1 **Title: Emerging tools to investigate bromodomain functions**

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Abstract:

Bromodomains (BRDs) are evolutionarily conserved protein domains that specifically recognize acetylated lysine, a common epigenetic mark on histone tails. They are found in 61 human proteins, including enzymes, scaffolding platforms, and transcriptional co-activators. BRD-containing proteins play important roles in chromatin remodeling and the regulation of gene expression. Importantly, disruptions of BRD functions have been reported in various diseases. The premise of BRD-containing proteins as therapeutic targets has led to the development of multiple BRD inhibitors, many of which are currently being investigated in clinical trials. Thus, in the last decade significant efforts have been devoted to elucidating BRD biology. Here, we review the emerging tools that contributed to these efforts, from the structural definition of BRDs to their functional characterization. We further highlight the methods that have allowed the systematic screening of BRD targets and the identification of their endogenous interactors. Interactome mapping tools, such as affinity purification and proximity-based biotinylation, have contributed to the elucidation of BRD functions and their involvement in signaling pathways. We also discuss how recent progress in proteomics may further enhance our understanding of the biology of BRDs.

34 **Abbreviations:**

- 35 AP: Affinity purification
- 36 BiFC: Bimolecular fluorescence complementation
- 37 BRD: Bromodomain
- 38 BRET: Bioluminescence resonance energy transfer
- 39 IP: Immunoprecipitation
- 40 Kac: Acetylated lysine
- 41 KAT: Lysine acetyltransferase
- 42 KDAC: lysine deacetylase
- 43 LC-MS/MS: Liquid chromatography coupled with tandem mass spectrometry
- 44 MS: Mass spectrometry
- 45 MS/MS: Tandem mass spectrometry
- 46 NCP: Nucleosome core particles
- 47 PPI: Protein-protein interaction
- 48 PTM: Posttranslational modification
- 49 RIME: Rapid IP mass spectrometry of endogenous protein
- 50 SAINT: Significance Analysis of INTeractome
- 51 SWI/SNF: Switch/sucrose nonfermenting
- 52 Y2H: Yeast two-hybrid

53 **Key words:**
54 Bromodomain
55 Acetyl lysine
56 Proteomics
57 Protein-protein interactions
58 Chromatin
59 Mass spectrometry
60

1. Introduction:

The genetic material of cells is organized through associations with proteins, forming a structure referred to as chromatin. At the most fundamental level, the DNA is wrapped around eight histone proteins into a structure termed the nucleosome, which is the basis of chromatin. Because of the overall positive charge of histone proteins, the presence of the histones counterbalances the negative charge of the DNA phosphate backbone, allowing for effective compaction and bending [1, 2]. For over 50 years, it has been known that histone proteins are readily acetylated in nuclei, allowing for effective transcription [3, 4]. Acetylation of the ϵ -amine of lysine results in its neutralization, and in the context of chromatin this allows for decompaction and DNA exposure, a necessity for transcription to occur [5, 6]. Approximately 40 years ago, sodium butyrate [7, 8] and subsequently trapoxin and trichostatin A [9, 10], were found to prevent histone deacetylation, further suggesting that lysine acetylation (Kac) is a posttranslational modification (PTM) regulated by a suite of enzymes in an analogous fashion to phosphorylation [11]. Indeed, in 1996, the first lysine acetyltransferase (KAT) and lysine deacetylase (KDAC) were identified from *Tetrahymena thermophila* and bovine thymus [12, 13], respectively, and were found to be homologs of *Saccharomyces cerevisiae* histone acetyltransferase GCN5 and histone deacetylase RPD3, key regulators of transcription. Three years later, bromodomains (BRDs), which are also evolutionarily conserved [14], were reported to specifically bind acetylated ligands [15]. Thus, by the year 2000, the foundation of a Kac-based signaling system was established, involving writer (KATs), reader (BRDs), and eraser (KDACs) modules [11, 16]. Since these early discoveries, the biological functions of acetylated lysines (Kac) have proved to be widespread, requiring the implementation of innovative technologies, particularly in the field of proteomics, for effective study. Here, we review how innovative biochemical and proteomics approaches have contributed to our current understanding of Kac signaling, with an emphasis on its main readers, the BRDs.

2. Lysine acetylation, a globally abundant PTM

Acetylation is an abundant PTM, with over 38,000 sites presently reported in the PhosphoSitePlus PTM repository [17]. Acetylation occurs posttranslationally through the actions of KATs, which transfer the acetyl moiety from acetyl-CoA to specific lysine residues. The acetyl group is later removed by KDACs. The posttranslational and highly dynamic nature of Kac was revealed by the work of Allfrey and coworkers through the use of radio-labelled acetate-2-C¹⁴ [18]. Following incorporation of acetate-2-C¹⁴ into calf thymus nuclei the authors reported that its transfer to histones continued following the inhibition of protein synthesis with puromycin. They also observed

that acetylation alleviated the ability of arginine-rich histones to reduce the activity of RNA polymerases [4]. A few years later, Panyim and Chalkley developed the acid-urea gel, which enabled them to resolve histone proteins based on their size and charge [19]. This allowed them to distinguish histone proteins from a variety of sources that differed by a single Kac site. The lack of effective tools to study Kac remained a challenge for ~30 years, and as a result the first report of a non-histone acetylated protein (tumor protein p53) was not until 1997 [20]. The development of effective anti-Kac antibodies enabled Kac mapping efforts to transition to proteome-wide studies in 2006, when Kim *et al.* used polyclonal antibodies generated in rabbits immunized with *in vitro* acetylated bovine serum albumin [21]. Thus equipped, they immunoprecipitated acetylated peptides from cytosolic and nuclear fractions of HeLa-S3 cells and employed mass spectrometry (MS) to identify 388 Kac sites, more than tripling the known Kac sites at the time. Three years later, Choudhary *et al.* revisited this line of inquiry using a more modern MS, off-line peptide fractionations, and KDAC inhibitor treatment to identify > 3,600 Kac sites [22]. State-of-the-art Kac mapping experiments are now capable of quantifying over 20,000 Kac sites, truly establishing Kac as a global PTM [23].

