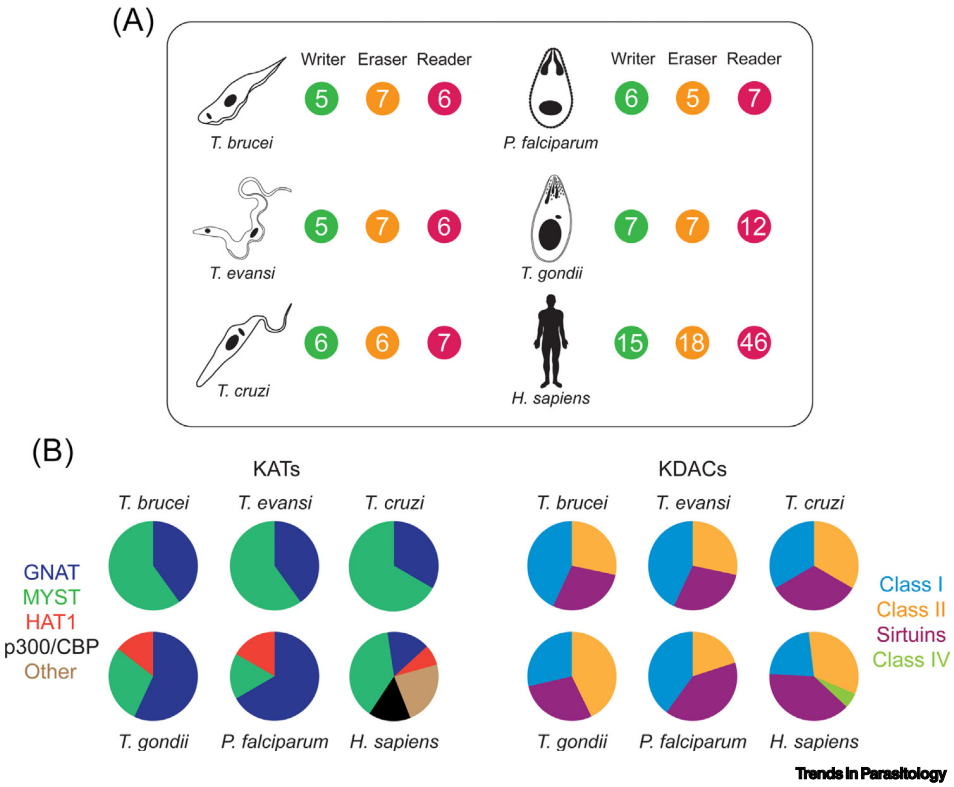


Figure 1. Regulatory lysine acetylation machinery of protozoan parasites. (A) Overview of 'writers', 'erasers', and 'readers' from protozoan parasites with described acetylomes compared to human. (B) Diversity of lysine acetylation machinery components of protozoan parasites. Although the repertoire of protozoan machinery is smaller, the diversity of components is comparable to that in humans, as observed by the families of enzymes from each species. Abbreviations: KAT, lysine acetyltransferase; KDAC, lysine deacetylase; *H. sapiens, Homo sapiens; P. falciparum, Plasmodium falciparum; T. gondii, Toxoplasma gondii, T. brucei, Trypanosoma brucei; T. cruzi, Trypanosoma cruzi; T. evansi, Trypanosoma evansi.*

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 [22].

T. gondii possesses four class I KDACs, but only TgHDAC3 has been characterized [26], while *P. falciparum* has only one class I KDAC. Trypanosomes have twice as many class II KDACs as *T. gondii* and *P. falciparum,* and of all the protozoan class II KDACs, only *T. brucei* enzymes have been characterized [27] (Figure 1 and Table S1).

The precise function of the *T. gondii* sirtuins has not yet been identified; however, the role of the *P. falciparum* sirtuins in regulating gene expression has been reported [28,29]. *T. brucei* has two mitochondrial sirtuins (TbSir2rp2 and TbSir2rp3) and one nuclear sirtuin (TbSir2rp1) [30,31]. The two sirtuins of *T. cruzi* – cytoplasmic TcSir2rp1 and mitochondrial TcSir2rp3 – have distinct functions in parasite multiplication and differentiation [32,33]. The three *T. evansi* sirtuins remain uncharacterized. (Figure 1 and Table S1). No class IV KDACs have been identified in apicomplexans or trypanosomes.

