FEBS Letters 586 (2012) 2692–2704

journal homepage: www.FEBSLetters.org

Review

The bromodomain interaction module

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ARTICLE INFO

Article history:
Received 12 March 2012
Revised 20 April 2012
Accepted 20 April 2012
Available online 3 May 2012

Edited by Marius Sudol, Gianni Cesareni, Giulio Superti-Furga and Wilhelm Just

Keywords:
Bromodomain
Lysine acetylation
Sequence specificity

ABSTRACT

ε-N-acetylation of lysine residues (K_{ac}) is one of the most abundant post-translation modifications (PTMs) in the human proteome. In the nucleus, acetylation of histones has been linked to transcriptional activation of genes but the functional consequences of most acetylation events and proteins recruited to these sites remains largely unknown. Bromodomains (BRDs) are small helical interaction modules that specifically recognize acetylation sites in proteins. BRDs have recently emerged as interesting targets for the development of specific protein interaction inhibitors, enabling a novel exiting strategy for the development of new therapies. This review provides an overview over sequence requirements of BRDs, known substrates and the structural mechanisms of specific K_{ac} recognition.

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1. Introduction

The concept of epigenetics was first introduced in 1939 by Waddington [1] to describe "the causal interactions between genes and their products, which bring the phenotype into being", and was later defined as heritable changes in gene expression that are not due to alterations in DNA sequence [2]. The physical correlate of these heritable changes is found in chromatin, composed of DNA, histones and other proteins that organize DNA topology. Over the past decade it has become increasingly clear that post-translational modifications (PTMs) of histones play a central role in regulating transcription of genes. The majority of histone modifications, sometimes referred to as 'histone code', are found mainly on the N-terminal histone tails that protrude from the globular core of the nucleosome [3] and include acetylation, methylation, phosphorylation, ribosylation, biotinylation, citruillination and SUMOylation [4,5]. Recently also a number of further modifications such as crotonylation have been described [6]. Each modification can affect chromatin structure, but the overall state of chromatin is ultimately determined by combinations of these modifications. This complex code of post translational modifications plays important roles in maintaining genome integrity, regulating transcription, and constitutes to the so called epigenetic memory, a mechanism which is poorly understood that inherits expression patterns in dividing cells [7].

2. Lysine acetylation – a prominent post translation modification

A discovery made more than 30 years ago revealed that ε-N-acetylation of lysine residues (K_{ac}) on histone tails is associated with an open chromatin architecture and transcriptional activation [8], although certain acetylation marks have been associated instead with chromatin compaction [9] and with other processes, such as metabolism, DNA repair [10], protein–protein interaction and protein stability [5]. Although lysine acetylation has been studied for a long time in the context of the histone code it is now recognized as a widespread post-translational modification occurring throughout the proteome [11,12]. So far, 18330 sites on 6870 proteins have been reported in total in human cells in the PhosphoSitePlus database (http://www.phosphosite.org) suggesting a wider role for this PTM, highlighting its importance. Surprisingly lysine-acetylation is also abundant in non-nuclear proteins. Given the widespread nature of acetylation together with the existence of enzymes that control it (acetyl-transferaces and de-acetylases) it has been suggested that it could be compared to phosphorylation as a regulatory mechanism for signal transduction in cells [13]. However, in contrast to protein phosphorylation no acetylation dependent pathways have been described but similarly to phosphorylation, dysfunctional acetylation levels have been linked to the development of a large number of diverse diseases.

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http://dx.doi.org/10.1016/j.febslet.2012.04.045

For example in cancer, deregulation of histone acetylation patterns often drives the aberrant expression of oncogenes resulting in proliferation and tumorigenesis. As a consequence enzymes that regulate lysine acetylation in histones, such as 'writers' or histone acetyltranferases (HATs), and 'erasers', histone deacetylases (HDACs) have emerged as attractive cancer targets. A rich literature has been established around the benefits of inhibiting K_{ac} 'erasing' events on chromatin in the past decades in order to affect gene expression, resulting in approved drugs as well as a large number of drug candidates, currently on clinical trials, targeting HDACs in cancer and neurodegenerative diseases such as Parkinson and Alzheimer [14–18]. Recently also proteins that are responsible for the 'readout' of K_{ac}, such as Bromodomains have emerged as potential drug targets and have been successfully targeted by small molecule inhibitors [19–21].

3. Bromodomains – readers of lysine acetylation

'Readers' of epigenetic marks are structurally diverse proteins each possessing one or more evolutionarily conserved effector modules, which recognize covalent modifications of histone proteins or DNA. Bromodomains (BRD) are the only interaction modules that specifically recognize ε-N-acetylation of lysine residues. All BRDs fold into an evolutionary conserved 120 residue structural four helix motif identified in the early 90s as a conserved domain in the Drosophila *brahma* gene [22]. The human genome encodes 46 diverse proteins that contain a total of 61 BRDs, present in many diverse transcriptional co-regulators and chromatin modifying enzymes, such as HATs and HAT associated proteins (GCN5, PCAF, BRD9) [23,24], ATP-dependent chromatin-remodelling complexes (BAZ1B) [25], helicases (SMARCA) [26], SET domain containing methyl-transferases (MLL and ASH1L) [27,28], transcriptional co-activators (TRIM/TIF1) [29] and mediators (TAF1) [30], nuclear scaffolding proteins (PB1) [31] and the BET family [32] (Table 1 [33]). Their biological functions as well as their potential as therapeutic targets have been recently reviewed [34]. Although BRD modules exhibit large sequence variations, they share a conserved fold that comprises a left-handed bundle of four alpha helices (αZ, αA, αB, αC), linked by diverse loop regions of variable length (ZA and BC loops), which line the K_{ac} binding site. Co-crystal structures with peptides demonstrated that acetyl-lysine epitopes are recognized by a central deep hydrophobic cavity, where the K_{ac} is anchored by a hydrogen bond to a conserved asparagine residue present in most BRDs [35,36] (Fig. 1A). It is interesting to point out that the diversity of the rim region lining up the K_{ac} binding site determines the surface properties thus driving binding specificity (Fig. 1B) [33]. A recent large scale structure-based analysis of the human BRD family using 34 high resolution crystal structures and 4 NMR models, together with secondary structure prediction algorithms, clustered the 61 available BRD modules into eight distinct families [33] (Fig. 1C). Briefly, models (XRay and NMR) were structurally superimposed and the resulting sequence alignment was used as a template to further align sequences of BRDs of unknown structure, using their predicted secondary structure elements as a footprint. This manually-curated sequence alignment was then used as a seed for generating a distance matrix employing the Neighbour-joining algorithm implemented in the ClustalW2 program. Given the structural conservation of the bromodomain fold, this method clustered the modules according to subtle differences manifested in their three dimensional structure in a similar fashion to plain sequence alignments. Importantly this method highlighted structurally conserved motifs which can be used together with secondary structure elements to identify new BRDs.

4. Bromodomain substrates

To date there are no systematic studies that describe substrate specificity of BRDs and only a few histone acetylation marks have been associated with a specific BRD. In total there have been reports describing usually single interactions (and in some reports affinities) for 20 out of the 46 BRD-containing proteins. In addition, published affinities of BRD substrate interactions are usually weak and have been determined by a variety of different biophysical techniques employing various lengths for the studied peptidic substrates. Methods used for substrate identification comprise classical pull-down experiments, peptide arrays (SPOT), biophysical techniques such as isothermal titration calorimetry (ITC), fluorescent polarization (FP) spectroscopy, Surface Plasmon Resonance (SPR) and NMR. For example, the bromodomains of BRD2 have been shown to bind acetylated histone H4 at lysine 12 ($H4K12_{ac}$) in cells [37]. A dissociation constant (K_D) of 360 μM for the di-acetylated substrate peptide $H4K5_{ac}/K12_{ac}$ has been determined in vitro by SPR [36] and a K_D for the mono-acetylated $H4K12_{ac}$ peptide of 2.9 mM has been determined by NMR titrations [38]. Furthermore, closely spaced multiple acetylation sites have been shown to increase affinity of the H4 tail for the murine BET family member BRDT [39] and this observation has been studied in detail for the entire subfamily of BET proteins [33] suggesting a cooperative role of neighbouring sites for substrate binding. Although there have been reports linking BRDs to acetylation sites in proteins other than histones, current research has been focussed on histone proteins given the significance of BRDs in chromatin biology [34]. Lastly, despite the large number of post translation modifications found on histones [5,6] studies on the effect that combinations of these PTMs may have on binding are limited only to a few marks.

