Review

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Beating the odds: BETs in disease

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Bromodomains (BRDs) are evolutionarily conserved protein interaction modules that specifically recognise acetyl-lysine on histones and other proteins, facilitating roles in regulating gene transcription. BRD-containing proteins bound to chromatin loci such as enhancers are often deregulated in disease leading to aberrant expression of proinflammatory cytokines and growth-promoting genes. Recent developments targeting the bromo and extraterminal (BET) subset of BRD proteins demonstrated remarkable efficacy in murine models providing a compelling rationale for drug development and translation to the clinic. Here we summarise recent advances in our understanding of the roles of BETs in regulating gene transcription in normal and diseased tissue as well as the current status of their clinical translation.

Introduction: recognition of acetylated lysine

Lysine acetylation (Kac) is a regulatory post-translational modification that, in the context of chromatin biology, is associated with open chromatin structure and transcriptional stimulation. Deregulation of acetylation levels has long been associated with disease and the enzymes responsible for the removal of this modification [histone deacetylases (HDACs)] have been successfully targeted in the past decade, resulting in approved drugs. However, it was only recently recognised that the protein domains that read acetyl-lysine – the evolutionarily conserved protein–protein interaction modules of the BRD class [1] – can also be targeted. The BET class of the BRD family in particular has attracted much attention following the disclosure of highly potent and selective inhibitors that attenuate BET function [2], which stimulated research that evaluated their biological roles and deregulation in disease. Here we review recent advances in our understanding of BET proteins, their structural organisation, and their involvement in disease as well as the translation of their small-molecule inhibitors to the clinic.

Architecture of BET proteins

BET proteins (BRD2, BRD3, BRD4, and the testis-specific BRDT) have a conserved modular architecture including two N-terminal tandem BRD effector modules (BRDs), an extra-terminal recruitment domain (ET), several conserved motifs (A,B, SEED motifs), and, in the case of BRD4 and BRDT, a C-terminal motif (CTM) (Figure 1A). The BRD module is shared by a conserved family of 42 proteins (in humans) that contains a total of 61 BRDs, including the eight BET BRDs (two per BET protein). Large-scale structural analysis of the human BRD family offered insight into their conserved architecture, comprising a four-helix bundle (helices αZ, αA, αB, and αC) linked by diverse loop regions (ZA and BC loops) (Figure 1B) [1]. A conserved region of 12 amino acids (KGVKRKADTTTP, found at the end of the A motif) between the two BET BRD modules was recently shown to act as a nuclear localisation signal and its deletion results in mislocalisation of the mutant proteins in HEK293T cells [3]. In addition, the conserved B motif is thought to be responsible for homo- and hetero-dimerisation, at least in the case of BRD2, and its deletion results in dissociation from mitotic chromosomes [4]. The ET domain shared between the four BET proteins also exhibits a helical architecture (Figure 1C), including an acidic surface shaped in a continuous ridge [5], and has been shown to participate in protein–protein interactions [6]. Although no structural information has been obtained for a complete CTM of BRD4/T, a complex structure of the human papillomavirus (HPV) E2 protein was characterised bound to a short peptide portion of the BRD4 CTM [7], demonstrating that the C-terminal tail binds to a highly basic region of the viral protein (Figure 1D). In the past few years, complete structural coverage of the eight BET BRDs has also offered insight into their similarities and plasticity [1,8–11] (Figure 1E), which has facilitated the design of inhibitors, as we discuss below.

Interactions initiated by BET proteins

The modular architecture of BET proteins is responsible for key interactions that facilitate the assembly of the transcriptional machinery. These include interactions initiated by the BRD modules and the ET domain (found in all four BET proteins) as well as the CTM motif found in BRD4 and BRDT.

Interactions with acetylated histones initiated by the BRD modules

BET BRDs recognise and bind to acetylated lysine sequences found on histones or other proteins, an interaction initiated by a conserved asparagine residue found at the beginning of the BC loop. A high-resolution crystal structure of a histone H4 peptide acetylated at lysine 12 (H4K12ac) bound to the first BRD of BRD2 showed the acetylated lysine inserted deep into the Kac recognition

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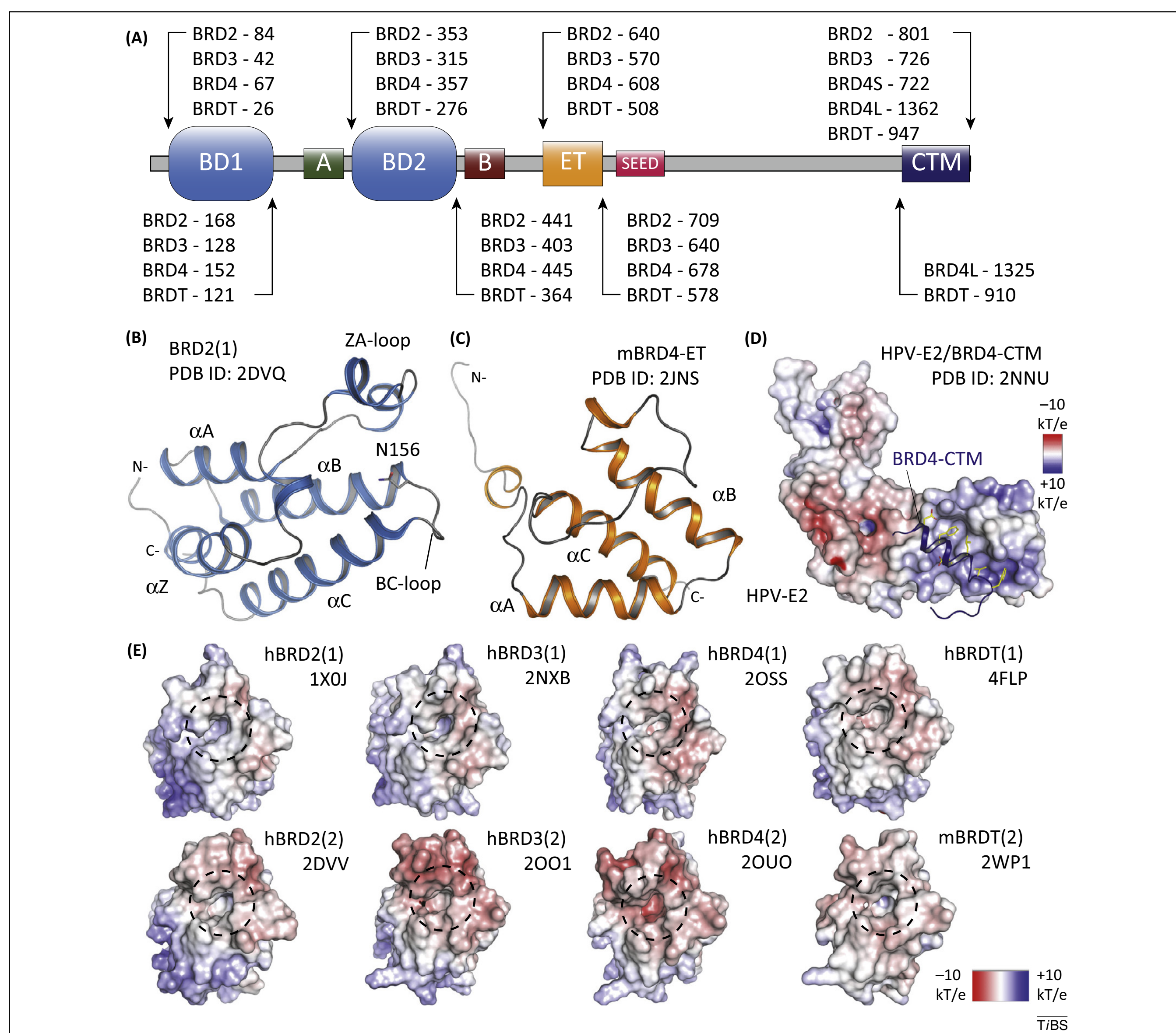

