



Bromodomains in Protozoan Parasites: Evolution, Function, and Opportunities for Drug Development

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SUMMARY Parasitic infections remain one of the most pressing global health concerns of our day, affecting billions of people and producing unsustainable economic burdens. The rise of drug-resistant parasites has created an urgent need to study their biology in hopes of uncovering new potential drug targets. It has been established that disrupting gene expression by interfering with lysine acetylation is detrimental to survival of apicomplexan (*Toxoplasma gondii* and *Plasmodium* spp.) and kinetoplastid (*Leishmania* spp. and *Trypanosoma* spp.) parasites. As “readers” of lysine acetylation, bromodomain proteins have emerged as key gene expression regulators and a promising new class of drug target. Here we review recent studies that demonstrate the essential roles played by bromodomain-containing proteins in parasite viability, invasion, and stage switching and present work showing the efficacy of bromodomain inhibitors as novel antiparasitic agents. In addition, we performed a phylogenetic analysis of bromodomain proteins in representative pathogens, some of which possess unique features that may be specific to parasite processes and useful in future drug development.

KEYWORDS *Toxoplasma*, *Plasmodium*, malaria, trypanosomes, *Leishmania*, epigenetics

INTRODUCTION

The epigenetic regulation of gene expression requires a wide spectrum of enzymes with functions including the alteration of posttranslational modifications (PTMs) on histones or reconfiguration of nucleosomes. The vast array of different PTMs and histone combinations has been postulated to constitute a code drafted by “writer” and “eraser” enzymes (1). Importantly, there are also “reader” proteins that interpret this

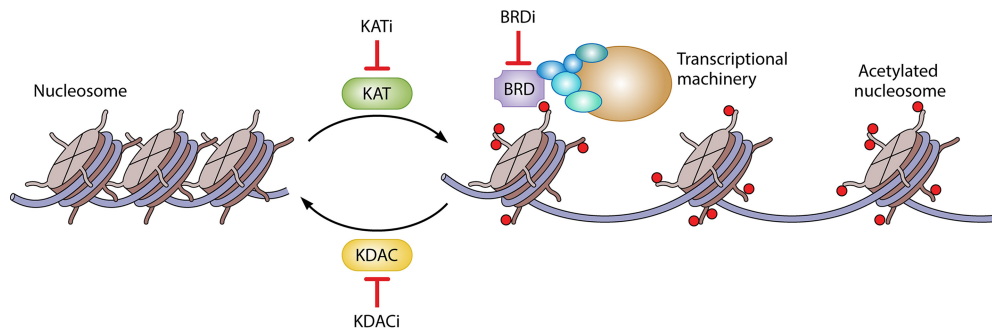


FIG 1 Regulation of transcription by lysine acetylation. Nucleosomes in the deacetylated state form tightly packed chromatin where transcription factors do not have access to regulatory sequences encoded in the DNA. The action of lysine acetyltransferases (KATs) adds acetyl groups to lysine residues in the histone tails, leading to a more open conformation of the chromatin and allowing access to the transcriptional machinery. Transcription may be further enhanced by recruitment of bromodomains to specific acetylated lysines and subsequent recruitment of additional transcriptional enhancers. Removal of the acetyl groups by lysine deacetylases (KDACs) restores chromatin to the closed state that is restrictive to transcription.

code by recognizing certain PTMs on specific residues of histone proteins. These reader domains may recognize acetylated lysines (bromodomains [BRDs]) or methylated lysines or arginines (e.g., chromodomains, PHD fingers, and Tudor domains) (2). These different factors work in concert to elicit changes in gene expression in response to developmental or environmental changes. Pathogenic protozoan parasites often move through complex life cycles involving multiple stages in more than one host. Consequently, pathogenic parasites rely on epigenetic control mechanisms to fine-tune gene expression, facilitating adaptation to different hosts/tissues or stressful environments. Emerging evidence has suggested that bromodomain-containing proteins are important mediators of gene expression that may represent novel drug targets in protozoal pathogens (3–5). This review discusses the various bromodomain proteins in representative parasites from the Apicomplexa and Euglenozoa phyla, providing novel insight into the evolution of this protein family and how it may contribute to parasite biology in the context of lysine acetylation signaling.

LYSINE ACETYLATION AND EPIGENETIC REGULATION OF GENE EXPRESSION

It is now well established that gene expression is regulated through covalent modification of histones. Addition or removal of chemical modifications such as acetylation, methylation, or phosphorylation can alter the structure and charge of the nucleosome (and thereby its interaction with DNA) or influence the recruitment of transcriptional regulatory machinery (1). Acetylation of histones is one of the best-studied modifications and is generally associated with transcriptional activation, although recent evidence suggests that in some cases histone acetylation may also mediate gene repression (6). Lysine acetyltransferases (KATs) generally facilitate gene activation by opening the chromatin structure to provide access for various protein complexes involved in gene transcription. Lysine deacetylases (KDACs) remove acetyl groups, restoring the heterochromatic structure that is refractory to gene activation (Fig. 1). Acetylation marks on histone tails can also serve as docking points for transcriptional regulators, recruited via their acetyl-lysine “reader” domain (Fig. 1). One of the best characterized “readers” that mediates protein-protein interaction through acetyl-lysine recognition is the bromodomain.

BROMODOMAINS: THE ACETYLATED LYSINE READERS

The bromodomain was first described in the characterization of the *Drosophila melanogaster* nuclear remodeling brahma protein, a SWI/SNF ATPase homologue known to activate gene expression (7), and was noted to be conserved on a number of other proteins involved in transcriptional activation in humans and yeasts (8). The structure of the bromodomain was first resolved for the KAT p300/CBP-associated factor (PCAF), which led to the discovery that it can act as a “reader” of acetylated lysine

residues (9). Comprised of ~110 amino acids, the bromodomain folds into a left-handed bundle of four α helices linked by two variable-loop regions (BC and ZA) that form a hydrophobic pocket for the acetylated lysine (10). The oxygen of the acetyl carbonyl group of the acetyl-lysine is typically anchored by a hydrogen bond to the amide nitrogen of an asparagine residue in the BC loop (11), and a tyrosine in the ZA loop binds the acetyl-lysine with four water molecules (10).