Following the recent structure-based classification of human BRDs there are eight families that span all 61 domains. The first subfamily (I) contains the acetyl-transferase containing proteins *P300/CBP-associated factor* (PCAF) [24] and the *general control of amino-acid synthesis 5-like 2* (GCN5L) [23] as well as the transcription factor *Fetal Alzheimer antigen* (FALZ) [25] and the chromatin remodelling factor *cat eye syndrome chromosome region 2* (CECR2) [40], all of which localize in the nucleus (Table 1). The solution structure of PCAF established in the late 90s the ability of BRDs to bind acetylayted lysine and supported the hypothesis that BRD-containing proteins contribute to highly specific histone acetylation by targeting HATs to specific chromosomal sites [24]. The BRD of PCAF has been shown to interact in vitro with acetylated histone H3 sites ($K9_{ac}$ [41], $K14_{ac}$ [33,41], $K36_{ac}$ [41]) as well as histone H4 sites ($K8_{ac}$ [24], $K16_{ac}$ [33,41] and $K20_{ac}$ [33,41]). It has also been shown to bind to the HIV-1 transcriptional trans-activator Tat ($K50_{ac}$ [41]), an interaction required to stimulate transcription of the integrated HIV-1 genome. All PCAF/K_{ac} interactions have been quantified and although there are small discrepancies in the determined affinities they all fall in the low micromolar range. The BRD of GCN5L2 has also been studied in the context of histone binding and has been shown to interact with histone H2A ($K5_{ac}$ [42]), histone H3 ($K9_{ac}$, $K14_{ac}$, $K9_{ac}/K14_{ac}$ [43]) and histone H4 ($K8_{ac}/K14_{ac}$ and $K16_{ac}$ [35], $K5_{ac}/K8_{ac}/K12_{ac}/K16_{ac}$ [43]) although the binding affinities of interactions have not been quantified yet. FALZ was shown to interact with histone H4 ($K5_{ac}$ [33]) as well as a poly-arginine 11-mer peptide that carried a central acetylated lysine epitope, demonstrating how affinity for this class of modules is mainly driven by electrostatic attractions of the rim-regions that flank the central K_{ac} docking site. CECR2 also has affinity for histone H3 peptides ($K9_{ac}$, $K14_{ac}$, [33]) and binds to single marks even on diacetylated peptides ($K9_{ac}/K14_{ac}$ [33]). Interestingly binding to histone H3 $K9_{ac}/K14_{ac}$ was found to be attenuated by phosphoryla-

Table 1
Human BRD Containing Proteins [33].

Protein	Name	Alias	Protein function	Sub-cellular localization[1]	UniProt ID	Reference
ASH1L	ash1 (absent, small, or homeotic)-like	ASH1, KMT2H	Methyltransferase	N, C	Q9NR48	[28]
ATAD2	Two AAA domain containing protein	ANCCA	Transcriptional regulator	N	Q6PL18	[67]
BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	ACF1, WALp1, WCRF180	Chromatin remodeling factor	N	Q9NRL2	[102]
BAZ1B	Bromodomain adjacent to zinc finger domain, 1B	WSTF, WBSCR9	Chromatin remodeling factor, Transcriptional regulator	N	Q9UIG0	[25]
BAZ2A	Bromodomain adjacent to zinc finger domain, 2A	TIP5, WALp3	Transcriptional repressor	N, C	Q9UIF9	[77]
BAZ2B	Bromodomain adjacent to zinc finger domain, 2B	WALp4	Unknown	N, C	Q9UIF8	[78]
BRD1	Bromodomain containing protein 1	BRL, BRPF2	Transcriptional regulator, Scaffold protein	N, C	O95696	[46]
BRD2	Bromodomain containing protein 2	FSH, RING3	Transcriptional regulator	N	P25440	[45]
BRD3	Bromodomain containing protein 3	ORFX, RING3L	Transcriptional regulator	N	Q15059	[45]
BRD4	Bromodomain containing protein 4	CAP, MCAP, HUNK1	Transcriptional regulator	N	O60885	[46]
BRD7	Bromodomain containing 7	BP75, NAG4, CELTIX1	Transcriptional regulator	N	Q9NPI1	[65]
BRD8B	Bromodomain containing 8 B	SMAP, SMAP2	Transcriptional regulator	N	Q9H0E9-2	[55]
BRD9	Bromodomain containing 9		Unknown	N, C	Q9H8M2	NA
BRDT	Bromodomain, testis-specific	BRD6	Chromatin remodeling factor	N	Q58F21	[39]
BRPF1	Bromodomain and PHD finger-containing protein 1A	BR140, Peregrin	Transcriptional activator	N, C	P55201-1	[66]
BRPF3	Bromodomain and PHD finger containing, 3		Unknown	N	Q9ULD4	NA
BRWD3	Bromo domain-containing protein disrupted in leukemia	BRODL	JAK-STAT signaling	N, C	Q6RI45	[58]
CECR2	Cat eye syndrome chromosome region		Chromatin remodeling factor	N	Q9BXF3	[40]
CREBBP	CREB binding protein	CBP, KAT3A	Histone acetyl transferase	N	Q92793	[56]
EP300	E1A binding protein p300	p300, KAT3B	Histone acetyl transferase	N	Q09472	[56]
FALZ	Fetal Alzheimer antigen	BPTF, FAC1	Transcription factor	N	Q12830	[25]
GCN5L2	General control of amino-acid synthesis 5-like 2	KAT2A, GCN5	Histone acetyl transferase	N	Q92830	[23]
KIAA1240	KIAA1240 protein	ATAD2B	Not known	N	Q9ULI0	NA
LOC93349	SP140 -like	SP140L	Unknown	U	Q13342	NA
MLL	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	HRX, TRX1, CXXC7, ALL-1	Histone Methyl transferase	N	Q03164	[80]
PB1	Polybromo 1	PBRM1, BAF180	Chromatin remodeling factor	N, C	Q86U86	[31]
PCAF	P300/CBP-associated factor	KAT2B	Histone acetyl transferase	N	Q92831	[24]
PHIP	Pleckstrin homology domain interacting protein	WDR11, ndrp	Insulin signalling	N	Q8WWQ0	[59]
PRKCBP1	Protein kinase C binding protein 1	ZMYND8, RACK7	Transcriptional regulator	N	Q9ULU4	[103]
SMARCA2	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2	BRM, SNF2L2	Chromatin remodeling factor, Splicing regulator	N	P51531	[86]
SMARCA4	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4	BRG1, SNF2L4, SNF2LB	Chromatin remodeling factor	N	P51532	[87]
SP100	Nuclear auto-antigen Sp-100		Transcriptional regulator	N, C	P23497	[74]
SP110	Nuclear auto-antigen Sp-110 A Nuclear auto-antigen Sp-110 C	IPR1	Transcriptional regulator	N	Q9HB58	[75]
SP140	SP140 nuclear body protein	LYSP100	Transcriptional regulator	N, C	Q13342	[76]
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor	TAFII250	Transcription initiation	N	P21675	[83]
TAF1L	TAF1-like RNA polymerase II, TATA box binding protein (TBP)-associated factor	TAF(II)210	Transcription initiation	N	Q8IZX4	[84]
TIF1α	Transcriptional intermediary factor 1	TRIM24, PTC6, RNF82,	Transcriptional regulator	N, C	O15164	[73]
TRIM28	Tripartite motif-containing 28	KAP1, RNF96, TIF1β	Transcriptional regulator	N	Q13263	[81]
TRIM33	Tripartite motif-containing 33 A	PTC7, RFG7, TIF1γ	Control of Transcription elongation	N	Q9UPN9	[72]
TRIM66	Tripartite motif-containing 66	TIF1δ	Transcriptional repressor	N	O15016	[71]
WDR9	WD repeat domain 9	BRWD1	Chromatin remodeling factor	N	Q9NSI6	[57]
ZMYND11	Zinc finger, MYND domain containing 11	BS69, BRAM1	Transcriptional repressor	N	Q15326	[82]

[1] Nuclear or Cytoplasmic.

tion at serine 10 (pS10), a modification required for mitotic progression suggesting that this PTM can influence binding to the surrounding acetylation marks. Notably, pS10 has also been linked to increased HAT activity resulting in elevated levels of $H3K14_{ac}$ [44].

The bromo and extra terminal (BET) proteins belong to the subfamily **II** of BRDs, sharing a common architecture comprising two N-terminal BRDs that exhibit high levels of sequence conservation as well as an extra-terminal (ET) domain and a more divergent C-terminal recruitment domain. There are four proteins in this subfamily, BRD2 [45], BRD3 [45], BRD4 [46] and BRDT [39]. Interestingly BET proteins are recruited to transcriptional start sites during mitosis [37,47,48] and the BET protein BRD4 has been shown to tether the positive transcription elongation factor (P-TEFb) to these sites via its unique C-terminus. As such, their interactions with histones have been studied in detail by several groups (Table 2). Most importantly BET proteins have been shown to bind to multiple adjacent acetyl sites with increased affinity and the structural basis of this interaction has been explained in detail in the case of the

