Lysine Acetylation Is Widespread on Proteins of Diverse Function and Localization in the Protozoan Parasite *Toxoplasma gondii*

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While histone proteins are the founding members of lysine acetylation substrates, it is now clear that hundreds of other proteins can be acetylated in multiple compartments of the cell. Our knowledge of the scope of this modification throughout the kingdom of life is beginning to emerge, as proteome-wide lysine acetylation has been documented in prokaryotes, *Arabidopsis thaliana*, *Drosophila melanogaster*, and human cells. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify parasite peptides enriched by immunopurification with acetyl-lysine antibody, we produced the first proteome-wide analysis of acetylation for a protozoan organism, the opportunistic apicomplexan parasite *Toxoplasma gondii*. The results show that lysine acetylation is abundant in the actively proliferating tachyzoite form of the parasite, which causes acute toxoplasmosis. Our approach successfully identified known acetylation marks on *Toxoplasma* histones and $\alpha$-tubulin and detected over 400 novel acetylation sites on a wide variety of additional proteins, including those with roles in transcription, translation, metabolism, and stress responses. Importantly, an extensive set of parasite-specific proteins, including those found in organelles unique to *Apicomplexa*, is acetylated in the parasite. Our data provide a wealth of new information that improves our understanding of the evolution of this vital regulatory modification while potentially revealing novel therapeutic avenues. We conclude from this study that lysine acetylation was prevalent in the early stages of eukaryotic cell evolution and occurs on proteins involved in a remarkably diverse array of cellular functions, including those that are specific to parasites.

Protozoan parasites are responsible for significant morbidity and mortality around the world, and new drug targets are sorely needed. Using the intracellular apicomplexan parasite *Toxoplasma gondii* as a model, we and others have established that histone acetylation is a critical posttranslational modification involved in parasite viability and development (11, 36). Lysine acetylation is also a validated drug target in protozoan parasites based on the antiparasite effects of lysine deacetylase (KDAC) inhibitors, such as apicidin and FR235222 (4, 9).

Recent studies have demonstrated that lysine acetylation occurs on a multitude of other proteins beyond histones (22, 31). Not only are there nonhistone proteins acetylated in the nucleus, but proteins in the cytoplasm and mitochondria contain acetylated residues as well. The development of specific acetyl-lysine antibodies to enrich acetylated tryptic peptides prior to identification by mass spectrometry has allowed lysine acetylation to be mapped at the whole-proteome level. So-called “acetylomes” have been described for prokaryotes (15, 42, 50), plants (12, 45), mapped at the whole-proteome level. So-called “acetylomes” have been described for prokaryotes (15, 42, 50), plants (12, 45), and eukaryotes (12, 45). Proteins involved in nearly every facet of cell biology, particularly proteins with roles in metabolism, translation, folding, DNA packaging, and the cytoskeleton, have been discovered bearing acetyl groups. The abundance of reversible $\varepsilon$-lysine acetylation found on such a wide variety of proteins underscores the regulatory potential of this modification, which has led to the idea that acetylation may rival phosphorylation (22, 28).

The scope of lysine acetylation has yet to be explored in early-branching eukaryotes, such as *Toxoplasma*. Lysine acetylation occurs on *Toxoplasma* histones, but studies suggest that lysine acetylation may be more extensive. A proteomics study mapping the posttranslational modifications of *Toxoplasma* tubulins revealed acetylation of lysine 40 (K40) on $\alpha$-tubulin (46), which is a conserved modification in most eukaryotes excluding yeasts (40). We have also noted that several *Toxoplasma* lysine acetyltransferases (KATs) are predominantly cytoplasmic, suggesting that they may have substrates in the parasite cytosol.

Here we report a proteome-wide analysis of lysine acetylation in *Toxoplasma*, the first such analysis completed for protozoa. We find that lysine acetylation is abundant and occurs in multiple subcellular compartments in tachyzoites, parasites in the proliferative stage that cause acute toxoplasmosis. Our data establish that lysine acetylation was prevalent in the early stages of eukaryotic cell evolution and continues to evolve along parasite-specific trajectories, occurring on proteins that are involved in a remarkably diverse array of cellular functions. Potentially important for future drug design, we found lysine acetylation on a striking number of parasite-specific proteins as well as proteins residing in specialized secretory organelles.