The variability in sequence and length of the ZA and BC loops among bromodomains likely contributes to recognition of specific targets dictated by the amino acids flanking acetylated lysine residues. Screening of 33 representative bromodomains against histone-peptide arrays supports the idea that not all bromodomains are created equal and that their binding activities can be influenced by amino acids flanking the target acetyl-lysine (10). Some bromodomains have affinity for a broad range of peptides, such as the bromodomain from PCAF and the transcriptional regulator SP140. Other bromodomains are far less promiscuous; the bromodomain from the transcriptional regulator tripartite motif containing 28 (TRIM28) specifically recognizes histone H3 acetylated at lysine 37 (H3K37ac), while the bromodomain from the mixed-lineage leukemia (MLL) methyltransferase has the highest affinity for histone H2A acetylated at lysine 15 (H2AK15ac). Finally, some bromodomains did not bind any histone peptides at all, consistent with recent studies showing that lysine acetylation is much more widespread within cells than first appreciated, linking metabolism and cellular signaling (12). Instances of bromodomains binding nonhistone acetyl-lysines include CREB binding protein (CREBBP), recognizing the acetylated lysine 382 on p53 in response to cellular stress (13). Another example involves modulating the association of SNF2 with the nucleosomal chromatin. Nucleosome-bound SNF2 can be acetylated by GCN5 on K1493 and K1497; the SNF2 bromodomain has a higher affinity for its own acetylated lysines than for acetylated histones and will dissociate from the nucleosome and bind to itself (14).

Bromodomain-containing proteins can further regulate transcription through modification of chromatin structure. Recently shown to possess KAT activity, BRD4 targets residues H3 K122, resulting in nucleosome eviction and chromatin decompaction (15, 16). Appropriate chromatin structure in sperm is maintained by the related bromodomain and extraterminal (BET) family member BRDT (17). Functions of bromodomain proteins also extend to DNA damage repair, which relies on rapid changes in chromatin structure to repress normal gene transcription and provide access to DNA repair complexes (18–21; reviewed in reference 22). The significant contribution of bromodomain proteins to DNA repair is underscored by their redistribution in response to DNA damage (18).

DEVELOPMENT OF BROMODOMAIN INHIBITORS

Given their importance in gene expression regulation, it is not surprising that a number of bromodomain-containing proteins have been linked to disease, including cancer, atherosclerosis, inflammation, cardiovascular disease, and diabetes (Table 1) (reviewed in references 23 and 24). Consequently, bromodomains are being investigated as potential drug targets. Several classes of bromodomain inhibitors that bind within the hydrophobic pocket, resulting in the exclusion of acetyl-lysine, have been reported. Many of the bromodomain inhibitors act as acetylated lysine analogues, forming hydrogen bonds in the hydrophobic pocket in a manner similar to the binding of the acetylated lysine (25–27).

The BET proteins contain tandem bromodomains in the C terminus and are major regulators of gene transcription by tethering transcriptional complexes to acetylated histones; as such, BET proteins have been a major focus of efforts to develop bromodomain inhibitors (25, 26, 28). The observation that thienodiazopines bind bromodomain protein BRD4 led to the development of JQ1, which binds with high selectivity and competes with acetylated histone peptides for the BRD4 bromodomains (25). JQ1 is able to displace the BRD4-nuclear protein in testis (NUT) oncogene fusion protein from chromatin, resulting in growth arrest of BRD4-NUT background carcinoma cells (25).

TABLE 1 Commercially available bromodomain inhibitors and their targets

Inhibitor	Target BRD(s)	Disease(s) and/or activity(ies)	Reference(s)
BET inhibitors			
I-BET151	BRD2, BRD3, BRD4	Endotoxic shock and sepsis, MLL-fusion leukemia, inhibits proliferation of <i>T. cruzi</i> and <i>T. gondii</i> , causes aberrant stage switching in <i>T. brucei</i>	3, 5, 26, 103
I-BET762	BET	Septic shock, neuroblastoma	104
(+)-JQ1	BET	Hematological malignancies, solid tumors, activates latent HIV, inhibits <i>T. cruzi</i> proliferation	5, 25, 49
PFI-1	BRD4	Anti-inflammatory, anti-lymphoma	105
OTX015	BET	Leukemia and lymphoma, NMC, castration-resistant prostate cancer, glioblastoma multiforme, activation of latent HIV	106–109
RVX 208	BET	Atherosclerosis, cardiovascular disease, diabetes	110–112
CPI-203	BET	Leukemia, pancreatic neuroendocrine tumors	113, 114
MS417	BET	Anti-inflammatory, colorectal cancer, activation of latent HIV	48, 115, 116
MS 436	BRD4	Anti-inflammatory	117
XD 14	BRD4	Leukemia	118
OXF BD 02	BRD4	Leukemia	119
Non-BET BRD inhibitors			
Ischemin	CREBBP		39
SCG-CBP30	CREBBP/EP300		120
I-CBP 112	CREBBP/EP300		
PF CBP1	CREBBP/EP300	Anti-inflammatory	40
BAZ2-ICR	BAZ2A/BAZ2B		43
GSK 2801	BAZ2A/BAZ2B		44
GSK 5959	BRPD1		121
PFI-4	BRPF1		121
IACS-9571	TRIM24/BRPF1		122
LP99	BRD7/BRD9	Anti-inflammatory	123
BI 9564	BRD7, BRD9	Antitumor	124
I-BRD9	BRD9		125
PFI-3	SMARCA2, SMARCA4, Polybromo 1		45, 126
Pan-BRD inhibitors			
Bromosporine	Broad spectrum		127
ISOX DUAL	CREBBP/EP300, BRD4		40

JQ1 has been applied to other human diseases, including liver cancer, inflammatory responses, and angiogenesis-related disease (29–31). Another BET family inhibitor, I-BET151, was shown to disrupt expression of genes activated in the inflammatory response to bacterial liposaccharide (LPS). Treatment of bone marrow-derived macrophages with I-BET151 led to downregulation of key LPS-stimulated cytokines and chemokines (26). I-BET151 has also shown promise for treatment of many different cancers in which cMYC is overexpressed due to the close regulatory relationship between this oncogene and BET proteins (32–36). The initial studies with both JQ1 and I-BET151 demonstrate that these synthetic compounds compete with acetylated lysines for bromodomains, resulting in gene expression changes that provide promising outcomes for several human diseases.