BDPs

Apicomplexans and trypanosomes have a limited repertoire of BDPs compared to humans. With 12 BDPs, *T. gondii* 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 *T. gondii* and TcBDF5 in *T. cruzi,* each of which have two bromodomains. Importantly, apicomplexans and trypanosomes have many parasite-specific BDPs with no similarity to human BDPs; they have been seen as promising drug targets [34] (Table S1).

Acetylated protein repertoire of protozoan parasites

General overview on published acetylomes

To date, acetylomes have been reported for five protozoan parasites: *P. falciparum* [16,18,35], *T. gondii* [14,15,19], *T. brucei* [17,20], *T. evansi* [20], and *T. cruzi* [17]. For more details about the methods used to describe their acetylomes see Box 2.

From the five parasite acetylomes addressed herein, those of *P. falciparum* and *T. brucei* were more comprehensively studied and combine several recent studies with revised and improved

Box 2. Proteome-wide analysis used for describing the acetylomes of protozoan parasites

Although there are differences in the approaches used to describe protozoan parasite acetylomes, it generally follows similar methodologies applied to other 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 *Trypanosoma cruzi* and *Trypanosoma 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 *Trypanosoma 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-ultra performance liquid chromatography-mass spectrometry (nano-UPLC) 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 (see Figure 2A,B in main text).

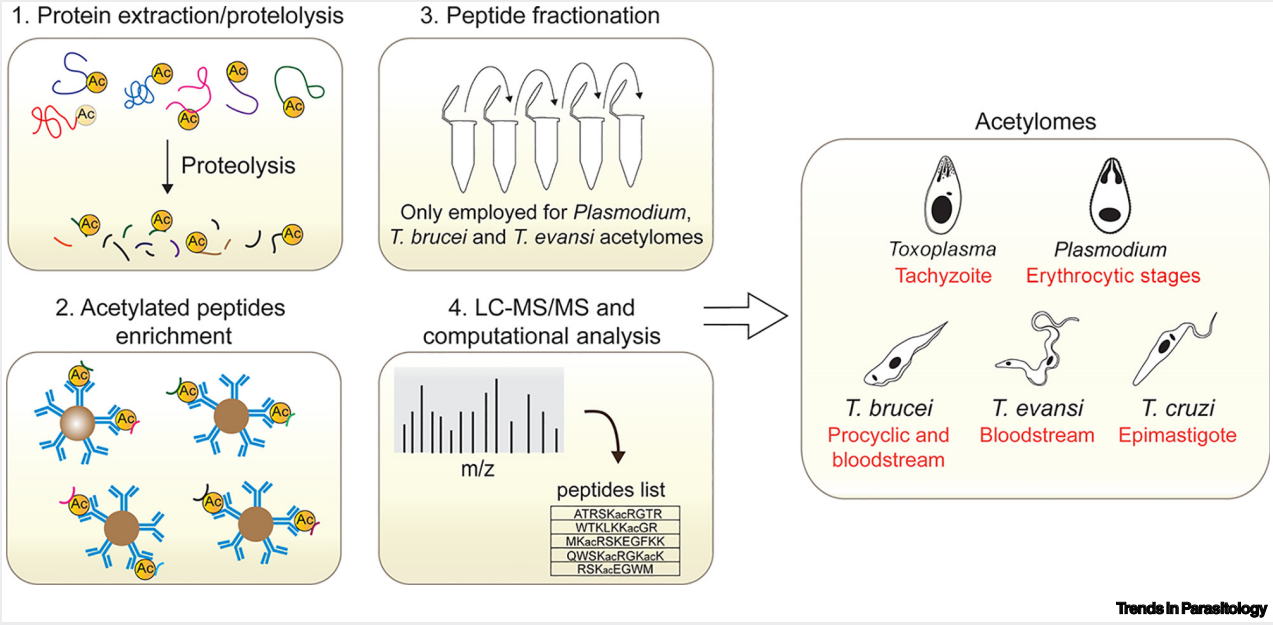


Figure I. Methodological steps used to describe the acetylomes of protozoan parasites. Abbreviation: m/z, mass divided by charge number.

new methodologies [20,35]. Twelve hundred and 2756 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,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,B). These differences might reflect the use of distinct protocols and tandem mass spectrometry (MS/MS) technologies. Notably, the *T. cruzi* acetylome protocol did not include the K-ac immunoaffinity enrichment step [17]. Hence, the number of acetylated proteins identified in some of these parasites might be underestimated (as depicted in Figure 2A–C).

