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Bromodomain biology and drug discovery

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Abstract

The bromodomain (BrD) is a conserved structural module found in chromatin- and transcription-associated proteins that acts as the primary reader for acetylated lysine residues. This basic activity endows BrD proteins with versatile functions in the regulation of protein-protein interactions mediating chromatin-templated gene transcription, DNA recombination, replication and repair. Consequently, BrD proteins are involved in the pathogenesis of numerous human diseases. In this Review, we highlight our current understanding of BrD biology, and discuss the latest development of small-molecule inhibitors targeting BrDs as emerging epigenetic therapies for cancer and inflammatory disorders.

Lysine acetylation and methylation were the first post-translational modifications (PTMs) on histones to be discovered. Allfrey and colleagues postulated in 1964 that they function as an 'on-off switch' to regulate chromatin-templated gene expression[1]. The seminal identification of GCN5 as the first histone acetyltransferse (HAT; 'writer') by Allis and colleagues[2,3] and HDAC1 as the first histone deacetylase (HDAC; 'eraser') by Schreiber and colleagues[4] in 1996 revealed an active control mechanism of histone acetylation that works through counteracting enzymatic activities, thereby substantiating Allfrey's vision of the role of histone acetylation in gene expression.

The question of whether lysine acetylation simply reduces interactions between histones and DNA by neutralizing the positive charges on histones or actively participates in gene expression remained unanswered until the discovery of the bromodomain (BrD) as the acetyl-lysine (Kac) binding domain by Zhou and colleagues[5] in 1999. The BrD, regarded as the first chromatin 'reader' and found in many chromatin and transcription-associated proteins[6], has now been established as a fundamental mechanism by which histone acetylation acts in trans to regulate cellular gene transcription in response to physiological and environmental cues. The general concept of chromatin 'reading' in a PTM-sensitive

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Competing interests

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manner played a key role in the 'histone code' hypothesis initially proposed by Allis and colleagues[7] in 2000, which predicted that "multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions." Owing to the increasing number and types of histone PTMs discovered ever since[8], this prediction has yet to be fully confirmed, but it has contributed to major advances in our current understanding of epigenetic gene regulation in the past two decades.

The paramount importance of BrDs in gene transcription was highlighted by two independent studies reported in 2010, which showed that the potent BrD inhibitors JQ1 and I-BET762, both selectively targeting BrDs of the bromodomain and extra-terminal (BET) family proteins, effectively blocked cell proliferation in nuclear protein in testis (NUT) midline carcinoma[9], and suppressed expression of inflammatory genes in macrophages activated by lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis[10], respectively. These two studies catalyzed an incredible surge of drug discovery efforts targeting BrDs as a new therapeutic strategy for a wide range of human diseases, including cancers and inflammatory disorders[11,12]. Currently, there are over 30 clinical trials evaluating efficacy of various BrD inhibitors for different human disorders.

In this Review, we highlight recent advances in BrD biology, focusing on the role of BrD-mediated Kac binding in gene transcription. We also discuss the latest in development of small-molecule BrD inhibitors as emerging epigenetic targeted therapies for human diseases.

Discovery of the BrD as the acetyl-lysine binding module

The BrD was first reported by Tamkun et al.[13] in 1992 in their study of the *Drosophila* gene Brahma and female-sterile homeotic proteins. Later, it was established as an evolutionarily conserved protein module found in many chromatin and transcription-associated proteins[6]. However, the specific molecular function of the BrD remained elusive until 1999 when the structure of the BrD from human transcriptional co-activator PCAF (p300/CBP-associated factor) was determined using nuclear magnetic resonance (NMR) spectroscopy[5] (Fig. 1a). The unique structural fold consists of a left-handed four-helix bundle (helices α_Z, α_A, α_B and α_C) and two inter-helical loops (ZA and BC) at one end of the helical bundle connecting the α_Z and α_A, and α_B and α_C helices, respectively. The ZA and BC loops form a hydrophobic pocket and likely have a role in stabilizing the left-handed four-helix bundle, dubbed the bromodomain fold, which is distinct from the more commonly seen right-handed four-helix bundle fold in proteins such as hemery-thrin and cytochrome b_{562}[14]. Using 2D ^{1}H-^{15}N-heteronuclear single quantum coherence (HSQC) spectra[5], Zhou and colleagues demonstrated that a lysine-acetylated histone H4 peptide perturbed NMR resonance of the residues in the binding pocket between the ZA and BC loops (Fig. 1b). This provided direct biochemical evidence that acetylated lysine is bound at this location. The structure of the PCAF BrD in complex with acetyl-histamine, a small-molecule mimic of Kac provided further evidence that the BrD is a Kac recognition domain[5].

In 2000, the crystal structure of the tandem BrDs of $TAF_{II}250$, the largest subunit of the RNA polymerase II transcription factor D (TFIID) complex, demonstrated their preference

for binding to multiple acetylated histone H4 peptides[15]. In that same year, Owen et al.[16] published the crystal structure of the BrD of the HAT GCN5p bound to a lysine 16-acetylated histone H4 peptide (H4K16ac), revealing that Kac recognition is achieved by a key hydrogen bond formed between the carbonyl oxygen of the acetyl group of K16ac and the amide nitrogen of Asn407, a highly conserved residue in the BrD family. This crystal structure also uncovered several water molecules at the base of the Kac binding pocket that form a network of water-mediated hydrogen bonds with the Kac and protein residues[16].

Structure and Kac recognition by BrDs

In 2012, Filippakopoulos et al.[17] presented a comprehensive structural characterization of human BrD family proteins and their binding preference for various lysine acetylation sites in core histones. That study used available sequence databases to identify 61 BrDs in the human genome in 46 diverse proteins that are grouped into eight subfamilies based on protein sequence similarity[17]. Currently, more than 400 high-resolution X-ray crystal structures of BrDs are available in the PDB, confirming the conserved left-handed four-helix bundle bromodomain fold as initially seen in the PCAF BrD[5] (Fig. 1a).

