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Metabolically-derived lysine acylations and neighboring modifications tune BET bromodomain binding to histone H4

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Abstract

Recent proteomic studies discovered histone lysines are modified by acylations beyond acetylation. These acylations derive from acyl-CoA metabolites, potentially linking metabolism to transcription. Bromodomains bind lysine acylation on histones and other nuclear proteins to influence transcription. However, the extent bromodomains bind non-acetyl acylations is largely unknown. Also unclear are the effects of neighboring post-translational modifications, especially within heavily modified histone tails. Using peptide arrays, binding assays, sucrose gradients, and computational methods, we quantified ten distinct acylations for binding to the bromodomain and extraterminal domain (BET) family. Four of these acylations – hydroxyisobutyrylation, malonylation, glutarylation, and homocitrullination – had never been tested for bromodomain binding. We found *N*-terminal BET bromodomains bound acetylated and propionylated peptides consistent with previous studies. Interestingly, all other acylations inhibited BET bromodomain binding to peptides and nucleosomes. To understand how context tunes bromodomain binding, effects of neighboring methylation, phosphorylation, and acylation within histone H4 tails were determined. Serine-1 phosphorylation inhibited BRD4 *N*-terminal bromodomain binding to polyacetylated H4 tails by >5-fold whereas methylation had no effect. Furthermore, BRDT and BRD4 *N*-terminal bromodomain binding to H4K5acetyl was enhanced 1.4- to 9.5-fold by any neighboring acylation of lysine-8, indicating a secondary H4K8acyl binding site that is more permissive of non-acetyl acylations than previously appreciated. In contrast, *C*-terminal BET bromodomains exhibited 9.9- to 13.5-fold weaker binding for polyacetylated compared to monoacetylated H4 tails indicating the *C*-terminal bromodomains do not cooperatively bind multiple acylations. These results suggest acyl-CoA levels tune or block BET bromodomain recruitment to histones, linking metabolism to bromodomain-mediated transcription.

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Notes

The authors declare no competing financial interests.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting methods, additional ITC data for histone H4 peptides containing propionyl, crotonyl, formyl, hydroxyisobutyryl, succinyl, and homocitrulline modifications (Supplementary Figure 1), and expected and observed masses of synthesized peptides (Supplementary Table 2) (PDF).

Quantitation of BRD4-BD1 binding to the EpiTitan histone peptide array (Supplementary Table 1) (Excel).

Graphical Abstract

INTRODUCTION

Bromodomains are ~110 amino-acid protein modules that recognize acetyl-lysine residues within histones and other nuclear proteins.¹ The bromodomain and extraterminal domain (BET) family consists of BRDT, BRD2, BRD3 and BRD4, each containing two bromodomains (referred to herein as BD1 and BD2) located *N*-terminal to the extraterminal domain, which is located near the *C*-terminus. Small molecules targeting the acetyl-lysine binding pockets of BET bromodomains (*e.g.* JQ1)² have rapidly progressed to clinical trials for subsets of cancer and cardiovascular disease.³ Due to the broad therapeutic promise associated with BET bromodomain inhibition, a firm understanding of the binding specificity of BET bromodomains is critical to predict and avoid potential adverse side effects of BET bromodomain inhibition.⁴ Towards this end, a handful of histone acetylation sites have been demonstrated to bind BET bromodomains with varying degrees of confidence.^{1, 5–9} BET bromodomain binding affinity has also been shown to be regulated combinatorially by neighboring serine/threonine phosphorylation and lysine/arginine methylation within histone H3 as well as arginine citrullination within E2F-1.^{1, 9} However, the effects of neighboring modifications on BET bromodomain binding to the histone H4 *N*-terminal tail, hypothesized to be the primary binding partner of the *N*-terminal (BD1) BET bromodomains,^{1, 6} remain unclear.

Bromodomain binding specificity is complicated by the recent discovery of a wide variety of histone lysine acylations beyond acetylation including formylation,¹⁰ homocitrullination,¹¹ propionylation,¹² butyrylation,¹² crotonylation,¹³ hydroxyisobutyrylation,¹⁴ malonylation, succinylation¹⁵ and glutarylation.¹⁶ Accumulating evidence indicates these acylations possess roles in transcriptional regulation distinct from acetylation.¹⁷ For example, butyrylation of histone H4 at gene promoters was recently found to block BRDT binding whereas acetylation induced BRDT binding leading to specific gene expression programs and chromatin reorganization.¹⁸ The proteomic discovery of 246 unique sites of lysine acylation within histones¹⁷ suggests an intricate relationship between metabolic regulation of acyl-CoA levels and epigenetic regulation of gene transcription through selective bromodomain binding of distinct histone acylations. Illustrative of the connection between acyl-CoA metabolism and lysine acylation, clinically relevant enzyme deficiencies such as propionyl-CoA carboxylase, short-chain acyl-CoA dehydrogenase, malonyl-CoA decarboxylase and acyl-CoA synthetase 2 are associated with increased protein lysine propionylation, butyrylation, malonylation and crotonylation.^{19, 20}

Critical to determining how metabolism controls gene transcription is understanding how these novel histone acylations recruit bromodomains to chromatin. While none of the bromodomains tested to date bind lysine succinylation and only one (PHIP) binds lysine formylation,²¹ several bromodomains (*e.g.* BRD7, BRD9, CECR2, and TAF1) bind lysine propionylation, butyrylation, and crotonylation.^{21, 22} However, the ability of bromodomains to interact with homocitrullination, hydroxyisobutyrylation, malonylation, and glutarylation remained unknown. Here, we combine peptide arrays, isothermal titration calorimetry (ITC) and fluorescence polarization (FP) binding assays, sucrose gradients with acylated nucleosomes, and computational methods to quantify the BET bromodomain binding specificity towards recently discovered histone lysine acylations as well as the combinatorial effects of neighboring methylation, phosphorylation and acylation within the histone H4 *N*-terminal tail.

MATERIALS AND METHODS

Materials

Acetonitrile, trifluoroacetic acid and acyl anhydrides were purchased from Sigma. Fmoc amino acids and Rink-amide resin were purchased from Chem-Impex. JQ1 was purchased from eNovation. Ni-NTA superflow resin was purchased from 5 PRIME.