Comparative analyses of protozoan parasite acetylomes

Gene ontology (GO)-based enrichment analyses of several parasite acetylomes via the VEuPathDB (www.veupathdb.org) [36] revealed that chromatin and nucleosome GO cellular component (CC) terms are present in all three genera: *Trypanosoma*, *Toxoplasma*, and *Plasmodium* (Figure 2D and Table S2); moreover, it showed that glucose metabolism is a GO biological process (BP) also common to both apicomplexan and trypanosomatid species studied herein (Figure 2D and Table S2). Furthermore, processes related to nucleotide metabolism/biosynthesis were shared between *P. falciparum* and *T. gondii*, whereas *T. evansi* and *T. cruzi* have 'tRNA aminoacylation for protein translation' (BP) and 'proteasome complex' (CC) terms in common. Finally, 'microtubule-based transport' (BP)-associated K-ac-containing proteins were enriched in *T. evansi* and *T. brucei* acetylomes.

A survey using parasite acetylated proteins by the Markov Clustering algorithm [37] indicated that those acetylome datasets form 20 distinct clusters that vary in size from five to 45 proteins each, and they contain at least one protein from each species (Figure 2E and Table S3). Putative functional clusters of orthologous groups (COGs) corroborate the GO-based enrichment analysis (Figure 2D), notably, the chromatin, glucose metabolism, and protein synthesis/degradation-related COGs (Figure 2E). Chaperones, cell division, oxidative stress, and RNA degradation-associated COGs also display functional groups of acetylated proteins in all analyzed species (Figure 2E). Details on all 20 COGs are found in Table S3. Hence, there are conserved functional groups of acetylated proteins in protozoans, and they may play a role in specific processes of parasite biology.

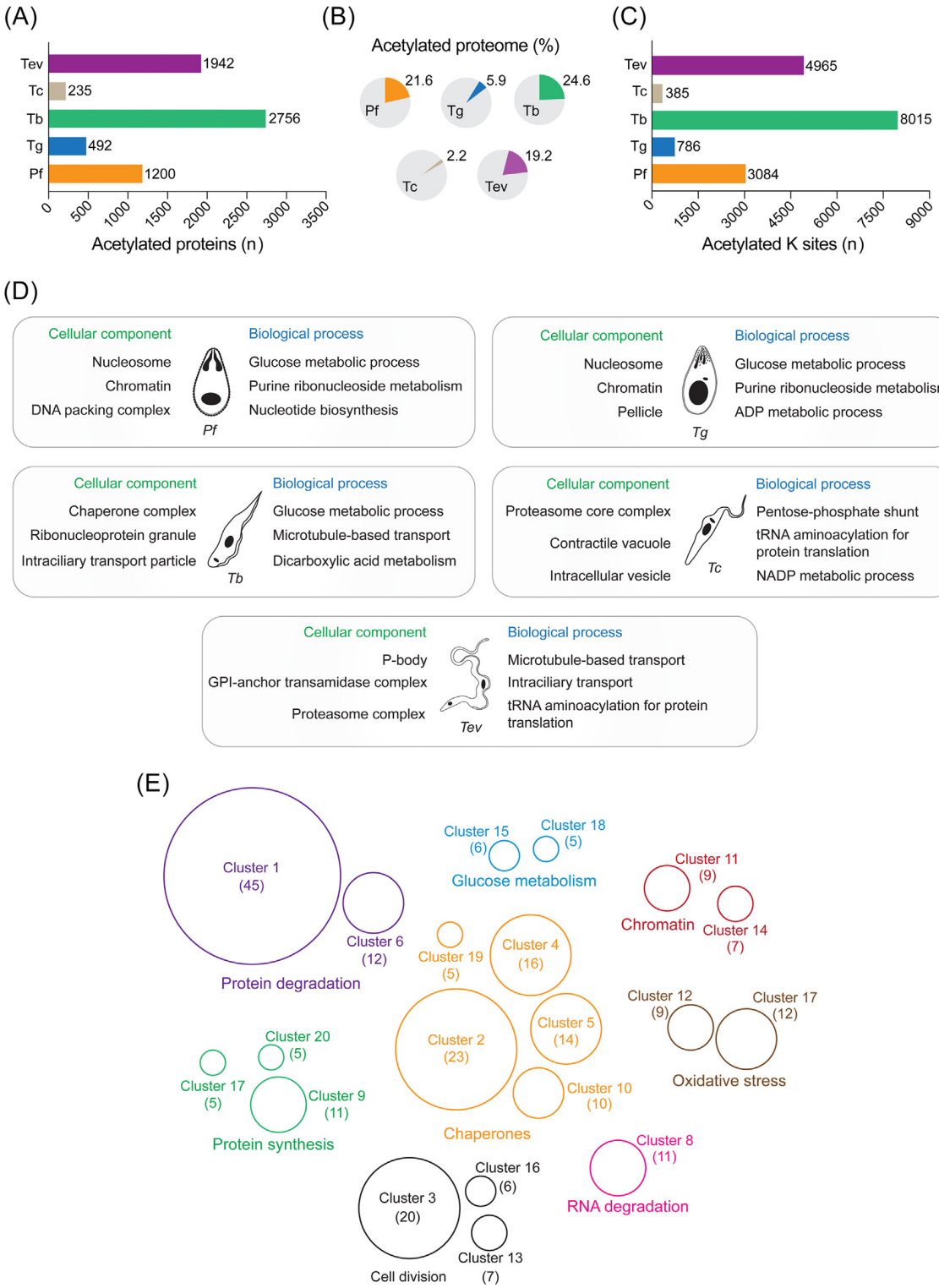
Physiological roles of protein acetylation in protozoan parasites