The high degree of sequence variations in length and amino acid composition of the ZA and BC loops, which engage in interactions with residues flanking the Kac, attributes to the binding preference of different BrDs for distinct lysine acetylation sites in histones and non-histone proteins. Nevertheless, the acetyl-lysine recognition by BrDs is conserved, involving two hydrogen bonds formed between the carbonyl oxygen of Kac and the side-chain NH_2 of the conserved Asn, and the side-chain OH of the conserved Tyr (mediated by water), as seen in 48 of the 61 BrDs[11] (Fig. 1c). In the other 13 BrDs, the conserved Asn is replaced by a Tyr, Thr or Asp, suggesting that although they adapt the conserved bromodomain fold, these ‘atypical BrDs (aBrDs) are likely not able to bind Kac. Functions of these atypical bromodomains are possibly related to adjacent protein modules and warrant further investigation. Another distinct feature of the Kac binding pocket of BrDs is the presence of an array of water molecules bound to the protein residues at the bottom of the Kac binding pocket that engage hydrogen-bond-mediated interactions with Kac or provide a solvation shell shielding the hydrophobic and aromatic residues in the Kac pocket in the free state.

Classification and functions of BrD proteins

BrD proteins have diverse activities, including histone modifications, chromatin remodeling, transcription factor recruitment and enhancer or mediator complex assembly, and they affect both transcription initiation and elongation. Notably, the majority of BrD proteins also contain other structurally conserved modular domains that function independently or work in concert with the BrD to exert effects on protein-protein or protein-nucleic acid interactions. Below, we propose a function-based classification of human BrD proteins (Fig. 2), which, in our view, provides a more direct way to appreciate the roles of BrDs in the diverse cellular functions of BrD proteins than the conventional structure-based clustering[17,18]. It is, however, interesting to note that both functional and structural organizations show some overlap. A summary of biological functions and disease implications of BrD proteins is presented in Supplementary Table 1.

Group Ia.

Group Ia comprises HAT-containing transcriptional co-activators PCAF, GCN5L2, p300/CBP; TFIID subunits TAF1 (TBP-associated factor 1, also known as $TAF_{II}250$) and TAF1L. In the HAT-containing co-activators, BrDs lie adjacent to the HAT catalytic domain and have a role in manifesting the multifaceted functions of these proteins. For instance, in PCAF, GCN5 and p300/CBP, the BrD can aid substrate recruitment and facilitate HAT-mediated acetylation of multiple lysine residues both on histones and transcription factors, thereby promoting transcriptional activation[19]. Additionally, the BrD can directly engage in the control of p300 HAT activation through intra- and/or intermolecular interactions with the autoinhibitory lysine-rich loop of p300, which undergoes hyper-acetylation[20] (Fig. 3a). The double BrDs of $TAF_{II}250$ (Fig. 3b) link lysine acetylation of histones and basal transcription factors to core promoter recognition by the TFIID complex that consists of TAF subunits and TATA-binding protein (TBP)[21]. Through its ability to couple lysine acetylation via the HAT domain and Kac binding via the BrDs, $TAF_{II}250$ can also recruit other HATs, including p300/CBP, to work cooperatively to promote transcription initiation, and assembly and activation of regulatory enhancer or mediator complexes at target genes.

Group Ib.

Group Ib comprises BRPF1/2/3 and BRD8, which function as accessory subunits of the conserved eukaryotic MYST family HATs (MOZ/MORF, MOF, TIP60 and HBO1). These HATs are responsible for acetylating free histones, and are also implicated in cancer, particularly leukemia[22]. BRPF1/2/3 consist of a PHD finger, a BrD and a C-terminal PWWP domain[23]. In the monocytic leukemic zinc finger (MOZ) HAT quaternary complex, BRPF1 enhances acetylation activity of MOZ[24] through its BrD and thus promotes expression of homeobox (*HOX*) genes. The MOZ HAT is essential for the development and maintenance of hematopoietic stem cells and for hematopoiesis[25], and it is involved in chromosomal translocations in a subtype of acute myeloid leukemia (AML)[26]. The HBO1-BRD1/BRPF2 complex is the major HAT for histone H3K14 acetylation, mediating transcriptional expression of erythroid developmental regulator genes[27].

BrD8 is an accessory subunit of the NuA4 HAT (TRRAP/TIP60) complex and binds thyroid hormone receptor β or retinoid X receptor α as a coactivator for nuclear hormone receptor-mediated transcription[28]. BRD8 is involved in sensitivity to spindle poisons and proteasome inhibitors in aggressive colorectal cancers and is found overexpressed in human metastatic colorectal cancer, with possible association to tumor progression toward advanced stages[29].

Group II.

Group II comprises SET domain-containing histone lysine methyltransferases (HKMTs) ASH1L and MLL. These proteins contain an aBrD, which lacks the conserved Asn residue required for Kac binding. Unlike in HATs, the aBrD is not located adjacent to the SET catalytic domain in sequence, but rather in tandem with a PHD finger and bromodomin adjacent homology (BAH) domain in ASH1L or a phenylalanine- and tyrosine-rich (FYRN) domain in MLL. Although the PHD finger can bind to histones in a PTM-sensitive manner, functions of BAH or FYRN domains remain undefined. Roles of triple-domain tandem

modules of aBrD-PHD-BAH and aBrD-PHD-FYRN also warrant further investigation, especially considering that a conserved structural fold can mediate diverse functions.

Group IIIa.