Protein purification

Recombinant His₆-tagged BET bromodomain constructs (Addgene, pNIC28-Bsa4 vector) were purified from BL21(DE3) *E. coli* using nickel affinity chromatography. Cells were transformed and grown in 2–4 L of LB in the presence of 50 µg/mL kanamycin to an optical density of 0.6–0.8 at 600 nm. Protein expression was induced with 0.1 mM IPTG and the cells were incubated overnight at 18 °C. Cells were harvested by centrifugation at 5,000 × g and re-suspended in 30 mL of lysis buffer (50 mM HEPES pH 7.5 at 20 °C, 500 mM NaCl, 5% v/v glycerol and 5 mM imidazole) supplemented with protease inhibitors (0.3 µM Aprotinin, 1 µM E-64, 1 µM Leupeptin, 1 µM Bestatin, 1 µM Pepstatin and 100 µM PMSF). Cells were lysed by sonication and lysates were cleared by centrifugation for 30 min at 30,000 × g. The lysates were then applied to Ni-NTA resin (0.75 mL resin/L of bacterial culture) and rocked for 1 h at 4 °C. The supernatant was discarded and the Ni-NTA resin was applied to a column and washed twice with 50 mL of lysis buffer. The protein was eluted using a step gradient of increasing concentrations of imidazole in lysis buffer (5 mL of 50, 100, 150, 200 and 250 mM imidazole). Fractions were monitored by SDS-PAGE and those containing recombinant protein were concentrated to a volume of 1 mL and applied to an Enrich SEC 70 10 × 300 mm column (Bio-Rad) to exchange the buffer to 25 mM HEPES (pH 7.5 at 20 °C), 150 mM NaCl and 5% v/v glycerol. Samples containing recombinant protein were identified by SDS-PAGE and concentrated to 5–10 mg/mL, flash frozen in liquid nitrogen and stored at –80 °C until used.

Histone modification peptide array

The EpiTitan histone peptide array (EpiCypher 11-2001) was hybridized and analyzed according to the manufacturer's instructions. BRD4-BD1 was added to the array at a concentration of 5 µM. BRD4-BD1 binding to the array with an anti-His₆ tagged primary

antibody (Millipore 05-949 clone HIS.H8) (1:500 dilution) followed by a goat anti-mouse IgG2b secondary antibody conjugated with Alexa Fluor 647 (Invitrogen A-21242) (1:1000 dilution). The array was scanned using a Typhoon Trio Imager and quantified using ImageQuant software (GE healthcare).

ChIP-seq data analysis

H4K5acetyl (ENCFF017IQV) and H4K8acetyl (ENCFF827EYY) ChIP-seq datasets in H9 human embryonic stem cells (hESCs) were downloaded from the ENCODE database in Browser Extensible Data (BED) format. BRD2 (GSM1466837), BRD3 (GSM1466839) and BRD4 (GSM1466835) ChIP-seq data sets in H9 hESCs²³ were downloaded from the GEO database through NCBI in BED format. H4K5acetyl and H4K8acetyl peak occupancy within 3 kb of hg19 TSS overlapped by BET protein peaks was determined using BEDTools²⁴ in conjunction with Pybedtools.²⁵ Finally, average peak profiles surrounding these TSS were visualized using ChIPseeker.²⁶

Solid-phase peptide synthesis

12-residue histone H4 tail peptides (base sequence Boc-SGRGKGGKGLGY) were synthesized (0.05 – 0.1 mmol scale) using standard tBu/Fmoc solid-phase peptide synthesis by the Protein Chemistry Core at the Blood Center of Wisconsin on a CEM Liberty1 microwave assisted peptide synthesizer. The protecting groups used were tBu for serine and tyrosine, Pbf for arginine and Dde or Boc for lysine. Peptides containing acetyl- and formyl-lysine and homocitrulline were synthesized from the corresponding commercially available Fmoc-Lys(acetyl)-OH, Fmoc-Lys(formyl)-OH or Fmoc-L-hCit-OH building blocks. For propionyl-, butyryl-, crotonyl-, hydroxyisobutyryl-, malonyl-, succinyl- and glutaryl-lysine containing peptides, Fmoc-Lys(dde)-OH was incorporated at the desired site(s) of acylation for subsequent on-resin derivatization. Dde-protected lysines on the full-length peptides were deprotected twice with ~3 mL of 2% v/v hydrazine in DMF. For peptides containing propionyl-, butyryl-, crotonyl-, succinyl- or glutaryl-lysine, deprotected lysine residues were coupled using ~10 equiv of the corresponding acyl anhydride and ~20 equiv DIEA in DMF. For peptides containing hydroxyisobutyryl-lysine, deprotected lysines were coupled using ~10 equiv of α -hydroxyisobutyric acid activated with 9 equiv HBTU, 9 equiv HOBt and 20 equiv DIEA in DMF. For the malonyl-lysine containing peptide, deprotected lysines were coupled using ~10 equiv of tbutyl-malonate-NHS ester (see below for synthesis) in DMF. To confirm complete coupling, Kaiser tests²⁷ were performed by heating a small amount of resin in equal parts solutions A, B and C for 4 min at 120 °C and checking for lack of color change due to the presence of free amines. Solution A was prepared by mixing 40 g of phenol with 10 mL of ethanol. Solution B was prepared by dissolving 64 mg of KCN in 100 mL of water and then diluting 2 mL of the KCN solution to 100 mL with pyridine. Solution C was prepared by dissolving 2.5 g of ninhydrin in 50 mL of ethanol. After completion of the synthesis, the resin was rinsed with dichloromethane and dried. Full-length peptides were fully deprotected and cleaved from the resin with 95% v/v TFA, 2.5% v/v H₂O and 2.5% v/v triisopropylsilane. Peptides were precipitated with 10–20 mL of cold (–20 °C) diethyl ether and pelleted by centrifugation for 5 min at 4,000 rpm followed by washing twice with cold diethyl ether. The precipitate was dried, dissolved in water, and lyophilized. Crude peptides were purified by semipreparative HPLC on a 5 μ m particle size Hypersil

GOLD C18 column (ThermoFisher, 4.6×250 mm) using an Agilent 1100 series HPLC eluting with a gradient of 5–60% v/v acetonitrile in water with 0.1% v/v TFA. Fractions collected were lyophilized to yield final peptides as dry white powders. High-resolution masses of the cleaved and deprotected peptides were confirmed by direct injection ESI mass spectrometry (QExactive, Thermo Scientific) (Table S2). Peptide concentrations were determined from tyrosine absorbance at 280 nm (extinction coefficient $1480 \text{ M}^{-1}\text{cm}^{-1}$).

Synthesis of tButyl-malonate-NHS ester

tButyl-malonate-NHS ester was synthesized as previously described.²⁸ Briefly, to mono-tert-butyl-malonate (480 mg, 3.0 mmol) in anhydrous DMF (2.0 mL) was added *N*-hydroxysuccinimide (365 mg, 2.9 mmol) with stirring at room temperature. Then *N,N'*-dicyclohexylcarbodiimide (598 mg, 2.9 mmol) in anhydrous DMF (3.0 mL) was added to the reaction. After stirring for 2 h, the reaction mixture was filtered and stored at -20°C for use in malonyl-lysine peptide coupling (see Solid-phase peptide synthesis).