The tandem BRD arrangement of the BET family of proteins has prompted efforts to develop even more potent BET inhibitors by linking two BET inhibitor molecules, producing bivalent inhibitors that can simultaneously bind both BRDs in the BET protein. Encouragingly, these molecules appear to retain their specificity to the BET family but have significantly improved potency over the single BRD binding compounds (37, 38).

Small-molecule inhibitors of other bromodomain subtypes are now under development, not only for treatment of human disease but also as useful tools to delineate the functions of various bromodomain proteins. The association of the CREBBP bromodomain with acetylated p53 (13) has elicited interest in the utility of specific inhibitors of this interaction. A compound library screen using nuclear magnetic resonance (NMR) to assess binding to recombinant CREBBP bromodomain, followed by lead compound

optimization, led to the identification of ischemin, which displays at least 5-fold-greater selectivity for the CREBBP bromodomain than for other human bromodomains (39). An independent study to determine CREBBP bromodomain ligands identified the compound PF-CBP1, which downregulates a specific subset of inflammatory genes in macrophages that were unaffected during treatment with a BET-specific inhibitor (40).

The two closely related bromodomain-adjacent zinc finger (BAZ) bromodomain proteins BAZ2A and BAZ2B play essential regulatory roles in chromatin remodeling and have also been implicated in the regulation of noncoding RNAs (41, 42). Two independent attempts at structure-based compound optimization based on two initially weak inhibitors led to development of GSK2801 and BAZ2-ICR, selectively potent inhibitors of both BAZ2A and BAZ2B (43, 44).

SMARCA2 and SMARCA4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 2 or 4) are mutually exclusive members of a SWI/SNF chromatin-remodeling complex implicated in cellular differentiation and proliferation and are both linked to a variety of different cancers. The inhibitor PFI-3 selectively binds the SMARCA4 bromodomain and displaces it from chromatin; however, these studies have shown that inhibition of the bromodomain binding to the nucleosome alone is not sufficient for preventing proliferation of tumor cells and that inhibition of the ATPase activity of SMARCA4 is also required (45).

Pan-bromodomain inhibitors such as bromosporine and [1,2,4]triazolo[4,3-a]phthalazines have demonstrated inhibitory activity for many different bromodomains (46, 47). Their promiscuity suggests that they may be of limited value in the clinic, but these compounds represent useful tools in dissecting bromodomain function in many organisms.

The utility of bromodomain inhibitors in infectious disease has already been revealed with studies aiming to completely eradicate HIV from patients by reactivating the latent virus. The BET bromodomain BRD4 has been shown to suppress HIV transcription through interaction with the positive transcription elongation factor b (P-TEFb), sequestering P-TEFb from the viral protein transactivator of transcription (Tat), which is essential for viral gene expression. Another BET family member, BRD2, was also found to contribute to HIV latency through a Tat-independent mechanism that has yet to be determined (48). When cells harboring latent HIV were treated with inhibitors of the BET family, it led to reactivation of viral gene transcription (48–53).

The concept of targeting bromodomains to thwart human disease might also be applicable to treating infections caused by eukaryotic pathogens. We and others have identified numerous bromodomain-containing proteins in protozoa that are essential for viability, including a subset of KATs, offering a new opportunity for the development of antiparasitics. The study of bromodomains in these early-branching eukaryotic cells is also revealing new insights into the evolution of this critical acetyl-lysine recognition motif.

LYSINE ACETYLATION IN PROTOZOAN PARASITES

Parasitic protozoa present a devastating burden to global human and veterinary health. As most of these diseases hit impoverished nations, they are neglected in terms of research; there are few effective treatments, and those that do exist are seeing their utility evaporate in the face of rampant drug resistance. Some of the major parasitic diseases include malaria (caused by *Plasmodium* spp.), toxoplasmosis (*Toxoplasma gondii*), the diarrheal disease cryptosporidiosis (*Cryptosporidium* spp.), African trypanosomiasis/sleeping sickness (*Trypanosoma brucei*), American trypanosomiasis/Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania* spp.).

The importance of histone acetylation in apicomplexan parasites (*Plasmodium* spp., *Toxoplasma gondii*, and *Cryptosporidium* spp.) was first demonstrated in 1996 with the discovery of apicidin, a natural product that proved “cidal” against apicomplexan parasites and was subsequently found to be a histone deacetylase inhibitor (54). Later studies revealed extensive lysine acetylation machinery in these parasites that could be targeted with additional deacetylase inhibitors (55–59) or KAT inhibitors (60–63). More

recent discoveries suggest that, in addition to perturbing gene expression patterns, these inhibitors could be interfering with cellular signaling in the parasite nucleus and beyond. Proteome-wide surveys have uncovered hundreds of parasite proteins in *T. gondii* and *Plasmodium falciparum* that are subject to lysine acetylation in every subcellular compartment (64–66).

There is also evidence that lysine acetylation is critical in kinetoplastids (*Trypanosoma* spp. and *Leishmania* spp.), although these parasites have a very unusual mechanism of gene regulation. The kinetoplastid genome is arranged in gene clusters, and transcription produces polycistronic transcripts. Regulation of gene expression appears to be primarily posttranscriptional, via modulation of the splicing machinery that generates the mature mRNAs (67). Moreover, their histone sequences and the PTMs that decorate them deviate from what is observed in other eukaryotes (68–72). For example, histone H3 is relatively free of acetylation, the majority of modifications that have been identified are found on H4, and the C-terminal tail of histone H2A is heavily acetylated in *T. brucei* (71). Nevertheless, the kinetoplastids clearly use histone acetylation to regulate synthesis of the polycistronic transcripts (73, 74). Underscoring the pharmacological relevance of lysine acetylation in kinetoplastids are studies showing efficacy of some KDAC inhibitors against *T. brucei*, *T. cruzi*, and *Leishmania donovani* (75–78).