Group IIIa comprises chromatin remodeling factors SMARCA2 (BRM), SMARCA4 (BRG1), BRD7, BRD9 and PBRM1 (polybromo). These proteins are key subunits of the mammalian SWI/SNF (mSWI/SNF) ATP-dependent chromatin remodeling complexes. The ATPase component of mSWI/SNF, BRG1/BRM, is composed of multiple functional modules and possesses DNA helicase activity via its DEAD domain and histone binding activity mediated by its BrD. Recent studies by Kadoch and colleagues[30] show that mSWI/SNF chromatin remodeling complexes exist in three distinct classes: canonical ATPase BRG1/BRM-associated factor (BAF), polybromo-associated BAF (PBAF) and the newly defined ncBAF complexes. Notably, all three forms of the mSWI/SNF complex have one anchoring BrD-containing ATPase. The PBAF complex contains BRD7 and a unique PBRM1 component, which comprises four tandem BrDs flanked by two aBrDs. The smallest ncBAF complex contains BRD9. The ability to bind Kac via the BrD likely contributes to multiple aspects of mSWI/SNF function. These include facilitating the assembly of PBAF and ncBAF complexes through BRD7 and BRD9, respectively, propelling ATPase-driven movement of the mSWI/SNF complex along chromatin, and coordinating spatial and temporal control of multifaceted protein-protein interactions via PBRM1 for chromatin remodeling.

Group IIIb.

Group IIIb comprises ISWI family chromatin remodeling factors[31] BAZ1A (ACF1), BAZ1B (WSTF, William-Beuren syndrome transcription factor), BAZ2A, BAZ2B, BPTF and CECR2. In mammals, the core ISWI ATPase is either SNF2H or SNF2L, both of which form distinct remodeling complexes that differ in their accessory subunits[32]. SNF2H is present in nucleolar remodeling complex (NoRC), WSTF ISWI chromatin remodeling (WICH), ACF and human CHRAC complexes, whereas SNF2L is in NURF and CECR2-containing remodeling factor (CERF) complexes[33]. The BrD of CECR2 or the tandem PHD finger and BrD module of BPTF (Fig. 3c) may contribute to functions of NURF and CERF complexes in transcriptional activation and repression. Similarly, tandem PHD finger and BrD modules present in BAZ1A and BAZ1B or BAZ2A and BAZ2B may contribute to the activities of ACF, CHRAC and WICH complexes in nucleosome assembly and spacing, and in DNA replication through heterochromatin and chromosome segregation[33,34].

Group IV.

Group IV comprises ATPase family AAA domain-containing proteins ATAD2 and ATAD2B. ATAD2 has been shown to be recruited to DNA replication sites through direct interaction with newly synthesized di-acetylated histone H4K5acK12ac[35], demonstrating BrD function in replication-coupled chromatin reassembly.

Group V.

Group V comprises BET family proteins BRD2, BRD3, BRD4 and testis-specific BRDT. These proteins consist of two characteristic BrDs followed by the ET domain[36]. Arguably the most extensively characterized BrD proteins, BET proteins have multifaceted roles in the regulation of gene transcription. Their two tandem BrDs bind to lysine-acetylated histones and transcription factors, augmented by the ET domain, which also interacts with chromatin and transcription proteins[37-39]. BET proteins facilitate chromatin opening, recruit transcription factors and co-activators to target gene promoters and enhancers, and activate paused RNA Pol II complexes to promote transcriptional elongation[40]. Distinguished from other BrDs, BET BrDs exhibit preference for di-acetylated lysine residues closely located in protein sequence[17,41,42]. As shown in the crystal structure of a histone H4K5acK8ac peptide bound to the first BrD of BRD4 (BD1), H4K5ac is recognized through the canonical mode of Kac binding by hydrogen bonding interactions with the conserved Asn140 and Tyr97, whereas the adjacent H4K8ac is engaged in binding to hydrophobic and aromatic residues, reinforcing H4K5ac recognition (Fig. 1c). This distinct specificity enables BRD4 to recruit di-acetylated transcription factors and cyclin T1 of the pTEFb complex through its second BrD (BD2). In addition, BRD4 interacts with paused RNA Pol II complexes at target loci marked with di-acetylated H4K5acK8ac (recognized by the BD1 of BRD4), leading to phosphorylation and activation of RNA Pol II for productive transcription elongation[43]. As a representative BET protein, BRD4 acts in the key steps of transcription factor recruitment, enhancer and mediator complex assembly, and transcription elongation of oncogenes and pro-inflammatory cytokines or chemokines[43]—and is thus seen as an attractive drug target. Pharmacological inhibition of BET BrDs indeed blocks oncogenic *cMyc* expression in multiple myeloma[44,45], MLL-fusion leukemia and AML[46,47].

Group VI.

Group VI comprises Tripartite-motif-containing (TRIM) family proteins TRIM24 (TIF1α), TRIM28 (TIF1β, KAP1), TRIM33 (TIF1γ) and TRIM66 (TIF1δ). These proteins are also known as transcriptional intermediary factor 1 (TIF1) proteins, characterized by an N-terminal TRIM domain consisting of a RING domain, one or two B boxes and a coiled coil as well as a C-terminal PHD finger and BrD. Despite their similar overall domain organization, these proteins have diverse functions in gene regulation. TIF1α is a ligand-dependent nuclear receptor coregulator and has been implicated in regulating p53 stability[48]. TIF1β is a component of the corepressor complex N-CoR1 and the NuRD nucleosome-remodeling complex, and promotes heterochromatin formation by interacting with the heterochromatin protein HP1. PHD finger of TIF1β does not bind histones[49]; instead the PHD finger-aBrD tandem module (Fig. 3c) acts as a SUMO E3 ligase, sumoylating the adjacent aBrD and likely histones as well and mediating transcriptional silencing[50,51]. The PHD-BrD tandem module of TIF1γ binds H3K9me3 and H3K18ac of the same histone H3 tail (Fig. 3c), thereby recruiting TRIM33-Smad2/3 to the promoters of mesendoderm regulators *Gsc* and *Mixl1* for transcriptional activation[52]. As a tumor suppressor, TIF1γ also possesses ubiquitin E3 ligase activity, and ubiquitylates nuclear β-catenin, targeting it for degradation, thereby blocking tumor cell proliferation and tumorigenesis[53]. TIF1δ is testis-specific and interacts with HP1γ[54].

Group VII.

