Cellular Target Engagement Approaches to Monitor Epigenetic Reader Domain Interactions

Supporting Information

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Table S1. Fluorescence Polarisation (FP) assay dissociation constant (K_d) values determined for BRD4 wild-type (WT), single site-directed mutants or double site-directed mutant proteins binding to either BET-647 or BSP-647.

BRD4 [a]	BET-647 K_d (nM) [b]	BSP-647 K_d (nM) [c]
WT	11.21 ± 0.63	20.50 ± 2.35
Y97A	18.24 ± 1.11	87.5 ± 10.10
Y390A	19.19 ± 1.47	28.55 ± 3.54
Y97A Y390A	N.D	N.D.

[a] BRD4 protein titrated, either WT, single side-directed mutant (Y97A or Y390A) or dual site-directed mutant (Y97A Y390A). [b] BET-647 is an Alexa Fluor 647 labelled small molecule based on a proprietary GSK BET inhibitor. [c] BST-647 is an Alexa Fluor 647 labelled small molecule based on the bromosporine compound disclosed by Picaud *et al.* in 2016[49].

Table S2. FP assay K_d values determined for BRD2 WT, single site-directed mutants or double site-directed mutant proteins binding to BET-647.

BRD2 [a]	BET-647 K_d (nM) [b]
WT	30.12 ± 1.02
Y113A	49.97 ± 4.01
Y386A	38.56 ± 2.75
Y113A Y386A	31014.10 ± 3796.34

[a] BRD2 protein titrated, either WT, single side-directed mutant (Y113A or Y386A) or dual site-directed mutant (Y113A Y386A). [b] BET-647 is an Alexa Fluor 647 labelled small molecule based on a proprietary GSK BET inhibitor.

Table S3. FP assay K_d values determined for BRD3 WT, single site-directed mutants or double site-directed mutant proteins binding to BET-647.

BRD3 [a]	BET-647 K_d (nM) [b]
WT	19.23 ± 0.42
Y73A	28.19 ± 1.12
Y348A	14.99 ± 0.35
Y73A Y348A	1205.44 ± 49.17

[a] BRD3 protein titrated, either WT, single side-directed mutant (Y73A or Y348A) or dual site-directed mutant (Y73A Y348A). [b] BET-647 is an Alexa Fluor 647 labelled small molecule based on a proprietary GSK BET inhibitor.

Table S4. FP assay K_d values determined for BRDT WT, single site-directed mutants or double site-directed mutant proteins binding to BET-647.

BRDT [a]	BET-647 K_d (nM) [b]
WT	5.57 ± 0.26
Y66A	211.63 ± 9.15
Y309A	19.16 ± 0.64
Y66A Y309A	8568.15 ± 709.50

[a] BRDT protein titrated, either WT, single side-directed mutant (Y66A or Y309A) or dual site-directed mutant (Y66A Y309A). [b] BET-647 is an Alexa Fluor 647 labelled small molecule based on a proprietary GSK BET inhibitor.

Table S5. Results of testing titrations of (+) – JQ1 and I-BET151 against either WB or isolated PBMCs and detecting the subsequent effect on secreted MCP-1 concentrations.

Inhibitor [a]	WB MCP-1 release pIC_{50}	WB MCP-1 release n^H	PBMC MCP-1 release pIC_{50}	PBMC MCP-1 release n^H
(+) - JQ1	6.18 ± 0.19	1.23 ± 0.12	8.16 ± 0.30	1.27 ± 0.23
I-BET151	6.35 ± 0.28	1.38 ± 0.35	7.67 ± 0.30	1.54 ± 0.47

[a] Experiments carried out using the methodologies outlined in the Materials and Method section. [b] Compounds initially prepared as 10 mM stocks, in 100% DMSO, prior to titration in the same solvent and transfer to the test microplate. [c] All data presented are mean values, determined from at least three independent replicates.

Table S6. FP and NanoBRET Tracer K_d value determination for CECR2 binding to BSP derived tracers.

Assay methodology [a]	BSP derived Tracer K_d (nM)	BSP derived Tracer n^H
FP	184.15 ± 11.10	0.92 ± 0.05
NanoBRET Tracer	110.23 ± 12.32	1.00 ± 0.12

[a] Experiments carried out using the methodologies outlined in the Materials and Method section. [b] All data presented are mean values, determined from at least three independent replicates.

Table S7. Results of testing titrations of iCECR2-1, -2 and -3 against either CECR2 TR-FRET or NanoBRET Tracer assays.