The identification of Kac sites by MS is made possible by the +42.01056 Da mass shift conferred by the additional acetyl group, which is readily detected in tandem mass spectra (MS/MS). As with other PTMs that result in similar mass shifts (e.g., lysine trimethylation, +42.04695 Da), high resolution mass spectrometers should be used to unambiguously identify Kac peptides. Alternatively, the identification of Kac residues may be accomplished by searching for the presence of “diagnostic ions” on the peptide spectrum; specifically, the ammonia-lost derivative (126.0913 Da) of the unstable immonium ion of Kac (143.1179 Da) [24, 25]. Another technical consideration for the identification of Kac sites by MS is that the lysine charge neutralization caused by acetylation prevents trypsin cleavage [22]. Therefore, identified Kac sites should not be located at the peptide C-terminus but rather at an internal missed cleavage lysine site. This was elegantly demonstrated by Choudhary *et al.* by using D₃C₁-labeled acetic acid during Kac peptide sample preparation, which confirmed that C-terminal Kac sites were *in vitro* artefacts and not representative of endogenous Kac [22]. A widely used approach to effectively study Kac is to first derivatize unacetylated lysine using heavy labelled acetic anhydride (e.g., the *d*₆ deuterated form). This results in the acetylation of all lysines, and allows the precise quantification of endogenous (light) and exogenous (heavy) Kac peptides and determination of their stoichiometry [26]. The stoichiometry of Kac sites is extremely low (median 0.02%) in mammalian cells, except for in

nuclear proteins involved in transcriptional regulation, suggesting that not all Kac sites function in the same manner [27]. Intriguingly, the first proteome-wide studies of Kac also revealed that in addition to the nucleus, the mitochondria were a prevalent source of acetylated proteins [21, 22, 28]. These results were consistent with the observation that increased concentrations of acetyl-CoA result in enhanced KAT activity [29]. Consequently, processes that enhance the acetyl-CoA concentration, such as those mediated by acetate-dependent acetyl-CoA synthetase 2 and citrate-dependent ATP-citrate lyase, may lead to protein hyperacetylation and disrupt cellular functions [30-32]. Furthermore, some drugs, such as acetylsalicylic acid (aspirin), may act as acetyl donors and have been associated with enhanced Kac levels [33]. Conversely, reduced acetyl-CoA pools may diminish Kac levels due to a lack of substrate. In addition, Kac may compete with non-acetyl acylation for same lysine residues. Thus, ketogenesis (which provides β -hydroxybutyryl-CoA), β -oxidation, and fatty acid synthesis have been suggested to influence Kac by releasing significant amount of non-acetyl acyl-CoA [34]. Non-enzymatic Kac has been shown to occur due to direct reaction with acetyl-CoA in eukaryotes [35, 36] and through metabolic intermediates, such as acetyl-phosphate, in prokaryotes [37]. Because of this, the cellular Kac landscape is now recognized to contain background noise and to be closely related to metabolic conditions.

3. BRD-containing proteins: the readers of Kac residues

The first bromodomains were reported in 1992 following a comparative analysis which identified six genes in humans, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* with a shared protein motif [14, 38]. Subsequent analysis revealed that BRDs are found throughout the eukaryotes, with 42 BRD-containing proteins encoded in the human genome, classified into eight families and containing 61 BRDs [39]. In 1999, Zhou and colleagues were the first to define the BRD structure, by performing NMR-based structural studies combined with site-directed mutagenesis on the BRD histone acetyltransferase co-activator KAT2B (p300/CBP-associated factor) to elucidate its structure and binding ability [15]. Shortly after, Tjian and colleagues reported the first crystal structure of a BRD and highlighted the affinity human TAF1 tandem BRDs toward poly-acetylated histone H4 tails [40]. Structurally, BRDs are organized into four left handed antiparallel α -helices (α A, α B, α C, and α Z) linked by two loop regions (the BC and ZA loops). This organization forms a central hydrophobic pocket that specifically recognizes Kac-containing motifs. In 2000, the crystallography-based elucidation of the yeast Gcn5p bromodomain in

complex with an acetylated H4 peptide highlighted a canonical asparagine residue contained in the binding pocket, which forms a hydrogen bond with Kac [41].

Human BRD-containing proteins are mostly localized to the nucleus [42] and are found in diverse protein groups, including ATP-dependent chromatin remodelers, helicases, histone acetyltransferases, methyltransferases, transcriptional mediators, and nuclear scaffolding proteins [39]. Some BRD-containing proteins, such as histone acetyltransferases, have intrinsic enzymatic activity, while others act as chromatin scaffolds, assembling protein complexes into functional modules on the DNA. Thus, BRD-containing proteins facilitate numerous facets of chromatin biology. For instance, BRDs in chromatin remodeler complexes (e.g., the mammalian switch/sucrose nonfermenting (mSWI/SNF) complex) can bind to modified histones and use ATP to act upon nucleosomes and modulate chromatin compaction. A better understanding of the roles of BRD-containing proteins can be obtained by characterizing the interactions they establish with their acetylated substrates and other interacting proteins, as well as the chromatin itself. Below, we will highlight proteomics approaches that have contributed to the elucidation of interactions involving BRD-containing proteins, and how they have informed our understanding of their biological functions.

4. An experimental toolkit to identify and quantify BRD substrates

Our understanding of BRD biology has benefited greatly from the establishment of experimental protocols allowing the study of their direct interaction partners, such as Kac substrates. Numerous techniques that identify binary interactions involving biophysical contact between a BRD and its cognate Kac substrates are now available.

4.1 Cell based screening of BRD substrates

4.1.1 Yeast two-hybrid (Y2H) Y2H is a genetic tool used to highlight interactions between pairs of proteins through the transcriptional activation of reporter genes (**Figure 1A**). In this system, a key transcription factor regulating a specific reporter gene(s) is split into its DNA binding and activation domains such that neither of the fragments can activate the promoter alone. One of the fragments is fused to a bait protein (e.g., a BRD-containing protein) and the second to the prey (a candidate BRD interaction partner). Transcription factor activity is reconstituted by direct interactions between the co-expressed bait and prey, thus allowing reporter gene expression and quantification of protein-protein interactions (PPIs). Early reports using the Y2H approach characterized interactions between TAF1 and the histone chaperone ASF1 [43], yeast

bromodomain factor 1 (Bdf1) and its homolog (Bdf2) and TBP-associated factor 67 (Taf67), and Taf67 and Bdf1 [44] as key regulatory elements in gene expression. Y2H has also been useful in the mapping of protein domains involved in specific interactions. For example, the CUL3 adaptor protein speckle-type POZ protein (SPOP), which contributes to the ubiquitination and proteasomal degradation of the BET family proteins BRD2, BRD3, and BRD4, was found to interact with conserved motifs by Y2H [45]. While Y2H has by now been used to successfully characterize interactions involving the majority of the human proteome, BRD-containing proteins remain underrepresented in Y2H studies, which may be due to the absence of proper human Kac signaling in the yeast cells in which the assay is based.