**MATERIALS AND METHODS**

Cell/parasite culture and parasite lysate preparation. hTERT-immortalized human foreskin fibroblast (HFF) cells (hTERT + HFF) were grown to confluence in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). RH strain *Toxoplasma gondii* parasites were used to infect the hTERT + HFF monolayers, and the infected cells were maintained in DMEM supplemented with 1% heat-inactivated fetal bovine serum (Invitrogen). Uninfected and infected cells were maintained in humidified incubators at 37°C under 5% CO$_2$. Actively growing tachyzoites were harvested before host cell lysis at a density of $\sim$64 to 128 parasites/vacuole. A 10 mM...
concentration of sodium butyrate (a KDAC inhibitor) was added to infected monolayers 30 min prior to the harvesting of tachyzoites. The parasites were physically separated from host cells by passage through 23G syringe needles and then purified from host cell debris using a 3.0-μm filter (29). To obtain the 20 μg of tachyzoite lysate used to map the acety-lome, we pooled multiple independent preparations. The parasites were washed in phosphate-buffered saline (PBS) and resuspended in urea lysis buffer (9.0 M urea, 20 mM HEPES [pH 8.0], 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate) freshly supplemented with 10 mM sodium butyrate. Sonicated lysates were centrifuged for 15 min at 4°C at 13,000 rpm (~20,000 × g) in a Beckman JA25.30 rotor. Supernatants were collected and reduced with 4.5 mM dithiothreitol (DTT) for 30 min at 55°C. Reduced lysates were alkylated with iodoacetamide (0.095 g per 5 mL H2O) for 15 min at room temperature in the dark. Samples were diluted 1:4 with 20 mM HEPES (pH 8.0) and digested overnight with 10 μg/mL 1:-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) (Worthington) in 1.0 mM HCl. Digested peptide lysates were acidified with 1% trifluoroacetic acid (TFA), and peptides were desalted over Sep-Pak Classic C18 columns (Waters). Peptides were eluted with 40% acetonitrile in 0.1% TFA, dried under a vacuum, and stored at −80°C.

Western blotting. Protein lysate (25 μg) generated from intracellular tachyzoites was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Acetylated lysines were detected using an anti-acetyl-lysine rabbit polyclonal antibody (CST number 9895; Cell Signaling Technology) at 1:1,000 and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (CST number 7074; Cell Signaling Technology) at 1:2,000.

Acetylated lysine peptide enrichment. Acetyl-lysine peptide immunoaffinity purification and identification were carried out as previously described (30, 32). Briefly, acetylated peptides were enriched using a pan-specific anti-acetylated lysine antibody (CST number 9895; Cell Signaling Technology) bound to 50 μl packed protein G agarose beads (Roche). Lyophilized peptides were resuspended in MOPS (morpholino propane-sulfonylic acid) IAP buffer (50 mM MOPS [pH 7.2], 10 mM KH2PO4, 50 mM NaCl) and centrifuged for 5 min at 12,000 rpm. Supernatants were mixed with anti-acetylated lysine slurries for 2.5 h at 4°C and then centrifuged for 30 s at 3,400 rpm at 4°C. Beads were washed 2 times with 1.0 ml MOPS IAP buffer and 4 times with 1.0 ml water (Burdick and Jackson). Peptides were eluted from beads with 0.15% TFA (sequential elutions of 65 μl followed by 55 μl for 10 min each at room temperature). Eluted peptides were desalted over tips packed with Empore C18 (Sigma) and eluted with 60% acetonitrile in 0.1% TFA. Eluted peptides were dried under a vacuum.