Group VIII comprises speckled protein (SP) family proteins SP100, SP110, SP140 and SP140L. The molecular functions of these proteins remain elusive, but the proteins share high sequence similarity with the autoimmune regulator (AIRE)[55]. SP family proteins are components of subnuclear promyelocytic leukemia nuclear bodies (PML-NBs)[56,57], which form in response to a variety of cellular stresses, including viral infection, interferon signaling and DNA damage. The presence of aBrDs in tandem with a SAND domain (SP100, AIRE, NucP41/P75 and DEAF)[58] and a PHD finger, which are both capable of DNA and histone binding suggests that the SP proteins may be involved in regulation of chromatin structure mediated by combined protein-protein and protein-DNA interactions.

Group VIII.

Group VIII comprises myeloid, Nervy and DEAF-1 (MYDN) proteins ZMYND8 and ZMYDN11. ZMYND8 (PRKCBP1, RACK-7 and Spikar) was first identified as a protein kinase C binding partner[59] and as a transcriptional regulator[60]. ZMYND8 (PRKCBP1) and ZMYND11 (BS69), both reported tumor suppressors[61], have similar domain organization, consisting of an N-terminal nuclear localization sequence, a PHD-BrD-PWWP tandem module and a C-terminal MYND domain. ZMYND11 was shown to bind histone H3.3K36me3 via its N-terminal BrD-PWWP module[62]. Coordinated interaction of the PHD-BrD-PWWP module with H3K4me1, H3K14ac and DNA enables ZMYND8 to be recruited to DNA damage sites and to control gene transcription[63]. A more recent study revealed that ZMYND8 can also bind non-histone proteins through its N-terminal PHD-BrD-PWWP tandem module, specifically the cytoplasmic actin binding protein Drebrin[64]. Because the majority of BrD proteins function in nucleus, the biological importance of ZMYDN8 cytoplasmic sequestration via its association with Drebrin awaits further investigation.

Group IX.

Group IX comprises WD-repeat proteins BRWD1 (WDR9), BRWD3 and PHIP (WDR11). The WD repeat-containing proteins have been implicated broadly in cellular functions, including transcription, RNA processing, signal transduction, cytoskeleton assembly and the cell cycle regulation[65]. Notably, distinct from the BET family proteins, the tandem BrDs in these BRWD proteins consist of a canonical Kac-binding BrD followed by an aBrD of unknown function. WDR9 acts as a transcriptional regulator involved in chromatin remodeling through association with BRG1, a BrD-containing subunit of the core SWI/SNF chromatin remodeling complex[66]. Moreover, WDR11 (PHIP), which is implicated in congenital hypogonadotropic hypogonadism and Kallmann syndrome, both human developmental genetic disorders defined by delayed puberty and infertility, has been reported to function by modulating the Hedgehog signaling pathway and is essential for ciliogenesis[67].

Small-molecule inhibitors of BrDs

The importance of BrDs in chromatin-based gene transcription makes it appealing to develop small molecules capable of selectively modulating acetylation-mediated protein-protein interactions as tools to study BrD functions in biology and diseases. This idea

became feasible when the structure of the PCAF first revealed a structurally defined Kac binding pocket well suited for small-molecule binding, as shown with acetyl-histamine, a Kac mimic[5]. Indeed, the first BrD inhibitor (N1-aryl-propane-1,3- diamine NP1; Supplementary Table 2), discovered in 2005 in an NMR spectroscopy-based screen, was shown to block PCAF BrD recruitment by the HIV-1 trans-activator Tat, resulting in inhibition of transcriptional activation of latent HIV-1 in host cells[68]. The CBP BrD inhibitor MS7972 (tetrahydrocarbazolone) reported[69] in 2006 disrupted CBP binding to Lys382-acetylated p53, a key association required for p53-induced transcription of the cell cycle inhibitor *p21* in response to DNA damage. This direct evidence of BrD druggability provided the impetus for further development of BrD inhibitors[69].

As previously reviewed (ref. [11]), the publication of two landmark patents[70,71] filed in 2006 and 2008 by Mitsubishi Tanabe Pharmaceutical Corporation was a seminal moment for BrD drug development. These patents reported a series of potent thienotriazolodiazepines targeting BrDs of BET family proteins. Notably, these diazepine compounds are analogs of benzodiazepines that have been in use in the clinic as anxiolytic and sedative agents for years[72]. The compounds' safety, bioavailability and efficaciousness in humans combined with the potential to tailor them to BET BrDs made them an ideal class to be developed into drug molecules for BET BrDs.

The crystal structure of one of these compounds, the thienotriazolodiazepine JQ1, bound to the BD1 of BRD4, showed that the triazole ring serves as a Kac mimic. It sits deep in the hydrophobic binding pocket and forms hydrogen bonds with bound water molecules and the conserved Asn[9] (Fig. 1d). The ligand-binding residues are common in BD1 and BD2, therefore JQ1 and other related diazepine compounds are pan-BET bromodomain inhibitors. Functionally, JQ1 was shown to displace BRD4-NUT fusion oncoproteins from chromatin. This resulted in tumor regression, squamous cell differentiation, and growth arrest in patient-derived xenograft models of NUT midline carcinoma (NMC), a rare and aggressive form of cancer[73]. The benzodiazepine I-BET762 (ref. [10]), disclosed at the same time as JQ1 (Fig. 4) was shown to disrupt chromatin complexes responsible for the expression of key inflammatory genes in activated macrophages. Additional thienotriazolodiazepines have been reported since, underscoring the robustness of this scaffold for a BET BrD inhibitor. For instance, MS417 shows improved potency compared to JQ1 because its methyl ester moiety at the chiral carbon on the diazepine ring engages in additional interactions with protein residues. This compound blocks HIV-induced activation of the NF-κB transcription factor in HIV-associated nephropathy[74]. Two other compounds showed clinically relevant activity in cancer models. CPI-203 has synergistic effects against mantle cell lymphoma and pancreatic neuroendocrine tumors[75,76]. OTX015 was efficacious in inhibiting proliferation of multiple human cancer cell lines[77].