4.1.2 Bimolecular fluorescence complementation (BiFC) assay An alternative protein complementation assay to Y2H is BiFC, in which a fluorescently tagged protein is genetically split into two non-fluorescent fragments that are separately fused to a bait BRD and a candidate interactor (**Figure 1B**). Direct interaction between the BRD and its candidate substrate allows the reconstitution of the fluorescent protein due to the spatial proximity of the two fragments. The resultant fluorescence can be quantified to infer a direct interaction between the BRD bait and the indicated partner. Wu *et al.* applied a yellow fluorescent Venus-based BiFC to BRD4 domain mapping. They fused the C- and N-terminal fragments of Venus to p53 and full length or truncation-mutant BRD4, respectively, and identified two conserved regions, BID and PDID, which were involved in direct interactions with p53 [46]. Farina *et al.* also employed BiFC to characterize interactions between the BRD4 BRDs and their substrates, as well as the subcellular localization shifts that can result from these interactions [47]. To explore the interaction between BRD4 and the GTPase-activating protein SPA-1 in NIH 3T3 cells, they split enhanced yellow fluorescent protein and fused its N- and C-terminal fragments to SPA-1 and BRD4 respectively. In doing so, they revealed that BRD4, a nuclear protein, interacts with SPA-1, which was previously described as cytoplasmic, in the nucleus. They found that BRD4 and SPA-1 can mutually regulate their subcellular localization through domain-specific PPIs. Subsequently, Jang *et al.* employed BiFC to detail the interaction between BRD4 and the positive transcription elongation factor b (P-TEFb) subunits cyclin T1 and CDK9 [48]. The BiFC-based domain analysis showed that this interaction involved multiple domains of BRD4, notably its BRDs and CTD domain. Finally, the authors highlighted that this interaction, which occurs in the nucleus (excluding the nucleoli), allows the transcription through the phosphorylation of RNA polymerase II.

226 **4.1.3 Bioluminescence Resonance Energy Transfer with Bright NanoLuc luciferase**

227 **(nanoBRET)** PPIs can be effectively quantified in cells using BRET [49]. Machleidt *et al.* recently
228 introduced nanoBRET, which employs the engineered NanoLuc luciferase to catalyze
229 luminescence production by its substrate, furimazine [50] (**Figure 1C**). The nanoBRET approach
230 benefits from the enhanced signal intensity of NanoLuc, enabling the effective quantification of
231 small amounts of analytes, especially when linked to a HaloTag derivatized with fluorophores. To
232 date, nanoBRET has been employed to investigate the potency of small chemical BRD inhibitors
233 for CREBBP/EP300 [51, 52], BRD7/BRD9 [53-56], BRD4 [57], and BRPF1 [58]. The nanoBRET
234 system is now commercially available for a number of predefined pairs of BRD-containing proteins
235 and histones but could potentially be applied to the entire family of BRD-containing proteins.

237 **4.2 In vitro BRD binding assays to screen BRD substrates**

238 Recombinant versions of most BRDs in the human genome can be readily generated, and a
239 myriad of approaches have been employed to make use of these reagents.

241 **4.2.1. Peptide microarray** A commonly employed strategy to investigate BRD substrates involves
242 the use of peptide arrays, in which recombinant BRDs are incubated with peptides arrayed on a
243 solid support. Through this method, thousands of peptides containing a myriad of PTM
244 combinations may be used to screen for interactions with multiple BRDs in a cost-effective
245 manner. One of the most widely used peptide microarray systems is the SPOT array, in which
246 interacting BRDs and other histone PTM binding domains are revealed by western blot analysis
247 [59] (**Figure 2**). This tool has proved its usefulness in mapping the specificity of histone-binding
248 domains. Historically, the SPOT array is rooted in the work of Edwin Southern, who pioneered the
249 microarray concept in 1988 by arraying oligonucleotide probes on ice to analyze nucleic acid
250 samples by hybridization. The success of this approach has led to its extension to various probes,
251 including peptides. The SPOT-synthesis technique was developed in 1990 by Frank *et al.*, and
252 initially consisted of Fmoc-based peptide synthesis spotted on pure cellulose chromatography
253 paper [60]. This technique contributed significantly to investigating the binding specificity of
254 epigenetic reading domains (BRDs and chromodomains) to modified histone tails [59, 61, 62].
255 Technically, a tagged recombinant BRD is incubated with a preblocked peptide array in a mild
256 buffer (e.g., phosphate-buffered saline + 0.1% Tween-20) for sufficient time to allow interactions
257 between the BRDs and their substrates (~ 1 h). After incubation, noninteracting BRDs are washed
258 away and the bound spots are revealed by fluorescence [63] or western blotting [64]. To allow for

the identification of PTM-specific interactions, the binding assay can easily be performed in parallel using modified and nonmodified peptides in a single experiment.

By combining SPOT arrays of core histone acetyl and methyl marks with structural analysis, Ruthenburg *et al.* highlighted the PHD-BRD modules of the human BPTF protein as binders of specific combinatorial patterns of histone marks [65]. Subsequently, Filippakopoulos *et al.* systematically screened more than 30 BRDs, including representatives of all eight BRD subfamilies, using SPOT arrays covering most histone acetyl marks [39]. The authors observed that BRDs were flexible binders of Kac peptides. Furthermore, this work allowed the identification of poly-acetylated substrates and revealed the strong impact of PTMs adjacent to the targeted Kac motif on BRD binding ability. For example, citrullinated residues close to E2F1 Kac sites in the promoters of inflammatory genes result in enhanced BRD4 binding [66]. Consequently, the ability of E2F1 to regulate the inflammatory response is modulated by the interplay between citrullinated and acetylated residues.