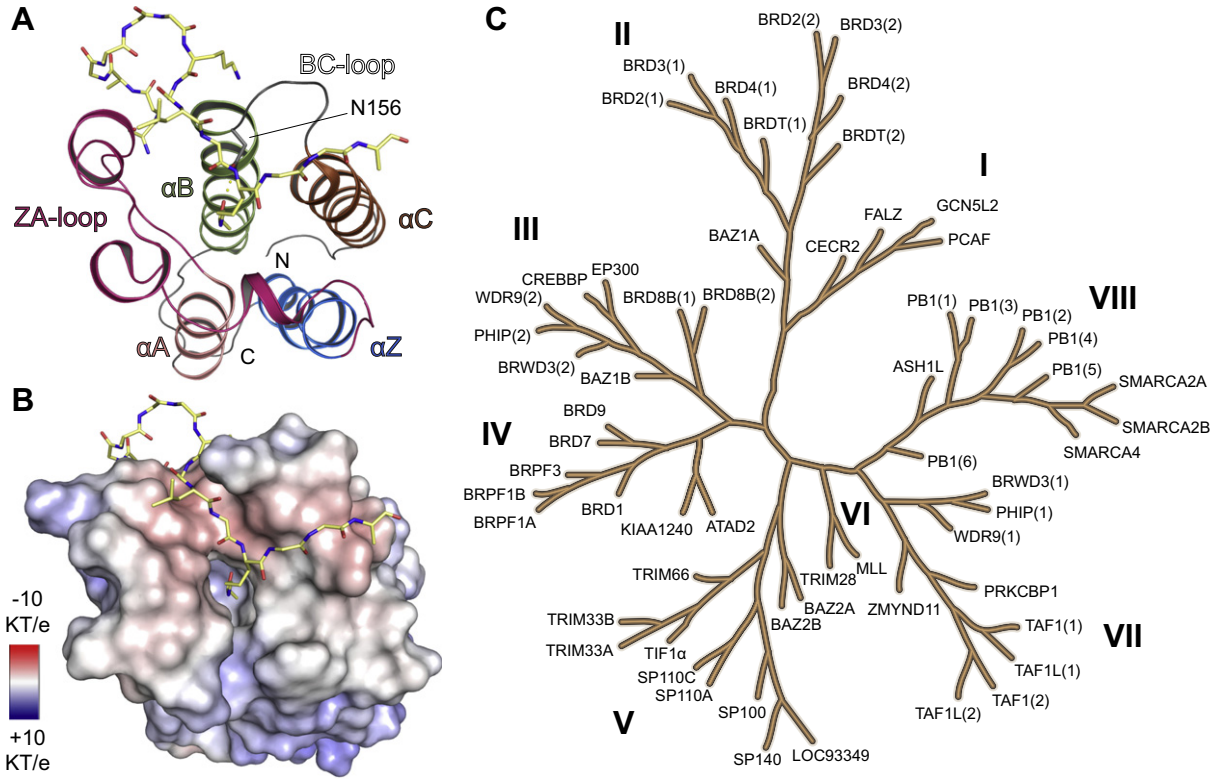

Fig. 1. Bromodomain Overall Fold, Substrate Binding Mode and Phylogeny. (A) Overall structure of the BRD2(1) bromodomain. N-, C-termini and secondary structure elements are labelled (PDB ID: 2DVQ, [36]). Binding of a histone H4 peptide acetylated at lysine 12 ($H4_{1-15}K12_{ac}$) on the central cavity of the module is also shown. (**B**) Surface of the BRD2(1) module (same orientation as in **A**) coloured according to its electrostatic potential as indicated in the inset, demonstrating the surface charge around the K_{ac} binding cavity. (C) Structure based phylogenetic tree of the human bromodomain family. The different families are named by Roman numbers (**I–VIII**).

murine BRDT [39] and the human BRD4 [33]. Tetra-acetylated histone H4 peptides ($K5_{ac}/K8_{ac}/K12_{ac}/K16_{ac}$) have single digit micromolar affinity for single or tandem BET BRDs and both bromodomains of each BET member exhibit affinity for histone H4 peptides with single or multiple acetylations although the N-terminal domain in each protein seems to bind with higher affinity than the C-terminal one [33,36–39,45,49]. Affinity for histone H3 peptides has also been demonstrated although it appears to be systematically stronger in the case of the C-terminal domains suggesting a mode of cooperativity between the tandem modules, either binding to the same nucleosome on different histone tails at the same time, or bridging adjacent nucleosomes by binding on histone H3 of the first and histone H4 of the second. It is however noteworthy that deletion of the first BRD in BRDT is sufficient to cause male sterility in mice by blocking development of mature sperm [50]. A link between BET proteins and increased expression of inflammatory genes via the transcriptional activation of NF-κB occurs through their interaction with the acetylated RelA subunit of NF-κB [51] as evidenced in the case of BRD4. This interaction is of particular interest as K310 acetylation is required for full transcriptional activity of RelA in the absence of effects on DNA binding [52]. BRD3 has been shown to interact with the acetylated transcription factor GATA1 recruiting this transcription factor to both active and repressed target genes in a histone acetylation-independent fashion [53]. The structural basis for this interaction was recently investigated in solution showing that BRD3(1) is responsible for acetylated-GATA1 association (diacetylated at lysines K312 and K315) while BRD3(2) does not seem to contribute to the interaction despite the existence of four distinct K_{ac} sites. A recent study also suggests that BRD4 can directly bind to P-TEFb via interactions of its second BRD with three acetyl-lysines found on Cyclin-T1 [54].

Subfamily **III** of BRDs contains the transcriptional regulator *bromodomain containing 8B* (BRD8B) [55], the HAT enzymes *CREB binding protein* (CREBBP) and *E1A binding protein p300* (EP300) [56], the C-terminal domain of the chromatin remodelling factors *WD repeat domain 9* (WDR9 domain 2) [57], the *bromodomain adjacent to zinc finger domain 1B* (BAZ1B) [25], the C-terminal domain of the JAK/STAT pathway related *bromodomain-containing protein disrupted in leukemia* (BRWD3 domain 2) [58] and the C-terminal domain of the insulin signalling related *pleckstrin homology domain interacting protein* (PHIP domain 2) [59]. Following the idea of targeting enzymatic HAT activity to specific sites via recognition of acetyl-lysine motifs, the BRDs of CREBBP and EP300 have been extensively studied in the context of histone binding. CREBBP binds histone H2B ($K85_{ac}$ [33]), histone H3 ($K9_{ac}/K14_{ac}$, $K14_{ac}$, $K36_{ac}$, $K56_{ac}$ [33,41,60]) as well as histone H4 ($K12_{ac}$, $K20_{ac}$, $K44_{ac}$ [33,41]). Importantly it was shown that serine 10 phosphorylation enhances binding to $H3K14_{ac}$ 6-fold [33]. CREBBP has also been shown to bind to acetylated p53 ($K382_{ac}$ [41]) inducing p53 acetylation-dependent coactivator recruitment after DNA damage, leading to p53-induced transcriptional activation of the cyclin-dependent kinase inhibitor p21. The structural basis for this interaction has been studied in solution [61]. Notable, the BRD of CREBBP binds to mono- and di-acetylated myogenic transcription factor MyoD ($K99_{ac}$, $K99_{ac}/K102_{ac}$ [62,63]) resulting in increased activity on muscle specific promoters, presumably via increased binding to DNA [64]. The BRD of EP300 also binds to acetylated histone H3 ($K36_{ac}$, $K56_{ac}$ [33]) and histone H4 ($K12_{ac}$, $K20_{ac}$, $K44_{ac}$ [33]) and interestingly only binds to diacetylated MyoD ($K99_{ac}/K102_{ac}$ [63]).

Subfamily **IV** of BRDs contains the transcriptional regulators *bromodomain containing 7* (BRD7) [65], *bromodomain containing protein 1* (BRD1) [46], *bromodomain and PHD finger-containing*

Table 2
Studied BRD/Peptide Interactions.