LC-MS/MS analysis and data deposition. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed by Cell Signaling Technology. Immunoprecipitated peptides were resuspended in 0.125% formic acid and separated on a reverse-phase C18 column (75-μm inner diameter [ID] by 10 mm) packed into a PicoTip emitter (~8-μm ID) with Magic C18 AQ (100 Å, 5 μm). Peptides were eluted using a 45-, 72-, or 90-min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nl/min. Tandem mass spectra were collected in a data-dependent manner with an LTQ-Orbitrap Velos mass spectrometer running XCalibur 2.0.7 SP1 using a top-twenty MS/MS method, a dynamic repeat count of one, and a repeat duration of 30 s. MS spectra were evaluated using SEQUEST 3G and the Sorcerer 2 platform from Sage-N Research (v4.0; Milipitas, CA) (26). Peptides were matched to Toxoplasma gondii RefSeq proteins (txd5811[Organism:exp] and scrcdb_refseq[Properties], 8,013 entries) with a false-positive rate of 5% using the SEQUEST search engine. Enzyme specificity was set to trypsin, with up to two missed cleavages permitted. High-scoring peptide identifications are those with cross-correlation (Xcorr) values of ≥1.5, delta CN values of ≥0.10, and precursor accuracy measurements within ±3 ppm in at least one injection. A mass accuracy of ±50 ppm was used for precursor ions and a mass accuracy of 1.0 Da was used for product ions. Carbamoylmethyl cysteine was specified as a fixed modification, with oxidized methionine and acetylation of lysine residues allowed. Reversed decoy databases were included for all searches to estimate false-positive rates and filtered using a 5% false-positive cutoff in the Peptide Prophet module of Sorcerer 2. Peptide assignments were further filtered with a mass accuracy of ±3 ppm. The mzXML, Datas, and Out files associated with this article may be downloaded from Proteome Commons at https://proteomecommons.org/dataset.jsp?i=G%2BZPb7%2BheFAnMGg9gdawYo51hGFnHAdshGBNEB%2FLisMHpq5KZKAnozw5xpQiQsc61m1pJ4lPagEgpSxYwqH7q8AAAAAABEQ%3D3D.

Data analysis and bioinformatic. Acetylated proteins were classified according to gene ontology (GO) annotations by ToxoDB 7.0 (www.toxodb.org) and Uniprot (http://www.ebi.ac.uk/uniprot/) (13). When a single peptide could be matched to two or more different proteins, manual inspection of gene expression and proteomics data from ToxoDB 7.0 usually allowed determination of the protein from which the peptide was likely derived. If such an inference was not possible, the peptide was omitted from the rest of the analysis. Cellular localization data were extracted from ApiLoc v3 (http://apiloc.biochem.unimelb.edu.au/apiloc/apiloc). SUMOylation consensus sequences were surveyed using SUMOplot (www.http://abgent.com/sumoplot_analysis_program) (47). Annotated Toxoplasma bromodomain-containing proteins were identified using the search term “bromodomain” on ToxoDB. BLAST analyses against the ToxoDB ME49 database with a number of human and yeast bromodomain sequences failed to identify additional unannotated bromodomains.

Motif analysis. Amino acid sequence motifs were compiled using WebLogo 3.1 (8). Heat maps were produced with ICELogo (7) using the Swiss-Prot Toxoplasma gondii protein database (TOXGO 5811; accessed 11 November 2011) to compare amino acid frequencies.

RESULTS AND DISCUSSION

Lysine acetylation in Toxoplasma. We and others have previously established that histone proteins are extensively acetylated in Toxoplasma (11). However, we have noted that both TgMST family KATs are cytoplasmic (34, 41), and a homologue of the cytosolic KAT called Hat1 is present in the parasite (36). We hypothesized that KAT enzymes present in the parasite cytoplasm may have additional substrates beyond histones. To address this hypothesis, we prepared parasite lysate made from intracellular tachyzoites for Western blotting using an antibody recognizing acetyl-lysine (Cell Signaling Technology). As expected, the abundant histone proteins (11 to 17 kDa) gave a strong signal on the Western blot (Fig. 1A). The data also revealed that a large number of nonhistone proteins with a wide range of molecular masses are acetylated in Toxoplasma tachyzoites.