Isothermal titration calorimetry

Binding affinities of diacylated histone H4(1-11)K5/8acyl peptides for BRD4-BD1 were determined using a VP-ITC instrument (MicroCal). Briefly, 0.5 mM H4K5/8diacyl peptide was injected ($1 \times 2 \mu\text{L}$ injection followed by $29 \times 8 \mu\text{L}$ injections) into the cell containing 20 μM BRD4-BD1, and heats of binding were measured. The buffer used for ITC analysis included 25 mM HEPES (pH 7.5 at 20°C), 150 mM NaCl and 5% v/v glycerol. Protein concentrations were determined using the method of Bradford.²⁹ The least-squares fits to the binding parameters ΔH° , K_d , and N were determined from the raw data using Origin (OriginLab).

Synthesis of JQ1-TAMRA fluorescence polarization probe

JQ1 (5.29 mg, 13.42 μmol) was added to 1 mL of 4 M HCl in dioxane and the mixture was stirred overnight. The reaction was dried under reduced pressure then redissolved and dried three times with ~ 1 mL dichloromethane to remove residual HCl and dioxane. The resulting solid was dissolved in 300 μL anhydrous DMF and to the solution was added HBTU (5.60 mg, 14.8 μmol), DIEA (8.58 μL , 49.3 μmol) and 5(6)-TAMRA ethylenediamine (6.00 mg, 8.56 μmol). The resulting JQ1-TAMRA was purified by semipreparative HPLC on a 5 μm particle size Hypersil GOLD C18 column (ThermoFisher, 4.6×250 mm) using an Agilent 1100 series HPLC eluting with a gradient of 5–95% v/v acetonitrile in water with 0.1% v/v TFA. Mass of JQ1-TAMRA was confirmed by direct injection ESI mass spectrometry (QExactive, Thermo Scientific). HRMS (ESI): Exact mass calculated for $\text{C}_{46}\text{H}_{44}\text{ClN}_8\text{O}_5\text{S}$ [M] 854.2766, found 854.2731. JQ1-TAMRA concentration was determined from TAMRA absorbance at 556 nm using an extinction coefficient of $89,000 \text{ M}^{-1}\text{cm}^{-1}$.

Fluorescence polarization

For the direct binding experiments, recombinant BET bromodomains were titrated at concentrations ranging from 1 nM to 10 μM against 20 nM of JQ1-TAMRA in 100 μL total volume. Fluorescence polarization values at each BET bromodomain concentration were converted to fraction JQ1-TAMRA bound (FB) using the following equation:

$$FB = \frac{P_{obs} - P_F}{P_B - P_F}$$

where P_{obs} is the measured polarization at the particular BET bromodomain concentration, P_F is the polarization of free JQ1-TAMRA, P_B is the polarization of fully bound JQ1-TAMRA. No correction was necessary for different fluorescence intensity of the free and bound forms of JQ1-TAMRA.

The K_d values for JQ1-TAMRA binding to BET bromodomains were then calculated from plots of fraction JQ1-TAMRA bound versus BET bromodomain concentration using the following equation:

$$FB = \frac{K_d + [L] + [B] - \sqrt{(K_d + [L] + [B])^2 - 4[L][B]}}{2[L]}$$

where $[L]$ is the concentration of JQ1-TAMRA and $[B]$ is the BET bromodomain concentration.

For the histone peptide competition experiments, recombinant BET bromodomains (0.6–1.5 μ M) were incubated with 20 nM JQ1-TAMRA and various concentrations of acylated H4(1-11) peptides ranging from 3 μ M to 3 mM in 30 μ L total volume. In both cases, fluorescence polarization was measured at 25 °C using a FlexStation 3 Multi-Detection Reader with 556 nm excitation and 575 nm emission wavelengths. Fluorescence polarization IC_{50} values of histone peptides in competition with JQ1-TAMRA were calculated as previously described.³⁰ These IC_{50} values were used to calculate K_d values as previously described³¹ using the following equation:

$$K_d^{peptide} = \frac{[I]_{50}}{\frac{[L]_{50}}{K_d} + \frac{[P]_0}{K_d} + 1}$$

where $[I]_{50}$ is the concentration of the free acylated H4 peptide at 50% competition, $[L]_{50}$ is the concentration of free JQ1-TAMRA at 50% competition, $[P]_0$ is the bromodomain protein concentration added to the assay, K_d is the dissociation constant of the JQ1:BET bromodomain complex, and $K_d^{peptide}$ is the dissociation constant of the acylated H4 peptide:BET bromodomain complex.

Chick erythrocyte nucleosome purification

100 μ L of chick erythrocyte nuclei (10 mg/mL) in 0.25 M sucrose, 10 mM $MgCl_2$ and 10 mM Tris (pH 8.0) was added to 200 μ L of 100 mM NaCl, 1 mM $CaCl_2$ and 40 mM Tris (pH 8.0). After a 3 min equilibration at 35 °C, 2 μ L of micrococcal nuclease 5 U/mL) was added and the solution was incubated at 35 °C for 12.5 min. The nuclease reaction was then quenched with 6 μ L of 250 mM EDTA and the mixture was pelleted for 4 min at 13,200

rpm. The pellet was resuspended in 300 μ L of 1 mM EDTA and pelleted for 4 min at 13,200 rpm and 200 μ L of the resulting supernatant was applied to a 4 mL sucrose gradient (5–20% w/v sucrose with 1 mM EDTA, pH 8.0) and centrifuged at $55,000 \times g$ for 3.5 h. The sucrose gradient was then collected in fractions and nucleosome populations were separated by DNA agarose gel electrophoresis and identified by ethidium bromide staining according to the length of histone-bound DNA. Concentrations of purified nucleosomes were determined according to DNA absorbance at 260 nm using an extinction coefficient of $6,600 \text{ M}^{-1}\text{cm}^{-1}$.

Sucrose gradient binding assay

Following sucrose gradient purification, chick erythrocyte nucleosomes were chemically acylated as previously described³² with 1 mM acetic, propionic, butyric or glutaric anhydride in the presence of 100 mM NaCl for 1 h at room temperature. Acylation reactions were quenched with 100 mM Tris (pH 8.0). Nucleosomes were precipitated by adding 1 reaction volume of 10% w/v trichloroacetic acid, pelleted by centrifugation at 15,000 rpm for 10 min, and resuspended in 25 mM Tris (pH 8.0) with 100 mM NaCl. 200 μ L of mononucleosomes (500 nM) combined with 2.5 μ M His₆-tagged BRD4-BD1 were applied to a 4 mL sucrose gradient (5–20% w/v sucrose with 1 mM EDTA, pH 8.0) and centrifuged at $55,000 \times g$ for 3.5 h. The sucrose gradient was then collected in fractions. Nucleosome-containing fractions were identified by agarose gel electrophoresis combined with ethidium bromide staining and BRD4-BD1 containing fractions were identified by anti-His₆ tag immunoblotting. Membranes were blocked with PBST with 3% w/v BSA and western blots were performed using an anti-His₆ tag primary antibody (Abgent, AM1010A) at a dilution of 1:1000 followed by anti-mouse IgG secondary antibody HRP (GeneTex, GTX213111-01) at 1:10,000 and detection by chemiluminescence.