Defining binding specificity is important for proteins containing several domains capable of mediating PPIs with independent substrate specificities, and multidomain architecture is very common amongst human BRD-containing proteins. For example, ZMYND8 has a triple reader module (PHD-BRD-PWWP) capable of simultaneously engaging DNA and multiple histone PTMs. Savitsky *et al.* used SPOT arrays to delineate the affinity of the ZMYND8 PHD-BRD-PWWP module toward multiple combinations of histone H3 PTMs [67]. More recently, we employed SPOT arrays to perform a systematic analysis of K-x-K; K-x-x-K, and K-x-x-x-K motifs to decipher whether flanking sequences influence BET BRD binding to Kac motifs [68]. Through this work, we identified novel modes of binding between BRDs and their acetylated substrates, including the identification of the KacY motif, which was strongly bound by BRD4 BD1. This work highlighted how residues flanking Kac marks can compensate for the absence of additional Kac marks by generating structural templates that can be specifically recognized by BRDs.

4.2.2 BRD pulldowns A number of researchers have performed BRD pulldown experiments to isolate their acetylated substrates. Using GST-tagged yeast BRD in pulldown experiments, Hassan *et al.* observed that recombinant BRD exhibited specificity toward specific acetylated histones [69]. Similarly, Gradolatto *et al.* investigated the atypical BRD of YTA7, a budding yeast protein with homology to human ATAD2 but lacking a key conserved tyrosine residue in the ZA loop of its BRD [70], using BRD pulldown assays coupled to *in vitro* d6-acetic anhydride labelling

[71]. They demonstrated that the absence of tyrosine prevented the anchoring of the Kac moiety within the YTA7 BRD, rendering it selective for unmodified histone H3 [72]. Bryson *et al.* furthered the BRD pulldown approach by systematically investigating the specificity of all *Saccharomyces cerevisiae* BRDs using degenerate peptide arrays [73]. They reported that recombinant BRDs were less potent at purifying acetylated peptides than anti-acetyl lysine antibodies but were effective nonetheless, particularly when engineered as tandem BRDs. BRD pulldowns assays have also been used to further our understanding of BRD substrate selection. For instance, we employed recombinant biotinylated BRD4 BD1 and BD2 to isolate salt-extracted histone proteins from HeLa cells [39]. The purified histones were digested on-bead with trypsin and the resulting peptides were quantified by MS at the MS1 level. Through this, we confirmed the presence of polyacetylation on the copurified histones, and furthermore, determined that BD1 and BD2 of BRD4 had distinct Kac selectivity.

BRD pulldowns can also be performed from nucleosome core particles (NCPs), rather than free ligands, to more accurately recapitulate the chromatin environment (Figure 3). Deng *et al.* used *in vitro* assembled NCPs to demonstrate that the BRD in CREBBP was necessary for its KAT activity toward nucleosomal histones and its coactivator activity in HEK293 cells. They further observed that the Epstein-Barr virus-encoded lytic activator Zta potentiated CREBBP activity on oligo- and dinucleosomes but not on mononucleosomes [74]. More recently, Miller *et al.* demonstrated that the nucleosome structure influences BRDT BRD binding to the histone tail [75]. Briefly, they assayed the binding of various human BET proteins to either engineered peptides or reconstituted NCPs containing wild type (WT) or H3-H4 chimeric histones. Using isothermal titration calorimetry, the authors observed that the association between BRDT and diacetylated H4K5acK8ac was enhanced when assayed using NCPs instead of peptides. They further reported that for BRDT, but not BRD4, the presence of DNA contributed to its BRD specificity. Wakamori *et al.* also took advantage of the NCP approach to investigate TAF1 double-BRD binding modalities [76]. Various customized H4 proteins, with single or multiple Kac marks at K5, K8, K12, and K16, were used for NCP reconstitution. Non-acetylated equivalent H4 proteins were used to validate the binding specificity. Interactions between the TAF1 BRDs and the NCPs N-terminal tail were strengthened by increased Kac levels, consistent with their previous results from TAF1 BRD binding tests performed with Kac peptides.

5. Investigating the interactomes of BRD-containing proteins

BRD-containing proteins are large, multidomain proteins that can act as scaffolds on the chromatin. Therefore, mapping their interactomes is an effective strategy to uncover their biological functions particularly when employing one of the bioinformatics pipelines that have been developed to ensure statistical robustness of these experiments (e.g., CompPASS [77] and SAINTexpress [78]). Proteomic approaches that have been used to do so are discussed below.

5.1.1. Endogenous immunoprecipitation The immunoprecipitation (IP) of endogenous complexes is an effective strategy to map PPIs if suitable antibodies are available to specifically target a protein of interest (**Figure 3**). Unfortunately, IP experiments using antibodies targeting BRDs can result in cross-reactivity between closely related BRD family members (data not shown). Therefore, we suggest that antibodies targeting regions not conserved between BRD-containing proteins be used for IP (e.g., [79]). An early example of the use of IP to investigate PPIs was the discovery of the BRD-containing constituents of the SWI/SNF complex [80, 81]. In 1995, Treich *et al.* identified a 19-kDa protein, which they named SNF11, as a new member of the yeast SWI/SNF complex that interacts with the BRD-containing SNF2 [82]. A year later, Wang *et al.* used column-immobilized polyclonal antibodies against different regions of BRG1 (SMARCA4) to purify complexes from various mammalian tissues and cell lines [83]. These purified complexes, called BRG1-associated factors (BAFs), contain 9-12 proteins depending on the cell line, and importantly, possess chromatin remodeling activity. The structural and functional similarity of BAF complexes with the yeast SWI/SNF complex demonstrated the heterogeneity of SWI/SNF-related complexes and their key roles in the regulation of chromatin activity [84]. A recent systematic effort by Mashtallir *et al.* followed a similar approach to investigate the ordered assembly of SWI/SNF complex subunits in greater depth [85]. Their results illustrated the modular nature of SWI/SNF complexes, and the various molecular paths required for their maturation into functional complexes.