Bromodomain	Sequence		Affinty (μM)	Method Used	Ref.
ATAD2	H3K14	N/A	Not quantified	IP	[68]
BAZ2B	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	7.6 ± 0.3	ITC	[33]
BPTF	H4K5	SGRG**K$_{ac}$**GGKGL***Y***	175 ± 13.3	ITC	[33]
	Poly-R	RRRRR**K$_{ac}$**RRRRR***Y***	3.1 ± 0.1	ITC	
BRD2(1/2)	H3K14	N/A	Not quantified	IP	[45]
	H4K5	N/A	Not quantified	IP	
	H4K12	N/A	Not quantified	IP	[37]
BRD2(1)	H4K5	SGRG**K$_{ac}$**GGKGL***Y***	130 ± 4.4	ITC	[33]
	H4K5/8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGY	13.5 ± 0.3	ITC	
	H4K5/12	SGRG**K$_{ac}$**GGKGLG**K$_{ac}$**GGA	360	SPR	[36]
	H4K8/12	G**K$_{ac}$**GLG**K$_{ac}$**GGAKR	65.8 ± 3.5	ITC	[33]
	H4K12/16	G**K$_{ac}$**GGA**K$_{ac}$**RHRKV	29.4 ± 1.0	ITC	
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	27.9 ± 0.7	ITC	
	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	3.7 ± 0.1	ITC	
BRD2(2)	H4K5/8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGY	13.5 ± 0.3	ITC	[33]
	H4K12	GKGLG**K$_{ac}$**GGAKR	2900	NMR	[38]
	H4K8/12	G**K$_{ac}$**GLG**K$_{ac}$**GGAKR	65.8 ± 3.5	ITC	[33]
	H4K12/16	G**K$_{ac}$**GGA**K$_{ac}$**RHRKV	29.4 ± 1.0	ITC	
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	27.9 ± 0.7	ITC	
	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	3.7 ± 0.1	ITC	
BRD3(1/2)	H3K14	N/A	Not quantified	IP	[45]
	H4K5	N/A	Not quantified	IP	
BRD3(1)	H4K8	RGKGG**K$_{ac}$**GLGKG***Y***	211 ± 5.2	ITC	[33]
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	31.4 ± 0.9	ITC	
	GATA1	**K312$_{ac}$/K314$_{ac}$/K315$_{ac}$/K316$_{ac}$**	Not quantified	IP	[53]
		KASG**K$_{ac}$**GK**K$_{ac}$**KRGSN	Not quantified	NMR	[92]
		KASG**K$_{ac}$**G**K$_{ac}$K$_{ac}$K$_{ac}$**RGSN	0.011 ± 0.006	NMR	
BRD3(2)	H3K18	GKAPR**K$_{ac}$**QLATK***Y***	67.1 ± 4.5	ITC	[33]
	H4K12	GKGLG**K$_{ac}$**GGAKR***Y***	149 ± 8.0	ITC	
	H4K20	AKRHR**K$_{ac}$**VLRDN***Y***	10.5 ± 0.2	ITC	
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	61.3 ± 1.9	ITC	
BRD4(1/2)	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	2.7 ± 0.2	ITC	[33]
	RelA-K310	N/A	Not quantified	IP	[51]
BRD4(1)	H3K9	KQTAR**K$_{ac}$**STGGK***Y***	301 ± 40.9	ITC	[33]
	H3K9/K14	KQTAR**K$_{ac}$**STGG**K$_{ac}$**APRK	Not quantified	NMR	[49]
	H4K5	SGRG**K$_{ac}$**GGKGLGKGGAK	810 ± 57	NMR	
	H4K8	RGKGG**K$_{ac}$**GLGKG***Y***	84.7 ± 8.2	ITC	[33]
	H4K12	SGRGKGGKGLG**K$_{ac}$**GGAK	650 ± 11	NMR	[49]
	H4K5/8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGY	6.8 ± 0.1	ITC	[33]
	H4K5/12	SGRG**K$_{ac}$**GGKGLG**K$_{ac}$**GGAK	Not quantified	NMR	[49]
	H4K8/12	G**K$_{ac}$**GLG**K$_{ac}$**GGAKR	27.4 ± 0.9	ITC	[33]
	H4K12/16	G**K$_{ac}$**GGA**K$_{ac}$**RHRKV	46.1 ± 0.9	ITC	
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	20.4 ± 0.8	ITC	
	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	2.8 ± 0.2	ITC	
	RelA-K310	N/A	Not quantified	IP	[51]
BRD4(2)	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	260 ± 32.2	ITC	[33]
	H3K9/14	KQTAR**K$_{ac}$**STGG**K$_{ac}$**APRK	Not quantified	NMR	[49]
	H4K5	SGRG**K$_{ac}$**GGKGLGKGGAK	1000 ± 126	NMR	
	H4K5	SGRG**K$_{ac}$**GGKGL***Y***	60.2 ± 2.5	ITC	[33]
	H4K12	SGRGKGGKGLG**K$_{ac}$**GGAK	1350 ± 78	NMR	[49]
	H4K5/12	SGRG**K$_{ac}$**GGKGLG**K$_{ac}$**GGAK	Not quantified	NMR	
	H4K31	IQGIT**K$_{ac}$**PAIRR***Y***	170 ± 10.1	ITC	[33]
	H4K5/8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGY	63.3 ± 2.3	ITC	
	H4K8/12	G**K$_{ac}$**GLG**K$_{ac}$**GGAKR	20.4 ± 0.8	ITC	
	H4K12/16	G**K$_{ac}$**GGA**K$_{ac}$**RHRKV	49.3 ± 1.1	ITC	
	H4K12/16/20	GK$_{ac}$GGAK$_{ac}$RHRK$_{ac}$V	22.7 ± 0.8	ITC	
	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	26.6 ± 0.1	ITC	
	RelA-K310	N/A	Not quantified	IP	[51]
	CyclinT1	**K380$_{ac}$/K286$_{ac}$/K390$_{ac}$**	Not quantified	NMR	[54]
BRDT(1)	H2BK12/15	PEPSKSAPAPK**K$_{ac}$**GS**K$_{ac}$**KAISKAQ	157 ± 15	ITC	[39]
	H3K9/14/18/23	H3(1–27)**K9$_{ac}$K14$_{ac}$K18$_{ac}$K23$_{ac}$**	390 ± 36	ITC	
	H4K12	GKGLG**K$_{ac}$**GGAKR***Y***	150 ± 14.6	ITC	[33]
	H4K5/8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGKGGAKRHRK	21.9 ± 1.7	ITC	[39]
	H4K8/12	SGRGKGG**K$_{ac}$**GLG**K$_{ac}$**GGAKRHRK	193 ± 22	ITC	
	H4K12/16	SGRGKGGKGLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	117 ± 22	ITC	
	H4K5/12	SGRG**K$_{ac}$**GGKGLG**K$_{ac}$**GGAKRHRK	340 ± 67	ITC	
	H4K5/8/12/16	SGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	28.0 ± 6.1	ITC	
	H4K5/8/12/16	YSGRG**K$_{ac}$**GG**K$_{ac}$**GLG**K$_{ac}$**GGA**K$_{ac}$**RHRK	23.0 ± 0.5	ITC	[33]
BRDT(2)	H3K9/14/18/23	H3(1–27)**K9$_{ac}$K14$_{ac}$K18$_{ac}$K23$_{ac}$**	214 ± 48	ITC	[39]
	H3K14/18	H3(1–27)**K14$_{ac}$K18$_{ac}$**	217 ± 28	ITC	
	H3K18/23	H3(1–27)**K18$_{ac}$K23$_{ac}$**	176 ± 52	ITC	
	H3K9/18	H3(1–27)**K9$_{ac}$K18$_{ac}$**	360 ± 140	ITC	
	H3K18	H3(1–27)**K18$_{ac}$**	251 ± 62	ITC	
BRD7	H3K9	QTAR**K$_{ac}$**STGG	3960 ± 440	NMR	[70]

Table 2 (*continued*)