Figure 1. Domain organisation of bromo and extra-terminal (BET) proteins and module structures. (**A**) BET proteins contain a modular architecture, including two N-terminal tandem bromodomain (BRD) modules (BD1, BD2; shown in blue) responsible for acetyl-lysine recognition and an extra-terminal protein–protein interaction domain (ET; shown in orange). BRDT, as well as the long variant of BRD4 (BRD4L), contains a C-terminal motif (CTM; shown in dark blue) that interacts with the positive transcription elongation factor b (P-TEFb). The CTM is sometimes referred to as P-TEFb-interacting domain (PID). Several conserved motifs are also present (A, B; motifs shown in green and dark red, respectively) as well as a serine–glutamate–aspartate-rich region (SEED). Domain boundaries for all family members are annotated in the figure. (**B**) Crystal structure of the human BRD2 first BRD module [BD1 or BRD2(1); from PDB ID: 2DVQ] [10]. Structural elements are annotated in the figure and the conserved asparagine is shown in stick representation. (**C**) Solution structure of the murine BRD4 ET domain (from PDB ID: 2JNS) [5]. Structural elements are annotated in the figure. (**D**) Crystal structure of human papillomavirus protein E2 in complex with a CTM peptide of BRD4 (residues 1343–1362) showing binding of the BRD4 CTM on a basic groove of the E2 protein [7]. The electrostatic potential of the viral protein is coloured as indicated in the inset. (**E**) Electrostatic potential of BET BRDs calculated from high-resolution crystal structures of the human [or murine in the case of BRDT(2)] modules [1,8–11]. The structures are oriented with the acetyl-lysine-binding cavity facing outwards, annotated with a dotted circle, and the electrostatic potentials are coloured as indicated in the inset. PDB IDs (http://www.pdb.org) are given for each protein module.

cavity and coordinated with the conserved asparagine (N156) (Figure 2A). The protein–peptide interaction is driven mainly by the high electrostatic character of the surface surrounding the hydrophobic pocket where the neutralised Kac inserts (Figure 2B) [10]. In addition to binding single acetylated lysines, BET BRDs were recently found to bind to histone sequences bearing two acetylated lysines. This was first described in the case of the murine BRDT, which was crystallised with the diacetylated histone H4 peptide H4K5acK8ac [9]. The diacetyl interaction was later found to be conserved and shared between the eight BET BRDs, for which both Kacs insert in the BRD cavity but only the N-terminal one initiates contacts with the conserved asparagine. The second Kac participates in a network of water-mediated interactions that further stabilise the first Kac within the cavity (Figure 2C). This mode of binding is conserved for other combinations of two acetylated lysines in histone H4, as seen in the crystal structure complexes of the first BRD of BRD4 with different H4 Kac mark combinations (Figure 2D) [1]. Interestingly, modified histone H3 peptides bearing propionylated or butyrylated lysines are also recognised by and bind to

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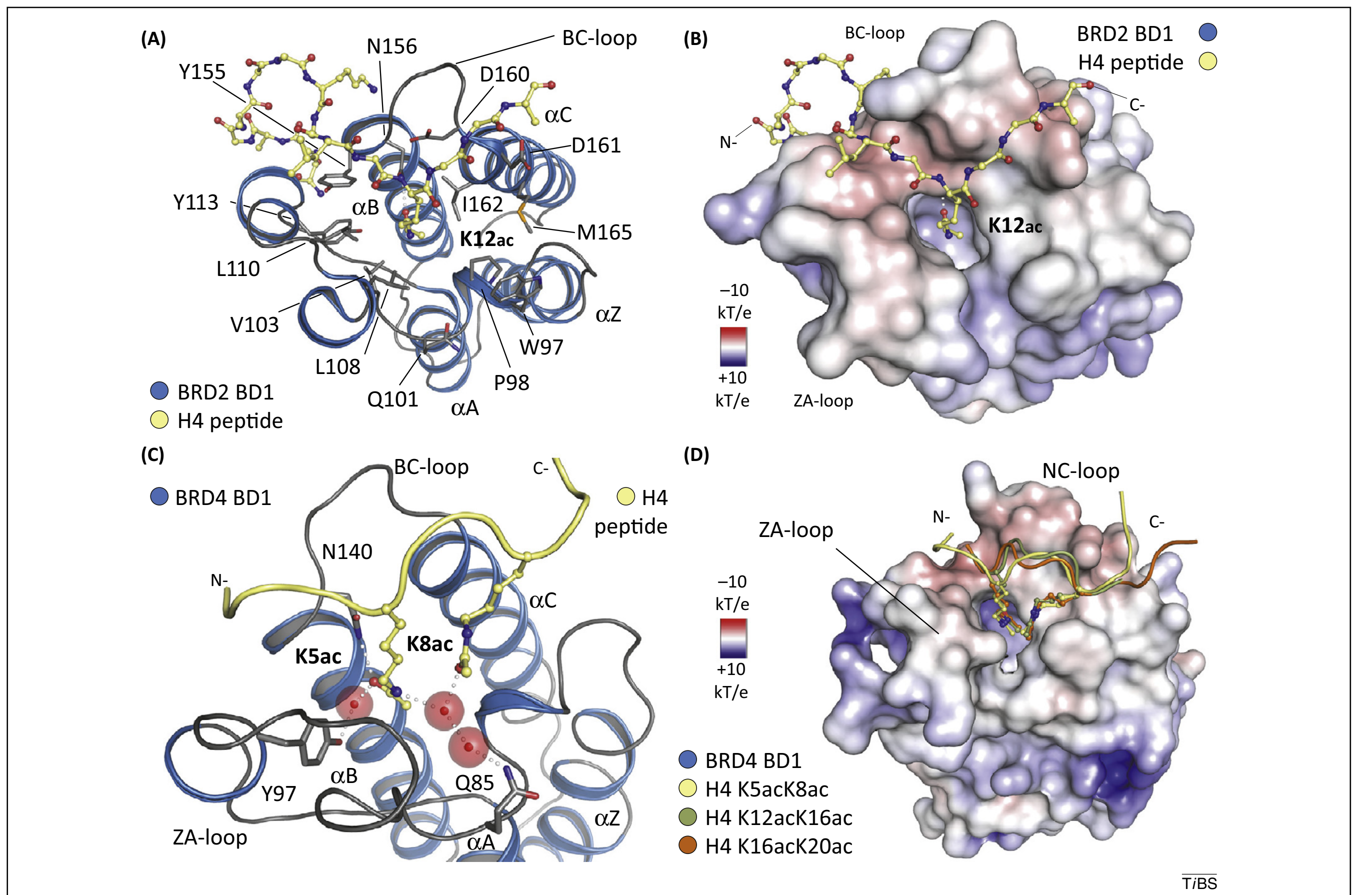