Molecular modeling and peptide docking

A co-crystal structure (PDB ID: 3UVW) of BRD4-BD1 complexed with an H4 peptide diacetylated at H4K5 and H4K8 (H4K5/8diacetyl) was used as the initial model for all calculations in Schrödinger (2016-3 release). BRD4-BD1 and peptide ligands were prepared in Maestro (version 10.6.014) using the Protein Preparation Wizard. BRD4-BD1/histone tail peptide complexes were minimized in Maestro using Embrace in energy difference mode and binding energies were calculated using the Prime MMGBSA function (v3.000).

RESULTS AND DISCUSSION

Binding of the *N*-terminal bromodomain of BRD4 to acetylated H4 tails is enhanced by neighboring acetylation, unperturbed by neighboring methylation, and inhibited by neighboring phosphorylation

To identify potential neighboring histone modifications that alter recruitment of the *N*-terminal bromodomain of BRD4 (BRD4-BD1) to chromatin, binding of hexahistidine (His₆)-tagged BRD4-BD1 to the EpiTitan Histone Peptide Array was measured using an anti-His₆ antibody followed by fluorescent detection of the secondary antibody. The EpiTitan array contains 265 multiply modified histone peptides encompassing 95 unique individual modifications including lysine acetylation, serine phosphorylation, and arginine/lysine methylation. Each peptide is spotted in triplicate on the array. Through this unbiased

peptide array-based approach, the detected signal reflective of BRD4-BD1 binding for polyacetylated histone H4 tail peptides was 40-fold higher compared to polyacetylated histone H2A, H2B or H3 tail peptides (Figure 1A; Table S1) consistent with previous histone tail peptide-based FP and ITC binding studies that implicated polyacetylated H4 as the highest affinity histone binding target of BRD4-BD1.^{1, 6, 33} In particular, acetylation of histone H4 at both Lys5 and Lys8 (H4K5/8diacetyl) was necessary and sufficient for high affinity interactions of polyacetylated histone tail peptides with BRD4-BD1 (Figure 1A; Table S1). Triacetylated histone H4 tail peptides containing only one of H4K5acetyl or H4K8acetyl (*e.g.* H4K5/12/16triacyl and H4K8/12/16triacyl) displayed significantly decreased (>5 -fold; $p < 0.004$) binding to BRD4-BD1 compared to polyacetylated histone H4 tail peptides containing both H4K5acetyl and H4K8acetyl. These results are consistent with previous time-resolved fluorescence resonance energy transfer and ITC binding studies performed by Jung *et al.*⁶ and Morinière *et al.*,⁵ respectively, showing that BRD4-BD1 and BRDT-BD1 binding to H4K5/8diacetyl peptides is enhanced by 2- to 11-fold compared to monoacetylated H4K5acetyl or H4K8acetyl peptides. Crystal structures solved by Filippakopoulos *et al.* (PDB ID 3UVW) and Morinière *et al.* (PDB ID 2WP2) demonstrate that the individual BRD4-BD1 and BRDT-BD1 bromodomains simultaneously and cooperatively bind both H4K5acetyl and H4K8acetyl through primary and secondary acetyl-lysine binding sites within a single bromodomain (Figure 4A/B).^{1, 5} BRD4-BD1 binding was further enhanced by additional acetylation beyond H4K5/8diacetyl at Lys12, Lys16 or Lys20 on the histone H4 tail (Figure 1A). Since previous studies by Filippakopoulos *et al.* of neighboring phosphorylation and methylation influencing bromodomain binding to acetyl-lysine were limited to histone H3,¹ we extended their analysis to histone H4. Interestingly, bromodomain binding to polyacetylated histone H4 tail peptides was significantly reduced by *N*-terminal phosphorylation at Ser1 (H4S1p) ($41 \pm 17\%$ decreased binding; $p = 0.04$ comparing H4K5/8/12/16tetraacetyl $\pm$ H4S1p) but was not significantly affected by the methylation status at Arg3 (H4R3me1, H4R3me2s or H4R3me2a) or Lys12 ($p = 0.59$ comparing H4K5/8/12/16tetraacetyl $\pm$ H4K12me1) (Figure 1A). These results suggest not only that BRD4-BD1 chromatin binding is dependent on the bivalent interaction with acetylated Lys5 and Lys8 of histone H4 as demonstrated in the crystal structure solved by Filippakopoulos *et al.* (PDB ID 3UVW) (Figure 4A),¹ but also that BRD4-BD1 binding to the histone H4 tail is tuned by nearby modifications, as BRD4-BD1 binding is enhanced by neighboring acetylation, unperturbed by neighboring methylation, and inhibited by neighboring phosphorylation. The effect of neighboring phosphorylation on bromodomain binding is likely sequence and bromodomain dependent as neighboring Thr3, Ser10 or Thr11 phosphorylation was previously shown by Filippakopoulos *et al.* to enhance binding of BRD4-BD2 to histone peptide arrays containing H3 peptides acetylated at Lys4, Lys9 or Lys14.¹ To investigate whether the observed binding selectivity of BRD4-BD1 toward polyacetylated peptides containing H4K5/8diacetyl translates into a cellular context and to the other proteins in the BET bromodomain family, the colocalization of BRD2, BRD3 and BRD4 with H4K5acetyl and H4K8acetyl was examined using the chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq) data deposited in the Encyclopedia of DNA Elements (ENCODE) database. As predicted, transcription start sites (TSS) bound by BRD2, BRD3 and BRD4 each correlated with both H4K5acetyl and H4K8acetyl genome occupancy (Figure 1B).

