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Citation: Bryson, Bryan D. et al. "Engineered Bromodomains to Explore the Acetylproteome." PROTEOMICS 15.9 (2015): 1470–1475.

As Published: http://dx.doi.org/10.1002/pmic.201400401

Publisher: Wiley Blackwell

Persistent URL: http://hdl.handle.net/1721.1/105868

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

HHS Public Access

Author manuscript

Proteomics. Author manuscript; available in PMC 2016 May 01.

Published in final edited form as:

Proteomics. 2015 May ; 15(9): 1470–1475. doi:10.1002/pmic.201400401.

Engineered Bromodomains to Explore the Acetylproteome

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Abstract

Mass spectrometry-based analysis of the acetylproteome has highlighted a role for acetylation in a wide array of biological processes including gene regulation, metabolism, and cellular signaling. To date, anti-acetyllysine antibodies have been used as the predominant affinity reagent for enrichment of acetyllysine-containing peptides and proteins; however, these reagents suffer from high non-specific binding and lot-to-lot variability. Bromodomains represent potential affinity reagents for acetylated proteins and peptides, given their natural role in recognition of acetylated sequence motifs *in vivo*. To evaluate their efficacy, we generated recombinant proteins representing all known yeast bromodomains. Bromodomain specificity for acetylated peptides was determined using degenerate peptide arrays, leading to the observation that different bromodomains display a wide array of binding specificities. Despite their relatively weak affinity, we demonstrate the ability of selected bromodomains to enrich acetylated peptides from a complex biological mixture prior to mass spectrometric analysis. Finally, we demonstrate a method for improving the utility of bromodomain enrichment for mass spectrometry through engineering novel affinity reagents using combinatorial tandem bromodomain pairs.

Keywords

bromodomain; post-translational modification; protein engineering; affinity reagent; lysine acetylation

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Analysis of lysine acetylation by mass spectrometry has led to the identification of lysine acetylation sites on a diverse array of proteins, including metabolic enzymes, signaling and scaffold proteins, and well-characterized structural and chromatin-interacting proteins such as histones [1 – 3]. These findings suggest novel roles for acetylation in regulating cellular metabolism and DNA damage responses, among other biological processes, several of which have been confirmed experimentally [4 – 7].

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B.D.B., A.M.D., M.B.Y., J.G. performed experiments and analyzed data. B.D.B., A.M.D., J.G., M.B.Y., F.M.W. designed the experiments and wrote the manuscript.

The authors have no conflict of interest to declare.

To date, identification of lysine-acetylated peptides has relied almost exclusively on pan-specific anti-acetyllysine antibodies for immunoaffinity enrichment prior to tandem LC MS/MS analysis [1 – 3]. This global approach is highly dependent on antibody quality and is thus susceptible to variability introduced by antibody source, lot, specificity, and affinity. Furthermore, due to the combination of poor affinity of the antibody and potentially low stoichiometry of acetylation sites, many studies have required large amounts of protein and antibody, limiting the ability to perform replicate experiments and preventing the analyses of biological samples with limited availability [8]. To address the issues with anti-acetyllysine antibodies, other biochemical approaches have been proposed, including top-down proteomics of specific proteins, or a modified form of the biotin switch assay [9 – 10]. While these options have been successfully implemented, top-down analysis is not generally applicable to large numbers of proteins and is limited in the total size of the protein to be analyzed, and the biotin switch assay tends to suffer from incomplete reactivity leading to potential false positives and false negatives.

In vivo, acetylation of lysine residues on selected proteins creates binding sites for bromodomains, naturally occurring protein domains with affinity for acetylated lysine. These bromodomain:acetyllysine interactions facilitate protein-protein interactions that modulate subsequent intracellular events. Since mapping of phosphorylation signaling networks has significantly benefited from biochemical tools such as recombinant SH2, PTB, and methyllysine domains, which have been used to profile subsets of the phosphoproteome [11 – 12], we reasoned that recombinant bromodomains may be an alternative approach to selectively enrich for lysine acetylation sites.

To investigate the potential of these domains as affinity reagents for acetylated peptides, we generated fourteen N-terminally GST-tagged and C-terminally 6xHis-tagged bromodomains representing all yeast bromodomains, along with an empty vector encoding a GST-6xHis tag control. Bromodomains were prepared by PCR from yeast genomic DNA, and cloned into the pGEX 4T-1 vector for recombinant protein expression in Rosetta-gami (DE3)pLysS cells (Primer sequences are provided in Supplemental Table S1). Following induction of protein expression using IPTG in log-phase culture, cells were incubated overnight at 20°C with shaking. Bromodomains were extracted using sonication. Lysate was centrifuged, proteins were recovered from the supernatant and subsequently enriched on glutathione sepharose followed by enrichment with NTA agarose (where necessary). All proteins were similarly expressed and recovered.