Figure 2. Mode of histone sequence recognition by bromo and extra-terminal (BET) bromodomains (BRDs). BET BRDs can recognise and bind to single as well as double acetyl-lysine marks. (**A**) Crystal structure of the first BRD of human BRD2 in complex with a histone H4 peptide acetylated at K12 (PDB ID: 2DVQ) [10]. The acetylated lysine engages the protein directly via the conserved asparagine (N156 in BRD2 BD1) and fits within the cavity formed by several conserved residues highlighted and annotated on the structure. (**B**) Complex of BRD2 BD1 with the histone peptide shown in (A), with the protein surface coloured according to the electrostatic potential of the residues found on the module's surface (as indicated in the inset), highlighting the charged nature of the area surrounding the central hydrophobic acetyl-lysine-recognition site. The acetylated lysine and the peptide termini have been annotated in the inset. (**C**) Crystal structure of the first BRD of human BRD4 in complex with a histone H4 diacetylated peptide (at K5 and K8; PDB ID: 3UVW) [1]. While the first acetyl-lysine (K5ac) directly engages the protein via the conserved asparagine residue (N140 in BRD4 BD1) (and is further stabilised via a water-mediated interaction to Y97), the second acetyl-lysine (K8ac) stabilises the peptide fold via water-mediated bridges to K5ac and to BRD4 Q85. (**D**) Surface representation of the electrostatic potential of human BRD4 BD1 (from PDB ID: 3UVW) in complex with a histone H4 peptide (H4K5acK8ac from PDB ID: 3UVW) superimposed to other double-acetylated peptide complexes of BRD4 BD1 (H4K12acK16ac from PDB ID: 3UVX and H4K16acK20ac from PDB ID: 3UVY). The surface is coloured as indicated in the inset. All three complexes retain the mode of interactions seen in (C) with both acetylated lysines filling the entire recognition cavity. All PDB codes shown are taken from the Protein DataBank (www.pdb.org).

BET BRDs [12], suggesting that other lysine modifications may play a role in signalling; however, this remains to be determined.

Interactions with other acetylated proteins initiated by the BRD modules

Beyond histones, BET BRDs have been recently found to bind to diverse acetylated proteins, including the RelA subunit of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and the transcription factors GATA1 and TWIST as well as cyclin-T1, a component of positive transcription elongation factor b (P-TEFb). RelA is acetylated at lysine K310 by the histone acetyl transferase (HAT) CBP/p300, which *trans*-activates NF-κB without affecting RelA binding to DNA or its association with the inhibitory protein IκBα. RelA acetylated at K310 can be recognised by the BRDs of BRD4, resulting in the recruitment of the BRD4/NF-κB complex to the promoter region of NF-κB-responsive genes such as E-selectin and the proinflammatory cytokine tumour necrosis factor alpha (TNF-α), stimulating their transcription [13].

Two acetylated lysines in close proximity, found on non-histone proteins, can also be recognised by BET BRDs in a similar mode to that observed for histones, as demonstrated for the erythroid- and megakaryocyte-specific transcription factor GATA1. When acetylated, two conserved lysines on the C-terminal tail of GATA1 (K312 and K315) are recognised by the first BRD of BRD3 in a similar fashion to the interaction between BET BRDs and diacetylated histone H4 peptides, albeit the GATA1 lysines bear different flanking residues [14]. This interaction, which seems to be initiated only by the first BRD of BRD3, is essential for erythropoiesis, as it results in stabilisation of acetylated GATA1 bound onto chromatin, thus affecting the expression of GATA1-controlled genes [15]. A similar interaction was described recently in the case of the epithelial–mesenchymal transition (EMT) transcription factor TWIST, which is a transcriptional activator that induces mesodermal gene expression. TWIST was found to interact with BRD4 in cells, an interaction that is driven by acetylation at two conserved lysines (K73 and K76) found at a region with sequence similarity to histone H4. This interaction

was further shown to be initiated exclusively by the second BRD of BRD4 and was necessary for the expression of WNT5A, a critical ligand of both canonical (controlling pluripotency) and noncanonical (regulating motility and planar cell polarity) Wnt pathways. Thus, interaction between BRD4 and TWIST facilitates invasion, cancer stem cell-like properties, and tumourigenicity in basal-like breast cancer cells [16] (although the exact roles of WNT5A are debated [17]).

Interactions involving acetylated cyclin-T1 (a component of P-TEFb) and the second BRD of BRD4 have also been shown to be necessary to stabilise the P-TEFb/BRD4 complex, resulting in activation of P-TEFb-responsive genes. Interestingly, *in vitro* experiments suggested that cyclin-T1 should carry three acetylated lysines to be sufficiently recognised by the second BRD of BRD4 (K380, K386, and K390) [18].