Bromodomain	Sequence		Affinty (μM)	Method Used	Ref.
	H3K14	STGG**K$_{ac}$**APRK	1190 ± 20	NMR	
	H4K8	SGRGKGG**K$_{ac}$**GLGK	1790 ± 100	NMR	
	H4K12	KGLG**K$_{ac}$**GGAK	3420 ± 140	NMR	
	H4K16	GGA**K$_{ac}$**RHRK	2560 ± 110	NMR	
CECR2	H3K9	KQTAR**K$_{ac}$**STGGK***Y***	189 ± 11.6	ITC	[33]
	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	49.3 ± 0.8	ITC	
	H3K9/14	KQTAR**K$_{ac}$**STGG**K$_{ac}$**APRKQ***Y***	57.8 ± 1.9 164.5 ± 5.5	ITC	
	H3K9/S10/14	KQTAR**K$_{ac}$pS**TGG**K$_{ac}$**APRKQ***Y***	51.0 ± 1.8 609.8 ± 48	ITC	
CREBBP	H2BK85	AHYN**K$_{ac}$**RSTITSRE	66.2 ± 3.8	ITC	[33]
	H3K9/14	N/A	Not quantified	IP	[60]
	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	734 ± 90.6	ITC	[33]
	H3K36	ATGGV**K$_{ac}$**KPHR***Y***	71.4 ± 1.0	ITC	
	H3K36	APATGGV**K$_{ac}$**KPHRYRP	122 ± 9	NMR	[41]
	H3K56	GTVALREIRRYQ**K$_{ac}$**S	21.3 ± 1.4	ITC	[33]
	H3K56	IRRYQ**K$_{ac}$**STELL***Y***	13.8 ± 0.4	ITC	
	H3S10/K14/18	KpSTGGK$_{ac}$APRK$_{ac}$Q*Y*	131 ± 6.5	ITC	
	H4K12	GKGLG**K$_{ac}$**GGAKR***Y***	48.1 ± 1.9	ITC	
	H4K20	GGAKRHR**K$_{ac}$**VLRDNIQ	218 ± 13	NMR	[41]
	H4K20	AKRHR**K$_{ac}$**VLRDN***Y***	41.5 ± 1.8	ITC	[33]
	H4K44	GGV**K$_{ac}$**RISGLI	82.0 ± 3.8	ITC	
	H4K44	RRGGV**K$_{ac}$**RISGL***Y***	25.3 ± 0.6	ITC	
	p53K382	QSTSRHK**K$_{ac}$**LMFKTEG	187 ± 23	NMR	[41]
		Fl-βA-RHK**K$_{ac}$**LMFK-NHC$_2$H$_5$	155 ± 5	FP	
	MyoD-K99	N/A	Not quantified	IP	[62]
	MyoD-K99	Biotin-CLLWAC**K$_{ac}$**ACKRKTTN	Not quantified		[63]
	MyoD-K99/102	Biotin-CLLWAC**K$_{ac}$**AC**K$_{ac}$**RKTTN	Not quantified		
EP300	H3K36	ATGGV**K$_{ac}$**KPHR***Y***	109 ± 3.8	ITC	[33]
	H3K56	GTVALREIRRYQ**K$_{ac}$**S	13.4 ± 0.5	ITC	
	H4K12	GKGLG**K$_{ac}$**GGAKR***Y***	59.5 ± 1.4	ITC	
	H4K20	AKRHR**K$_{ac}$**VLRDN***Y***	54.6 ± 1.3	ITC	
	H4K44	GGV**K$_{ac}$**RISGLI	132 ± 6.3	ITC	
	MyoD-K99/K102	Biotin-CLLWAC**K$_{ac}$**AC**K$_{ac}$**RKTTN	Not quantified		[63]
GCN5	H2AK5	SGRG**K$_{ac}$**QGGKARAKAKTRSSR	Not quantified	NMR	[42]
	H3K9	N/A	Not quantified	IP	[43]
	H3K14	N/A	Not quantified	IP	
	H3K9/K14	N/A	Not quantified	IP	
	H4K8/16	A**K$_{ac}$**RHRKVLRDNIQGITKPAI	≈900	NMR	[35]
	H4K16	A**K$_{ac}$**RHRKILRNSIQGI	Not quantified	NMR	
	H4K5/8/12/16	N/A	Not quantified	IP	[43]
KIAA1240	H4K5	N/A	Not quantified	IP	[69,75]
	H4K5	RGKGG**K$_{ac}$**GLGKG***Y***	150 ± 6.4	ITC	[33]
PB1 (all 6 single domains)	H3K4	ART**K$_{ac}$**QTARKSTGGKAPRKQLATKAA	1.1 – 34	FA	[91]
	H3K9	ARTKQTAR**K$_{ac}$**STGGKAPRKQLATKAA			
	H3K14	ARTKQTARKSTGG**K$_{ac}$**APRKQLATKAA			
	H3K18	ARTKQTARKSTGGKAPR**K$_{ac}$**QLATKAA			
	H3K23	ARTKQTARKSTGGKAPRKQLAT**K$_{ac}$**AA			
PB1(1)	H3K4	ART**K$_{ac}$**QTARKSTGGKAPRKQLATKAA	0.39	FP	[90]
PB1(2)	H3K9	ARTKQTAR**K$_{ac}$**STGGKAPRKQLATKAA	0.36	FP	
PB1(3)	H3K9	ARTKQTAR**K$_{ac}$**STGGKAPRKQLATKAA	0.71	FP	
PB1(4)	H3K23	ARTKQTARKSTGGKAPRKQLAT**K$_{ac}$**AA	0.12	FP	
PB1(5)	H3K14	ARTKQTARKSTGG**K$_{ac}$**APRKQLATKAA	0.79	FP	
PCAF	H3K9	RTKQTAR**K$_{ac}$**STGGKAP	1051 ± 90	NMR	[41]
	H3K14	ARKSTGG**K$_{ac}$**APRKQLA	128 ± 12	NMR	
	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	188 ± 14.6	ITC	[33]
	H3K36	APATGGV**K$_{ac}$**KPHRYRP	402 ± 93	NMR	[41]
	H4K8	SGRGKGG**K$_{ac}$**GLGK	346 ± 54	NMR	[24]
	H4K16	GLGKGGA**K$_{ac}$**RHRKVLR	365 ± 10	NMR	[41]
	H4K16	GKGGA**K$_{ac}$**RHRKV***Y***	103 ± 11.2	ITC	[33]
	H4K20	GGAKRHR**K$_{ac}$**VLRDNIQ	247 ± 53	NMR	[41]
	H4K20	AKRHR**K$_{ac}$**VLRDN***Y***	90.9 ± 9.6	ITC	[33]
	TATK50	LGISYGR**K$_{ac}$**KRRQRRA	84 ± 12	NMR	[41]
		Fl-GGGGSYGR**K$_{ac}$**KRRQRC	212 ± 17	FP	
SMARCA2	H3K9	N/A	Not quantified	IP	[43]
	H3K14	N/A	not quantified	IP	
	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	287 ± 35.2	ITC	[33]
	H3K9/14	N/A	Not quantified	IP	[43]
	H4K8	N/A	not quantified	IP	
	H4K12	N/A	Not quantified	IP	
	H4K16	GKGGA**K$_{ac}$**RHRKV***Y***	86.2 ± 3.0	ITC	[33]
	H4K5/8/12/16	N/A	Not quantified	IP	[43]
SMARCA4	H2BK5	SDPA**K$_{ac}$**SAPAPKK	Not quantified	NMR	[88]
	H3K14	KSTGG**K$_{ac}$**APRK	1200	NMR	
	H3K9/14	N/A	Not quantified	IP	[60]

(*continued on next page*)

Table 2 (*continued*)

Bromodomain	Sequence		Affinty (μM)	Method Used	Ref.
	H3K9/14	TKQTAR**K$_{ac}$**STGG**K$_{ac}$**APR	≈500	NMR	[89]
	H4K8	SGRGKGG**K$_{ac}$**GLGK	4000	NMR	[88]
	H4K12	GKGLG**K$_{ac}$**GGAKR	3600	NMR	
	H4K16	GGA**K$_{ac}$**RHRK	Not quantified	NMR	
	H4K16	A**K$_{ac}$**RHRKVLRDNIQGI	Not quantified	NMR	[89]
TAF1(1/2)	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	178.9 ± 6.4	ITC	[33]
	H3K14	N/A	Not quantified	IP	[37]
	H3K9/14	KQTAR**K$_{ac}$**STGG**K$_{ac}$**APRKQ***Y*	339 ± 21	ITC	[33]
	H3K9/S10/14	KQTAR**K$_{ac}$pS**TGG**K$_{ac}$**APRKQ***Y*	150 ± 87	ITC	
	H4K8	N/A	Not quantified	IP	[37]
	H4K12	N/A	Not quantified	IP	
	H4K16	N/A	Not quantified	IP	
	H4K16	H4(1–36)**K16$_{ac}$**	39 ± 7	ITC	[30]
	H4K8/16	H4(1–36)**K8$_{ac}$K16$_{ac}$**	5.6 ± 0.2	ITC	
	H4K5/12	H4(1–36)**K5$_{ac}$K12$_{ac}$**	1.4 ± 0.3	ITC	
	H4K5/8/12/16	H4(1–36)**K5$_{ac}$K8$_{ac}$K12$_{ac}$K16$_{ac}$**	5.3 ± 0.2	ITC	
	p53K373/382	p53(364–389)**K373$_{ac}$K382$_{ac}$**	Not quantified	IP	[85]
TAF1(2)	H3K14	KSTGG**K$_{ac}$**APRKQ***Y***	306 ± 21.4	ITC	[33]
TIF1α (BRD)	H3K9	ARTKQTAR**K$_{ac}$**STGGKAPRKQL	232 ± 33	ITC	[73]
	H3K14	ARTKQTARKSTGG**K$_{ac}$**APRKQ	229 ± 32	ITC	
	H4K16	GKGGA**K$_{ac}$**RHRKV***Y***	92.6 ± 1.9	ITC	[33]
TIF1α (PHD/BRD)	H3K23	H3(13–32)**K23$_{ac}$**	8.8 ± 0.1	ITC	[73]
	H3K27	H3(13–32)**K27$_{ac}$**	206 ± 44	ITC	
	H4K16	SGRGKGGKGLGKGGA**K$_{ac}$**RHRK	26 ± 2	ITC	
	H3K4	H3(1–33)K4	1.4 ± 0.3	ITC	
	H3K4/K23	H3(1–33)K4/**K23$_{ac}$**	0.07 ± 0.01	ITC	
	H3K4/K23	H3(1–33)**K4$_{me3}$K23$_{ac}$**	0.34 ± 0.04	ITC	
TRIM33A (PHD/BRD)	K3K9	H3(1–28)**K9$_{me3}$**	0.20 ± 0.03	ITC	[79]
	H3K18	H3(1–28)**K18$_{ac}$**	0.21 ± 0.02	ITC	
	H3K9/K18	H3(1–28)**K9$_{me3}$K18$_{ac}$**	0.06 ± 0.01	ITC	
	H3K14/18/23	H3(1–28)**K9$_{me3}$K14$_{ac}$K18$_{ac}$K23$_{ac}$**	0.20 ± 0.03	ITC	

protein 1 (BRPF1) [66] and *two AAA domain containing protein* (ATAD2) [67] as well as the *KIAA1240 protein* (KIAA1240) the *bromodomain containing 9* (BRD9) and the *bromodomain and PHD finger-containing protein 3* (BRPF3). BRDs of this family have not been extensively studied for binding to acetylated proteins although their involvement in disease has been described and their target sites will contribute to better understanding of their function [34]. The BRD of ATAD2 has been shown to immunoprecipitate histone H3 ($K14_{ac}$ [68]) but this interaction has not been quantified yet, while the closely homologous KIAA1240 binds to histone H4 ($K5_{ac}$ [33,69]). Solution binding experiments have established weak micromolar binding of the BRD of BRD7 to histone H3 ($K9_{ac}$, $K14_{ac}$ [70]) as well as histone H4 ($K8_{ac}$, $K12_{ac}$, $K16_{ac}$ [70]).