Regulation of chromatin structure and gene expression in apicomplexan parasites

Protozoans possess the same basic components and assembly of chromatin as higher eukaryotes. Acetylation weakness the histone tails' affinity to DNA, allowing chromatin to relax and trans factors to access DNA. *T. gondii* and *P. falciparum* both rely heavily on PTMs to regulate gene expression [38]. They lack typical regulatory mechanisms present in metazoans, such as a conserved TATA box in promoters, the linker histone H1, DNA methylation, and diverse DNA-binding transcription factors.

Analysis of the repertoire of PTMs in *Plasmodium* showed a prevalence of histone acetylation [18]. Histone H3 and H4 acetylation upstream of active genes has consistently been observed in *T. gondii* and *P. falciparum* [16,18,35,39–41]. The presence of histone acetylation in **euchromatin** and its 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 [42,43]. Moreover, the acetyl marks H3K9ac, H3K14ac, H4K5ac, and H4K12 are present in intergenic regions of transcribed genes [39,44,45]. The correlation between H3K9ac and transcript levels has been established in *P. falciparum* asexual blood stages, **sporozoites**, and male **gametocyte ookinetes** [46].

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



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stages of *P. falciparum* H3K27ac is enriched in intergenic euchromatin regions but is not associated with gene expression [47]. Also, it was found that the H3K9ac mark does not correlate with increased transcript levels in female gametocytes [46], and low-passage strains of *T. gondii* have significant histone acetylation at inactive genes [48].

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* and *P. falciparum* asexual replication requires the KATs TgGCN5B and PfGCN5, respectively, to regulate gene expression via histone acetylation [49,50]. The *P. falciparum* KDACs PfSir2A, PfSir2B, and PfHda2 play an essential role in heterochromatin formation and silencing *var* genes [51,52]. 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 [53].

More recently, BDPs have surfaced as critical players in protozoan gene regulation. Studies suggest that they might be essential for *T. gondii* growth [54] and verified to be critical for *P. falciparum* growth and invasion [55]. Recently, TgGCN5B was identified as essential for *T. gondii* viability [56]. 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 BDP PfBDP2 [55,57].