BRD4-BD1 binds acetylation and propionylation but not other known acylations within histone tail peptides and nucleosomes

BET bromodomains contain a conserved network of water molecules at the base of the acyl-lysine binding site (Figure 2E).¹ A recent report by Crawford *et al.* indicated these water networks can be rearranged and stabilized by small-molecule BET bromodomain inhibitors that bind within the acyl-lysine binding site and contain acyl mimic groups of varying size and polarity.³⁴ As a result, we hypothesized that a subset of recently identified histone lysine acylations (Figure 2A) may similarly interact with and rearrange the conserved water network within the BET bromodomain acyl-lysine binding pockets. To begin to determine the acyl-lysine binding specificity of BET bromodomains, the binding affinity of histone H4 peptides diacylated at Lys5 and Lys8 (H4K5/8diacyl) to BRD4-BD1 was determined by ITC. These ITC studies revealed that the binding affinity of BRD4-BD1 toward the H4K5/8dipropionyl ($K_d = 72 \pm 26 \mu\text{M}$) peptide is within 4.2-fold that of the H4K5/8diacetyl ($K_d = 17.2 \pm 3.8 \mu\text{M}$) peptide (Figure 2B and S1A; Table 1), with no observed binding for the H4K5/8dibutyryl and H4K5/8dicrotonyl (Figure 2B and S1B) peptides. These acyl-lysine binding trends are consistent with previous ITC results from Vollmuth and Geyer²² that BRD4-BD1 and BRD4-BD2 bromodomains bind acetyl- and propionyl- but not butyryl-lysine containing histone H3 tail peptides from Flynn *et al.*²¹ that BRD4-BD2 binds propionyl-lysine but not butyryl- or crotonyl-lysine containing H4K5/8diacyl peptides. Building upon these previous studies, further addition of a hydroxyl group to the acyl chain of butyryl-lysine in hydroxyisobutyryl-lysine did not recover binding as determined by ITC (Figure S1E). Although we hypothesized that acyl-lysines containing carboxylic acids may yield favorable interactions with the conserved water network, binding was not observed for the H4K5/8disuccinyl peptide (Figure S1F) consistent with recent histone acylation peptide array results from Flynn *et al.* that found that none of the 49 bromodomains they tested bind lysine succinylation.²¹ Peptides carrying modifications smaller than acetylation including formyl-lysine and the previously uninvestigated homocitrulline, also did not bind to BRD4-BD1 (Figure S1C/D). Consistent with our findings, the non-canonical second bromodomain of PHIP is the only bromodomain reported by Flynn *et al.* using histone acylation peptide arrays to bind formylation.²¹ As all previously reported non-acetyl acylation binding trends were only tested in the context of isolated histone tail peptides, we extended these bromodomain binding studies to their native nucleosome environment. Importantly, the acylation specificity observed with histone tail peptides was maintained within nucleosomes as BRD4-BD1 co-migrated in a sucrose gradient with acetylated and propionylated chick erythrocyte nucleosomes and not butyrylated, glutarylated or unmodified nucleosomes (Figure 2C). These results are consistent with a recent study by Miller *et al.* demonstrating that BRD4-BD1 binds to the H4K5/8diacetyl modification on both peptides and nucleosomes with equal affinity by ITC.³⁵ To gain structural and mechanistic insight into the experimentally observed binding specificity of BRD4-BD1 for acetyl- and propionyl-lysine over all other known lysine acylations, ligand-receptor complex minimizations were performed using the published crystal structure of BRD4-BD1 bound to H4K5/8diacetyl (PDB ID: 3UVW) solved by Filippakopoulos *et al.*¹ as an initial scaffold. For modeling studies, this bound H4K5/8diacetyl peptide was used to build the other H4K5acyl modifications into the BRD4-BD1 acyl-lysine binding site and the resulting protein-ligand complexes were subjected to force field minimization and the free energies of ligand binding

were calculated. In agreement with experimental results, the H4K5acetyl and H4K5propionyl peptides displayed the lowest predicted free energies of binding toward BRD4-BD1 (−107 and −105 kcal/mol) relative to all other known lysine acylations (Figure 2D). Lengthening the acyl chain from propionylation to butyrylation introduced significant ligand strain (17 versus 30 kcal/mol) when the binding pocket water network was kept intact (Figure 2D). Unfavorable steric interactions for butyrylation and larger acylations likely arise from steric clash with a conserved phenylalanine^{21, 22} (Phe83 in BRD4-BD1; Figure 2E) consistent with crystal structures solved by Vollmuth and Geyer of mouse BRD4-BD1 bound to a histone H3 tail peptide either propionylated at Lys23 (PDB ID 3MUK) or butyrylated (PDB ID 3MUL) at Lys14.²²

Assessment of the generality of BET bromodomain binding to lysine acetylation and propionylation

Previous K_d value measurements by Flynn *et al.* and Vollmuth and Geyer of BET bromodomain binding to acylations beyond acetylation were limited to the bromodomains of BRD4.^{21, 22} Thus to extend measurement of acyl-lysine binding affinity to the remaining untested BET family members BRD2, BRD3 and BRDT, a high-throughput FP competition binding assay was developed. A probe for FP competition assays was synthesized through substitution of the t-butyl ester of the pan-BET bromodomain inhibitor JQ1² with a fluorescent tetramethylrhodamine (TAMRA) group connected via an amide to yield JQ1-TAMRA (Figure 3A), which contains a shorter linker length (two carbons instead of five carbons) compared to the “JQ1-TAMRA” previously reported by Jung *et al.*⁶ Modification of the t-butyl ester was previously shown to minimally affect JQ1 binding affinity toward BET bromodomains,⁶ and we observed K_d values for JQ1-TAMRA binding to BET bromodomains ranging from 154 to 432 nM as determined by direct FP binding assays (Figure 3B; Table 2). Binding affinity of H4K5/8diacyl peptides was quantitatively determined by measuring the decrease in FP signal resulting from competition for binding of the JQ1-TAMRA probe at the bromodomain acyl-lysine binding pocket. In these FP competition experiments, all BET *N*-terminal bromodomains tested demonstrated binding of H4K5/8diacyl ($K_d = 15 \pm 3 \mu\text{M}$ for BRDT-BD1, $93 \pm 11 \mu\text{M}$ for BRD3-BD1 and $55 \pm 7 \mu\text{M}$ for BRD4-BD1) and H4K5/8dipropionyl ($K_d = 58 \pm 8 \mu\text{M}$ for BRDT-BD1, $360 \pm 90 \mu\text{M}$ for BRD3-BD1 and $71 \pm 7 \mu\text{M}$ for BRD4-BD1) peptides (Figure 3C; Table 3). Importantly, the K_d values determined by our FP competition assay agree with the K_d values determined by ITC reported in this study for BRD4-BD1 and previously for BRD4-BD1 and BRDT-BD1 toward H4K5/8diacyl peptides. For example, the K_d value of $15 \mu\text{M}$ determined by our FP competition assay is similar to the K_d value of 21.9 reported by Morinière *et al.*⁵ using ITC for binding of H4K5/8diacyl peptides to BRDT-BD1. Overall, the *C*-terminal bromodomains of BET proteins bound to the H4K5/8diacyl ($K_d = 920 \pm 140 \mu\text{M}$ for BRD3-BD2 and $740 \pm 110 \mu\text{M}$ for BRD4-BD2) peptide with 9.9- to 13.5-fold weaker affinity compared to the *N*-terminal bromodomains of the same BET proteins (BRD3-BD1 and BRD4-BD1). Similar >9-fold weaker binding affinity for the *C*-terminal bromodomains was previously observed by Morinière *et al.*⁵ and by Filippakopoulos *et al.*¹ in ITC studies of a H4K5/8/12/16tetraacetyl peptide binding to individual bromodomains from BRD4 and BRDT suggesting that polyacetylated histone H4 tails are not the physiologically relevant binding site of the *C*-terminal BET bromodomains. The *C*-terminal BET bromodomains

may instead bind acetylated transcription factors such as STAT3³⁶ (BRD2), RelA³⁷ (BRD4) and TWIST³⁸ (BRD4). In addition, the observation that BRD4-BD1 binds propionyl- and acetyl-lysine with comparative affinities by ITC is conserved among BET bromodomains as the K_d values measured by FP for the H4K5/8diacetyl and H4K5/8dipropionyl peptides were within error for all BET bromodomains tested except for BRDT-BD1 and BRD3-BD1 that bound the H4K5/8diacetyl peptide up to 3.9-fold tighter than the H4K5/8dipropionyl peptide (Table 3). This conservation of acylation specificity is consistent with the observation that most small molecules targeting the acyl-lysine binding sites of BET bromodomains bind similarly to all BET bromodomains.^{2, 39}