Inhibitor [a]	NanoBRET Tracer IC_{50} (nM)	NanoBRET Tracer release n^H	TR-FRET pIC_{50} (nM)	TR-FRET n^H
iCECR2-1	64.77 ± 9.05	1.03 ± 0.12	25.11 ± 5.23	0.97 ± 0.09
iCECR2-2	248.90 ± 31.28	0.82 ± 0.07	199.52 ± 26.34	1.11 ± 0.13
iCECR2-3	239.20 ± 97.04	0.78 ± 0.21	398.10 ± 70.07	1.03 ± 0.16

[a] Experiments carried out using the methodologies outlined in the Materials and Method section. [b] Compounds initially prepared as 10 mM stocks, in 100% DMSO, prior to titration in the same solvent and transfer to the test microplate. [c] All data presented are mean values, determined from at least three independent replicates.

Table S8. Protein construct table detailing the residues in the BET bromodomain (BRD) acetyl-lysine binding pockets that were mutated using site-directed mutagenesis.

BET [a]	Truncate length [b]	Bromodomain mutated [c]	Bromodomain residue mutated [d]
BRD4	1-477	N/A	N/A
BRD4	1-477	BD1	Y97A
BRD4	1-477	BD2	Y390A
BRD4	1-477	BD1, BD2	Y97A, Y390A
BRD2	1-473	N/A	N/A
BRD2	1-473	BD1	Y113A
BRD2	1-473	BD2	Y386A
BRD2	1-473	BD1, BD2	Y113A, Y386A
BRD3	1-435	N/A	N/A
BRD3	1-435	BD1	Y73A
BRD3	1-435	BD2	Y348A
BRD3	1-435	BD1, BD2	Y73A, Y348A
BRDT	1-397	N/A	N/A
BRDT	1-397	BD1	Y66A
BRDT	1-397	BD2	Y309A
BRDT	1-397	BD1, BD2	Y66A, Y309A

[a] Experiments carried out using the methodologies outlined in the Materials and Method section. [b] Construct covering the stated residues of the endogenous BET protein (BRD2, Entrez gene ID 6046; BRD3, Entrez gene ID 8019; BRD4, Entrez gene ID 23476; BRDT, Entrez gene ID 676). [c] Bromodomain to be mutated to either enable isolation of a specific domain or inactivation of both domains. [d] Bromodomain residue mutated to Ala using site-directed mutagenesis.