We performed a proteome-wide survey of Toxoplasma acetylated proteins using lysate made from intracellular tachyzoites that were physically separated from their host cells and extensively purified (see Materials and Methods). The enrichment of acetylated tryptic peptides using acetyl-lysine antibody and identification of those peptides by LC-MS/MS were performed using a strategy outlined in Fig. 1B. Proteomics screening identified 411 lysine acetylation sites across 274 tachyzoite proteins (see Table S1 in the supplemental material). The fidelity of the data set was evident with the identification of acetylation on known substrates, such as Toxoplasma histones and α-tubulin (discussed below). Each acetylated site was assigned to a functional group based on gene ontology (GO) (see Table S2 in the supplemental material). The 274 acetylated sites span a wide variety of functions, with the majority clustering into metabolism, translation, chromatin biology, and the stress response (Fig. 1C). The most heavily acetylated proteins are the eukaryotic translation elongation factor 1 alpha 1 (EF1-α; TGME49_086420, TGME49_
and the porin, voltage-dependent anion channel 1 (VDAC1; TGME49_063300), each acetylated at 7 independent lysine residues. Homologues of these proteins are also heavily acetylated in human cells (6), strongly suggesting an important, conserved role for lysine acetylation in the activities of these proteins.

Acetylomes mapped for bacterial and plant species have revealed 100 to 150 acetylated proteins, while up to 1,000 to 1,700 proteins are acetylated in *Drosophila* and humans (6, 12, 42, 43, 45, 50, 51). The number of acetylated *Toxoplasma* proteins in our data is consistent with what one might expect for a protozoan, which contains a number of acetylated substrates closer to those of prokaryotes than to those of metazoans. Inherent in studies of this nature, the number of detected acetylated proteins very much depends on the depth of the analysis. However, the data presently available strongly suggest that lysine acetylation is a critical modification found throughout all kingdoms of life and that there is selective pressure to maintain this activity even after adaptation to a parasitic lifestyle.

The *Toxoplasma* acetylome reveals novel histone modifications. Previously documented histone acetylation marks in *Toxoplasma* include K9, K14, and K18 on H3 and K5, K8, K12, and K16 on H4, most of which were confirmed in our data (Table 1). Acetylation of K23 and K79 on H3 and K31 on H4 are novel histone acetylation marks that have yet to be described in *Apicomplexa*. In very recent reports, low levels of H3 K79 acetylation have been detected in HeLa cells and *Saccharomyces cerevisiae* yeast (3, 14), but the role of this novel mark has yet to be defined. The majority of H3 K79 is methylated in other species by Dot1, but intriguingly, *Toxoplasma* does not appear to possess a Dot1 homologue (36). It will be of interest to determine if H3 K79 is methylated in *Toxoplasma* or if acetylation is the predominant mark found on this residue.
The failure to detect K5 and K8 on H4 may be due to the transient nature of this modification or a limitation associated with the affinity of the antibody used to enrich acetylated peptides. Consistent with analyses of *Plasmodium* histones (27, 39), we found extensive histone acetylation among the unusual H2A and H2B variants expressed in *Toxoplasma* (Table 1). While no acetylation was detected on canonical H2A or H2AX, six different lysines were acetylated on H2AZ. No modifications have been reported to date for the near-identical pair of lysine-rich H2B histones, but we detected lysine acetylation occurring at 3 positions within the H2B variants.

**Extensive acetylation of chromatin-modifying machinery.** A substantial portion of the acetylated substrates detected in tachyzoites is comprised of proteins that constitute and modulate chromatin. In addition to the histone proteins mentioned above, many KATs are acetylated themselves, including both pairs of TgGCN5 and TgMYST family KATs. A summary figure of acetylation of *Toxoplasma* KATs, along with recently discovered phosphorylation sites (38), is shown in Fig. 2. There are two MYST family KATs in *Toxoplasma*, TgMYST-A and TgMYST-B, and each is expressed in long and short forms (34, 41). Both TgMYST-A and TgMYST-B are acetylated within the N-terminal extension of the long form of each protein (Fig. 2). TgMYST-A is also acetylated at a conserved lysine residue within the MYST domain, present in both the long and short form. The analogous residue is acetylated in the yeast MYST homologues Esa1 and Sas2 (49) as well as in the human MYST family member MOF, in which the modification decreases the ability to bind to nucleosomes (24).