Crosslinking has been used to fix protein complexes and facilitate their subsequent purification for many years. For example, the rapid IP mass spectrometry of endogenous protein (RIME) method [86, 87] relies on formaldehyde-crosslinking of endogenous protein complexes prior to IP to enhance their recovery with immobilized antibodies. The captured complexes are then digested on-bead and analyzed by MS. The formaldehyde-crosslinking helps to maintain weaker binding partners that may otherwise be lost during the purification process. For chromatin-associated proteins, including most BRD-containing proteins [42], the method requires isolation of the nuclei

and chromatin shearing prior to IP. RIME was used in a recent report by Raisner *et al.* describing their efforts to analyze EP300 interacting partners on the chromatin in the presence of GNE-049, a CREBBP/EP300 bromodomain inhibitor [88]. They treated MOLM-16 cells with either GNE-049 or dimethyl sulfoxide for 4 h prior to EP300 IP using RIME. EP300 interaction partners were successfully purified, but GNE-049 treatment resulted in no significant loss of PPIs, suggesting that its BRD is not required to mediate these interactions. The RIME approach was also employed by Shu *et al.* to investigate how BET BRD inhibition by JQ1, a pan-BET BRD inhibitor, affected the BRD4 interactome in SUM159 breast cancer cells that were resistant or sensitive to JQ1 [89]. JQ1 reduced the interaction between BRD4 and MED1 in JQ1-sensitive cells but not in JQ1-resistant ones. Additional experiments revealed the hyperphosphorylation of BRD4 by CK2 in JQ1-resistant but not JQ1-sensitive SUM159 cells. Furthermore, knockdown or inhibition of the PP2A phosphatase, which opposes CK2 at these sites, promoted the growth of breast cancer cells, indicating an important role of BRD4 phosphorylation in breast cancer cell proliferation.

Potential pitfalls of endogenous IP include the masking of key interaction surfaces by the antibody used, as well as off-target effects. In our recent characterization of the BET protein family [68], we encountered both situations while validating our results by IP-MS (Figure 4). The antibody we used to IP BRD4 masked its C-terminal motif, thus preventing copurification of the P-TEFb kinase complex, a well-characterized partner of BRD4 (Figure 4A). Furthermore, our purification of BRD2 was enriched for numerous cross-reacting proteins, including lysosomal components of the mTOR amino acid sensing pathway that we were unable to confirm with orthogonal assays (Figure 4B). Together, these results reinforce the need for caution when employing antibodies that have not been systematically characterized in functional proteomics assays.

5.1.2. Affinity purification The use of epitope tags, instead of endogenous epitopes, has allowed the standardization of purification protocols and helped reduce the reporting of off-target contaminants (i.e., background proteins) in functional proteomics experiments. Commercially available affinity matrices exist for many commonly employed epitope tags, including FLAG, Strep-tag II, hemagglutinin (HA), and green fluorescent protein (GFP), allowing for experimental reproducibility. Another benefit of employing epitope tag for functional proteomics is that parental, untagged, cell lines or organisms make for good negative controls to properly model the non-specific binders of a given affinity matrix. Using epitope tags, large consortiums have begun to systematically map PPIs in model organisms (e.g., [90]) and in human cells [91-93]. However, as

BRD-containing proteins are for the most part associated with chromatin, their solubility remains low in traditional buffers [94]. One solution is to use a high salt concentration in lysis buffers (e.g., 420 mM NaCl [95]) to effectively solubilize the chromatin. The downside of this approach, however, is that high salt concentrations disrupt numerous PPIs. For example, Yang *et al.* reported that the binding of BRD4 to P-TEFb through the kinase CDK9 was salt-sensitive [96]. By washing the affinity-precipitates of FLAG-tagged CDK9 with increasing concentrations of KCl (150 to 300 mM) before western blotting, they revealed that BRD4 recovery was significantly reduced in the presence of 250 mM KCl. Critically, they observed that some interactions remained unperturbed (such as those involving CCNT1 and HEXIM1) in up to 300 mM KCl, confirming that interactions involving the FLAG epitope itself were unaffected. An alternative approach to enhance the solubility of chromatin-associated proteins is DNA shearing. Both mechanical shearing (using a sonicator or Bioruptor) and enzymatic digestion (with DNase) can enhance the solubility of BRD-containing proteins in mild lysis buffers. Importantly, we previously observed that the presence of large chromatin fragments (> 500 base pairs) in a lysate augmented the number of interaction partners purified with a given bait, presumably due to the presence of indirect associations occurring through chromatin [97]. Based on this observation, we developed an effective sample preparation method for mammalian cells combining a mild buffer (100 mM KCl), sonication, and enzymatic chromatin digestion, which enabled the effective purification of BRD-containing proteins by AP-MS [94]. We recently used this method to map the BET protein interactome and its modulation upon treatment with JQ1 [68]. To do so, we engineered inducible Flp-In T-REx HEK293 cell lines allowing the expression of 3xFLAG-tagged BRD2, BRD3, BRD4, and BRDT as well as a nuclear localization signal-containing control. Using these cells, we performed a 4 h JQ1 time course and analyzed the interactions by AP-MS. We quantified 3,503 proteins in 32 distinct samples and 4 controls, identifying 556 proteins as specific interaction partners of BET proteins ($\text{FDR} \leq 1\%$) in at least one condition using the SAINTexpress algorithm [78] (**Figure 5A**). After a 1 hour treatment with JQ1, BET BRD inhibition drastically remodeled the BET interactome, with 367 interaction partners either reduced or lost (**Figure 5B**). As many of the interaction partners negatively impacted by JQ1 were BRD substrates, we used this information to better define the acetylated substrates of BET BRD and their modes of binding. Our AP-MS results also revealed a group of 262 proteins that were not significantly affected by JQ1 treatment (**Figure 5B**). Additional experiments allowed us to define short linear motifs in some members of this group that mediated interactions with BET ET domains. Lastly, we found 248 proteins whose association with BET proteins was enhanced upon JQ1 treatment (**Figure 5B**). Through follow-up experiments we

were able to determine that JQ1 resulted in the displacement of BRD3 from ribosomal DNA repeats, resulting in its association with the master ribosome biogenesis regulator, TCOF1, and numerous other nucleolar proteins. These new biological insights revealed unforeseen roles for BRD3 into the regulation of ribosomal RNA transcription and proliferation in general.