The 14 yeast bromodomains selected in this study have diverse amino acid sequences (Supplemental Figure S1). To assess how bromomodomain sequence diversity may affect the specificity of binding, we employed a generic strategy to explore bromodomain binding using a degenerate peptide library array [13]. Although previous work has focused on the binding preferences of bromodomains for histone proteins and peptides, lysine acetylation has been found to occur on a vast array of proteins in addition to histones, and thus the degenerate peptide library enables the identification of binding motifs with minimal *a priori* assumptions [14]. In this assay, 11-mer degenerate peptide libraries were synthesized in an array format on a solid support membrane with the central position of the 11-mers corresponding to either unacetylated or acetylated lysine (AcK), one other position fixed as

one of the 20 natural amino acids as well as pS, pT, pY, methylated lysine (MeK), or AcK, and the remaining positions degenerate (Supplemental Figure S2). For example, the peptide sequence of the spot in the top left corner of the membrane is AXXXX-AcK-XXXXX. To test for the affinity of the bromodomains for different histone peptide sequences, the final two columns of the degenerate peptide array consist of replicates of selected histone peptides in either the acetylated or non-acetylated form (Supplemental Table S2). To assess binding preferences of the bromodomains, degenerate peptide array membranes were pre-blocked with 2% milk and 0.1% tween in TBS (TBST) for 1 hour and incubated with purified recombinant GST-bromodomain-His_6 proteins (50 nM in TBST) for 1 hr at room temperature. Following three washes with TBST, bound bromodomains were electro-transferred to nitrocellulose membranes, blocked with 2% milk in TBST, and visualized by incubation with anti-GST antibody (Millipore; 1/1000 in 2% milk/TBST) for 1 hour followed by washing, and development using IR-fluorescent secondary antibodies (1:15000; Licor). After a final wash, the blots were visualized using an Odyssey scanner (Figure 1A).

The degenerate peptide binding assay identified specific bromodomain preferences for acetyl-lysine recognition in a position- and amino-acid dependent context. As shown in Figure 1B, some bromodomains such as the second bromodomain from BDF1 (BDF1-B) require the presence of acetyl-lysine for binding, but show little specificity in the surrounding sequence, other than negative discrimination against the acidic residues D and E, and phosphorylated Ser/Thr and Tyr residues that would be created by additional PTMs within the acetyl-lysine containing motif. In contrast, other bromodomains such as the first bromodomain from RSC4 (RSC4-A) only has detectable binding to a subset of peptides – in this case corresponding to the histone sequences representing 11-mers centered around the following human histone acetylation marks: H3K57, HIST1H2AEK37, and H2BK86. Based on this result, RSC4-A may not be a good choice as a pan-acetyllysine antibody alternative, but instead it may be a strong candidate to selectively enrich a small subset of histone peptides.

Other interesting binding patterns and trends emerge from the degenerate peptide array screen suggesting unique characteristics about particular bromodomains (Figure S3). For instance, particular bromodomains (c.f. RSC2-B and RSC4-A) show minimal library-wide binding and instead bind strongly to a subset of histone peptides. Intriguingly, in some cases this sequence selectivity was unaffected by the acetylation state of the peptide (e.g. SNF-2). Acetylation may actually reduce binding in some cases. It appears as though the non-acetylated histone peptides are more tightly bound by RSC1-A and RSC2-A, though a more detailed characterization of binding across a range of bromodomain concentrations would better highlight these differences. Further supporting the widely held notion that bromodomains bind histone proteins, we observed a strong general selection for lysine and arginine, two amino acids that are quite abundant in the N-terminal regions of histone proteins. We observe this lysine and arginine selectivity most dramatically in the case of GCN5 in the acetylated library, and for BDF2-B, RSC1-A, and RSC2-A in the non-acetylated library. Interestingly, RSC1-A and RSC2-A demonstrated increased binding to methylated lysine rather than acetylated lysine in the non-acetylated part of the array, suggesting that these domains might also function as methyl-lysine binding domains in some contexts; however, more characterization is necessary. Finally, the STH1 bromodomain

showed minimal selection for any of the peptides when assayed at at 50 nM and may therefore have very weak affinity, or may not have folded correctly in this assay; additional investigation of this bromodomain would be required to understand the cause of the poor binding. Overall, the degenerate peptide arrays revealed novel features of bromodomains binding selectivity that would not have been captured in a focused analysis of histone-bromodomain binding relationships.