Acylation of H4K8 enhances binding of BRDT-BD1 and BRD4-BD1 but not other BET bromodomains to acetylated or propionylated H4K5

Crystal structures of the *N*-terminal bromodomains of BRD4 (PDB ID 3UVW) solved by Filippakopoulos *et al.*¹ (Figure 4A) and BRDT (PDB ID 2WP2) solved by Morinière *et al.*⁵ (Figure 4B) in complex with H4K5/8diacetyl peptides indicate the acyl-lysine binding sites of BRD4-BD1 and BRDT-BD1 recognize both acetyl groups cooperatively through two distinct binding sites within each individual bromodomain. In these structures, the acetyl amide oxygen of H4K5acetyl forms a hydrogen bond with a conserved Asn residue (Asn140 in BRD4-BD1) and the methyl group of H4K5acetyl makes hydrophobic contacts with Phe83, Val87 and Ile146 at the base of the acyl-lysine binding pocket (Figure 4A). The aliphatic carbons of the H4K8 lysyl side chain are stabilized through hydrophobic contacts with the conserved Trp, Pro and Met (Trp81, Pro82 and Met149 in BRD4) adjacent to the acetyl-lysine binding pocket, facilitating formation of an intramolecular hydrogen bond between the H4K8acetyl carbonyl oxygen and the H4K5acetyl amide hydrogen. The H4K8 acetyl group resides in an apolar environment formed by the conserved WPF shelf (Trp81, Pro82 and Phe83 in BRD4) on one side and a leucine residue (Leu92 in BRD4) on the other. These hydrophobic interactions with H4K8acetyl likely underlie the 20- to 2.3-fold weaker affinity (Table 3) of BRDT-BD1 and BRD4-BD1, respectively, for the monoacetylated H4K5acetyl peptide compared to the diacetylated H4K5/8diacetyl peptide as localization of the positively-charged protonated amine of H4K8 in this apolar environment is unfavorable.^{1, 5} As a result, as long as H4K5 is acetylated, we hypothesized that any acylation that both neutralizes the positive charge of the H4K8 sidechain and allows for a hydrogen bond with the H4K5acetyl amide nitrogen would be accommodated and increase BRDT-BD1 and BRD4-BD1 binding affinity. Indeed, recent pull-down experiments using biotinylated peptides performed by Goudarzi *et al.* demonstrated that BRDT-BD1 binding to H4K5/8diacetyl peptides is inhibited when H4K5acetyl is replaced with butyryl-lysine, but BRDT-BD1 binding is maintained when H4K8 is butyrylated in the presence of H4K5acetyl.¹⁸ Furthermore, a recent peptide array study of BRD4-BD1 and BRD4-BD2 binding to the acetylated transcription factor E2F-1 by Ghari *et al.* demonstrated that binding of both BRD4 bromodomains was enhanced when a positively charged arginine residue next to the acetylation site was converted to a neutral citrulline residue, thereby demonstrating the detrimental effect of neighboring positive charge to the acetyl-lysine site on BET bromodomain binding.⁹ To investigate if our hypothesis is correct that all acylations at H4K8 enhance BRDT-BD1 and BRD4-BD1 binding to H4K5acetyl, a series of histone H4 peptides monoacetylated at H4K5 and either unmodified or modified with a non-acetyl

acylation at H4K8 were synthesized. Relative to the binding affinity of the monoacetylated H4K5acetyl peptide to BRD4-BD1 ($K_d = 129 \pm 22 \mu\text{M}$), acylation of H4K8 (H4K5acetylK8acyl) increased the binding affinity of BRD4-BD1 1.4-fold with K_d values ranging from 17 to 92 μM , comparable to that of the diacetylated H4K5/8diacetyl peptide ($K_d = 55 \pm 7 \mu\text{M}$) (Figure 4C; Table 3). Similar 2.3-fold increases in BRDT-BD1 binding affinity (K_d values ranging from 32 to 131 μM for H4K5acetylK8acyl peptides compared to $303 \pm 101 \mu\text{M}$ for monoacetylated H4K5acetyl) were observed upon acylation of H4K8 in the context of H4K5acetyl (Figure 4D; Table 3). To investigate the histone sequence dependence of this enhancement of BET *N*-terminal bromodomain binding to lysine acetylation by additional adjacent lysine acylation, the analysis described above was repeated in reverse using histone H4 peptides monoacetylated at H4K8 and differentially acylated or unmodified at H4K5. In this case, almost all H4K8acetyl peptides that were modified with a non-acetyl/propionyl acylation at H4K5 displayed equivalent or reduced binding affinity to BRD4-BD1 and BRDT-BD1 compared to the monoacetylated H4K8acetyl peptide (Figure 4E/F; Table 3). The only exceptions were the H4K5crotonylK8acetyl ($K_d = 162 \pm 40 \mu\text{M}$) and H4K5hydroxyisobutyrylK8acetyl ($K_d = 99 \pm 18 \mu\text{M}$) peptides that displayed increased binding affinity to BRD4-BD1 compared to the monoacetylated H4K8acetyl peptide ($K_d = 338 \pm 171 \mu\text{M}$) suggesting that the BRD4-BD1 is more accommodating to larger acylations compared to the BRDT-BD1. Surprisingly, diacylation did not increase binding affinity for the other BET bromodomains tested (Table 3). Instead, BRD3-BD1, BRD2-BD2, BRD3-BD2 and BRD4-BD2 bound the H4K5acetyl peptide with 1.3- to 2.3-fold tighter affinity compared to the diacetylated H4K5/8diacetyl peptide indicating a binding mode distinct from BRDT-BD1 and BRD4-BD1. Taken together, these results implicate acetylated and propionylated H4K5 as the primary histone acylations that recruit BRDT and BRD4 to chromatin. Additional acylation of H4K8 increases BRDT-BD1 binding affinity 2.3- to 9.5-fold and BRD4-BD1 1.4- to 7.6-fold. Furthermore, BRD3-BD1, BRD2-BD2, BRD3-BD2 and BRD4-BD2 likely possess distinct specificity for single acetylation/propionylation sites within histones, transcription factors, or other nuclear proteins. In the future, it will be crucial to elucidate the functions of *C*-terminal BET bromodomains in the context of the tandem BET bromodomains. It is possible that the *N*- and *C*-terminal BET bromodomains simultaneously bind two distinct protein targets to scaffold nucleosomes, transcription factors, and other nuclear proteins in an acylation-dependent manner.