Subfamily **V** of the human BRDs contains the transcriptional repressor *tripartite motif-containing 66* (TRIM66) [71], the *tripartite motif-containing 33* (TRIM33) [72], the transcriptional regulator *transcriptional intermediary factor 1* (TIF1α) [73], the transcriptional regulators *nuclear auto-antigen Sp-100* (SP100) [74], *nuclear auto-antigen Sp-110* (SP110) [75] and *SP140 nuclear body protein* (SP140) [76] as well as the *SP140-like protein* (LOC93349), the transcriptional repressor *bromodomain adjacent to zinc finger domain 2A* (BAZ2A) [77] and the *bromodomain adjacent to zinc finger domain 2B* (BAZ2B) [78]. The main characteristic of this sub-class of bromodomains is the existence of a PHD/BRD tandem module which seems to be a necessary structural motif for peptide recognition as well as protein stability [73,79]. The BRD of TIF1α binds acetylated histone H3 peptides with weak micromolar affinity which is enhanced when longer peptides and the tandem PHD/BRD module are used, suggesting a cooperativity between modules even though peptides may not be methylated in order to promote binding to the PHD finger [73]. It is noteworthy that a monoacetylated histone H3 peptide ($K23_{ac}$) spanning residues 1–28 binds with double digit nM affinity to the PHD/BRD tandem module of TIF1α. This observation suggests a binding mode which most likely involves peptide interactions with the charged surface of the protein. TIF1α also binds to histone H4 ($K16_{ac}$ [33]). The PHD/BRD tandem modules of TRIM33A have also recently been shown to bind with sub-micromolar affinities to acetylated and poly-acetylated histone H3 peptides although a trimethylated peptide spanning residues 1–28 was also shown to bind with similar affinity suggesting that the charge neutralization of lysine upon acetylation might be overcome by surface interactions with the extended interface presented to the substrate [79]. A single digit micromolar affinity for histone H3 ($K14_{ac}$) has also been reported for the BRD of BAZ2B while the unacetylated peptide did not interact with the BRD [33].

No acetyl lysine mediated interactions with histones or other proteins have been reported for the BRDs of subfamily **VI** which includes the histone methyl-transferase *myeloid/lymphoid or mixed-lineage leukemia* (MLL) [80] and the transcriptional co-regulator *tripartite motif-containing 28* (TRIM28) [81].

Subfamily **VII** of the human BRDs contains the transcriptional repressor *zinc finger MYND domain containing 11* protein (ZMYND11) [82], the transcription initiators *TAF1 RNA polymerase II TATA box binding protein (TBP)-associated factor* (TAF1) [83] and *TAF1-like RNA polymerase II TATA box binding protein (TBP)-associated factor* (TAF1L) [84] and the N-terminal BRDs of the chromatin remodelling factors *WD repeat domain 9* (WDR9 domain 1) [57], the JAK/STAT pathway related *bromodomain-containing protein disrupted in leukemia* (BRWD3 domain 1) [58] and the insulin signalling related *pleckstrin homology domain interacting protein* (PHIP domain 1) [59]. TAF1 is of particular interest as it was the first reported structure of a tandem BRD module which demonstrated the ability of the modules to cooperatively bind to multiple K_{ac} marks on histone H4 with low micromolar affinity ($K8_{ac}$/$K16_{ac}$, $K5_{ac}$/$K12_{ac}$, $K5_{ac}$/$K8_{ac}$/$K12_{ac}$/$K16_{ac}$ [30]) although binding to single histone H4 marks has also been reported ($K8_{ac}$, $K12_{ac}$, $K16_{ac}$ [37]). Histone H3 binding has also been demonstrated ($K14_{ac}$ [37], $K9_{ac}$/$K14_{ac}$ [33]) and interestingly phosphorylation of serine 10 doubles the affinity of the BRD for the target peptide [33]. TAF1 has also been shown to bind di-acetylated p53 ($K373_{ac}$/$K382_{ac}$ [85]) resulting in recruitment to the p21 promoter.

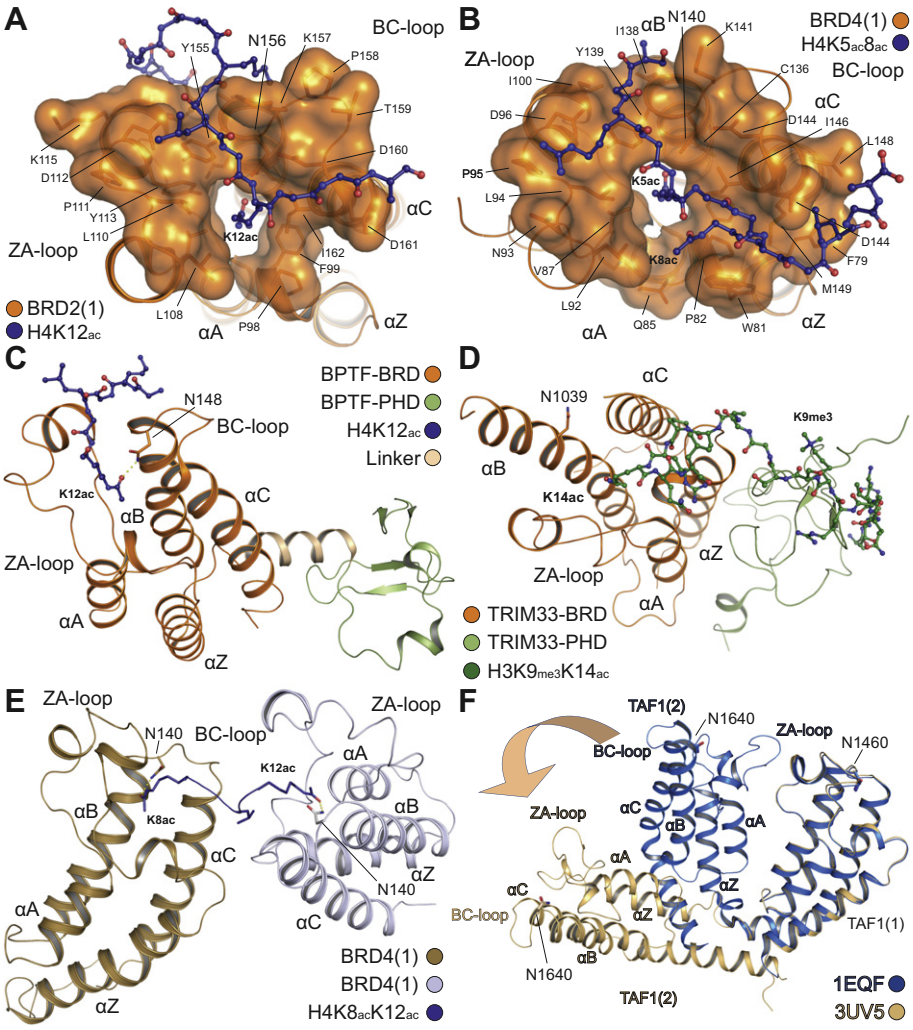

Fig. 2. Peptide Binding Modes and Module Cooperativity. (A) Binding of a single K_{ac} ($H4K12_{ac}$) onto BRD2(1) [36]. Key interacting residues and secondary structure elements are labelled. (B) Co-operative binding of two K_{ac} marks ($H4K5_{ac}K8_{ac}$) onto BRD4(1) [33]. Key interacting residues and secondary structure elements are labelled. (C) Binding of a single K_{ac} peptide ($H4K12_{ac}$) to the PHD/BRD tandem module of BPTF [93]. The arrangement of the two modules suggests a non-cooperative mode of peptide binding between them. (D) Binding of a tri-methylated and acetylated peptide ($H3K9_{me3}K14_{ac}$) to the tandem PHD/BRD modules of TRIM33 [79]. The two modules cooperatively bind the peptide demonstrating an avidity effect. (E) Binding of a diacetylated H4 peptide ($H4K8_{ac}K12_{ac}$) to two distinct BRD4(1) BRD modules [33], suggesting that the linker length between marks can allow for more than one BRD modules to bind the same peptide. (F) Structures of the tandem BRD modules of TAF1 [30,33] demonstrating the flexibility of the linker region which can allow for distant marks to be recognized by the tandem domains. Secondary structure elements have been annotated for the second domain (TAF1(2)) which exhibits a different orientation between the two structures.

The last subfamily of human bromodomains (**VIII**) contains the methyl-transferase *ash1 (absent, small, or homeotic)-like* (ASH1L) [28], the chromatin remodelling factors *SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2* (SMARCA2) [86] and *SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4* (SMARCA4) [87] as well as the *Polybromo 1* (PB1) [31]. The BRD of SMARCA2 has been shown to bind to histone H3 ($K9_{ac}$ [43], $K14_{ac}$ [33,43] and $K9_{ac}/K14_{ac}$ [43]) as well as histone H4 ($K8_{ac}$, $K12_{ac}$ [43], $K16_{ac}$ [33] and $K5_{ac}/K8_{ac}/K12_{ac}/K16_{ac}$ [43]). SMARCA4 has been reported to bind histone H2B ($K5_{ac}$ [88]), histone H3 ($K14_{ac}$ [88], $K9_{ac}/K14_{ac}$ [60,89]) as well as histone H4 ($K8_{ac}$ & $K12_{ac}$ [88], $K16_{ac}$ [88,89]). BRDs of the PB1 protein have also been shown to bind to any of the N-terminal histone H3 lysine marks ($K4_{ac}$, $K9_{ac}$, $K14_{ac}$, $K18_{ac}$, $K23_{ac}$ [90,91]) employing long peptides (residues 1–25) and fluorescent polarization or fluorescent anisotropy. It is noteworthy that these results could not be confirmed by isothermal titration calorimetry employing shorter peptides (11-mers with a central K_{ac} epitope) [33].