Interactions initiated by the ET domain

The ET domain of BET proteins is a protein–protein interaction motif that initiates interactions with viral proteins such as the latent nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus (KSHV) [19], an interaction that was recently structurally characterised by NMR [20]. Importantly, recent evidence suggests that this domain can initiate protein–protein interactions with transcriptional effectors such as the methyltransferase NSD3, resulting in stimulation of transcription in a P-TEFb-independent manner [6]. This is in the case of the short variant of BRD4, which lacks the CTM necessary for P-TEFb interactions (see next section). It remains to be seen whether P-TEFb-independent stimulation of transcription driven by the ET domain is a feature shared by all BET proteins.

Interactions initiated by the CTM

The CTM of BRD4 interacts with the HPV E2 protein, resulting in tethering of viral genomes to host mitotic chromosomes supporting low-level replication maintenance of the viral genome [7,21] and transcriptional stimulation [22] as well as transcriptional repression of the E6 and E7 viral oncoproteins that antagonise p53 and pRB tumour-suppressor activity [23]. This interaction was recently found to be regulated by phosphorylation at S243 of the E2 protein [24]. The CTM also interacts with the P-TEFb complex, resulting in the dissociation of the inhibitory protein HEXIM1, thus enabling the active form of P-TEFb [18]. Recent data suggest that a region comprising two basic clusters and a hydrophobic stretch on the CTM of BRD4 is able to stimulate the kinase activity of CDK9 above basal levels, even in the presence of the inhibitory protein HEXIM1; however, the precise mechanism of CDK9 stimulation remains not well understood [25]. Interestingly, the HPV E2 interaction with BRD4 is necessary for the recruitment of P-TEFb to the HPV early promoter [26]; however, there is also evidence that E2 binding competitively inhibits the BRD4/P-TEFb complex [27]. The precise contribution of BRD4 to the mechanisms of E2-controlled transcriptional stimulation therefore remains unknown.

The modular architecture of BET proteins facilitates multipoint interactions resulting in the assembly of complexes tethered to chromatin. BET complexes with diverse protein partners resulting through interactions initiated by the ET domain and CTM are tethered to acetylated chromatin via the BRD modules. At the same time the BRD modules may act to stabilise secondary interactions with the same or other acetylated protein partners. Ultimately these large assemblies affect transcriptional programmes and can be tightly controlled. It will be interesting to see how the specific readout of two acetylated lysines correlates with the transcriptional attenuation conferred by BETs. A common theme in the assembly of such complexes is the recognition of acetyl-lysine by the BRD modules, an event that can be targeted by small-molecule inhibitors (Box 1). Next we discuss how BETs participate in transcription in the context of these protein assemblies.

BET roles in transcription

BET proteins play a central role in chromatin biology by acting as tissue-specific recruitment platforms that tether complexes to acetylated histones and chromatin. BRD2, for example, plays a key role in neuronal development, controlling neuronal differentiation, cell-cycle progression, and cell-cycle exit in neuroepithelial cells [28]. For example, in mitogen-stimulated primary B cells BRD2 controls cyclin A expression by directly binding to the cyclin A promoter, affecting S-phase progression [29]. In HeLa cells BRD2 has yet another function; it was found to affect alternative splicing of multiple genes without affecting RNA polymerase II (Pol II) processivity downstream of the alternatively spliced elements. The affected genes included some involved in MAPK signalling and the response to growth factor stimuli [30].

BRD3 plays a role in erythropoiesis by directly interacting with the transcription factor GATA1, which induces all known erythroid-specific genes while repressing genes associated with the proliferative, immature state. The complex of BRD3 with GATA1 stabilises the association of the transcription factor with chromatin, affecting the transcription of its responsive genes [14,15]. Although BRD3 binds to most GATA1-regulated genes, its displacement from chromatin by employing the BET inhibitor (+)-JQ1 (Box 1) affected only GATA1-mediated transcriptional activation and not transcriptional repression on a genome-wide scale [31]. More importantly, BRD2 and BRD4 were also found to bind to a subset of GATA1-related genes and their depletion blunted erythroid gene activation. Depletion of BRD3 affected erythroid transcription only if BRD2 was also deficient [31], suggesting that BET proteins may have overlapping roles that need to be further investigated.

BRD4 and more recently BRDT have been established as central regulators of transcriptional elongation by recruiting the P-TEFb complex to chromatin [32–34]. BRD4 (and most likely BRDT) facilitate the dissociation of the inhibitory factor HEXIM1 resulting in an active form of P-TEFb, which in turn phosphorylates and activates RNA Pol II [18]. The recent use of BET inhibitors helped establish that BETs mainly control lineage-specific genes. For instance, when BRD4 is displaced from chromatin in $CD4^{+}$ T cells, RNA Pol II phosphorylation is affected

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Box 1. Inhibition of BET BRD–peptide interactions

The hydrophobic nature of the acetyl-lysine cavity allowed for the development of small-molecule compounds that disrupt BRD/Kac interactions. Following disclosure of a triazolothienodiazepine scaffold in the patent literature in 2009 [110], two key compounds were characterised, the triazolothienodiazepine (+)-JQ1 [111] and the triazolobenzodiazepine I-BET762 [112]. These highly potent and selective inhibitors target only BET BRDs and are capable of dissociating BET proteins from chromatin, affecting the transcriptional programs that they control. Since 2010 several potent and selective compounds have been developed (recently reviewed elsewhere [2]) and widely distributed allowing thorough validation of BET function in chromatin biology and disease. Some of the most advanced BET BRD inhibitors (in terms of potency and selectivity against other BRDs) are summarised in Figure I. Interestingly, certain advanced kinase inhibitors that were thought to be highly selective have been found to target BET BRDs [113–116]. Of particular interest are the JAK2/FLT3 kinase inhibitor fedratinib and the PLK1 inhibitor BI-2536, which exhibited low-nanomolar affinity for the first BRD of BRD4. These inhibitors were used in cellular assays to target BRD4 at clinically relevant concentrations, leading to potential applications in developing polypharmacology aiming to attenuate both BET and kinase phenotypes in diverse diseases [113]. Kinase inhibitors that have been shown to be highly potent toward BET BRDs are also shown in Figure I (lower panel). The plasticity and features of the BRD modules of BETs are well established so it remains to be seen whether specific and selective inhibitors will emerge for each of the four proteins or for individual domains within proteins. Such selective compounds would help delineate the roles of each BET protein in biology.