BROMODOMAIN PROTEINS IN PROTOZOAN PARASITES

Toxoplasma gondii

The first bromodomain-containing protein in protozoan parasites was identified with the discovery of a GCN5 family KAT in *T. gondii* (79, 80). *T. gondii* is unique in that it possesses two nuclear GCN5 KATs, each harboring a bromodomain near the C-terminal end of the protein (81). *T. gondii* GCN5a (TgGCN5a), while not essential in the replicative stage (tachyzoite) of the parasite, appears to contribute to the transition to the latent tissue cyst stage (bradyzoite) (82). The discovery that bromodomain proteins are involved in parasite differentiation is particularly important given the profound role that tissue cysts have in transmission and pathogenesis of *T. gondii* (83). In contrast, TgGCN5b is refractory to genetic disruption, suggesting that it is essential for tachyzoite replication. Inducible expression of a KAT-dead form of GCN5b acts as a dominant negative, leading to dysregulation of gene expression and parasite growth arrest (84). GCN5b is found in association with AP2 proteins (plant-like transcription factors present in Apicomplexa), the ADA2-A coactivator, and members of the TFIID complex, including another bromodomain-containing protein (TAF1). It is presumed that the TgGCN5 bromodomains function to bind acetylated lysines on histone tails (10, 85) in order to anchor the GCN5 complex to nucleosomes at target promoters, as seen for the yeast GCN5 complex (86).

In addition to those for GCN5a, GCN5b, and TAF1, there are nine other genes encoding proteins with predicted bromodomains in the *T. gondii* genome (ToxoDB.org) (Table 2). Another putative member of the TFIID complex, TAF2, contains a C-terminal bromodomain. There is also a predicted SET1 homologue with lysine methyltransferase activity, in addition to a number of PHD finger domains that may also function as PTM “readers.” These domains may act in concert with the bromodomain to target the SET1 methyltransferase activity to specific loci on the chromatin.

Plasmodium falciparum

There are seven bromodomain-containing proteins in the *P. falciparum* genome, and two have been characterized to date. Unlike for *T. gondii*, only a single GCN5 (PfGCN5) has been identified in *Plasmodium* species, but it does contain a single C-terminal bromodomain. As seen for both *T. gondii* GCN5s, PfGCN5 also displays a preference to acetylate lysine residues in the histone H3 tail, and it appears to be essential for blood-stage replication (87).

The domain architecture of *P. falciparum* bromodomain protein 1 (PfBDP1) contains a single C-terminal bromodomain and a number of ankyrin repeats; this architecture is

TABLE 2 Bromodomain proteins in protozoan parasites

Species and gene ID	Annotation	Protein name	Localization	Other domain(s)	Function	Reference
<i>P. falciparum</i>						
PF3D7_0629700	SET domain protein, putative	PfSET1				
PF3D7_0823300	Histone acetyltransferase GCN5	PfGCN5	Nuclear	KAT	Histone acetylation	87
PF3D7_1234100	Bromodomain protein, putative	TAF1	Nuclear	KAT	TFIID complex of RNA polymerase II	94
PF3D7_1033700	Bromodomain protein 1	PfBDP1	Nuclear	Ankyrin repeats	Regulation of invasion-related genes	4
PF3D7_1212900	Bromodomain protein 2, putative	PfBDP2			Associated with PfBDF1	4
PF3D7_0110500	Bromodomain protein, putative	PfBDP3				
PF3D7_1475600	Bromodomain protein, putative	PfBDP4				
<i>T. gondii</i>						
TGME49_254555	Histone lysine acetyltransferase GCN5-A	TgGCN5a	Nuclear	KAT	Histone acetylation	82
TGME49_243440	Histone lysine acetyltransferase GCN5-B	TgGCN5b	Nuclear	KAT	Essential, histone acetylation	84
TGME49_276180	Histone acetyltransferase TAF1/250	TAF1/250		KAT	TFIID complex of RNA polymerase II	95
TGME49_214240	Bromodomain-containing protein	TAF2/150			TFIID complex of RNA polymerase II	
TGME49_226810	Histone lysine methyltransferase SET1	TgSET1		SET, PHD		
TGME49_278440	SWI2/SNF2 brahma-like putative	TgBRG1		DEXDc, helicase		
TGME49_263580	Bromodomain-containing protein	TgBDP1		Ankyrin repeats		
TGME49_264640	Bromodomain-containing protein	TgBDP2				
TGME49_258990	Bromodomain-containing protein	TgBDP3				
TGME49_306460	Bromodomain-containing protein	TgBDP4				
TGME49_264020	Hypothetical protein	TgBDP5				
TGME49_311625	WD domain, G-beta repeat-containing protein	TgBDP6		WD40, PHD		
<i>T. cruzi</i>						
TcCLB.506247.80	DNA binding protein, putative	TcBDF1	Glycosome			90
TcCLB.506553.20	Bromodomain factor 2 protein	TcBDF2	Nuclear		Binds acetylated H4, accumulates after UV irradiation	88
TcCLB.510719.70	Bromodomain-containing protein 3	TcBDF3	Cytoplasm, flagellum			91
TcCLB.504213.70	Hypothetical protein, conserved	TcBDF4	Nuclear			
TcCLB.506755.120	hypothetical protein, conserved	TcBDF5	Nuclear			
<i>T. brucei</i>						
Tb927.10.8150	Predicted bromodomain protein	TbBDF1				
Tb927.10.7420	Bromodomain factor 2 protein, putative	TbBDF2			Regulation of VSG expression	3
Tb927.11.10070	Bromodomain, putative	TbBDF3			Regulation of VSG expression	3
Tb927.7.4380	Bromodomain, putative	TbBDF4		Zinc finger		
Tb927.11.13400	Bromodomain, putative	TbBDF5	Nuclear		Regulation of VSG expression	93
Tb927.1.3400	Hypothetical protein, conserved	TbBDF6				