In addition to histones, KATs, KDACs, BDPs, and other transcriptional components are themselves acetylated. The effects of acetylation on the function of these proteins are poorly understood. Acetylation of ApiAP2 transcription factors has been found to alter their interactions with DNA and other proteins [35]. Moreover, the KAT inhibitor garcinol decreased acetylation of the KAT TgGCN5B itself, in addition to its substrate H3, resulting in disrupted tachyzoite growth [58]. 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 [59]. 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 that of other eukaryotes [60]. Proteomic analysis has identified over 170 PTMs in trypanosome histones [17,20,61–63]. 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 [61–63]. Notably, acetylation was also detected in the globular domains of several histones.

Much less is known about histone acetylation in *Leishmania*, but H4 is acetylated at K4 and K10, whereas H3 is acetylated at the N-terminal tails [64]. KATs and KDACs are also encoded in the

Figure 2. Protein lysine acetylation repertoire of protozoan parasites. (A) The number of acetylated proteins detected from *Plasmodium falciparum* (Pf), *Toxoplasma gondii* (Tg), *Trypanosoma brucei* (Tb), *Trypanosoma cruzi* (Tc), and *Trypanosoma evansi* (Tev). (B) The percentage of acetylated proteomes over their respective total predicted proteomes. (C) The 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 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. (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.

trypanosomatid parasite genomes (Table S1). The *T. brucei* TbSir2rp1 acetylates histones H2A and H2B *in vitro* [31]. *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 [65,66].

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) [66]. 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. By contrast, the knockdown of HAT1 decreased total mRNA levels by half, implying that these histones' acetylation plays a role in RNA polymerase II transcription [66]. 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 [67].

Little is known about the role of histone acetylation in *T. cruzi* and *Leishmania* spp. The expression of mutant nonacetylated forms of H4, which prevents acetylation at positions K10 or K14, affected DNA replication and repair and indicated a role in chromatin assembly/remodeling required for gene expression or DNA replication in *T. cruzi* [68]. 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 [69,70]. Conventionally, RNP associations are mediated by RNA-binding domains (RBDs) [71,72]. Of great interest, many RNA-associated proteins lack traditional RBD motifs [73]. 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 [74].

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 [75,76]. Arginine monomethylation impacts both RBP stability and RNA-binding capacity [75]. 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 [73,75].

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 [77,78]. 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 [14,16,17,20]. For example, Pumilio homology domain family member 8 (PUF8) in *T. brucei* (Tb927.3.2470) [20]; RNA-binding

protein 42 (TcCLB.509167.140) in *T. cruzi* [17]; RNA-binding protein (TGME49_105850) in *T. gondii* [14], and RNA-binding protein NOVA1 (Pf7G8_140020700) in *P. falciparum* [16]. 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 trypanosomatid parasites present an excellent eukaryotic system to accomplish such an investigation due to their relatively high abundance of RBPs and emphasis upon post-transcriptional gene regulation [73,75].

Regulation of glycolytic metabolism by differential acetylation

During their life cycle, protozoan parasites must adapt their metabolism in response to nutrient sources available in the different hosts [6]. Metabolic enzymes are among the most prevalent acetylated proteins detected in the acetylomes of both prokaryotes and eukaryotes [79,80]. In protozoans, this is evident in the glycolytic pathway, where most of the enzymes were detected acetylated at different lysine sites (Box 3), except for glucose phosphate isomerase (PGI). The number of lysine acetylated sites identified varies between protein homologs in each species [14–17,20,35] (Box 3), 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 for *T. brucei* and *T. gondii* [15,81]. The *T. brucei* **procyclic form**, which develops in the insect gut, relies on amino acids as the primary carbon source and obtains adenosine triphosphate (ATP) by **oxidative phosphorylation**. By contrast, the **bloodstream form** that replicates in the blood faces high glucose levels and generates ATP mainly by glycolysis in the **glycosomes** [6,82]. Comparing the acetylation profile of both parasite forms, Moretti *et al*. found higher levels of acetylation on procyclic glycolytic enzymes compared to bloodstream forms [17] (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 [79,80].

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 [81] (Figure 3B).