**Figure 2** shows that both GCN5 family members, TgGCN5-A and TgGCN5-B, are acetylated within the ADA2-binding domain (TgGCN5-A, K1057 and K1060; TgGCN5-B, K857), similar to the human GCN5 homologue PCAF (6). TgGCN5-A is also acetylated at K1080 in its bromodomain, a domain that recognizes acetylated lysine residues (10). In contrast, no acetylation of the TgGCN5-B bromodomain was detected in our data. However, TgGCN5-B possesses a unique 50-amino-acid C-terminal tail that is acetylated at 3 lysines. The *Toxoplasma* homologue of TAF1/TAF250 (16) is acetylated within the KAT catalytic domain at K1881.

**TABLE 1** Detected lysine acetylation on *Toxoplasma* histones

| ToxoDB accession no. | Protein name | Acetylation sites |
|-----------------------|--------------|-------------------|
| TGME49_100200         | Histone H2AZ  | 9, 13, 17, 23, 26, 28 |
| TGME49_009910         | Histone H2B variant | 8, 13, 14, 18 |
| TGME49_051870/105160  | Histone H2Bb/H2Ba | 33/34*, 98/99*, 110/111* |
| TGME49_061240/018260  | Histone H3/histone H3.3 variant | 9, 14, 18, 23, 79/58* |
| TGME49_039260         | Histone H4     | 12, 16, 31        |

*Positions of the lysine residues detected as acetylated in this study. An asterisk indicates that due to high homology between some histone variants, it was not possible to discern if these acetyl marks are present on one or both of the histones listed.

**FIG 2** Acetylation sites on *Toxoplasma* lysine acetyltransferases. Acetylation sites (red spots) and phosphorylated sites (blue spots) (38) found in the TgGCN5 and TgMYST family KATs. Acetylation was found within the ADA2 interaction domain of each TgGCN5 (the domain between the KAT and the bromodomain [BRM]). TgGCN5-A was also acetylated within the BRM, and TgGCN5-B was found to be acetylated at multiple lysines within its C-terminal tail. The TgMYST KATs contain a chromodomain (ChRM) and a zinc finger (ZnF) and are expressed as two forms (long and short) due to two translational start sites (the second start codons are marked by a dark green line). Both TgMYST-A and TgMYST-B are acetylated within the N-terminal extension of the longer form of the proteins. An acetyl mark on K288 was also found on TgMYST-A, which is conserved in a number of MYST homologues and may regulate KAT activity of the enzyme. Acetylation was also detected on the TAF1/TAF250 homologue at K1881. No acetylated residues were detected on predicted homologues of Elp3 or Hat1.
Components of chromatin remodeling complexes, such as K671 of TgADA2-A, a coactivator in GCN5 KAT complexes, are acetylated as well. Additional proteins involved in chromatin biology that were found to be acetylated include SWI/SNF factors, PHD finger proteins, bromodomain proteins, a JmjC domain-containing protein, and a protein with a histone deacetylase-interacting domain (see Table S2 in the supplemental material). The extensive acetylation of KATs and other chromatin remodeling components suggests that an elaborate network has evolved early in the course of eukaryotic evolution to maintain precise control of gene expression.

**Acetylation of cytoskeletal components.** Our data set validates the previously documented acetylated K40 residue on α-tubulin but also reveals that a second lysine is acetylated in α-tubulin (K326). This modification was previously reported for human α-tubulin, but its function remains unknown (6). A number of other cytoskeletal proteins, including an actin-depolymerizing factor and multiple acetylations on actin itself, were found to be acetylated in *Toxoplasma*. Actin and a number of actin nucleation and actin-interacting factors are acetylated in human cells (6), and an actin-depolymerizing factor is acetylated in *Arabidopsis* (ADF2, At3g4600) (12).