Interestingly, fusion events involving BET proteins are commonly observed in nut midline carcinoma, an aggressive subtype of squamous cell carcinoma [98]. The results of these fusions are large (> 250 kDa) proteins that encompass most of BRD4 (and less often BRD3) and NUTM1, which are difficult to effectively purify. To circumvent this issue, Alekseyenko *et al.* employed the BioPLEX-TAP strategy [99], in which nuclei are isolated from formaldehyde-crosslinked cells and the chromatin is sheared prior to a tandem purification scheme, to quantify changes in BRD4 interactions caused by the NUTM1 fusion [100]. The zinc finger protein ZNF532 was found to interact with the BRD4-NUTM1 fusion, but not BRD4, in patient-derived NUT midline carcinoma cells. Knockdown of ZNF532 in these cells resulted in morphological changes, induction of the terminal squamous differentiation marker involucrin, and reduced proliferation.

In addition to its success in yeast and other eukaryotic model organisms, AP-MS has also been successfully used to study BRD-containing proteins in protist models. For instance, *Tetrahymena thermophila* is a unicellular organism in which the transcriptionally active and the silent chromatin are segregated into two distinct nuclei. Intriguingly, many *Tetrahymena* BRD-containing proteins are short, with simple domain architectures [101], in stark contrast to the human BRD-containing proteins [39]. To define *Tetrahymena* SWI/SNF complexes, Saettone *et al.* performed AP-MS of BRG1, the only ATPase subunit found in *Tetrahymena*, and copurified a single BRD-containing protein, IBD1 [101]. Subsequent AP-MS of IBD1 revealed interactions with the *Tetrahymena* SWR and SAGA complexes, suggesting that it acts as a molecular scaffold on the chromatin.

5.1.3 Proximity-dependent biotinylation Proximity-dependent biotinylation is a complementary interactome mapping approach to antibody-based methods [102]. Initially introduced as BioID by Roux *et al.* [103] in 2012, proximity-dependent biotinylation has since been widely adopted (Figure 3A). By fusing an abortive biotin ligase (as in BioID [103] or a peroxidase (as in APEX [104]) to a bait protein of interest, it is possible to covalently mark proteins in close spatial proximity with biotin. Following harsh lysis conditions that can effectively solubilize most of the cellular components, including the chromatin [102], biotinylated proteins are isolated using

streptavidin resin and quantified by MS. Proximity-dependent biotinylation thus effectively circumvents the solubility issues impacting the study of chromatin-associated proteins. In addition, because the biotinylation reaction occurs over time (generally 24 h for BioID), a signal amplification effect occurs, allowing for temporally transient interactions to be effectively identified (see [105] for additional discussion of this point). For optimal proximity-dependent biotinylation experiments, the control samples employed should be selected with care to possess similar levels of biotinylation, as assessed by western blots or immunofluorescence, to the samples and to localize to the same environment. In our own work, we have successfully combined multiple types of controls to better model the background signal of our BioID experiments [102].

To comprehensively define the interactome of ZMYND8, which contains a triple reader module composed of PHD-BRD-PWWP domains, we used both AP-MS and BioID [67]. Our aim was to elucidate the multivalent interactions of ZMYND8 with DNA and histones and test the contributions of its BRD. By comparing WT and BRD-deficient (N228F) ZMYND8 constructs, we observed that loss of BRD activity reduced associations between ZMYND8 and its chromatin-associated partners (e.g., CoREST and the Integrator complex). Interestingly, BioID revealed a significant gain in interactions for BRD-deficient ZMYND8. As these proteins were mostly detected outside the nucleus, these results suggested that the loss of BRD activity altered the localization of ZMYND8.

Proximity-dependent biotinylation has been demonstrated to be an effective interaction mapping tool for membrane-less organelles [106]. In our recent characterization of the BET protein family, we employed BioID to investigate the molecular mechanisms of BRD3 in the nucleolus [68]. We generated BirA*-tagged BRD3 constructs with point mutations in the first (BD1^{mut}), second (BD2^{mut}), or both (BD1:2^{mut}) BRDs, abrogating their Kac binding activity, and stably expressed them in Flp-In T-REx HEK293 cells. Thus equipped, we performed BioID after 24 h of incubation with biotin in the presence or absence of 500 nM JQ1. This analysis confirmed the association of BRD3 with many nucleolar protein partners, such as RNAPI and TCOF1, upon JQ1 treatment. In addition, we observed that loss of BD2 significantly reduced the recovery of nucleolar proteins in the BD2^{mut} and BD1+2^{mut} cell lines, below the level observed with WT BRD3 treated with JQ1, but higher than the basal level observed in the absence of treatment. These results highlighted the key role for BRD3 BRDs in the regulation of ribosomal RNA transcription.

The bromodomain and PHD finger (BRPF) proteins are scaffolds for the MYST family of histone acetyltransferases, which includes KAT6A, KAT6B, and KAT7, and enable their recruitment to specific chromatin sites. Three BRPFs have been described in eukaryotes, namely BRPF1, BRD1 (BRPF2), and BRPF3. To better compare and contrast the interactomes of the three BRPFs, Meier *et al.* performed BioID in HEK293 cells [107]. Cross-comparison of the resulting interactomes revealed flexibility in the tetrameric core complex formed by MYST/Esa1-associated factor 6 (MEAF6), inhibitors of growth proteins (ING4/5), histone acetyltransferases (KAT6A/B, KAT7), and BRPFs. Beside the core tetramer, large variations were observed in BRPF binding partners, including Ser/Thr phosphatases, CK1 and CK2 kinases, and 14-3-3 proteins. These results suggest the involvement of phospho-dependent signaling pathways in the functions of BRPFs.