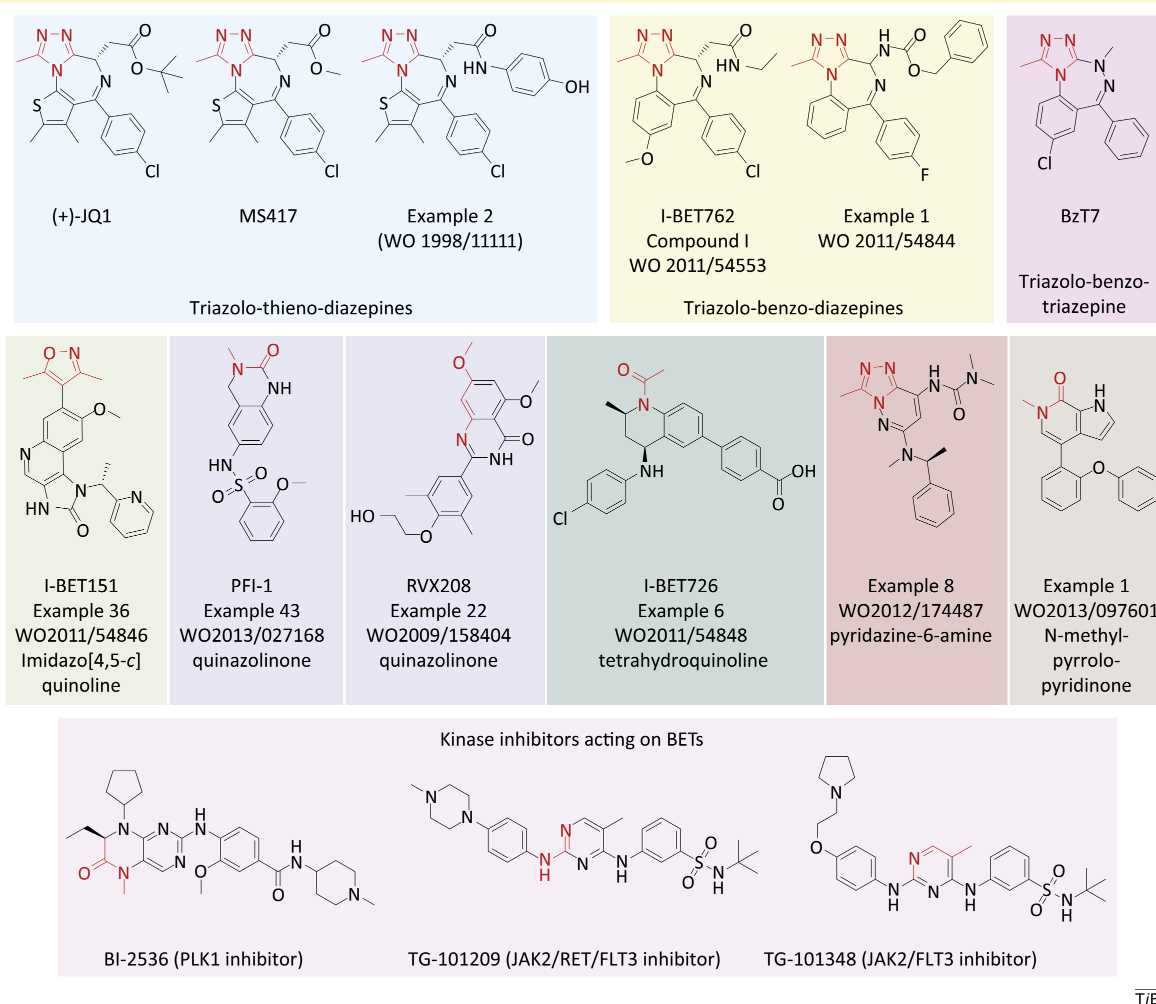

Figure I. Advanced bromo and extraterminal (BET) inhibitors. Several chemical series have been developed targeting BET bromodomains, with triazolothieno- and triazolobenzodiazepines currently in clinical testing. The most potent and advanced pan-BET inhibitors employing diverse chemical templates are shown here. The equivalent name from each patent (where applicable) as well as the chemical class is also given. Diverse kinase inhibitors were also shown to exhibit very high affinity toward BET bromodomains and the most potent of these compounds are shown on the bottom panel, annotated with their primary kinase targets. In all cases, the acetyl-lysine-mimetic part of the molecule is highlighted in red.

resulting in downregulation of genes that are largely T cell specific [35]. Transcriptional attenuation through P-TEFb is also achieved by physical interaction of BRD4 with the JmjC domain-containing protein JMJD6. This allows H4R3me$_{2(sym)}$ and 7SK snRNA demethylation, thus releasing the 7SK snRNA/HEXIM1 inhibitory complex imposed on the P-TEFb complex and leading to promoter-proximal Pol II pause release and transcriptional reactivation while maintaining association of the P-TEFb complex with chromatin [36]. Additional evidence suggests that BRD4 occupies widespread genomic regions to stimulate directly elongation of both protein-coding transcripts and noncoding enhancer RNAs, assisting Pol II in progression through hyperacetylated nucleosomes

while facilitating interactions with acetylated histones via its BRD modules [37]. Many M/G1 genes that are programmed to be expressed at the end of mitosis have been known to be bound by BRD4 [38] and additional evidence recently suggested a role for this protein in gene bookmarking – facilitating postmitotic transcriptional reactivation by accelerating the kinetics of transcription and helping to decompact chromatin in daughter cells [39]. Further evidence supports a model whereby BRD4 interactions with chromatin help maintain chromatin structure and morphology [40]. Interestingly, a short variant of BRD4 lacking the CTM responsible for P-TEFb interaction functions as an endogenous inhibitor of DNA damage-response signalling by recruiting the condensin II chromatin-remodelling complex to acetylated histones. This stimulated chromatin reorganisation and compaction while depletion of this variant resulted in rapid cell-cycle checkpoint recovery and enhanced cell survival [41]. A similar effect was previously reported for a C-terminal truncation of the testis-specific BET isoform (BRDT) that also stimulated chromatin compaction [42], suggesting that a regulatory role in chromatin decompaction may be attributed to the CTM found on the longer BET variants (BRD4 and BRDT).

BRDT has a central role in chromatin remodelling during spermatogenesis, as indicated by the importance of its first BRD in maintaining the chromatin architecture of the sperm head [43]. A recent study delineated BRDT's role in reorganising chromatin, facilitating histone eviction and replacement by transition proteins, establishing it as an essential factor for male germ cell differentiation [34]. BRDT was further shown to regulate meiotic divisions and postmeiotic genome repackaging, functions that were found to be dependent on its first BRD [34]. BRDT has also been suggested as a target for male contraception [11].