**Acetylation of *Toxoplasma* metabolic enzymes.** There are 25 acetylated proteins (9% of the total detected) that are involved in cellular metabolism. In addition to histone modifications altering gene expression to modulate metabolism, direct acetylation of metabolic enzymes can also impact cellular energy status (44, 51). Phosphoenolpyruvate carboxykinase (Pck1 in yeasts, PEPCK in humans), a key enzyme in gluconeogenesis, is acetylated at K514 in *S. cerevisiae* (23) and at K70, K71, and K594 in humans (51). The yeast K514 residue is conserved in the *Toxoplasma* PEPCK homologue (K643; TGME49_089650) but was not found to be acetylated in this study. In contrast, K591 on TgPEPCK is acetylated, which represents a novel modification for this enzyme. The activity and protein levels of PEPCK in human cells are regulated by the KAT p300, whereby acetylation of PEPCK by p300 is triggered by high glucose levels and targets the protein for degradation through the ubiquitination pathway (19). Although there is no homologue of p300 in *Toxoplasma*, it remains possible that one of the aforementioned cytoplasmic KATs is involved in regulation of gluconeogenesis through TgPEPCK acetylation.

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) II, found in the parasite apicoplast, is acetylated on a lysine that is analogous to an acetylated lysine in *Arabidopsis* GAPDH II (12). Cytosolic GAPDH I was found to be acetylated on two lysines, one of which matches an acetylation site on the human GAPDH (6). *Toxoplasma* phosphoglycerate mutase is acetylated on K115, which is conserved with an acetylation mark on the human, *Drosophila*, and *Escherichia coli* homologues (6, 43, 50). *Toxoplasma* phosphoglycerate kinase is acetylated on five lysine residues; similarly, the human homologue is heavily acetylated, with 13 lysines targeted (6). The three lysines acetylated in both human and *Toxoplasma* phosphoglycerate kinases are K75, K131, and K199.

Acetylation of aldolase at K121 in *Toxoplasma* may have additional relevance, as this metabolic enzyme also functions to bridge the actin cytoskeleton to cell surface adhesions (18). Aldolase has been subject to extensive mutational analysis but not at K121 (35). K121 is conserved in *Plasmodium* but not in metazoans, suggesting that acetylated K121 may play a parasite-specific role. Precisely how acetylation at K121 may affect aldolase activity and/or host cell invasion awaits further investigation.

A preponderance of acetylation occurs on ribosomal proteins and aminoacyl-tRNA synthetases in both prokaryotes and eukaryotes, and this trend holds true for *Toxoplasma*. In *E. coli*, 22% of the acetylated substrates identified were involved in translation machinery or processes (50). In humans, 75 ribosomal proteins (~4% of the acetylyome) were identified as acetylated (6). In *Toxoplasma*, 15% of the acetylated proteins we detected were proteins involved in translation, including multiple tRNA synthetases, translation initiation and elongation factors, and ribosomal proteins. We conclude from these analyses that acetylation of metabolic enzymes is a conserved phenomenon in early-branching eukaryotic cells.

**Lysine acetylation on proteins unique to Apicomplexa.** A number of lysine acetylation modifications were detected on proteins or protein families that lack clear homologues in metazoan species. *Toxoplasma* lacks the well-conserved transcription factors found in yeasts and humans; rather, apicomplexans appear to use a group of proteins harboring a plant-like Apetela-2 (AP2) DNA-binding domain (1). Five AP2 domain proteins in *Toxoplasma* harbor acetylated lysines: AP2IX-5, AP2IX-7, AP2X-5, AP2XI-4, and AP2XII-8. Four independent lysines were acetylated on AP2XI-4. AP2 domain proteins are common transcription factors in plants, but no AP2 protein has been detected as acetylated in *Arabidopsis* to date. In other species, lysine acetylation proximal to a DNA-binding domain can promote DNA binding and lysine acetylation within the DNA-binding domain can disrupt DNA binding (22, 48). The acetylated lysines on these *Toxoplasma* AP2 proteins are positioned at least 100 amino acids outside the DNA-binding domain, so it is unclear if these acetylation marks would directly influence DNA-protein interactions.