6. Characterization of BRD inhibitors

The involvement of BRD-containing proteins in various pathologies, including cancer, metabolic disorders, and inflammation, has made BRDs very interesting therapeutic targets. The first description of a weak small molecule inhibitor of a BRD was by Zeng *et al.* in 2005 [108]. They targeted the BRD of KAT2B (PCAF) with N1-aryl-propane-1,3-diamine compounds and reported compounds with half maximal inhibitory concentrations (IC₅₀) in the micromolar range in terms of their ability to displace a biotinylated Tat-AcK50 peptide. Four years later, triazolothienodiazepines, initially patented as potential anti-inflammatory agents [109, 110], were described as antitumor agents targeting the BRDs of BET proteins in a patent from the Mitsubishi Tanabe Pharma Corporation [111]. In 2010, two seminal reports described small molecule inhibitors targeting the BRDs of the BET protein family [112, 113]. Filippakopoulos and co-workers reported the thieno-triazolo-1,4-diazepine, JQ1, as a potent (IC₅₀ < 100 nM) and selective inhibitor of BET BRDs [112]. In an *in vivo* model of nut midline carcinoma, which is often molecularly defined by fusion of the NUTM1 gene with BRD4 or BRD3 [114], JQ1 treatment resulted in differentiation and growth arrest. Nicodeme *et al.* performed a positive chemical screen for activators of the apolipoprotein A1 (APOA1) gene, resulting in the identification of the benzodiazepine I-BET (GSK525762A) as a BET BRD inhibitor [113]. I-BET was effective at suppressing inflammation in lipopolysaccharide-treated mice. Together, these studies revealed that BRD inhibitors have therapeutic potential through their ability to displace BRD-containing proteins from their substrates, leading to transcriptional changes.

6.1 Screening platforms for BRD target identification The discovery of potent BET BRD inhibitors inspired the development of numerous other chemical probes targeting BRDs and highlighted the need for effective characterization platforms for these molecules. One such library screening tool is BROMOScan, a commercial chemical toolkit engineered by DiscoverX for bromodomain binding assays based on KINOMEScan [115]. The BROMOScan technology employs site-directed competition between a test compound and an immobilized BRD ligand for DNA-tagged BRDs. When binding to a test compound occurs with higher affinity than to the immobilized ligand, the recombinant DNA-tagged BRD is displaced. Therefore, the amount of BRD captured is inversely proportional to its affinity to the test probe, and following its elution, this can be measured by quantitative PCR for selectivity profiling and quantitative affinity assessment. For example, Wu *et al.* recently used the BROMOScan platform to systematically characterize 25 BRD inhibitors [116].

6.2 Chemoproteomics and BRD inhibitors A common source of clinical failure for novel chemical inhibitors is off-target effects, which lead to toxicity and /or poor efficacy. Therefore, precisely defining the targets engaged by a small molecule is crucial to transition inhibitors to the clinic and also to support fundamental research programs. For example, the widely employed phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 was recently reported to also inhibit BET BRDs [117]. These unsuspected off-target effects of LY294002 were uncovered by Dittmann *et al.* using a chemoproteomic approach, in which LY294002 and its PI3K-inactive analogue LY303511 were immobilized on NHS-activated Sepharose and used to purify target proteins. Through quantitative proteomics, the authors observed that BET proteins were the most abundant proteins copurified in these assays. We recently employed a similar chemoproteomic approach to investigate the cellular targets of bromosporine (BSP), a promiscuous BRD inhibitor [118]. We synthesized two biotinylated BSP adducts tethered by a flexible linker that we predicted would point away from the BRD cavity. The biotinylated BSPs were bound to magnetic streptavidin beads and subsequently used to purify BSP targets from HEK293 lysates. Using a high salt buffer (300 mM KCl) to minimize the co-purification of protein complexes, we observed 14 distinct BRD-containing proteins that co-purified with BSP in the absence of excess free BSP. As HEK293 cells do not express CECR2, the strongest BSP binder *in vitro*, we generated an inducible 3xFLAG-CECR2 HEK293 cell line, and confirmed the ability of BSP to purify it by western blot (**Figure 6A-B**). We also observed that exogenous CECR2 could outcompete other targets, such as BRD2, for BSP binding. The relative abundance of a drug target should be considered when performing chemoproteomics, as low abundance binders may be masked by higher abundance targets.

In 1996, Taunton *et al.* reported pioneering work in which they synthesized a “K-trap”, a trapoxin analogue linked to a solid support, which allowed the isolation of two proteins that copurified with KDAC activity [13]. They were able to identify RBBP4, a component of several chromatin-associated complexes, in addition to the deacetylase HDAC1. Their work was the first reported use of chemoproteomics to elucidate the composition of protein complexes, and was expanded upon by Bantscheff *et al.* to reveal the diversity of human KDAC-containing protein complexes [119]. Dawson *et al.* employed this antagonist-based AP approach to characterize two new BET inhibitors, I-BET151 and its analog I-BET762, in mixed lineage leukemia (MLL) cells [79]. To do this, they immobilized I-BET762 on a solid support and profiled its substrates in HL60 cells. These results, in conjunction with IP experiments, allowed them to discover that BET proteins reside in large protein complexes, including the super elongation complex and the polymerase-associated factor complex, which are both implicated in MLL. We employed a similar approach using a biotinylated analogue of JQ1 to investigate how BRD inhibition impacted the scaffolding functions of BET family members. Toward this goal, we used the same strategy as in the BSP study [118] but reduced the salt content in our buffer to 100 mM KCl to preserve the integrity of protein complexes in our HEK293 cellular extract (**Figure 6C**). We identified 97 proteins, 65 of which were BET protein interaction partners that were reduced in the presence of excess free JQ1 (**Figure 6D**; **Supplementary Table S1**). This highlights the fact that BRDs are not the domains responsible for sustaining most BET PPIs since pulldown with a BET inhibitor still allows for their co-purification.

7. Conclusions

In recent years, it has been demonstrated that the Kac signaling system can be effectively drugged for clinical benefit. In particular, the structural elucidation of most human BRDs has allowed us to begin to delineate the process of acetylated substrate recognition and binding, supporting the development of novel BRD inhibitors. These efforts have emphasized the notion that BRD binding to acetylated substrates depends on the number and distribution of Kac motifs contained in the ligand sequence. Additionally, the specificity of BRD binding is often impacted by the multivalence of their interactions, which involve the chromatin, surrounding non-Kac PTMs, and the spatial architecture of the binding site. Furthermore, while individual BRDs can bind different ligands, substrates can also be shared by multiple BRDs. The complexity of these interactions is further increased by the multidomain architecture of BRD-containing proteins.