conserved in the probable *T. gondii* orthologue (TGME49_263580) and appears to be unique to apicomplexan parasites. Inducible knockdown of PfBDP1 resulted in parasites that were unable to penetrate red blood cells (4). This invasion defect was concurrent with deregulation of invasion-associated genes after PfBDP1 knockdown. Chromatin immunoprecipitation sequencing (ChIP-seq) revealed that PfBDP1 associates with sequences upstream of these invasion-associated genes to activate their expression. Microscale thermophoresis experiments showed that PfBDP1 preferentially binds H3K9ac and H3K14ac, consistent with the role of this protein in gene activation. Coimmunoprecipitation revealed that PfBDP1 interacts with a set of nuclear proteins, including another bromodomain protein (PfBDP2) and an AP2 protein (PF3D7_0613800). Together, these findings are consistent with the idea that PfBDP1 acts like other bromodomain proteins in higher eukaryotes by tethering a transcriptional activator complex to acetylated H3 to control genes required for parasite invasion. It will be of interest to see if other bromodomain proteins in *Plasmodium* associate with discrete subsets of genes.

There are five additional predicted bromodomain proteins in *P. falciparum* (PlasmoDB.org) (Table 2). Similar to the case for *T. gondii*, the predicted SET1 homologue also contains an N-terminal bromodomain in combination with a number of PHD finger domains. The only other bromodomain-containing protein in *Plasmodium* with any known homology is the TFIID complex member TAF1.

Trypanosoma cruzi

Five bromodomain proteins (TcBDF1 to -5) have been identified in the *T. cruzi* genome (Table 2). The first to be described was TcBDF2, which is expressed throughout the life cycle in the parasite nucleus and associates with chromatin through interactions with H4K10ac and H4K14ac. Little is known about the biological function of TcBDF2 other than that its levels increase following UV irradiation (88), so it may be involved in coordinating a response to DNA damage.

While most bromodomain proteins are found predominantly in the nucleus, TcBDF1 and TcBDF3 are not nuclearly localized. TcBDF1 is expressed throughout the life cycle but is found at higher levels in the trypomastigotes, which are responsible for infection and dissemination in humans, than in the replicative, intracellular amastigotes or the epimastigotes, which infect the insect vector. Microscopy and cellular fractionation confirmed that TcBDF1 localizes to the parasite glycosome, a peroxisome-like organelle that is unique to kinetoplastids and required for biochemical processes related to glucose metabolism (89). Although the exact function and associated factors of TcBDF1 remain unknown, its importance for parasite viability was indicated by studies demonstrating that overexpression of the protein in epimastigotes led to growth arrest and parasite death (90). A mutant version of TcBDF1 had no effect on epimastigote growth but negatively impacted trypomastigote infection and amastigote replication (90). How these phenotypes relate to the localization of TcBDF1 at glycosomes, or the ability of the protein to bind acetylated lysines, remains to be elucidated.

TcBDF3 is also expressed in all *T. cruzi* life cycle stages and is notably associated with the parasite flagellum, possibly through association with acetylated α -tubulin (91). Acetylated α -tubulin is conserved across eukaryotes, yet no other bromodomain protein has been found to interact with this PTM. Overexpression of wild-type TcBDF3 had no effect on parasite replication, but overexpression of a TcBDF3 mutant unable to bind acetylated lysines impaired growth of insect-stage epimastigotes (5). The appearance of aflagellated parasites during overexpression of mutant TcBDF3 suggests that this bromodomain protein contributes to the formation and/or maintenance of flagella in epimastigotes. Curiously, mutant TcBDF3 still localized to microtubules, suggesting that its association with the cytoskeleton occurs independently of its ability to bind acetylated lysines (5).

Trypanosoma brucei

T. brucei possesses six bromodomain proteins (TbBDF1 to -6) (Table 2), at least four of which are nuclear: TbBDF1, TbBDF2, TbBDF3, and TbBDF5 (92). TbBDF3 colocalizes with H4K10ac, enriched in a narrow region at the 5' end of probable RNA polymerase II transcriptional start sites (TSSs). The authors proposed a model whereby TbBDF3 is recruited to RNA polymerase II TSSs by H4K10ac, which results in a subsequent recruitment of general transcription factors and chromatin-remodeling complexes to the TSS, in a manner similar to that for BDF1 in yeast. RNA interference (RNAi) knockdown of TbBDF3 led to a parasite growth defect and death (92).

Additional ChIP-seq studies performed by Schulz and colleagues confirmed the association of TbBDF3 with TSSs, along with TbBDF1 and TbBDF4; however, TbBDF2 did not localize to TSSs (3). Genetically engineered parasites lacking TbBDF2 or TbBDF3 exhibited impaired growth *in vitro*, with RNA-seq showing significant upregulation of variant surface glycoproteins (VSGs) located at silent bloodstream expression sites. VSGs are crucial for *T. brucei* to maintain a bloodstream infection in its mammalian host. The parasite carries a large repertoire of genes encoding variants of these surface proteins but expresses only a single variant at a time. The immune system of the host will mount a response against the VSG, upon which the parasite will switch to express an alternative VSG for immune evasion. Epigenetic regulation is critical for monoallelic expression of VSGs, and these data support an important role for bromodomains in maintaining monoallelic expression of VSG genes. Loss of either TbBDF2 or TbBDF3 also caused a dysregulation in parasite stage-specific gene expression, leading to upregulation of transcripts specific to the parasite insect stage. The importance of bromodo-