The modular interactions initiated by the ET domain, which is conserved in BETs, can also stimulate transcription in a P-TEFb-independent manner by affecting the chromatin microenvironment of BET target genes. This was demonstrated for BRD4-recruited NSD3, a methyltransferase that was shown to regulate the levels of histone H3 K36 methylation, a modification that is enriched in regions of active transcription [6]. It remains to be seen whether all BETs can adopt similar P-TEFb-independent roles in transcription.

BET proteins are key tissue-specific chromatin adaptors and recruitment platforms that help attenuate diverse transcriptional programs, from neuronal development to erythropoiesis and spermatogenesis as well as alternative splicing. Many of these functions seem to overlap, such as control of erythropoiesis, while others seem to be very specific, such as spermatogenesis, suggesting that complicated and non-overlapping mechanisms of regulation are in place. It is therefore unsurprising that BET functions are found to be perturbed in disease, including cancer, inflammation, and viral infection. We next discuss how these proteins are deregulated in such disease settings.

BET proteins in disease

As key drivers of transcription, BET proteins are often deregulated in disease, affecting numerous growth-promoting genes and cytokines.

BET roles in cancer

BET proteins are known to be deregulated in cancer [44] and the availability of potent inhibitors (Box 1) in the past 5 years has helped us to better understand the specifics behind diverse tumour types. For example, a role for BRD2 has recently been investigated in induced lung adenocarcinoma. In this system, BRD2 was found to form a complex with RUNX3 in a K-RAS-dependent fashion. This complex induced the expression of the cell-cycle inhibitor p21; thus, the study provided insight into K-Ras-driven oncogenic functions [45]. Oncogenic fusions involving BET proteins have also been identified, leading to tumourigenic functions. BRD3 and BRD4 have been involved in chromosomal translocations leading to tumourigenic fusions with nuclear protein in testis (NUT) leading to NUT midline carcinomas (NMCs), which are rare and aggressive carcinomas that have no organ or tissue of origin [46]. The *BRD4* locus (19p13.1) [or *BRD3* locus (9q34.2)] and *NUT* locus (15q14) translocate to create a *BRD4–NUT* fusion gene whose product is driven by the *BRD4* promoter. The *BRD4–NUT* fusion product blocks differentiation and maintains tumour growth, although the mechanism is unclear. Early studies established that the fusion product induces formation of hyperacetylated, transcriptionally inactive foci by direct recruitment of the acetyltransferase p300, resulting in local hyperacetylation as well as acetylation and therefore deactivation of p53 [47]. Dispersion of these foci by various methods, including incapacitating the ability of BRD4 to bind to acetylated chromatin via its BRD, was sufficient to restore cellular functions. Interestingly, global acetylation was shown to be decreased, which should result in downregulation of many genes [48]; however, hyperacetylation of chromatin results in subsequent recruitment of BRD4 and further transcriptional stimulation [49]. Although the precise mechanism of action remains to be uncovered, it is thought that the fusion product affects the transcription of proproliferative and antidifferentiation genes such as *MYC*, which is supported by recent evidence that the fusion protein is present at the *MYC* promoter, maintaining MYC expression [50]. Observation that genetic knockdown of the BRD4–NUT product results in squamous differentiation and G1 cell-cycle arrest, and that this effect is phenocopied by the pan-BET inhibitor (+)-JQ1 in mouse xenograft models of NMC [111] has led to several clinical trials employing thieno- and benzodiazepines in the past few years (Table 1).

Pan-BET inhibitors downregulate MYC expression in many diverse tumour settings. This was first described in myeloma [51,52] and later in Burkitt's lymphoma [53] and has now also been described for acute myeloid and mixed lineage leukaemia [54–58], acute lymphoblastic leukaemia (ALL), and T cell ALL [59–62], medulloblastoma [63–65], glioblastoma [66], prostate cancer [67–69], lung adenocarcinomas [70], lung cancer and non-small cell lung cancer [71], ovarian cancer [72,73], and skin cancers [74,75] as well as in oestrogen receptor-positive breast cancers [76]. The c-MYC isoform N-MYC was also found to be downregulated in neuroblastoma by BET inhibitors [77–79] and the generation of Cre-conditional human MYCN-driven mouse models that closely recapitulate

Table 1. BRD inhibitors in clinical development

Company	Compound	Trial[a]	Phase	Start date	Indication
Bayer	BAY1238097 (not disclosed)	NCT02369029	I	02/2015	Neoplasms
Constellation	CPI-0610 (triazolobenzodiazepine class)	NCT01949883	I	09/2013	Lymphoma
		NCT02158858	I	06/2014	Acute leukaemia [acute myeloid leukaemia (AML)] Myelodysplastic syndrome (MDS) Myelodysplastic/myeloproliferative neoplasms (MDS/MPNs)
		NCT02157636	I	07/2014	Multiple myeloma
GlaxoSmithKline	GSK525762 (triazolobenzodiazepine class)	NCT01587703	I	03/2012	Midline carcinoma
		NCT01943851	I	05/2014	Cancer
OncoEthix	OXT015 (triazolothienodiazepine class in license from Tanabe Mitsubishi)	NCT01713582	I/II	12/2012	Acute leukaemia Other haematological malignancies
		NCT02259114	I	11/2014	NUT midline carcinoma Triple-negative breast cancer Non-small cell lung cancer with rearranged ALK gene/fusion protein or KRAS mutation Castrate-resistant prostate cancer (CRPC) Pancreatic ductal adenocarcinoma
		NCT02296476	I/II	11/2014	Glioblastoma multiforme
	OXT015 and Vidaza (azacitidine)	NCT02303782	I/II	01/2015	AML
Resverlogix	RVX000222 (quinazolinone class)	NCT01058018 (completed)	II	12/2009	Atherosclerosis Coronary artery disease
		NCT01728467 (completed)	II	11/2012	Diabetes
Tensha Therapeutics	TEN-010 (triazolothienodiazepine class)	NCT01987362	I	10/2013	Solid tumours
		NCT02308761	I	10/2014	AML MDS

[a]Data taken from http://www.clinicaltrials.org.

the human disease with respect to tumour localisation, histology, marker expression, and genomic makeup has been reported [80]. However, recent studies have identified diverse tumours where MYC is not downregulated following BET inhibition, suggesting that MYC expression is driven by unique, tumour type-specific regulatory mechanisms [81,82].