Human aldolase is negatively regulated by the acetylation of lysine 147 (K147) present in the catalytic site [79]. 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* [81] (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 [81], similarly to mammalian aldolase [79]. 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 compared to unacetylated protein [81]. These findings identify lysine acetylation as a new regulatory mechanism of the *T. brucei* aldolase enzyme, and the conservation of key lysine residues for the enzyme activity among other species (Figure 3C) might indicate that this regulatory mechanism could be conserved.

Box 3. Acetylation of glycolytic enzymes from protozoan parasites

Glycolysis is an ancient and regulatory mechanism used by most organisms to break down 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) happen in specialized organelles called glycosomes.

The first reaction of the pathway is catalyzed by hexokinase (HK), which phosphorylates glucose, producing glucose 6-phosphate; one molecule of ATP is consumed. Glucose 6-phosphate is then isomerized into fructose 6-phosphate by glucose phosphate isomerase. (PGI), which is then 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-phosphate dehydrogenase (GAPDH), releasing $NADH^+$, and then adds a phosphate to generate 1,3-biphosphoglycerate. Phosphoglycerate kinase (PGK) transfers a phosphate from 1,3-biphosphoglycerate to ADP forming ATP and two molecules of 3-phosphoglycerate. These two molecules of 3-phosphoglycerate are converted to 2-phosphoglycerate by phosphoglycerate mutase (PGM), which has a water molecule removed by enolase (ENO) to obtain phosphoenolpyruvate. Finally, pyruvate kinase transfers a phosphate group from phosphoenolpyruvate to ADP, generating ATP and pyruvate.

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

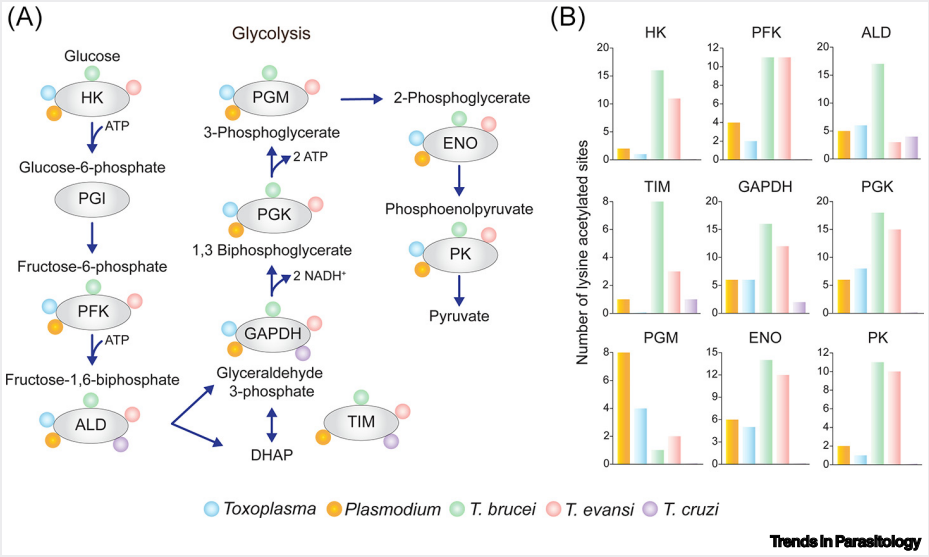

Figure I. Acetylation profile of glycolytic enzymes from protozoan parasites.

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 [15]. Interestingly, K216, the residue corresponding to K157 in *T. brucei* (Figure 3C), was not detected acetylated in *T. gondii* aldolase, suggesting that the enzyme is active, and this could explain the lower levels of F-1,6P. Also, K301, present in the catalytic site of *T. gondii* aldolase, involved in the Schiff reaction [83] and conserved among other parasites (Figure 3C), was detected hyperacetylated, suggesting that acetylation of this residue could activate the enzyme [15]. By