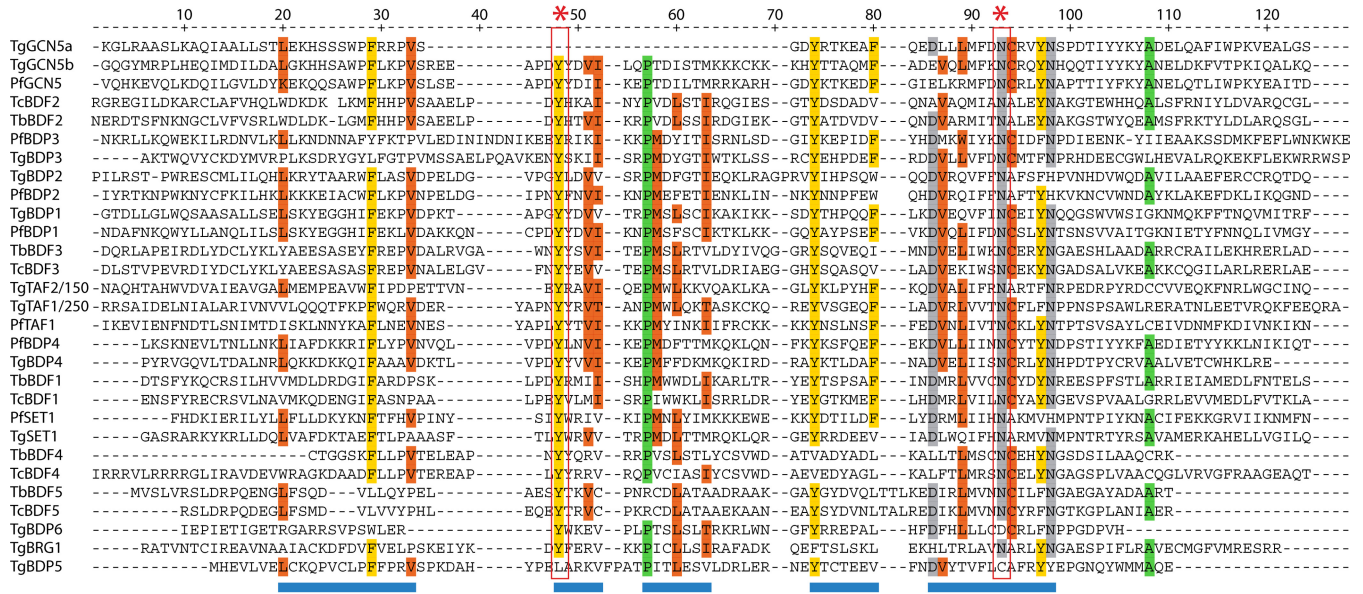


FIG 2 Alignment of bromodomain sequences found in representative parasites. Protein sequence alignments were performed in MUSCLE, and further alignments were modified by the predicted three-dimensional structure of each sequence with STRAP. The conserved blocks of the final alignments were selected by Gblocks 0.91b for tree-building (highlighted in blue). Phylogenetic analysis was performed with a maximum-likelihood method using PHYML 3.0 with the LG model. Branch support values were estimated by the approximate likelihood-ratio test (aLRT). The conserved tyrosine (Y) and asparagine (N) residues that mediate interaction with the acetylated lysine are highlighted with asterisks and shaded boxes. The first two letters of each accession number designate the species, as follows: Tg, *Toxoplasma gondii*; Pf, *Plasmodium falciparum*; Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*.

main proteins in maintenance of host-appropriate gene expression was validated by the observation that treatment with a bromodomain inhibitor severely compromised *in vivo* infection in mouse models (3).

Results from a genome-scale RNA interference viability screen revealed a potential role for TbBDF5 in regulating VSG gene expression sites (93). TbBDF5 localizes to the nucleus, and knockdown in both bloodstream and insect stages causes severe growth defects, indicating an essential regulatory function for TbBDF5 that has yet to be resolved.

Table 2 lists the bromodomain proteins for the species described above, along with what is currently known about localization and function. While there is some degree of conservation in function for some of the parasite bromodomain proteins, a number of species-specific bromodomains have arisen. We performed a phylogenetic analysis (see the next section) to shed more light on the bromodomain proteins among these parasite species.

CONSERVATION OF BROMODOMAINS IN PROTOZOAN PARASITES

To investigate the relationships of the 29 bromodomain-containing proteins from the four protozoan parasites *P. falciparum*, *T. gondii*, *T. brucei*, and *T. cruzi*, we performed a phylogenetic analysis of the bromodomain sequences, which are aligned in Fig. 2. Reciprocal BLAST searches of only the bromodomain sequence were combined with BLAST searches of the entire protein sequence, followed by manual curation of the two analyses to identify the most closely related bromodomain factors in these species. The final phylogenetic tree clustered these proteins into six groups, with three groups containing proteins from all four species (Fig. 3).

The first group contains the two GCN5s in *T. gondii* and the single GCN5 in *P. falciparum*, with each possessing one C-terminal bromodomain and one KAT domain. The bromodomains from both TgGCN5a (reciprocal blast E values, 3e−156 and 7e−162) and TgGCN5b (2.00e−173 and 2e−167) show even higher similarities with the bromodomain from PFGCN5 compared to each other (1e−144 and 3e−148). The close relationship of the essential KATs PFGCN5 and TgGCN5b suggests that these may carry out a housekeeping function, while TgGCN5a arose to perform a regulatory function