The observed bromodomain binding patterns share some binding characteristics with other protein domains that bind covalent post-translational modifications. For example, some SH2 domains bind unique phosphotyrosine-containing peptides similar to the way in which RSC4-A binds the histone peptides, while others, such as the Abl-SH2 domain, appear to be highly promiscuous [15]. As expected, conducting this assay at various concentrations of the BDF1-B bromodomain (Fig 1C) exhibited a dose-response relationship in which the highest affinity binding peptides were more readily distinguished at the lower bromodomain concentrations, while at high concentrations, the bromodomains displayed much more promiscuity. The results of this assay indicate that the 14 yeast bromodomains have variable binding specificity, and suggest that combinations of these domains might be particularly useful for affinity enrichment of subsets of the acetylproteome.

To assess the capability of the bromodomains to function as affinity enrichment reagents, we substituted bromodomains in place of a pan anti-acetyllysine antibody and used mass spectrometry to profile the acetylproteome. Lung adenocarcinoma A549 cells were grown to confluence in RPMI supplemented with 10% fetal bovine serum. Cells were serum starved in RPMI without FBS overnight prior to lysis. At the time of lysis, cells were rinsed once with cold PBS, and then lysed in ice-cold 8M urea. Protein lysates were reduced with DTT, alkylated with iodoacetimide, and digested with trypsin and prepared for mass spectrometry analysis as previously described [16]. Peptide samples were pre-cleared with Ni-NTA agarose for 1hr and then incubated with GST-His_6 tagged BDF1-B overnight. Ni-NTA beads were then added to the bromodomain-peptide mixture for two hours to capture bound complexes of bromodomain and peptides. A mock sample was prepared without the addition of GST-His_6 tagged BDF1-B. Beads were washed and peptides were eluted at low pH with 100mM glycine HCl. Eluted peptides were loaded onto a pre-column and were separated by reverse phase HPLC on custom microcapillary analytical columns with integrated electrospray emitter tips over a 150 minute gradient prior to MS/MS analysis on an Orbitrap Elite mass spectrometer (ThermoFisher Scientific). Raw mass spectrometry data files were analyzed using Proteome Discoverer (ThermoFisher Scientific). All resulting MS/MS spectra were searched against a SwissProt *Homo sapiens* database using Mascot version: 2.4 (Matrix Science). Trypsin enzyme specificity was applied with a maximum of nine missed cleavages to account for peptides containing multiple acetylated lysines. Mass tolerance was set at 20 ppm for the precursor ions (Orbitrap full scan mass spectra) and 0.8 Da for peptide fragments (LTQ CID MS/MS spectra). MS/MS spectra were searched with fixed carbamidomethylation of cysteine and variable modifications including oxidized methionine, and acetylation of lysine (A list of identified peptides is provided in Supplemental Table S3). With this approach, the BDF1-B bromodomain functioned relatively well as a pan-specific affinity enrichment reagent for acetyllysine-containing

peptides (Figure 2). As has been seen with immunoaffinity enrichment of acetylated peptides, BDF1-B precipitated peptides represent a diverse set of proteins. Querying the identified proteins for their biological roles revealed that BDF1-B binds to proteins with a wide array of biological functions, consistent with an expansion for the roles of bromodomains beyond transcriptional regulation to include metabolic regulation and signal transduction. The binding motif generated from these MS identified peptides (Figure 2B) demonstrates a lack of dramatic enrichment for any amino acid at any position, although lysine and glycine residues tend to be slightly more favored at many positions, in general agreement with the degenerate peptide library assay results. Interestingly, while the acidic peptides were slightly disfavored for bromodomain binding in the peptide library experiments, they were slightly enriched in the mass-spectrometry-based analysis of the bound peptides derived from cell lysates, perhaps as a function of enhanced ionization/fragmentation. Comparing the enrichment of BDF1-B to antibody-based approaches such as those employed by Kim and colleagues reveals that the antibody-based analysis demonstrated a slight enrichment for asparagine in some positions, while this enrichment is not observed with BDF1-B, suggesting that bromodomains may be able to enrich for a different subset of peptides compared to the pan-specific anti-aceyllysine antibody. Intriguingly, both approaches provide enrichment for glycine and lysine peptides, potentially due to the abundance of acetylated histone peptides which are rich in these amino acids. This observation suggests that the empiric binding motif specificities for bromo-domains established by the degenerate peptide library partially correlate, but are not necessarily co-linear with the collection of bound peptides subsequently detected by mass-spectrometry.