Despite significant advances in our understanding of BRD biology in recent years, many key questions still remain including the BRD contribution to many larger complex associations with chromatin and the detailed molecular mechanisms underlying much of their functions. Improving the spatiotemporal characterization of BRD interactions may provide deeper insight into the biology of BRD-containing proteins. Our own efforts have revealed that proximity-dependent biotinylation is a promising avenue to uncover the dynamics of BRD-containing complexes. To this end, recent innovations, such as the engineering of highly active abortive biotin ligases (e.g., TurboID and mini-Turbo), allow for robust biotinylation in minutes [120], a time frame permitting the performance of precise time course experiments. This approach will be useful for BRDs, as their complexes are built through cascades of interactions whose chronology has not been completely elucidated.

Alternatively, “enzyme splitting” approaches have been suggested to reduce off-target biotinylation, permitting interactome mapping with improved precision and specificity. For example, Han *et al.* demonstrated that using a split APEX2 peroxidase is an efficient way to counter nonspecific biotinylation [121]. Similarly, De Munter *et al.* demonstrated the benefit of using Split-BioID to survey transient interactors of protein dimers with increased labelling specificity [122]. Another way of reducing off-target effects (and their subsequent background signals) in proximity-dependent biotinylation was illustrated by Chojnowski *et al.*, who applied a protein dimerization approach to BioID in a system termed “two-component BioID” [123]. The use of these approaches to study BRD-containing proteins has the potential to enhance our understanding of their underlying biology.

Ultimately, the choice and implementation of a given technique depends on the question being asked. Despite the capabilities of each of the techniques discussed here, none is independently sufficient for the structural and functional elucidation of the biology of BRDs. Rather, it is the combination of several approaches that will provide systematic, meaningful insights. We believe that ongoing improvements to biochemical and proteomics technologies will allow for tremendous insights in BRD biology in the future, and that this fundamental research will uncover new therapeutic opportunities involving this critical domain.

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Figure Legends

Figure 1 – *Cell based screening methods for BRD substrates and interaction partners.* Protein complementation assay rely on the fragmentation of a particular protein into two non-functional fragments which are then fused to putative interaction partners. In the Y2H method (**A**), the two putative interaction partners with the activating domain (AD) and DNA binding domain (DBD) of a transcription factor and co-expressing them in a given cell. If the two proteins understudy interact, they will reconstitute the activity of the transcription factor allowing for the production of a reporter gene. In BiFC assays (**B**), two fragments of a fluorescent protein are fused to the putative interaction partners. If the protein interact, fluorescence will be detected. In nanoBRET assays (**C**), the putative interaction partners are fused to a nanoLuciferase (NL) and halo-tag (HT). Upon treatment with the nonchloroTOM (NCT), ligand for the HT, an intense light emission can be generated if the two proteins are interacting. Red dots represent putative acetylated lysine residues that may mediate protein-protein interaction.

Figure 2 – Using SPOT arrays to define BRD acetylated substrates. (A) Overview of the use of SPOT arrays to identify BRD acetylated substrates. (B) Unprocessed membrane of peptide SPOT validation of histone-like peptides containing a Kac-XX-Kac motif (left panel). The quantified membrane is reported as a heatmap showing binding intensities against the first (BD1) BRD of BRD4 (right panel). Panel B was adapted from [68].

Figure 3 – Functional proteomics approaches to characterize BRD-containing proteins. (A) Overview of the functional proteomics approaches discussed here to characterize the interactome of BRD-containing proteins. Methods enabling the purification of endogenous complexes includes the use of nucleosome core particles (NCPs) (i), peptides (ii), small molecules (iii), and antibodies (iv). Proximity-dependent biotinylation approaches, such as BioID (v), require the expression of a fusion protein coupled to an abortive biotin ligase to permit the generation of activated biotin capable of covalently marking protein in the vicinity of a bait of interest. While mild lysis and wash buffers should be employed in most functional proteomics experiments to maintain protein-protein interaction intact, proximity-dependent biotinylation approaches are amenable to the use of harsh lysis and wash conditions. (B) Following the isolation of protein of interests, the samples are digested into peptides and quantified using LC-MS/MS. Statistical tools should be employed to facilitate the identification of proteins over-represented in the samples compared to the control purifications.

Figure 4 – Common pitfalls encountered in IP-MS experiments. (A) Cartoon of 3xFLAG-BRD4 showing the epitopes recognized by the anti-FLAG and anti-BRD4 antibodies employed to map the interactome of BRD4. The anti-BRD4 antibody mask BRD4's CTM preventing the co-purification of the P-TEFb and NELF complexes. (B) Cartoon of 3xFLAG-BRD2 showing the epitopes recognized by the anti-FLAG and anti-BRD2 antibodies employed to map the interactome of BRD2. The anti-BRD2 antibody employed recognized both BRD2 as well as non-specific proteins. Dot plots were generated from previously published data [68].

Figure 5 – Detailed characterization of the modulation of the BET protein interactome by BET BRD inhibitors using AP-MS. (A) Experimental pipeline deployed to characterize the BET interactome during a JQ1 time-course by AP-MS. (B) Overview of the alterations caused by JQ1 to the BET interactome and how this new knowledge was employed. Figure adapted from previously published data [68].

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698 **Figure 6 – Applications of chemoproteomics to the study of BRD inhibitors.** (A) Structures of
699 biotinylated BSP probes used. (B) Western blots showing the levels of co-purified 3xFLAG tagged
700 CECR2 and endogenous BRD2 from HEK293 cell lysate. (C) Overview of the bio-JQ1 structure
701 and experimental scheme employed to identify BET proteins interaction partners. (D) Scatter plot
702 overview of proteins associated with the biotinylated-JQ1 probe. Proteins co-purified with
703 biotinylated-JQ1 are displayed as circles. Circle size corresponds to the SAINT FDR. Inside circle
704 color corresponds to the indicated complex or functional categories. BET interaction partners
705 previously identified are labelled with a black circle edge. Complete data generated can be found
706 in **Supplementary Table S1**. Bromosporine data and figures are adapted from previously
707 published data [[118](#)].

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