Chemotypes distinct from the diazepine scaffold were also used in the design of BrD inhibitors (see representative bromodomain inhibitors in Fig. 4 and Supplementary Table 2). For example, 3,5-dimethylisoxazole can act as a replacement for the triazole ring in the thienotriazolo-diazepine and benzodiazepine inhibitors[78]. The isoxazole ring is a main characteristic of the potent and selective BET inhibitor I-BET151, which features a quinoline core in place of a diazepine-based core[46]. I-BET151 was shown to block the

growth of MLL-fusion leukemia cells[46] and inhibited the progression of JAK2V617F-driven myeloproliferative neoplasms[79]. Additional quinoline-based BrD inhibitors were reported, including the representative tetrahydroquinoline I-BET726 (ref. [80]), the dihydro-quinazolinone PFI-1 (ref. [81]) and the quinazolinone RVX-208 (ref. [82]).

Despite their impressive selectivity for BET BrDs, diazepine-based compounds are nonselective within the BET family. Functional differences in the regulation of gene transcription among members of the BET family (BRD2 versus BRD4)[83], different BrDs of the same protein (BRD4-BD1 versus BD2)[84] or even analogous BrDs of different BET family members (BRD4-BD1 versus BRD2-BD1)[85], inspired the development of BET BrD selective inhibitors. These compounds have different phenotypic effects than pan-BET inhibitors (Fig. 4 and Supplementary Table 2). The quinazolinone RVX-208 shows 15–30-fold selectivity for BET BD2s over BD1s[82]. Inversely, the diazobenzenes MS436 and MS611 (ref. [86]), and the tetrahydro-pyrido indole olinone[86], show a preference for BET BD1s over BD2s. BD1-specific inhibition by olinone, with over 88-fold selectivity, promotes differentiation of oligodendrocyte progenitors, whereas pan-BET inhibitors, such as MS417, do not have the same effect[86]. The recently reported cyclic vinylogous amide MS402, which is selective for BRD4-BD1, was shown to inhibit lineage differentiation of T-helper 17 (Th17) cells with little or no effect on Th1, Th2 and regulatory T cells[85]. This contrasts sharply with the broad inhibitory effects on different T-helper cell lineages by pan-BET inhibitors[85]. MS402 ameliorates T cell transfer-induced colitis in mice by blocking Th17-specific gene expression and Th17 cell overproduction[85]. These studies highlight the therapeutic potential of selective inhibition of individual BET BrDs as new treatment for inflammatory disorders.

Small-molecule inhibitors have also been reported for non-BET BrDs, including those previously regarded to have low druggability because of shallower binding pocket or suboptimal hydrophobicity in the Kac binding pocket[87,88] (Supplementary Table 2). For instance, SGC-CBP30 and I-CBP112 are highly selective and potent inhibitors for CBP/p300 BrDs and interact with low-nanomolar affinity. For the BrD of BRPF1, a key subunit of the MOZ or MORF HAT complexes, a benzimidazolone inhibitor (compound **1**; Fig. 4) was shown to be potent and selective over BRPF2, BRPF3 or BET BrDs[89]. Another BRPF1 inhibitor, IACS-9571, shows nanomolar potency for BRPF1 and TRIM24 (dissociation constant (K_d) of 14 nM and 31 nM, respectively), and promising cellular activity (half-maximal effective concentration (EC_{50}) of 50 nM)[90]. This inhibitor is selective over other BrDs, but not in BRPF BrDs[90].

Inhibitors were reported for the BrD of ATAD2, which was initially regarded as an very challenging but attractive cancer target[87] owing to its role in the control of cell proliferation and survival[91]. An NMR spectroscopy-based fragment screen[92] yielded several moderate-affinity core chemotypes, and another study reported low-micromolar-affinity quinolinone- and napthyridone-based inhibitors for ATAD2 (ref. [91]). Recently, GlaxoSmithKline reported a number of potent and highly selective inhibitors for ATAD2 and ATAD2B BrDs, including GSK8814 (ref. [93]) (Supplementary Table 2). These BrD inhibitors might aid in gaining a greater understanding of the role of ATAD2 and ATAD2B in disease pathways.

As previously discussed[11], inhibitors for functionally less characterized BrDs were also reported. For instance, a tetrahydro-γ-carboline (THγC) inhibitor was reported for the BAZ2B BrD with K_d of 9 μM[94,95]. GSK2801 has shown high affinity and selectivity for BAZ2A and BAZ2B (K_d of 257 nM and 136 nM, respectively)[96], whereas BAZ2-ICR is selective for both BAZ2A and BAZ2B (half-maximal inhibitory concentration (IC_{50}) of 130 nM and 180 nM, respectively)[94]. Inhibitors were also reported for closely related BrDs of BRD9 and BRD7, which are components of SWI/SNF remodeling complexes. For instance, the quinolone-fused lactam LP99 (ref. [97]) and the imidazopyridine compound **2** (ref. [98]) (Fig. 4) have high affinity for BRD9 (K_d of 99 nM and 68 nM, respectively) with lower activity for BRD7, and little to no activity against other BrDs. Another series of 9H-purine-based compounds has nanomolar activity against the BRD9 BrD, with moderate selectivity for BRD4-BD1 (ref. [99]). The thienopyridine I-BRD9 is the most potent and selective BRD9 inhibitor developed thus far; it has low-nanomolar affinity for BRD9 with >200–700-fold selectivity over BRD7 and the BET BrD[100].

Given the functional importance of the tandem BrDs of the BET family, the notion of simultaneously inhibiting both using bivalent inhibitors, suggested by Arnold et al.[101] in a patent filing is attractive. The feasibility of this approach was demonstrated by two bivalent BET BrD inhibitors, the triazolopyridazine AZD5153 (ref. [102]) and diazepine-based MT1 (ref. [103]). Both exhibit much higher efficacy than monovalent BET inhibitors in growth inhibition of hematopoietic cancer cells. A recent study further showed that the linker length, composition and rigidity of a bivalent BET inhibitor (Fig. 4) are determinant factors for inhibition of BRD4 activity in cells[104]. Spatially constrained binding of the tandem BRD4 BrDs by a highly potent carbon-linker bivalent BET BrD inhibitor MS645 (Fig. 3d) affords a sustained inhibition of BRD4. The inhibitor blocks BRD4 interactions with the mediator protein MED1 and the transcriptional regulator YY1, which are required for accelerated proliferation of a panel of triple-negative breast cancer cell lines[104].