As described elsewhere, acetyllysine affinity reagents such as antibodies are subject to significant non-specific binding. Similar levels of non-specific binding were observed in the bromodomain pulldown assay, where only 10% of the identified peptides contained acetylated lysine. The difference in specificity for acetylated lysine versus non-acetylated lysine observed in the degenerate peptide library arrays (Figures 2 and S3) may be significantly offset in cell lysates where the concentration of non-acetylated peptides vastly exceeds the concentration of acetylated peptides. Multi-step enrichment at the protein and peptide level may lead to significant improvement in the fraction of acetylated peptides, while additional fractionation techniques such as basic reverse-phase fractionation, isoelectric focusing, or strong cation exchange may be useful to simplify the complex mixture prior to enrichment. It is worth noting that the enrichment studies with the BDF1-B bromodomain were performed with less sample (2 mg total protein) relative to most of the previously published immunoprecipitation studies; increasing the sample amount and fractionating the sample into dozens of fractions is likely to further increase the total number of acetylated peptides identified, although it is unlikely to change the percentage of non-specific binding. Optimization of other features of this protocol including the pulldown buffer, or the use of SILAC labeling followed by selection with wild-type and mutant bromodomains may also improve yield and/or specificity. Titrating the level of bromodomain-bead complex to sample may also improve yield, though this will likely need to be determined empirically on a sample-by-sample basis.

Many bromodomain-containing proteins contain multiple bromodomains within a single polypeptide, which may confer higher effective affinity for acetylated proteins due to an

avidity effect. To assess the effects of expressing bromodomains in tandem, we expressed a naturally occurring tandem pair occurring in BDF1 (combining BDF1-A and BDF1-B) and a hybrid pair containing the GCN5 bromodomain and BDF1-B (this tandem pair, which links a relatively sequence-nonselective bromodomain to a relatively sequence-selective bromodomain, will be referred to as GLB). We compared the binding affinities of the five protein constructs, three individual bromodomains and these two fusion proteins, to an acetylated histone peptide (H4K13) using surface plasmon resonance. In each case, the constructs with the tandem bromodomains featured increased binding affinity for the histone peptide compared to each single bromodomain (Table 1). The BDF1 construct containing the BDF1-A and BDF1-B tandem yielded approximately 10-fold and 100-fold improved affinity relative to BDF1-B or BDF1A alone, respectively. Furthermore, the sequence selective GCN5 bromodomain, which had the lowest affinity of any bromodomain tested for the H4K13 peptide, displayed a 200-fold increase in affinity when fused to the non-selective BDF-1B bromodomain, and displayed tighter binding than any of the isolated bromodomain singlets. Overall, binding constants for single bromodomains were in the micromolar affinity range, in agreement with previously published results [17 – 18]. These findings strongly suggest that improved affinities of the tandem bromodomains might lead to improved affinity enrichment performance, although the specificity of these reagents still needs to be established. Engineered protein constructs composed of tandem yeast bromodomains allows for almost 200 combinations, each of which may each have different acetyllysine binding kinetics and specificity; these combinations may lead to an expansive library of acetyllysine affinity reagents.

The bromodomains described here provide an additional tool to query the acetylproteome in both cells and tissues. Here we have demonstrated that bromodomains can bind to non-histone acetylated peptides and that they can function as affinity enrichment reagents for acetylated peptides. Furthermore, we have suggested a method to improve the affinity of the reagent by combining different bromodomains in tandem. Other protein engineering methods such as targeted or random mutagenesis followed by yeast surface display or phage display could further improve the affinity or specificity of these reagents. In the future, using bromodomains to selectively enrich biologically relevant subsets of the acetylproteome, akin to motif-specific phospho-antibodies, may enhance our understanding of lysine acetylation signaling networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The author would like to thank the White Lab for valuable conversations and Duaa Muhammad for supplying yeast DNA. We thank the KI Swanson Biotechnology Center for technical support, specifically the Biopolymer Core Facility for creating the degenerate peptide arrays. Funding for this work was supported by NIH grants R24DK090963, R01CA118705, U54CA112967, R01ES015339, R01 GM104047, U24CA159988 and P30CA14051. B.D.B. has been supported by the Charles S. Krakauer and Hugh Hampton Young Fellowships.