Another group of proteins that are specific to the apicomplexans includes rhoptry proteins, which are secreted into host cells during parasite invasion. Some rhoptry proteins, such as ROP16 and ROP18, serve as important virulence factors (33). We detected acetylation on ROP8 and ROP17 as well as rhoptry neck proteins RON2 and RON4. Four proteins predicted to be in the apicoplast (according to ApiLoc), including acetyl coenzyme A (acetyl-CoA) carboxylase, dihydriopiloyl dehydrogenase, (3R)-hydroxymyristoyl acyl carrier protein (ACP) dehydrase, and a protein with a 2-oxo acid dehydrogenase acyltransferase catalytic domain, contain acetylated lysine residues. A number of plastid proteins involved in photosynthesis are acetylated in *Arabidopsis* (12, 45). As the apicoplast is nonphotosynthetic, there was no overlap with acetylated plant plastid proteins. *Toxoplasma* possesses two acetyl-CoA carboxylases, one that is cytosolic and another that localizes to the apicoplast (TgACC1) (17). Interestingly, TgACC1 is acetylated on the acetyl-CoA-binding pocket within the carboxyl transferase domain at K2422, but the cytosolic TgACC is not acetylated. K2422 is highly conserved on the ACC protein throughout evolution (human K2127, plant K2041, and yeast K2034) but has yet to be reported as acetylated. The acetylation of TgACC1 at K2422, therefore, may be specific to the parasite apicoplast.

Notably, there is a significant number of hypothetical parasite-specific proteins (87, or 32% of the acetylome) that are subject to lysine acetylation. Considered with other examples mentioned above, these data show that lysine acetylation is not exclusive to a core set of proteins that are conserved throughout evolution.
presence of acetylated lysines on parasite proteins that have no homologues in the human host cell may hold great promise in new therapeutic opportunities.

**Acetylation motifs.** While acetylome data from other species have not revealed a definitive motif for lysine acetylation, patterns have emerged. In higher eukaryotes, acetylated lysines of nuclear and cytosolic proteins tend to be in the context of F, W, or Y at the +2 and Y at the +1 position, flanked on each side by additional lysine residues (6). In histones, a GK motif is commonly found. Mitochondrial proteins harbor a motif that bears striking resemblance to those of acetylated proteins in prokaryotes (F at +2 and Y or H at +1) (50). Analysis of the *Toxoplasma* lysine substrates revealed high conservation of the GK motif on histones (Fig. 3A), and the preference for G at the −1 position is preserved in non-histone proteins as well. Tyrosine at the +1 position is conserved, but unique to *Toxoplasma* is the proclivity for E and, to a lesser extent, D at this position. Stretches of lysines are not observed in the *Toxoplasma* nonhistone substrates, but G residues appear overrepresented, especially in the N-terminal regions of the targeted lysine. In *Toxoplasma* mitochondrial substrates, G is most commonly found at −2 and R is most commonly found at +1, a pattern which differs from those of prokaryotes and the mitochondrial substrates in higher eukaryotes (Fig. 3A). However, the paucity of mitochondrial proteins detected as acetylated in our study limits the confidence in this motif.

To assess if there is significant enrichment or depletion of specific amino acids with respect to the general amino acid composition of the entire *Toxoplasma* proteome, we generated IcLogo heat maps (7). As shown in Fig. 3B, it is evident that G is highly enriched in most positions N-terminal to the acetylated lysine, in addition to Y at the +1 position. Alanine is significantly enriched in the −2 position, while E exhibits a high abundance in the residues immediately surrounding the acetylated lysine. Additional characteristics of the acetylation site not evident from the sequence motifs include the depletion of R in the −1 and −2 positions, the depletion of P at the −1 position, and a general depletion of M, I, L, N, and V within the vicinity of the acetylation site.

Lysine residues are targeted for a number of other posttranslational modifications, including methylation and SUMOylation. A proteomics survey found 120 SUMOylated proteins in *Toxoplasma* tachyzoites (5), 29 of which cross-reference to a protein that can be acetylated (see Table S3 in the supplemental material). These proteins are involved primarily in translation and the stress response. On six of these proteins, the lysine that is acetylated is embedded within a SUMOylation consensus sequence, [FIVL]-xK[DE], suggesting that competition for lysine residues by different modification moieties exists beyond histone proteins (see Table S3 in the supplemental material). Acetylation may also influence the manifestation of posttranslational modifications (PTMs) occurring on other residues, such as phosphorylation of serine, threonine, or tyrosine. Recent computational modeling studies suggest that acetylation of a lysine can exert effects on nearby residues and influence the availability of the residue for phosphorylation and/or kinase binding (25). This “cross talk” between PTMs may be of importance in *Toxoplasma* since 78% (214 proteins) of the acetylated proteins reported here were also detected in a screen for phosphoproteins (38). The presence of multiple posttranslational modifications on individual proteins supports the idea that these marks interplay to constitute a dynamic regulatory program.