Given the wide distribution of acetylation in cells according to proteomics data analysis [11] it is surprising that mainly interactions between histones and BRDs have been extensively studied so far. There is evidence of interactions between BRDs and other 'hotspots' such as p53 [41,85] or transcription factors such as MyoD [62,63], RelA [51] and GATA1 [53,92]. Systematic studies are also lacking and reported affinities do not always agree between reports especially since multiple techniques have been used to determine binding as well as peptides of different sizes have been employed. Studies on the cross-talk between PTMs are also lacking although there is evidence that they affect binding, as seen by the effect of phosphorylation of serine 10 on histone H3 binding to the BRDs of CECR2, CREBBP and TAF1 [33]. A list of all studied BRD/K_{ac} interactions is given in Table 2.

5. Peptide binding modes and module cooperativity

The interactions of histones with almost half of the human BRD family have been studied but the structural basis of these interactions is limited, especially in light of their organization within large modular proteins that have a variety of domains that can facilitate or inhibit interactions with the target substrates. The readout of

Table 3
Structures of BRD/peptide complexes.

Protein	PDB code	Reso. [Å]	Histone mark	Peptide/ligand	Ref.
BPTF	2RI7	1.45	H3K4	H3(1–9)**K4$_{me2}$**	[104]
BPTF	2F6J	2.00	H3K4	H3(1–15)**K4$_{me3}$**	[104]
BPTF	2FSA	1.90	H4K4	H3(1–15)**K4$_{me2}$**	[104]
BPTF	3QZV	2.00	H4K12	H4(7–17)**K12$_{ac}$**	[93]
BPTF	3QZS	1.80	H4K16	H4(12–21)**K16$_{ac}$**	[93]
BPTF	3QZT	1.50	H4K20	H4(16–25)**K20$_{ac}$**	[93]
BRD2(1)	2DVQ	2.04	H4K12	SGRGKGGKGLG**K$_{ac}$**GGA	[36]
BRD2(1)	2DVR	2.30	H4K12	GGKGLG**K$_{ac}$**GGA	[36]
BRD2(2)	2E3K	2.30	H4K5/K12	SGRG**K$_{ac}$**GGKGLG**K$_{ac}$**GGA	N/A
BRD3(1)	2L5E	NMR	GATA1-K312K315	KASG**K$_{ac}$**GK**K$_{ac}$**KRGSN	[92]
BRD4(1)	3MUK	1.75	H3K23prop	AT**K$_{prop}$**AARK	[105]
BRD4(1)	3MUL	1.65	H3K14buty	GK$_{but}$	[105]
BRD4(1)	3UVW	1.49	H4K5K8	SGRG**K$_{ac}$**GG**K$_{ac}$**GLGY	[33]
BRD4(1)	3UVX	1.91	H4K12K16	G**K$_{ac}$**GGA**K$_{ac}$**RHRKV	[33]
BRD4(1)	3UVY	2.02	H4K16K20	A**K$_{ac}$**RHR**K$_{ac}$**VLRDN	[33]
BRD4(1)	3UW9	2.37	H4K8K12	G**K$_{ac}$**GLG**K$_{ac}$**GGAKR	[33]
CREBBP	1JSP	NMR	p53K382	SHLKSKKGQSTSRHK**K$_{ac}$**LMFK	[61]
MLL	3LQI	1.92	H3K4	H3(1–9)**K4$_{me2}$**	[106]
MLL	3LQJ	1.90	H3K4	H3(1–9)**K4$_{me3}$**	[106]
PB1(2)	2KTB	NMR	H3K14	ARTKQTARKSTGG**K$_{ac}$**APRKQL	[107]
PCAF	2RNW	NMR	H3K9	ARTKQTAR**K$_{ac}$**STGGKA	[41]
PCAF	2RNX	NMR	H3K36	STGGV**K$_{ac}$**KPHRYKC	[41]
PCAF	2RNY	NMR	H4K20	GGAKRHR**K$_{ac}$**VLRDNIQ	[41]
PCAF	1JM4	NMR	HIV/Tat	SYGR**K$_{ac}$**KRRQR	[108]
TIF1α	3O34	1.90	H3K23	H3(13–32)**K23$_{ac}$**	[73]
TIF1α	3O35	1.76	H3K27	H3(23–31)**K27$_{ac}$**	[73]
TIF1α	3O36	1.70	H4K16	H4(14–19)**K16$_{ac}$**	[73]
TIF1α	3O37	2.00	H3K4	H3(1–10)**K4$_{ac}$**	[73]
TRIM33A	3U5N	1.95	H3K14	H3(1–20)K9$_{me3}$**K14$_{ac}$**	[79]
TRIM33A	3U5O	2.70	H3K14K18	H3(1–22)K9$_{me3}$**K14$_{ac}$K18$_{ac}$**	[79]
TRIM33A	3U5P	2.80	H3K14K18K23	H3(1–28)K9$_{me3}$K14$_{ac}$K18$_{ac}$K23$_{ac}$	[79]

single acetylation sites has been investigated in detail and the structural motif has been demonstrated by high resolution crystal structures. A central hydrophobic cavity is presented to the peptide substrate, which docks via a hydrogen bond to the conserved asparagine residue found in most BRDs, with extensive back-bone hydrogen bonding stabilizing further the BRD/peptide interaction (Fig. 2A). However some BRDs contain a tyrosine, threonine or glutamate instead of an argentine suggesting a different mode of interaction with peptidic substrates which remains to be elucidated [33]. It has also been recognized that multiple acetylations, especially when they are closely spaced, may affect binding by enhancing affinity and this has also been structurally demonstrated in several cases [33,39,92]. The interaction of the diacetylated peptides is similar to the mono-acetylated ones, with one K_{ac} docking directly to the conserved asparagine and the second stabilizing it by direct hydrogen bonding to the peptide backbone, resulting in an orientation which sterically fits within the BRD cavity (Fig. 2B).

As part of larger multi-domain proteins, BRDs are usually found in tandem with a PHD module or another BRD module [33]. This suggests a functional arrangement whereby both domains can act in concert in order to bind to a specific acetylated site. It is still possible that there are no interactions between the modules of the tandem organization, as seen for example in the case of the complex of BPTF and an H4K12$_{ac}$ peptide [93], either because the linker between domains is not flexible or because the peptide presented is not long enough to span both modules (Fig. 2C). Interactions however between longer peptides and a PHD/BRD module have been reported and it is noteworthy that the affinity for such substrates is higher suggesting an avidity effect between modules [73,79]. The structure of TRIM33 with a methylated and acetylated histone H3 peptide (H3K9$_{me3}$K14$_{ac}$) [79] for example showed such an interaction, whereby the tri-methylated lysine 9 binds to the PHD module and the acetylated lysine 14 binds to the BRD module (Fig. 2D). The linker sequence between K_{ac} marks can also affect binding to multiple BRDs suggesting that distant marks can be recognized by two different domains in a tandem BRD/BRD system when the linker between modules is flexible or long enough to allow for such an organization to take place. The structure of BRD4(1) with a histone H4 diacetylated peptide (H4K8$_{ac}$K12$_{ac}$) demonstrated that two distinct BRD modules can bind at the two sites simultaneously [33] (Fig. 2E). Although the initially obtained crystal structure of the tandem TAF1 BRDs [30] showed the two modules in close proximity, a recent crystal structure [33] demonstrated the flexibility of the tandem system whereby one of the two domains can move in a twist motion away from the other, potentially allowing for longer substrates to be recognized, with K_{ac} marks at distant sites (Fig. 2F). A list of known BRD/peptide structures is given in Table 3.

A superimposition of the known BRD structures found in the public domain to the structure of BRD4(1) in complex with the diacetylated histone H4 peptide H4$_{(1–11)}$K5$_{ac}$K8$_{ac}$ (PDB ID: 3UVW [33]) suggests that several members of other subfamilies can bind to similar multiply acetylated motifs (Fig. 3). This suggests that the K_{ac} recognition cavities can be large enough to accommodate two marks, an observation that can be exploited for the development of specific inhibitors for this class of reader modules.