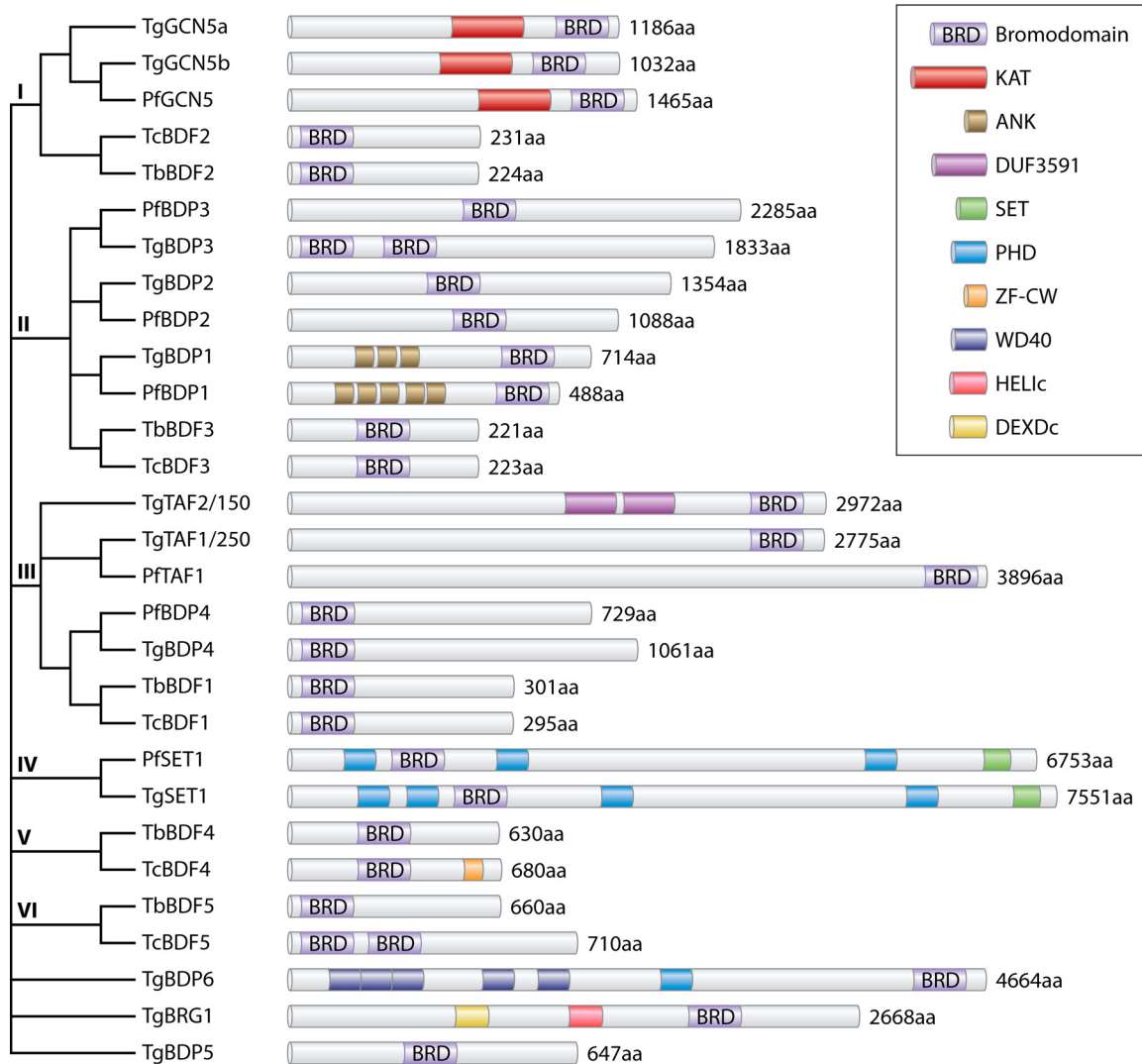


FIG 3 Phylogenetic analysis of protozoan bromodomains. The 29 bromodomains (BRDs) identified among the four parasite species shown in Fig. 2 clustered into six main clades with three outlier proteins from *T. gondii*. The domain architecture of each bromodomain protein is displayed to the right of the dendrogram. aa, amino acids.

that is unique to *T. gondii* (e.g., stage switching in response to stress [82]). We included the BDF2s from the two trypanosome parasites in this group because they reciprocally best match TgGCN5b and PfGCN5, suggesting some homology and potentially a comparable acetylated lysine substrate. Interestingly, trypanosome species lack a clear GCN5 homologue, making it tempting to speculate that BDF2 pairs with a different KAT to form a GCN5-like complex that has both a KAT domain and bromodomain. Group II is the largest grouping of parasite bromodomain proteins, containing three pairs of homologues between *T. gondii* and *P. falciparum*, including PfBDP1 and PfBDP2, and a pair of homologues from the two trypanosome species, TbBDF3 and TcBDF3. The proteins in this group do not show particularly high similarities but are most closely related compared with the other bromodomain sequences.

Group II members do not contain any other functional domains, with the exception of PfBDP1 and the related TgBDP1, which contain ankyrin domains in the N-terminal portion of the protein, presumably operating in protein-protein interactions.

Group III is comprised of the trypanosome BDF1 homologues, which cluster with two uncharacterized bromodomain proteins present in *P. falciparum* and *T. gondii*; members of this clade carry a single bromodomain in the N terminus, suggesting a common function. Also in this group are the apicomplexan TAF1 homologues (94, 95).

Two bromodomain proteins unique to the apicomplexan species also harbor C-terminal SET methyltransferase domains (group IV) that, based on domain architecture, appear to be homologues of the mixed-lineage leukemia (MLL) family of methyltransferases; these bromodomain proteins are the only ones examined that also have multiple PHD fingers, another domain that can act as a histone PTM “reader” (96).

Groups V and VI consist only of two homologous pairs of trypanosome proteins, BDF4 and BDF5, with significant similarities within each pair. The remaining three bromodomain proteins from *T. gondii* are not related to any other sequences investigated. It is surprising that only the *T. gondii* homologue of the putative SWI2/SNF2 protein TGME49_278440 contains a bromodomain. This family of chromatin remodelers contains a C-terminal bromodomain in higher eukaryotes, yet the homologues in the other protozoal species examined lack this domain. It is also significant that the remaining two bromodomains do not contain the conserved asparagine residue required for acetyl-lysine recognition. In TgBDP6, the position of the conserved asparagine is occupied by an aspartic acid (Fig. 2), which has been reported for some atypical bromodomain proteins such as MLL (97). Interestingly, a cysteine is located in the position of the conserved asparagine in TgBDP5, which has not been reported to date; experimental validation is required to confirm if this bromodomain binds acetylated lysines.

BROMODOMAIN INHIBITION IN PROTOZOAN PARASITES

As mentioned above, several bromodomain inhibitors have been developed in hopes of treating multiple types of disease by interfering with lysine acetylation signaling. We may be able to add infections caused by protozoan parasites to this list, as genetic studies have shown that most parasite bromodomain proteins are crucial for viability and virulence. Some studies have already begun to examine the effects of bromodomain inhibitors against these protozoan parasites, and these complement previous work showing the antiparasitic activities of KAT and KDAC inhibitors (55–63).