**Bromodomain proteins in Toxoplasma.** Bromodomains are well-conserved domains that recognize acetylated lysines, suggesting a role in protein-protein interactions (10). The two TgGCN5 KATs contain bromodomains, and we sought to determine if...
other bromodomain proteins exist in the parasite. Our survey of the ToxOxDB uncovered 12 putative bromodomain proteins present in Toxoplasma (Table 2), supporting the idea that this early-branching eukaryote has established a network capable of recognizing lysine acetylation signals. Seven of these bromodomain proteins are acetylated themselves, suggesting potential modes of autoregulation similar to those of the yeast SNF2 bromodomain protein (20).

Concluding remarks. Our data represent the first proteome-wide analysis of acetylated proteins for a single-celled eukaryote and parasite, highlighting the antiquity of this posttranslational modification in the course of evolution. Extensive protein acetylation takes place in intracellular tachyzoites among a wide variety of functional groups, likely impacting metabolism, transcription, translation, and stress responses. A large portion of these acetylation marks is found on parasite-specific proteins, demonstrating translation, and stress responses. A large portion of these acetylation marks is found on parasite-specific proteins, demonstrating that PTMs continue to evolve along species-specific trajectories. Moreover, we discovered novel acetylated lysines on Toxoplasma homologues of well-conserved proteins, including PEPCK, aldolase, and ACC.

The abundance of acetylation on nonnuclear proteins provides a probable explanation as to why several Toxoplasma KATs are present in the parasite cytosol. The inability to knock out or overexpress cytosolic KATs suggests that maintaining steady-state lysine acetylation is crucial in Toxoplasma tachyzoites (34, 41). Our results underscore the value of continued study of acetylation dynamics, as the machinery that governs the delivery, recognition, and removal of acetylation marks in protozoan parasites may contain promising new drug targets. Our findings also suggest that the mode of action for KDAC inhibitors like apicidin may involve more than dysregulation of parasite gene expression. Our studies demonstrate the technical feasibility of monitoring acetylated proteins in Toxoplasma, paving the way to a better understanding of how lysine acetylation may contribute to stress responses and parasite development. Due to inherent limitations of antibody affinity or the transient nature of the modification, it is likely that additional acetylated sites exist in Toxoplasma tachyzoite proteins. Lysine acetylation is found throughout all domains of life from bacteria to humans, but little progress has been made in understanding the physiological role of this modification. Our data will facilitate the generation of new hypotheses that will increase our understanding of lysine acetylation in the context of cell biology.

**TABLE 2 Predicted bromodomain proteins in Toxoplasma**

| ToxoDB accession no. | Product description | GenBank accession no. | (reference) |
|----------------------|---------------------|-----------------------|-------------|
| TGME49_063580        | 26S proteasome non-ATPase regulatory subunit, putative | XP_002365458          |            |
| TGME49_064640        | Bromodomain-containing protein | XP_002368593          |            |
| TGME49_058990        | Bromodomain-containing protein | XP_002365113          |            |
| TGME49_014240        | Bromodomain-containing protein | XP_002370757          |            |
| TGME49_076180        | Bromodomain-containing protein (TAFI/TAF250) | XP_002371624 (16)     |            |
| TGME49_043440        | Histone acetyltransferase GCN5 (GCN5-B) | XP_002366843 (2)      |            |
| TGGT1_004130         | Histone acetyltransferase (GCN5-A) | EEE21529.1 (37)       |            |
| TGME49_106460        | Hypothetical protein | XP_002370408          |            |
| TGME49_064020        | Hypothetical protein | XP_002365492          |            |
| TGME49_026810        | SET domain-containing protein/bromodomain-containing protein | XP_002366334          |            |
| TGME49_078440        | Transcription regulatory protein SNE2, putative | XP_002370547          |            |
| TGME49_111620        | WD repeat domain-containing protein | XP_002364394          |            |

* a Shading indicates that the protein was also acetylated, based on results from this study.
  b Where appropriate, literature references are provided.

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