The collective findings of many studies employing pan-BET inhibitors suggest that downregulation of antiapoptotic and growth-promoting genes is highly selective while the effect of BET inhibition is context dependent, suggesting that displacement of BETs from gene promoters is only in part responsible for the observed changes in transcription on BET inhibition. Recent ChIP-seq data suggest that both BRD4 and the Mediator complex are co-occupying large numbers of enhancer regions as well as a small set of exceptionally large super-enhancers [83] that seem to be responsible for the increased expression of key transcription factors in cancer, such as the MYC oncogene [84] (Figure 3A) although their exact role is still debated [118]. Intriguingly, BET inhibition seems to preferentially displace BRD4 from these super-enhancers, offering a potential strategy for selective targeting of certain oncogenes [85].

BET roles in inflammation

BET proteins play a central role in inflammatory responses by directly facilitating the transcriptional activation of proinflammatory cytokines. This occurs mainly through interactions with NF-κB and by direct recruitment of P-TEFb to NF-κB-dependent acetylated histones, resulting in stimulation of primary response genes [2,44]. Inhibition of BETs using pan-BET inhibitors also strongly affects the expression of many secondary response proinflammatory genes. Typically these are induced during the late stage of macrophage activation in response to lipopolysaccharide (LPS) stimulation and BET inhibition results in protection from LPS-induced endotoxic shock and bacteria-induced sepsis in mice [112]. Interestingly, inhibition of BRD4 also resulted in ubiquitylation and degradation of the constitutively active nuclear form of RelA [86]. NF-κB has been shown to rapidly migrate to *de novo* enhancers post-LPS treatment [87] suggesting a potential mechanism for the sequestration of BRD4 in super-enhancer-enriched regions, offering an explanation for the attenuation conferred on NF-κB-controlled genes by pan-BET inhibitors [88]. Additionally, enhancer RNAs (eRNAs) were shown to be dynamically induced at most of the innate immunity and inflammation driver genes, which are also associated with super-enhancer domains, following Toll-like receptor stimulation. The dynamic induction of eRNAs was shown to further attenuate gene transcription in a BRD4-dependent manner [89] (Figure 3B).

An intriguing link has also been established between BET proteins and *in vitro* cardiomyocyte hypertrophy and *in vivo* pathological cardiac remodelling [90]. The authors found that BRD4 is at least in part responsible for the expression of transcription factors that control pathological gene expression during heart remodelling and heart failure, such as GATA4 and NFAT. Direct BRD4 inhibition in murine models abolished many characteristics of pathological remodelling, including an increase in cardiomyocyte volume and activation of inflammatory pathways, offering a potential therapeutic target for heart-failure pathogenesis.