Another promising approach to develop novel therapeutics involves the design of molecules that in addition to BrD inhibition also can inhibit another key target function (that is, HDAC or kinase activity). Amemiya et al.[105] reported a novel benzo[*d*] imidazole inhibitor (compound **3**) that demonstrated potent BRD4 and HDAC dual-inhibitory activities (Fig. 4), and inhibited HL-60 cell growth and induced differentiation. Using the chemical scaffold of a JAK2 inhibitor, Ember et al.[106] developed and characterized indolizine compound **4** as a single agent (Fig. 4), which potently and simultaneously inhibits BRD4 and the oncogenic tyrosine kinases JAK2, FLT3, RET and ROS1. These studies underscore the promise of using a polypharmacological strategy for treating cancers.

Targeted protein degradation using proteolysis-targeted chimera (PROTAC) has also been applied to BrDs. In principle, a PROTAC binds a protein of interest and enforces proximity to an E3 ligase that results in polyubiquitination and degradation of the protein via the proteasome[107]. Cellular efficacy of a PROTAC is highly dependent on composition of the PROTAC linker (that is, its length, geometry and rigidity). This linker connects a ligand for the protein of interest to an E3 ligase ligand[108], for example, thalidomide, a ligand that targets the F box protein CRBN (cereblon), or a VHL ligand that provides the essential hydroxyproline to engage the F box protein VHL. PROTACs targeting both BET and non-

BET BrD proteins have been reported, including ARV-825 (ref. [109]), dBRD9 (ref. [110]) and dTRIM24 (ref. [111]) (Fig. 4). These molecules are another set of powerful chemical probes complementary to reversible small-molecule inhibitors to be used for dissecting molecular mechanisms underlying BrD protein functions and can aid drug target validation.

BrDs as therapeutic targets for human diseases

BrD proteins are intimately involved in the regulation of gene transcription, as illustrated by the BET family proteins. Therefore, pharmacological inhibition of certain BrDs was conceived as a strategy to target transcription factors, such as Myc, that have long been regarded as attractive drug targets but eluded direct modulation with small-molecule inhibitors. Indeed, BET BrD inhibition has been shown to block Myc association with BRD4 as well as *Myc* transcription[44]. Thus, BET BrD inhibition offers an effective means to target Myc oncogenic activity and was shown to block cell growth in a wide variety of cancers, including acute myeloid leukemia[44,112], Burkitt's lymphoma[112], multiple myeloma[44], lung adenocarcinoma[113], neuroblastoma[114], a genetically diverse glioblastoma[115], castration-resistant prostate cancer[116] and basal-like breast cancer[42]. BET inhibitors have also been used to study HIV latency[117], autoimmune disorders and Th17 pathology[118], and the role of transcriptional pause release in heart failure[119], among many other viral, autoimmune and inflammatory diseases.

As previously discussed[11], the success of BET BrD inhibitors in preclinical studies has prompted multiple pharmaceutical companies to enter these compounds into clinical trials. In 2012, the diazepine compound I-BET762 (GSK525762) was among the first BET inhibitors evaluated in human clinical trials. The trials were initiated by GlaxoSmithKline and targeted NUT midline carcinoma, multiple other types of cancer and relapsed, refractory hematologic malignancies (Supplementary Table 3). OncoEthix/Merck has been evaluating OTX015, a thienotriazolodiazepine, originally in-licensed from Mitsubishi Tanabe Pharmaceuticals, in multiple clinical trials for a variety of human cancers. Constellation Pharmaceuticals and Tensha Therapeutics have been conducting clinical trials evaluating their BET inhibitors CPI-0610 and TEN-010, respectively. Additionally, Resverlogix Corp. has been clinically testing their BET BD2-selective inhibitor, RVX-208, against indications other than cancer. This compound has completed Phase II clinical trials for diabetes, coronary artery disease, atherosclerosis, dyslipidemia and cardiovascular diseases. Data from these trials indicate that RVX-208 can lead to a reduction in major adverse cardiac events in high-risk individuals with cardiovascular disease and with diabetes mellitus, prompting plans for a Phase III clinical trial[120]. Details of these BrD inhibitor trials, including disease indications and ClinicalTrials.gov identifiers, are summarized in Supplementary Table 3. Notably, although clinical studies have confirmed BET inhibitor target engagement and validated BET BrD function in cancer pathogenesis[121], substantial dose-limiting toxicities have also emerged. These include thrombocytopenia, fatigue, gastrointestinal bleeding and hypertension in dose-escalation studies[121]. Therefore, further development is needed to mitigate these adverse effects and achieve beneficial clinical outcome of BET BrD inhibitors, especially under chronic dosing conditions.

Concluding remarks

The field of histone biology has seen many advances in our understanding of the role of histone lysine acetylation in the regulation of chromatin-templated gene transcription since the discovery of BrDs as the Kac readers 20 years ago[5]. BrD binding to Kac residues is at the heart of many acetylation-driven processes, including histone modifications, remodeling of chromatin structure, recruitment of transcription factors and assembly of transcription regulatory complexes (Fig. 5). All these effects are, of course, not mediated by BrDs alone. Rather they act in concert with many other chromatin reader and writer domains present in BrD proteins or their associating protein complexes. Neither are BrDs the only chromatin readers for Kac. Certain PHD fingers, such as the tandem PHD finger of the transcriptional regulator DPF3b[122], as well as the YEATS domains of human AF9, ENL, YEATS2 and GAS41/YEATS4 (ref. [123]) have been reported to be capable of binding acetylated lysine on histones. Notably, the YEATS domains consist of a conserved immunoglobin fold made of a two-layer β sandwich with eight antiparallel β strands, and can recognize other forms of acyl-lysine, including crotonyl-ation, propionylation and butyrylation[124]. This is in sharp contrast to BrDs, which solely bind acetylated lysine[11]. For more information on non-bromodomain chromatin readers, we refer readers to the Perspective by Arrowsmith and Schapira[125] in this issue. The existence of functional elusive atypical BrDs further highlights the versatility of the structurally conserved bromodomain fold in chromatin biology, which awaits future investigation.