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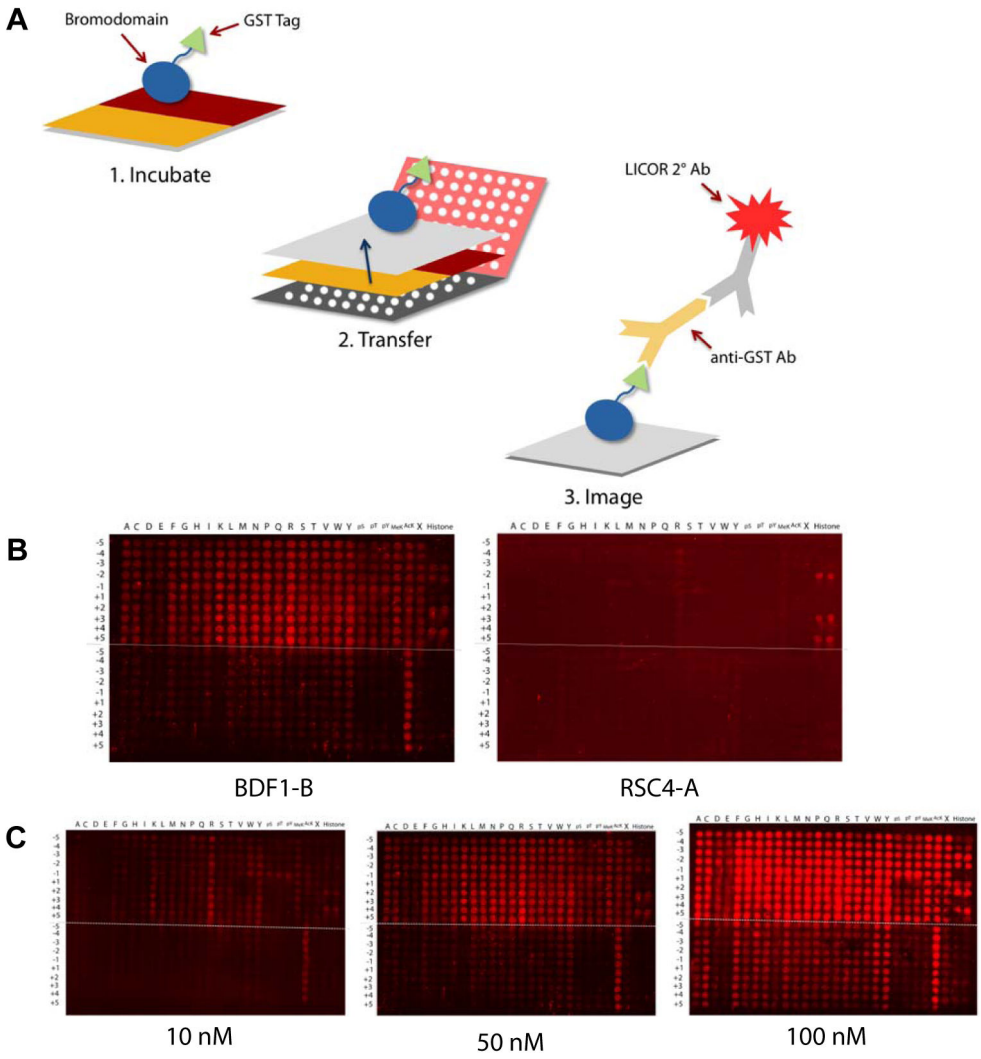

Figure 1. Bromodomain Binding Assay and Results

(A) Experimental scheme for bromodomain-binding motif determination. See text for details. **(B)** BDF1-B and RSC4-A binding motifs. Bromodomains were incubated with the degenerate peptide library array at a concentration of 50 nM. Unbound bromodomains were washed off; bound bromodomains were transferred to a nitrocellulose membrane and incubated with an anti-GST antibody and visualized using LICOR. **(C)** Concentration-dependent specificity. BDF1-B at 10nM, 50 nM, 100nM was incubated with the degenerate library arrays and bound protein visualized as in B.