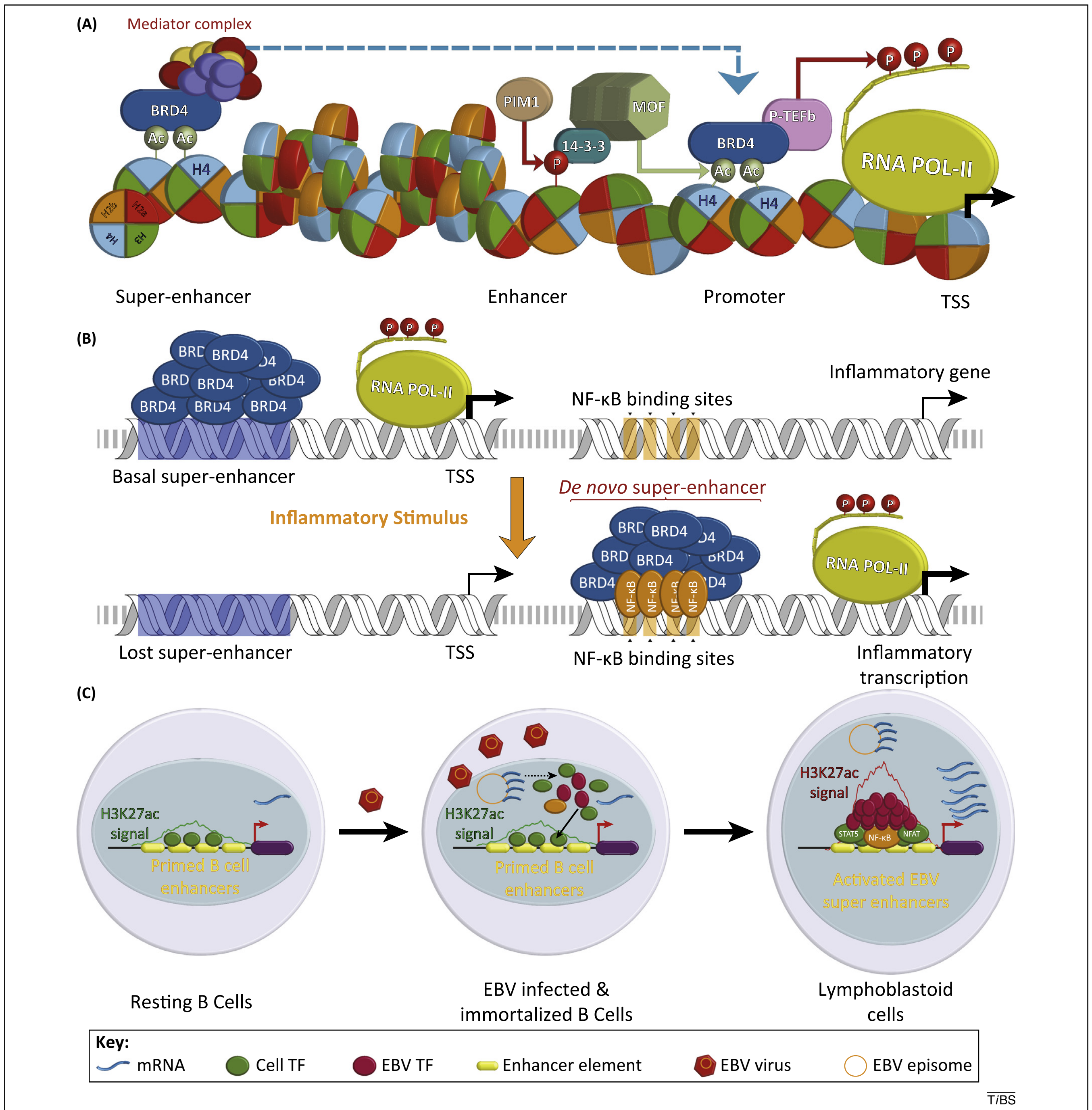

Figure 3. Transcriptional attenuation by bromodomain (BRD) 4. (**A**) BRD4 has been found to be particularly enriched at enhancer and super-enhancer regions strongly stimulating the expression of oncogenes in cancer. BRD4 is able to recruit the mediator complex to acetylated chromatin sites via its BRD modules, effectively stimulating transcription. In a cascade of events, the serine/threonine protein kinase PIM1 phosphorylates histone H3 at serine 10, an event that helps recruit a 14-3-3 protein that in turn recruits the acetyltransferase MOF, resulting in acetylation of histone H4 and thus generating new docking sites for BRD4. BRD4 also recruits (via its C-terminal motif and BRD) the positive transcription elongation factor b (P-TEFb) (the complex of CDK9 and cyclin-T1) to promoter regions leading to phosphorylation of the carboxy-terminal repeats of RNA polymerase II, which reactivates the paused polymerase complex stimulating elongation [117]. Adapted from [2]. (**B**) Inflammatory stimuli can result in redistribution of chromatin activators to *de novo* massive clustered enhancer domains (super-enhancers). An inflammatory stimulus (such as tumour necrosis alpha) can cause sequestration of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and other cofactors (including BRD4) from the basal super-enhancer, redistributing these factors to a new super-enhancer that stimulates the transcription of proinflammatory genes. Adapted from [88]. (**C**) Epstein–Barr virus (EBV) infection drives the formation of super-enhancers that drive transcription. On infection, viral oncoproteins are expressed and activate NF-κB resulting in viral and NF-κB subunits occupying primed B cell enhancer sites, recruiting additional cell transcription factors (TFs) (including adaptor proteins such as BRD4) and nucleating EBV super-enhancers effectively upregulating transcription of growth-promoting genes (e.g., *MYC*, *BCL2*). Adapted from [93].

BET roles in viral infection

Interactions of viral oncoproteins with BET proteins have been examined in the past, establishing a role for BETs in tethering viral genomes to mitotic chromosomes. This function of BETs contributes to the segregation of HPV, Epstein–Barr virus (EBV), KSHV, Merkel cell polyomavirus (MCV), and murine leukaemia virus (MLV) genomes [2]. For instance, BRD4 colocalises with the viral E1 and

E2 proteins at early stages of HPV infection, facilitating viral genome tethering to the host genome and the generation of foci that recruit additional factors. However, BRD4 is displaced from these foci during viral replication, suggesting that it plays a role in sequestering factors essential for viral replication at early stages but is not essential for viral DNA amplification [91]. In addition, BET interactions with viral proteins are at least in part responsible for the stabilisation of the viral partners within the nucleus, by preventing their cytosolic ubiquitylation and proteasomal degradation [92]. Analysis of ChIP-seq data also demonstrated interplay between EBV nuclear antigens (EBVNAs) (viral oncoproteins responsible for growth and survival) and NF-κB subunits, which were found to co-occupy sites with high H3K27ac density (a hallmark of super-enhancers). These so called EBV super-enhancer sites were shown to regulate key B-cell growth and survival genes such as *MYC* and *BCL2* and were sensitive to BET inhibition [93] (Figure 3C). It remains to be seen whether BET participation in super-enhancer function is a generic mechanism of viral infection.

Direct inhibition of BETs was also shown to reactivate latent HIV, although the precise mechanism differs between BET proteins. While BRD2 associates with E2F1 and NF-κB at the *HIV* promoter, repressing HIV transcription [94], BRD4 blocks the interaction of the HIV *trans*-activator Tat protein with the super-elongation complex at the *HIV* promoter [95]. Release of the BET proteins using pan-BET inhibitors resulted in latent reactivation of the virus and stimulated HIV replication, offering a potential therapeutic strategy for eliminating latent HIV [96].

Clinical validation of BETs

The disclosure of highly potent and selective BET inhibitors, and the remarkable follow-up and use of these tool compounds, has generated a wealth of information on BET function in multiple survival pathways. Several studies have already combined current treatment with BET inhibitors, demonstrating synergy and improved therapeutic potential [62,76,97–101]. At the same time many translational projects have been stimulated resulting in several clinical trials seeking to modulate BETs in diverse settings, particularly in oncology. Currently, several trials are in progress seeking to attenuate BETs in leukaemias, lymphomas, myelomas, glioblastomas, non-small cell lung cancer, and NUT midline carcinomas (Table 1). Although the BET inhibitors were discovered through a phenotypic screen for regulation of the expression of the APO-A1 protein, which is responsible for reverse cholesterol transport, the transcriptional attenuation of this gene by BETs is unclear [58]. The link between BETs and conditions such as diabetes and atherosclerosis is not entirely understood. Two clinical trials that sought to target BETs in this context (Table 1) have been completed; however, the precise outcome has not been disclosed and remains to be determined.

Concluding remarks

BET proteins are key players in transcription, controlling gene expression in inflammation, viral infection and cancer biology. The recent disclosure of pan-BET inhibitors that attenuate BRD function has allowed wide validation of these drug targets, shedding light on their roles in disease. Diverse transcriptional programmes are attenuated in a tissue-specific manner by BETs and preclinical targeting of BETs has had initial success, justifying the need for selective tools to better modulate their function. It will be challenging to target specific diseases without severe side effects, given the diverse and tissue-specific roles of individual BRDs. For instance, BRD2 is involved in differentiation in neurons and adipose tissue [28,102] but also haematopoiesis [31] and juvenile myoclonic epilepsy [103,104], BRD3 is involved in erythropoiesis [14,15], BRD4 is involved in transcriptional activation [32], non-coding enhancer RNA elongation [37], and chromatin structure and morphology [40] as well as the DNA-damage response [41], and BRDT is involved in spermatogenesis [11,34]. Despite the encouraging Phase I responses [105], concerns have been raised regarding the safety of pan-BET inhibition. When targeting BETs, care must be taken to monitor the effects on super-enhancer-controlled stem cell-specific transcription factors such as OCT4 and PRDM14, which control stem cell identity and cell fate decisions [106]. Direct inhibition of BRD4 in mice was recently shown to stimulate epidermal hyperplasia, alopecia, decreased cellular diversity, and stem cell depletion in the small intestine as well as sensitivity to organ stress [107]. In addition, BET inhibition resulted in strong modulation of the innate immune response, increasing the risk of infections when used clinically. For example, (+)-JQ1 treatment of mice infected with the bacterial pathogen *Listeria monocytogenes* reduced NO production and increased the animals' susceptibility to developing colitis due to strong downregulation of nitric oxide synthase 2 expression [108]. Lastly, Brd2-hypomorphic mice are severely obese [109], suggesting potential metabolic effects of BET inhibition. However, no *in vivo* data or clinical studies have demonstrated that targeting BRD2 may result in weight gain, although this must be further examined. These examples highlight the urgent need for novel selective tools that will improve understanding of the biology of each BET protein, to develop safe and effective novel drugs.

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