BrD inhibitors developed in recent years have had a crucial role in uncovering functions of BrD proteins. These insights have provided the rationale for therapeutic targeting of BrDs. Although this strategy has been validated to some extent by ongoing clinical trials, particularly for the BET family BrDs, serious adverse effects also emerged. These on-target dose-limiting toxicities include thrombocytopenia, fatigue, gastrointestinal bleed and hypertension in the dose-escalation studies. These challenges should motivate scientists to refine BrD inhibitors as precision medicine for specific diseases. Strategies might include intermittent dose scheduling, developing more disease-selective BrD inhibitors, as well as combination therapy with other therapeutic agents.

Looking into the future, the field of BrD biology and associated drug discovery are filled with great potential. Better understanding of the role of histone modifications in gene transcription coupled with next-generation BrD inhibitors is expected to fulfill the promise of Allfrey's groundbreaking discovery of histone lysine acetylation[1] by delivering new BrD-targeted epigenetic treatments for a wide array of human diseases, including cancer, autoimmune disease and inflammatory disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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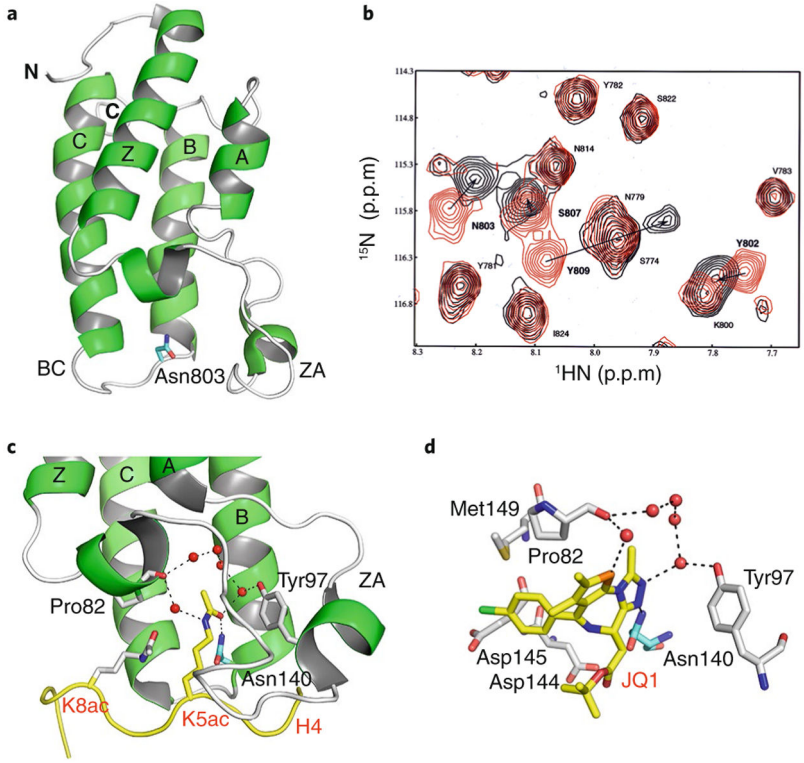

Fig. 1 |. Three-dimensional structure and Kac binding mode of the bromodomain.

a, NMR spectroscopy structure of the PCAF BrD (PDB 1N72), depicted in a ribbon representation. The four helices (αZ, αA, αB, αC) of the left-handed bundle BrD fold and the two inter-helical connecting ZA and BC loops are labeled. The conserved asparagine residue, Asn803, is highlighted in yellow. **b**, Superimposition of the two-dimensional ^{15}N-HSQC spectra of the PCAF BrD in the free form (red) and in complex with an acetylated histone H4 peptide (black). Image adapted from ref. [5]. Reprinted by permission from *Nature.* **c**, Ribbon diagram of the crystal structure of a H4K5ac/K8ac peptide bound to BRD4-BD1 (PDB 3UVW). Side chains of key protein residues involved in ligand recognition are labeled. **d**, Crystal structure of JQ1 bound to BRD4-BD1 (PDB 3MXF). Side chains of key protein residues involved in ligand recognition are labeled. The bound water molecules at the base of the Kac binding pocket are shown as red spheres.

Fig. 2 | Classification of BrD-containing proteins based on major known functions.
Key functional domains in each protein are highlighted, with the BrD in purple.

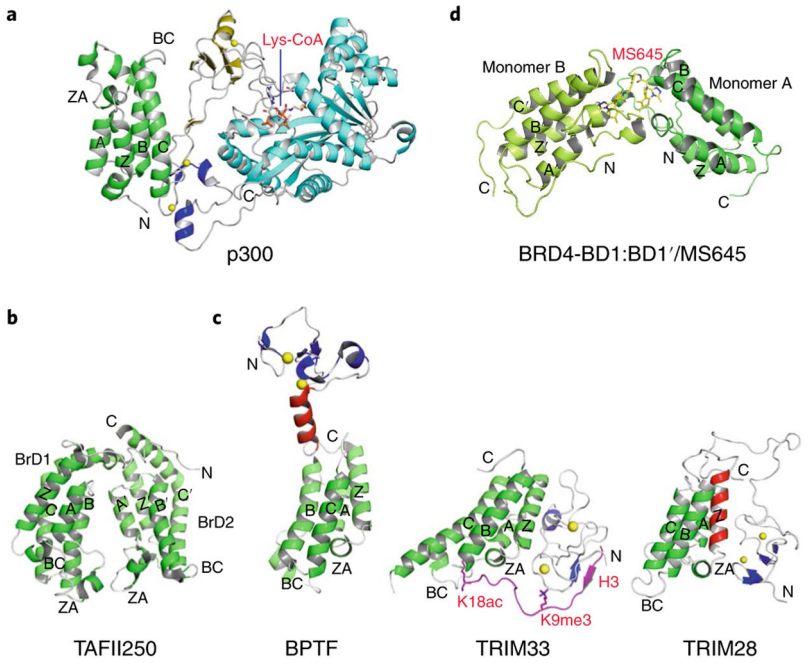

Fig. 3 |. 3D structures of representative BrDs in different forms.