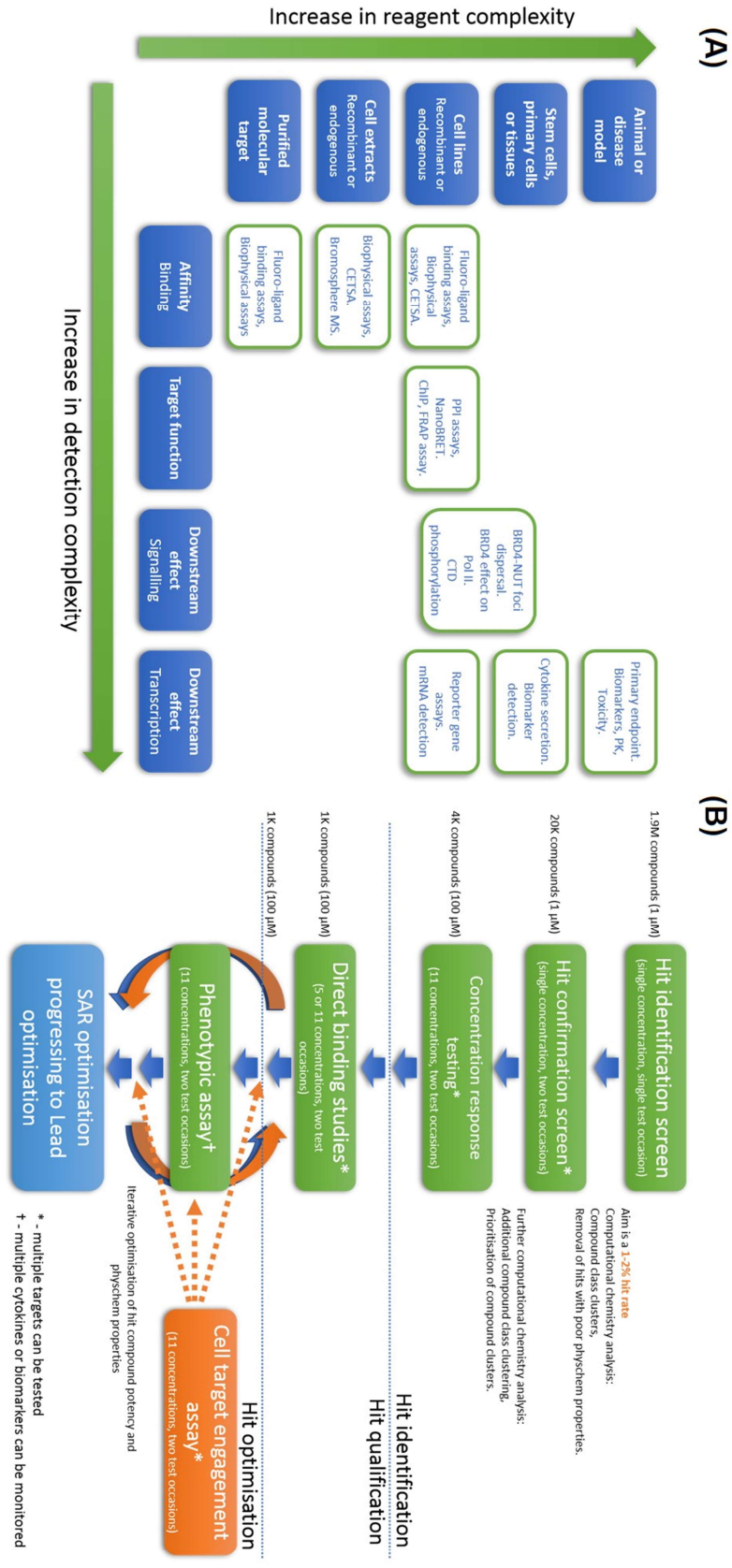

Figure S1. Small molecule drug discovery approaches for bromodomain proteins.

(A) Schematic representation of the assays utilized to assess compounds for inhibition of BRD4. This schematic represents how molecules can be tested in a range of assays, which vary from *in vitro* biochemical approaches to downstream signalling effects in clinically relevant disease models. (B) Typical bromodomain inhibitor identification strategy employed at GSK. The position for the possible implementation of cell target engagement assays is highlighted in orange.

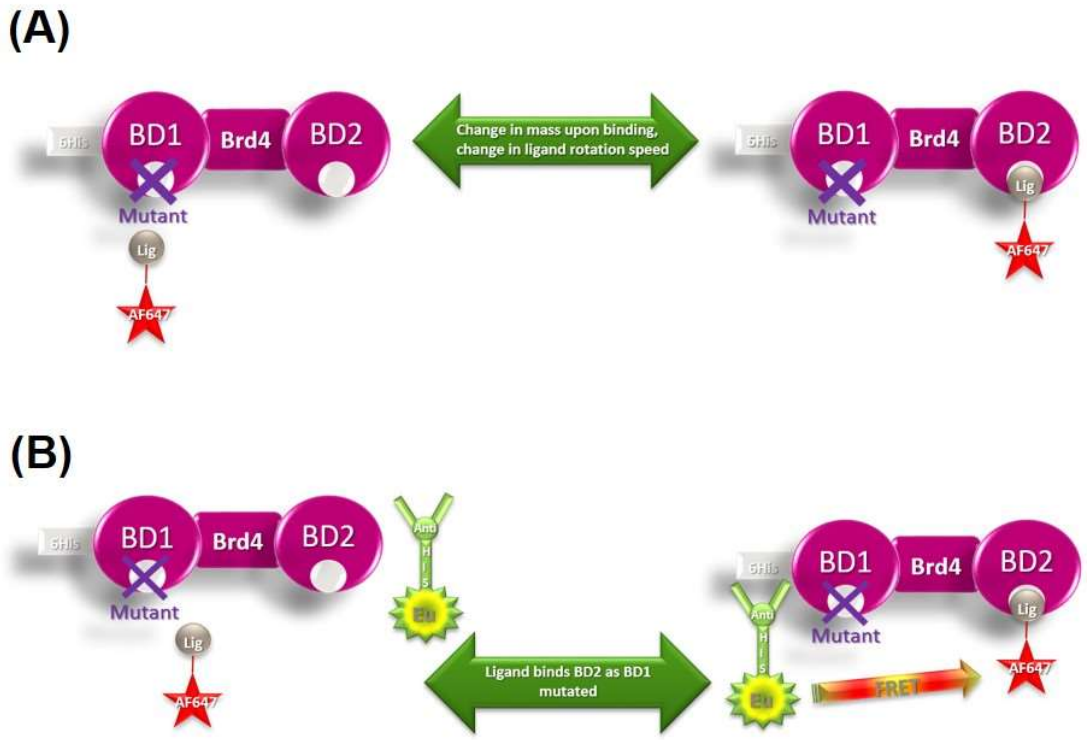

Figure S2. Schematics of fluorescence methodologies used to monitor binding at the tandem BDs of the BET proteins.