CONCLUSIONS

In this study, we quantify the binding affinity of bromodomains from all four BET family proteins toward acylated histone H4 tails. In particular, BRD4-BD1 selectively recognized histone H4 acetylation over the H2A, H2B and H3 acetylations represented in the EpiTitan peptide array (Figure 1A). BRD4-BD1 binding to polyacetylated histone H4 tails was attenuated by additional serine-1 phosphorylation, but not neighboring arginine or lysine methylation. Further, our results suggest potential mechanisms by which BET bromodomain binding to chromatin may be tuned by levels of diverse acyl-CoA metabolites, which are necessary intermediates to the newly discovered lysine acylations (Figure 2A). Specifically, lysine propionylation largely mimics acetylation in recruiting BET bromodomains to histone

H4, as all BET bromodomains tested bound acetyl- and propionyl-lysine with similar affinity (Figure 2B/C and 3C/D; Table 3). In contrast, all other known lysine acylations displayed 2.9-fold weaker BET bromodomain binding when present in isolation without acetylation (Figure 1A, 2B and 3E; Table 3). However, when present at H4K8, all lysine acylations enhanced BRD4-BD1 and BRDT-BD1 binding to H4K5acetyl (Figure 4C/E; Table 3). As a result, the balance between competition and coexistence of histone acetylation/propionylation with other lysine acylations may tune BET bromodomain recruitment to chromatin in diverse metabolic contexts. This interplay between levels of different acyl-CoA metabolites and histone lysine acylation represents a potential novel mechanism through which cellular acyl-CoA metabolism may directly impact gene expression. For instance, short-chain fatty-acids produced by the microbiome such as propionate and butyrate are incorporated into propionyl-CoA and butyryl-CoA, respectively, ⁴⁰ potentially leading to increased histone propionylation and butyrylation. Tricarboxylic acid cycle activity directly influences malonyl-⁴¹ and succinyl-CoA⁴² levels in a cell-type specific and context-dependent manner. Deficiencies in propionyl-CoA carboxylase, short-chain acyl-CoA dehydrogenase, malonyl-CoA decarboxylase and acyl-CoA synthetase 2 cause metabolic disorders associated with increased protein lysine propionylation, butyrylation, malonylation and crotonylation.^{18–20} Despite identification of these diverse histone lysine acylations, the transcriptional roles played by these newly identified histone lysine acylations relative to acetylation remain unclear. For instance, while the acetyltransferase p300 can also act as a propionyl-,^{21, 43} butyryl-,¹² crotonyl-,²⁰ and glutaryl-transferase,¹⁶ the mechanisms underlying the unique functions of non-acetyl histone lysine acylations are only beginning to be explored. Given the high selectivity of bromodomain interactions for specific histone acyl-lysine sites, for instance the selectivity of BET N-terminal bromodomains toward acetylation/propionylation at the H4K5 position and acylation at the H4K8 position, in the future it will be important to further define the broader bromodomain binding specificity for, the relative stoichiometry of, and the metabolic pathways leading to site-specific histone lysine acylation

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BED	browser extensible data
BET	bromodomain and extraterminal domain
ChIP-seq	chromatin immunoprecipitation with massively parallel DNA sequencing
DIEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N'</i> -dimethylformamide
ENCODE	encyclopedia of DNA elements
ESI	electrospray ionization
FP	fluorescence polarization
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	high-performance liquid chromatography
hESCs	human embryonic stem cells
IPTG	isopropyl β -D-1-thiogalactopyranoside
ITC	isothermal titration calorimetry
LB	luria broth
NHS	<i>N</i> -hydroxysuccinimide
NTA	nitrilotriacetic acid
PDB	protein data bank
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAMRA	tetramethylrhodamine
TFA	trifluoroacetic acid
TSS	transcription start sites

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Figure 1.

Selective recognition of histone H4 polyacetylation by the *N*-terminal bromodomain of BRD4 (BRD4-BD1). (A) Selective BRD4-BD1 binding to acetylated histone H4 tail peptides over H2A, H2B and H3 peptides in an EpiTitan Histone Peptide Array. The EpiTitan array includes 265 histone H2A, H2B, H3 and H4 peptides with multiple post-translational modifications (including acetylation, methylation and phosphorylation) encompassing 95 unique individual modifications. The data shown corresponds to a representative set of acetylated peptides and the full peptide array dataset is presented in Table S1 (n.d. = no detectable binding). (B) Average ChIP-seq peak profiles of H4K5acetyl and H4K8acetyl at transcription start sites (TSS) bound by BRD2, BRD3 or BRD4 demonstrate co-occupancy between acetylated histone H4 tails and BET proteins. BET protein and reference epigenome ChIP-seq data was obtained from the GEO and ENCODE databases, respectively (accession codes GSE60171 and ENCSR554TZE).

Figure 2.

The *N*-terminal bromodomain of BRD4 (BRD4-BD1) selectively binds acetyl- and propionyl-lysine over all other identified lysine acylations. (A) Chemical structures of the known histone lysine acylations tested in this study. (B) BRD4-BD1 binds to H4K5/8diacetyl and H4K5/8dipropionyl peptides but does not bind H4K5/8dibutyryl peptide by isothermal titration calorimetry (ITC). (C) Sucrose gradient of acylated mononucleosomes with BRD4-BD1. Comigration of BRD4-BD1 and nucleosomes was assessed by anti-His₆ blot for BRD4-BD1 and ethidium bromide staining for nucleosome DNA. BRD4-BD1 comigrated with acetylated and propionylated, but not butyrylated nor glutarylated nucleosomes. (D) Protein-ligand complex minimizations of BRD4-BD1 bound to H4K5acyl peptides (using PDB ID 3UVW as the starting structure) and subsequent free energy of binding predictions. H4K5acetyl and H4K5propionyl display the lowest predicted free energy of binding relative to all other H4K5acyl peptides (black). Lengthening of the hydrophobic acyl chain beyond propionylation induces an increase in the ligand strain component of the binding free energy calculation (gray). (E) Minimized poses of BRD4-BD1 bound to acetyl-, propionyl- and butyryl-lysine demonstrate steric clash between the H4K5butyryl group and Phe83 of BRD4-BD1 similar to that previously shown experimentally by Vollmuth and Geyer in X-ray structures of BRD4-BD1 bound to acetylated, propionylated, and butyrylated histone H3 peptides.²² The conserved network of water molecules in the back of the acyl-lysine binding pocket is shown as red spheres.