The effects of seven bromodomain inhibitors on *T. cruzi* have been assessed, and three (JQ1, I-BET151, and GSC-CBP30) displayed promising inhibitory effects on parasite replication. These inhibitors were shown to interact with the bromodomain of TcBDF3, and overexpression of the protein decreased parasite sensitivity to the compounds (5).

I-BET151 helped identify a critical role for *T. brucei* bromodomain proteins in the maintenance of the bloodstream stage, which includes immune evasion through retention of monoallelic VSG gene expression (3). However, I-BET151 impacts human bromodomain proteins that affect host immunity, which may preclude its use against infections (26). Further characterization of parasite bromodomains is needed to develop more specific inhibitors against those that are required for *in vivo* pathogenesis. Recently, TbBRD2 was crystallized with I-BET151 and revealed a novel, flipped binding mode of this inhibitor, underscoring the value of this approach toward rational drug design (26).

Preliminary studies of bromodomain inhibitors in *T. gondii* have also shown I-BET151 to be a potent inhibitor of tachyzoite replication (128). At present, bromodomain inhibitors have not been analyzed for potential activity against *Plasmodium*.

FUTURE DIRECTIONS

The functions of the bromodomain proteins in parasitic protozoa are just beginning to be uncovered. The development of high-throughput and sensitive proteomics technologies as well as the introduction of the genomic editing tool CRISPR/Cas9 will facilitate closer inspection of the biological processes that these proteins regulate. The remarkable discovery of bromodomain proteins outside the nucleus in *T. cruzi*, coupled with the growing catalogue of acetylation marks on proteins involved in many different functions, implies that bromodomain inhibitors could effectively perturb multiple cellular pathways in the parasites.

Protein binding arrays, such as those used for the human bromodomains (10), can be applied to parasite bromodomains in order to determine the acetylated lysine

substrates that these proteins recognize, which will help unravel the precise signaling pathways that are regulated by each factor. It will be of particular interest to identify the substrates of the nonnuclear bromodomain factors, as these functions will likely be unique to the protozoans.

There is emerging evidence that other domains are capable of acetylated lysine recognition, such as the double PHD finger and double pleckstrin domains and the YEATs domain (98–102). Combing the available parasite genome sequences will uncover any parasite homologues of these factors that may also prove to be valuable targets for inhibiting lysine acetylation pathways. Novel acetylation recognition domains may be revealed by probing parasite lysates with immobilized acetylated peptides to identify the parasite proteins that bind the acetyl mark.

The bromodomains and other reader domains that are essential to parasite proliferation and development could be screened against compound libraries to identify lead compounds. These inhibitors will be invaluable for drug development but will also be useful tools for further dissection of bromodomain function in protozoans. Ongoing development of bromodomain inhibitors for human disease will generate a multitude of bromodomain binding compound libraries that may then be screened for selective activity against parasite bromodomains. This offers a tantalizing opportunity to develop effective antiparasitic therapies with minimal side effects to help alleviate the heavy burden of parasitic disease.

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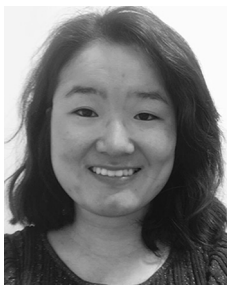
Victoria Jeffers obtained her B.A. Mod. in microbiology from Trinity College, Dublin, Ireland. She then studied at Dublin City University to receive her M.Sc. in bioinformatics before pursuing her Ph.D. studies through the Antimal PhD Programme, as part of the EMBL Network of Excellence. Her Ph.D. research was performed at the Biomedical Primate Research Centre, The Netherlands, and the University of Heidelberg, Germany, where she studied food vacuole formation and morphology in the primate malaria parasite *Plasmodium knowlesi*. She then moved to Indiana University School of Medicine (IUSM), Indianapolis, IN, USA, to carry out her postdoctoral research investigating the functional significance of lysine acetylation in *Toxoplasma gondii*, performing the first global proteomic survey of acetylated lysines in a protozoan organism. Now an Assistant Research Professor at IUSM, she continues to pursue her interests in lysine acetylation in *Toxoplasma*, studying the mechanistic roles of bromodomain proteins in regulating parasite proliferation and differentiation.



Chunlin Yang obtained his B.S. in biology from the Northeast Agricultural University in China. He then moved to Kunming Institute of Zoology, Chinese Academy of Sciences, for his graduate studies and received his M.S. in evolutionary biology. He continued his graduate studies at East Carolina University in North Carolina, where he investigated the evolution of the C-terminal domain of the RNA polymerase II large subunit. After he received his Ph.D. from East Carolina University, he joined the laboratory of Dr. Gustavo Arrizabalaga at the Indiana University School of Medicine (IUSM) as a postdoctoral fellow. Dr. Yang performed the phylogenetic analysis of parasite bromodomain proteins and is also analyzing kinases and phosphatases in *Toxoplasma gondii*.



Sherri Huang received her B.A. in biology from Washington University in St. Louis, MO. As an undergraduate, she worked in the laboratory of Dr. Petra Levin, investigating regulators of *Bacillus subtilis* cell division. After college, she worked in the laboratory of Dr. Nicole King at the University of California, Berkeley, where she helped study the microbial symbiosis driving choanoflagellate colony formation. She joined the Medical Scientist Training Program at Indiana University School of Medicine in 2011 to pursue her M.D. and Ph.D. degrees. Her Ph.D. studies involve the characterization of transcriptional mechanisms in *Toxoplasma gondii*.



William J. Sullivan, Jr., received his Ph.D. in cell and molecular biology at the University of Pennsylvania in Philadelphia, PA, based on his discovery of novel drug resistance genes in *Toxoplasma gondii*. He conducted some of the first investigations of epigenetic gene regulation in *T. gondii* during postdoctoral studies at Elanco/Eli Lilly and at the Indiana University School of Medicine (IUSM). He is now Showalter Professor in the Departments of Pharmacology & Toxicology and Microbiology & Immunology at IUSM. Dr. Sullivan continues to study gene expression control in *T. gondii* at the levels of transcription, translation, and epigenetics in hope of gaining a better understanding as to how parasites progress through their different life cycle stages.