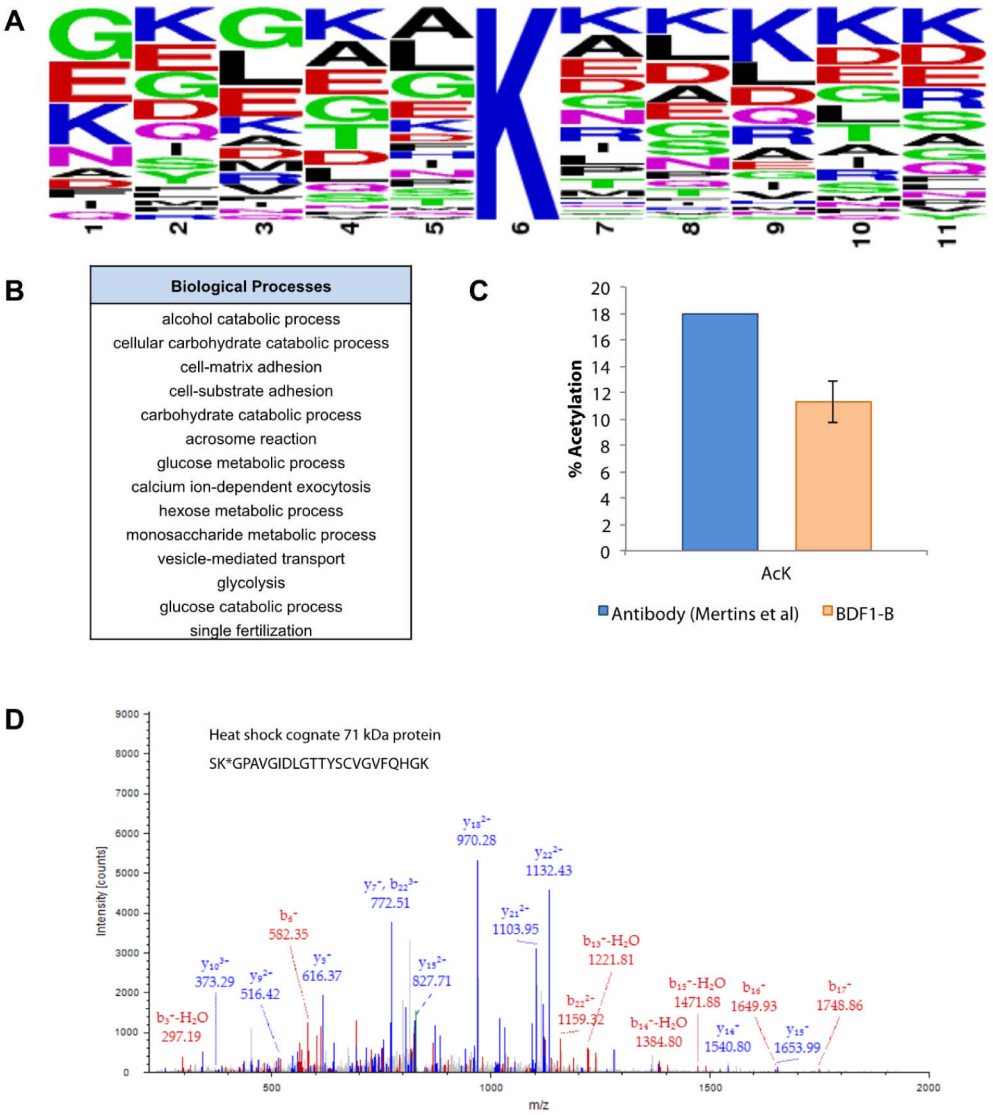

Figure 2. Results of BDF1B Peptide Pulldown

(A) Binding motif for cellular acetyl-lysine proteins bound to the second bromodomain of BDF1. Acetylated peptides detected by mass-spectrometry were aligned using a custom Python script and then submitted to WebLogo for sequence logo generation (shown for a representative experiment) **(B)** Table B shows the Gene Ontology biological processes associated with the proteins identified. **(C)** Percentage of specific binding in bromodomain peptide pulldowns. **(D)** Representative MS/MS spectra of acetylated peptide from BDF1-B pulldown.

Table 1

K_D Measurements of Engineered Bromodomain-Containing Proteins

Protein	K_D (M)
BDF1-A	1.11×10^{-6}
BDF1-B	2.14×10^{-7}
BDF1	1.67×10^{-8}
GCN5	2.25×10^{-5}
GLB	1.08×10^{-7}