Figure 3.

Fluorescence polarization (FP) competition assay to measure the generality of acylated histone H4 peptide binding to BET bromodomains. (A) An FP competition probe was developed by modifying JQ1 with tetramethylrhodamine (JQ1-TAMRA). This FP probe allows rapid determination of relative binding affinity to BET bromodomains through competition of acylated peptides with JQ1-TAMRA. (B) Direct BET bromodomain binding of JQ1-TAMRA measured by FP. All BET bromodomains tested bind to JQ1-TAMRA with similar nanomolar affinity. (C) Competitive JQ1-TAMRA FP binding curves with H4K5/8diacetyl (left) and H4K5/8dipropionyl (right) peptides and individual BET bromodomains. Coloring for each bromodomain is as shown in panel B. (D) Competitive JQ1-TAMRA FP binding curves with H4K5/8diacetyl peptides and BRD4-BD1.

Figure 4. Combinatorial recognition of diacylation of H4K5/8 by *N*-terminal BET bromodomains. Crystal structures of (A) BRD4-BD1 (PDB ID 3UVW) and (B) BRDT-BD1 (PDB ID 2WP2) bound to H4K5/8 diacylated peptide which forms a conserved intramolecular hydrogen bond (yellow dashes) between H4K5acetyl and H4K8acetyl. The primary binding site shown on the right recognizes the H4K5acyl group and is highly selective for acetyl- and propionyl-lysine while the H4K8acyl group interacts with the side of the binding pocket shown on the left in a manner that is permissive of larger acyl chains. (C) Competitive JQ1-TAMRA FP binding curves with BRD4-BD1 and histone H4 peptides acetylated at H4K5 and differentially acylated or unmodified at H4K8. (D) Competitive JQ1-TAMRA FP binding curves with BRD4-BD1 and H4 peptides acetylated at H4K8 and differentially acylated or unmodified at H4K5. (E) Competitive JQ1-TAMRA FP binding curves with BRDT-BD1 and histone H4 peptides acetylated at H4K5 and differentially acylated or unmodified at H4K8. (F) Competitive JQ1-TAMRA FP binding curves with BRDT-BD1 and H4 peptides acetylated at H4K8 and differentially acylated or unmodified at H4K5.

Table 1

BRD4-BD1 isothermal titration calorimetry (ITC) binding parameters ($\pm$ std. error of the fit, n.d. = no detectable binding).

Peptide	K_d (μ M)	N	H (kcal/mol)
H4K5/8diacetyl	17.2 ± 3.8	1.05 ± 0.004	-30.8 ± 1.6
H4K5/8dipropionyl	72 ± 26	0.74 ± 0.51	-29 ± 24
H4K5/8dibutyl	n.d.	-	-
H4K5/8diformyl	n.d.	-	-
H4K5/8dihomocitrulline	n.d.	-	-
H4K5/8dicrotonyl	n.d.	-	-
H4K5/8dihydroxyisobutyl	n.d.	-	-
H4K5/8disuccinyl	n.d.	-	-

Table 2

Direct JQ1-TAMRA fluorescence polarization (FP) K_d values for BET bromodomains ($\pm$ std. error of the fit of 2 replicates for each titration).

Bromodomain	K_d (nM)
BRDT-BD1	171 ± 31
BRD3-BD1	154 ± 36
BRD4-BD1	432 ± 125
BRD2-BD2	268 ± 69
BRD3-BD2	272 ± 94
BRD4-BD2	310 ± 71

Table 3

Fluorescence polarization (FP) JQ1-TAMRA competition K_d (μ M) values for histone H4 acylated peptides binding to BET bromodomains ($\pm$ std. error of the fit of 2 replicates for each titration).

Peptide	BRDT-BD1	BRD3-BD1	BRD4-BD1	BRD2-BD2	BRD3-BD2	BRD4-BD2
H4K5/8diacetyl	15 $\pm$ 3	93 $\pm$ 11	55 $\pm$ 7	182 $\pm$ 23	920 $\pm$ 140	740 $\pm$ 110
H4K5/8dipropionyl	58 $\pm$ 8	360 $\pm$ 90	71 $\pm$ 7	131 $\pm$ 11	> 1000	960 $\pm$ 110
H4K5/8diethyl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5/8dicrotonyl	> 1000	270 $\pm$ 50	656 $\pm$ 105	> 1000	> 1000	> 1000
H4K5/8diformyl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5/8dihomocitrulline	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5/8dihydroxyisobutyryl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5/8disuccinyl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5/8glutaryl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5acetyl	303 $\pm$ 101	44 $\pm$ 5	129 $\pm$ 22	145 $\pm$ 20	397 $\pm$ 89	525 $\pm$ 227
H4K5acetylK8propionyl	76 $\pm$ 25	59 $\pm$ 9	66 $\pm$ 10	187 $\pm$ 31	> 1000	> 1000
H4K5acetylK8butyryl	88 $\pm$ 11	58 $\pm$ 10	90 $\pm$ 22	337 $\pm$ 88	> 1000	> 1000
H4K5acetylK8crotonyl	32 $\pm$ 6	34 $\pm$ 4	17 $\pm$ 2	169 $\pm$ 32	> 1000	> 1000
H4K5acetylK8hydroxyisobutyryl	120 $\pm$ 12	60 $\pm$ 6	92 $\pm$ 21	223 $\pm$ 40	> 1000	> 1000
H4K5acetylK8succinyl	83 $\pm$ 16	87 $\pm$ 11	65 $\pm$ 14	310 $\pm$ 43	> 1000	> 1000
H4K5acetylK8glutaryl	131 $\pm$ 18	123 $\pm$ 22	47 $\pm$ 6	219 $\pm$ 27	> 1000	> 1000
H4K8acetyl	279 $\pm$ 45	109 $\pm$ 15	338 $\pm$ 171	361 $\pm$ 83	284 $\pm$ 40	> 1000
H4K5propionylK8acetyl	116 $\pm$ 14	92 $\pm$ 9	52 $\pm$ 11	206 $\pm$ 22	> 1000	> 1000
H4K5butyrylK8acetyl	> 1000	> 500	233 $\pm$ 57	> 1000	> 1000	> 1000
H4K5crotonylK8acetyl	> 1000	399 $\pm$ 132	162 $\pm$ 40	> 1000	> 1000	> 1000
H4K5hydroxyisobutyrylK8acetyl	> 1000	186 $\pm$ 38	99 $\pm$ 18	166 $\pm$ 29	> 1000	> 1000
H4K5malonylK8acetyl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5succinylK8acetyl	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
H4K5glutarylK8acetyl	> 1000	> 500	> 1000	> 1000	> 1000	> 1000