a, Crystal structure of the BrD-PHD-HAT module of human p300 (PDB 4BHW). The BrD-PHD module is rendered in green and yellow, RING domain in blue and HAT domain in cyan. Lys-CoA is displayed as a stick model. **b**, Crystal structure of double BrDs of TAF$_{II}$250 (PDB 1EQF). **c**, Left, crystal structure of the BPTF PHD-BrD (PDB 3QZV), with α-helix connecting the two domains (red). Middle, crystal structure of the TRIM33 PHD-BrD module in complex with the H3K9me3/K18ac peptide (magenta) (PDB 3U5P). Right, the solution structure of the PHD-BrD module of TRIM28 (PDB 2RO1). Three of the four BrD helices are depicted in green; the fourth, αZ, colored in red, is highlighted because it serves as the hydrophobic center of the tandem structure. **d**, Crystal structure of bivalent BET BrD inhibitor MS645 bound to the BRD4 BD1 dimer (PDB 6DJC), displayed in ribbon and spacefilled surface depictions. MS645 (yellow) is color-coded by atom type.

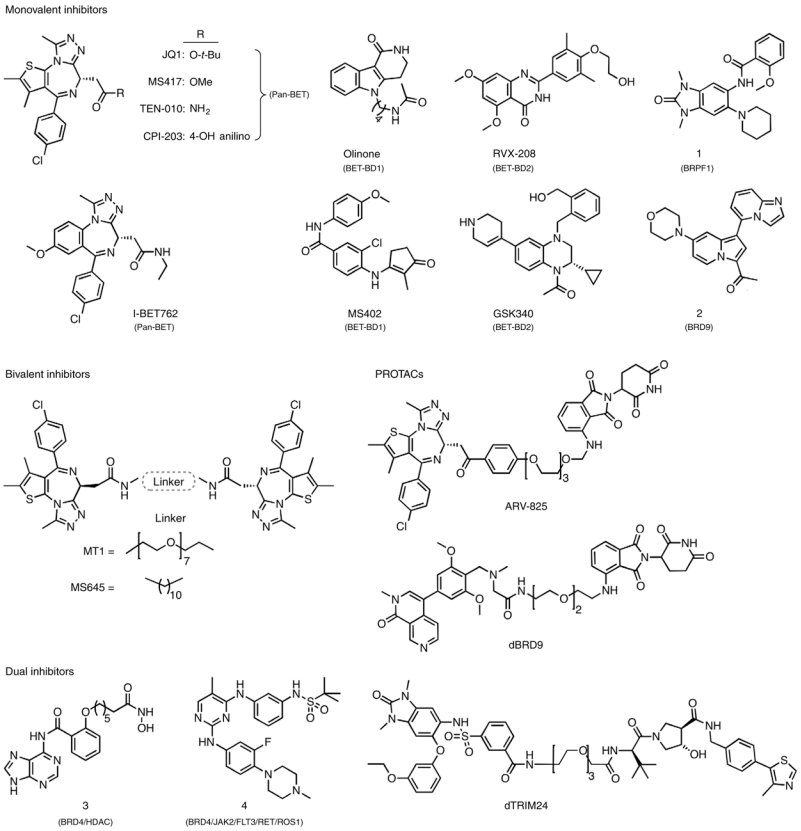

Fig. 4 |. Representative small-molecule inhibitors and PROTACs for selected BrDs.
Chemical structures of representative BrD inhibitors are shown. Additional examples are in Supplementary Table 2. Properties and cellular effects are discussed in main text.

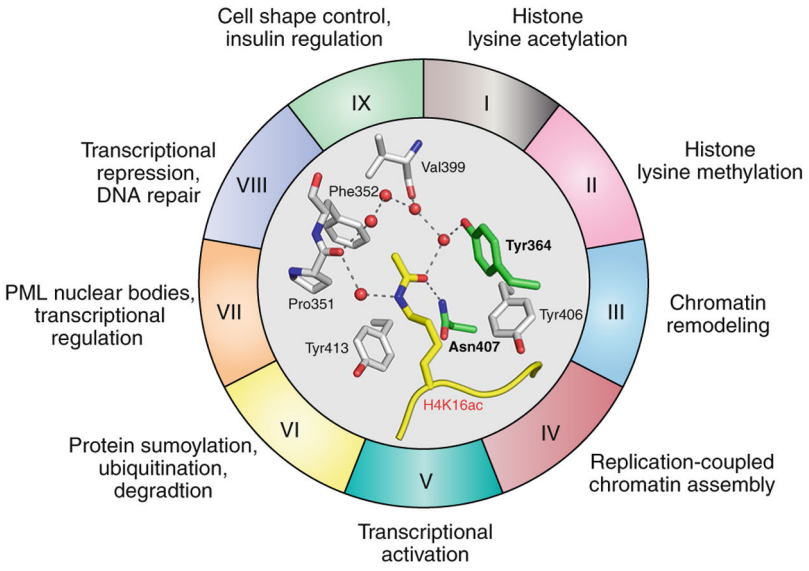

Fig. 5 |. Illustrative diagram highlighting representative functions of nine subclasses of human BrD proteins.

Center, stick diagram depicting atomic details of molecular recognition of the acetyl-lysine of the H4K16ac peptide by the BrD of GCN5L2 (PDB 1E6I). Side chains of key protein residues involved in Kac binding are color-coded by atom type. Conserved water molecules in the Kac binding pocket are in red.