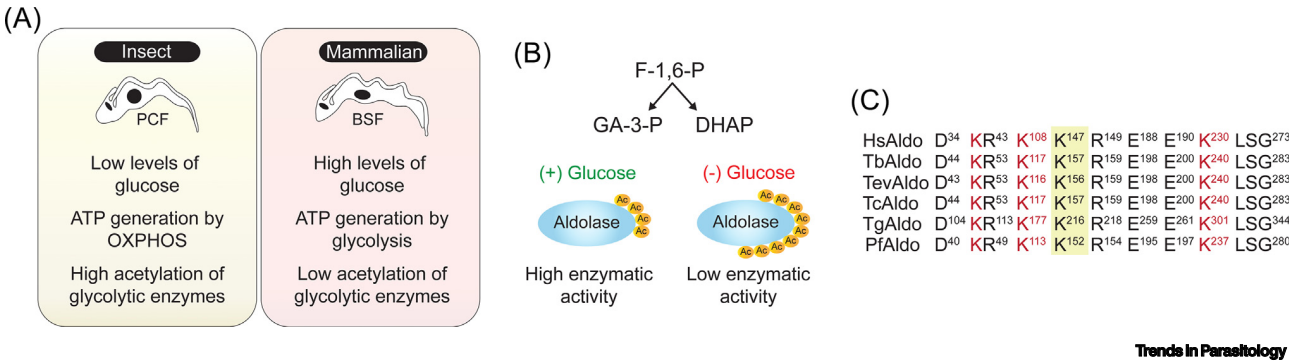

Figure 3. Regulatory function of acetylation on glycolytic enzymes from protozoan parasites. (A) Changes in metabolism during the life cycle of *Trypanosoma brucei*. The bloodstream of a mammalian host is a very rich environment, containing high levels of glucose, while the nutrient sources found in 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 are acetylated in some of these parasites.

contrast, the authors also investigated the impact of acetylation in the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK-1) *in vivo* but found no clear impact in the enzyme activity [15].

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 the acetylation level of glycolytic enzymes.

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 [84]. Since then, inhibitors of KATs, KDACs, and more recently, BDPs have been investigated for their potential as antiprotozoan therapeutic targets [85,86].

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-molecule 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 antiprotozoan drugs that target the parasite's lysine acetylation network. Multiple groups have performed parasite growth assay screens with known synthesized and natural compounds to identify those with cytotoxic effects [87–89]. 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 antimalarial primaquine and the KDAC inhibitor SAHA [90]. Another successful strategy has been rational drug design, using *in silico* molecular modeling and docking to identify inhibitors with a high likelihood of binding parasite-specific KAT, KDAC, and BDP domains [87,91,92].

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 [88]. 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 [32], and from further screenings of 33 chemically different modulators of human SIRTs [93]. For more information about the potential of KDAC inhibitors against protozoan see [86,94].

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 antimalarial and antitrypanosomal activity [95–97]. These compounds, while nonspecific, have been identified as binding and inhibiting KATs. Garcinol is another nonspecific KAT inhibitor identified as targeting GCN5 homologs and disrupting parasite growth [58]. With recent studies identifying BDPs as essential to parasites and their amenability to drug design, BDP inhibitors are being investigated for their antiprotozoan activity. Jeffers *et al*. showed that the human BDP inhibitor I-BET151 is cytotoxic to *T. gondii* at concentrations that do not affect host cells [98]. The compound L-Moses has been reported to inhibit the bromodomain of the GCN5 homologs in both *P. falciparum* and *T. gondii,* revealing a second potential route for drug inhibition of the critical GCN5 homologs in the apicomplexans [56,99]. The bromodomain of PfGCN5 was 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 [87]. Recently, GSK2801 was demonstrated to bind to *T. brucei* TbBDF2 and reduce parasite growth [100].

Tremendous progress has been made in the past 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 as 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. 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 nonhistone 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.

Outstanding questions

Is acetylation regulatory function important for other crucial biological processes in protozoan parasites?

How are acetylation writers, erasers, and readers targeted to specific proteins?

How is the activity of acetylation writers, erasers, and readers regulated in protozoan parasites?

Can KAT-, KDAC-, and BDP-specific inhibitors be an effective treatment for diseases caused by protozoans in the future?

How do the known protein acetylation profiles compare to those of other protozoan species, such as *Leishmania* spp. and *Bodo saltans*?

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 protozoa, 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 regulatory machinery acts within each specific process will support efforts to explore these enzymes as drug targets. Finally, it will be crucial to uncover the acetylome of other protozoan species, either human parasites or free-living organisms, as they might provide insights into how acetylation impacts the development of parasitism.

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Declaration of interests

The authors declare no competing interests.

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