6. Inhibition of BRD-specific K_{ac} readout

Disruption of the interaction between a BRD and its acetylated-lysine substrates could in principle provide an additional control over the disease-associated acetylome, when combined with HDAC inhibitors. The deep and relatively hydrophobic cavity where K_{ac} epitopes dock offers a drugable pocket for the development of small molecule inhibitors of the BRD interaction with acetylated proteins [94]. Additionally, the diversity of the loop regions surrounding the central cavity results in large variations in surface

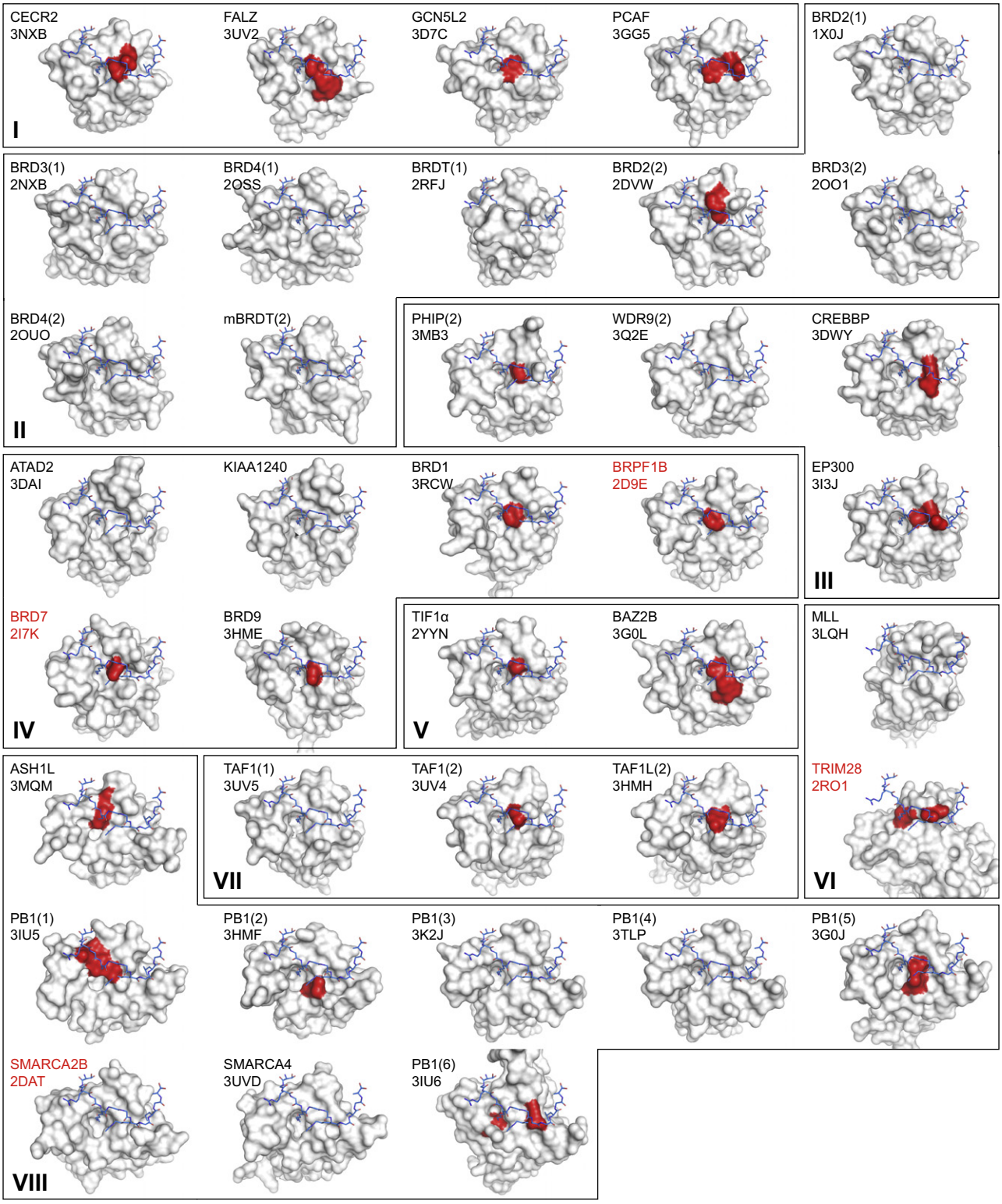

Fig. 3. Double Acetylated Lysine Binding onto BRD Modules. Superimposition of known holo-BRD structures (represented as white surfaces) to the complex of BRD4(1) with a diacetylated H4 peptide ($H4K5_{ac}K8_{ac}$) suggests that several BRDs can bind two K_{ac} marks at the same time. Residues that clash with the peptide backbone are coloured in red showing that several BRDs cannot recognize di-acetyl marks in this mode.

electrostatic properties suggesting that specific targeting of BRDs is possible [33]. Early BRD inhibitors with low micromollar affinities have been shown to disrupt the interaction of PCAF to the HIV-1 TAT with selectivity over the BRDs of CREBBP and TIF1β [95] or the interaction between CREBBP and p53 [96]. Interestingly the latter still functions effectively in cellular systems in modulating p53 transcriptional activity by suppressing recruitment of CREBBP to p53, ultimately leading to transcriptional activation of target genes such as the cell cycle inhibitor p21. The BRD of CREBBP has also been targeted by ischemin, a sulfonic acid based scaffold

which induces cellular protective activity against myocardial damage by down-regulating p53-induced apoptosis instigated by doxorubicin treatment of rat cardiomyocytes [95]. Cyclic peptides were also shown to disrupt binding of the CREBBP BRD to acetylated p53, thus inhibiting transcriptional activity and promoting p53 instability in colorectal carcinoma cells [97].

Benzo- and thieno-diazepines were initially reported in the patent literature as very potent and selective inhibitors of the BET subfamily of BRDs [98], leading us and others to investigate these promising chemotypes. We found that benzo-diazepine and benzo-triazepine scaffolds exhibit affinity for BET BRDs providing good starting points for rational inhibitor design [99]. We developed a cell-permeable and potent inhibitor (JQ1), based on a triazolo-thieno-diazepine scaffold with high affinity and selectivity for all BET BRDs [19]. Binding of JQ1 to the tandem bromodomains of BRD4 was shown to be K_{ac} competitive and displaced BRD4 from chromatin in human cells. Competitive binding of JQ1 to the BRD4-NUT fusion oncoprotein in NUT-midline carcinomas (NMCs) resulted in squamous differentiation exhibiting specific anti-proliferative effects both in BRD4-dependent cell lines as well as in patient-derived xenograft models. Additionally, JQ1 was shown to have therapeutic effects in multiple myelomas, through regulation of the Myc oncogenic driver in these tumors [100]. Another benzodiazepine scaffold (IBET) was shown to exhibit great potential as an anti-inflammatory agent, by reducing expression of pro-inflammatory genes in activated macrophages [20].

A series of weak K_{ac} mimetic inhibitors based on the isoxazole chemical fold has also been recently developed for BET BRDs [101]. This acetyl lysine mimetic scaffold has also been further optimized to yield the highly potent and specific BET inhibitor I-BET151 which also showed improved pharmacokinetics when compared to earlier benzodiazepines such as IBET and JQ1, exhibiting good efficacy against human and murine acute leukaemic cell lines, through the induction of early cell cycle arrest and apoptosis [21]. The developed chemical molecules provide excellent tools for proof-of-concept studies in different disease backgrounds. It will be now interesting to see if molecules of similar selectivity and potency can be developed for bromodomains outside the BET family.

BRD modules are part of larger multi-modular proteins and therefore it will be interesting to see how inhibitors targeting either single or combination of different modules will attenuate the overall protein function. For instance, PHD fingers are often found next to BRD modules and have been shown to contribute to increased affinity for peptide substrates in vitro. Inhibition of both the PHD and BRD function would completely abolish these interactions while inhibition of only one module would only decrease binding to one specific mark. One can envision cases where inhibitors of different functions (reading/writing/erasing of PTMs) could be combined in order to attenuate the overall protein behaviour. Furthermore, when binding sites are aligned one can envision larger molecules that could potentially bind to multiple sites at the same time, thus abolishing the readout of PTMs altogether.

7. Outlook

The availability of sequence data of the entire human genome made it possible to identify all members of large protein families. The bromodomain interaction module represents a large superfamily of functional acetyl lysine interaction domains that is present in a large diversity of proteins. High throughput crystallography enabled rapid generation of high resolution structural models and the BRD family is now well characterized from a structural biology viewpoint. However, so far only few substrates have been identified in particular for non-histone proteins that would allow functional annotation of BRDs. The large number of described acetylation sites and the importance of cross talk with neighbouring post translational modification makes systematic peptide array studies time consuming and expensive. Proteomic approaches may help to address the question of substrate recognition in a more effective way. These methods would also consider the multi-domain architecture of most BRD containing proteins which will contribute to the high affinity recognition of substrates. We believe that specific and potent BRD inhibitors will be the most effective tools to investigate the cellular functions of bromodomain proteins and their role in disease. Initial studies already indicated a large potential of BRD inhibitors for the development of new treatment strategies that target epigenetic reader domains.

Acknowledgements

The SGC is a registered charity (number 1097737) that receives funds from the Canadian Institutes for Health Research, the Canada Foundation for Innovation, Genome Canada, GlaxoSmithKline, Pfizer, Eli Lilly, the Novartis Research Foundation, the Ontario Ministry of Research and Innovation and the Wellcome Trust. PF is supported by a Wellcome Trust Career Development Fellowship (095751/Z/11/Z).

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