(A) Schematic depicting the basic principle of the domain mutant BET FP assays, which utilise a non-selective bromodomain binding tracer. (B) Schematic demonstrating the use of the same BET FP assay reagents to configure a TR-FRET binding assay, with the inclusion of a europium chelate labelled anti-6-His antibody.

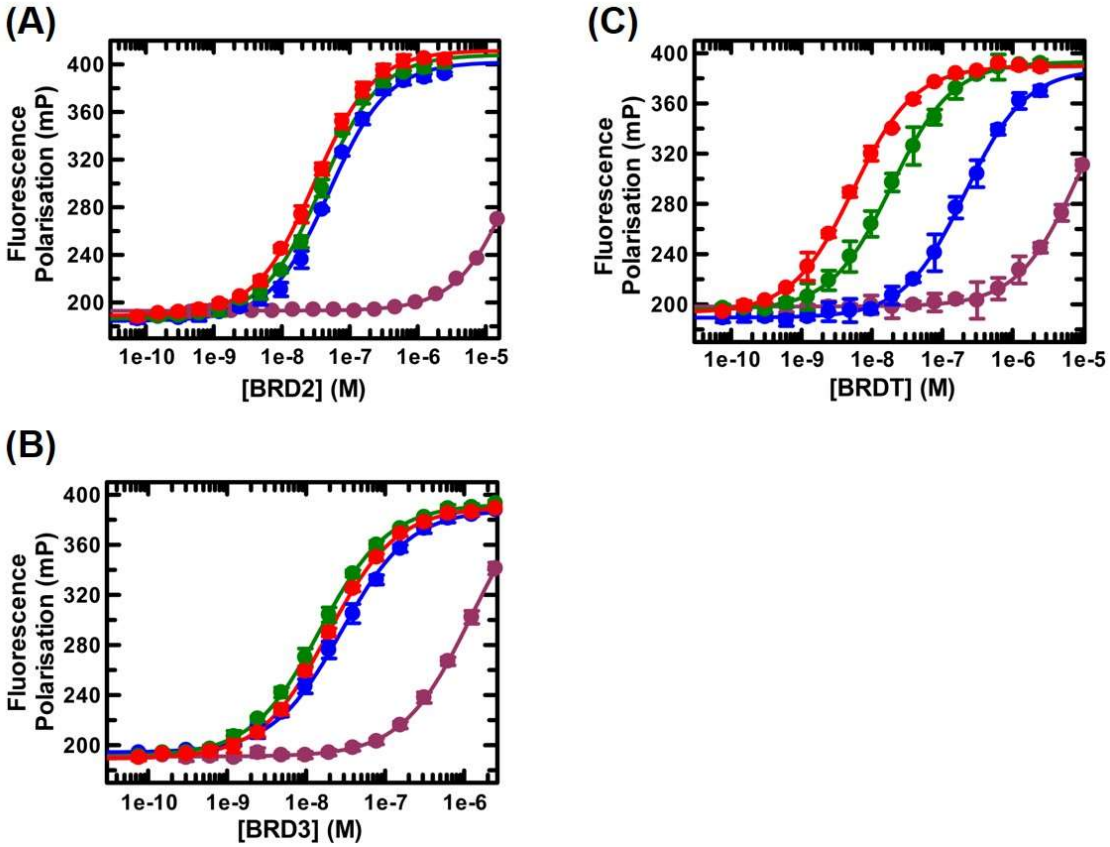

Figure S3. Further FP quantification of tracer interactions with BET proteins.

(A) BRD2 FP binding assay, carried out by titrating BRD2 WT (●), BRD2 Y113A (●), BRD2 Y386A (●) and BRD2 Y113A Y386A (●) from 2μM, prior to addition of 5 nM BET-647 and detection of FP signal change. Data fitted to the Hill-Langmuir equation (1). (B) BRD3 FP binding assay, carried out by titrating BRD3 WT (●), BRD3 Y73A (●), BRD3 Y348A (●) and BRD3 Y73A Y348A (●) from 2μM, prior to addition of 5 nM BET-647 and detection of FP signal change. Data fitted to the Hill-Langmuir equation (1). (C) BRDT FP binding assay, carried out by titrating BRDT WT (●), BRDT Y66A (●), BRDT Y309A (●) and BRDT Y66A Y309A (●) from 2 μM, prior to addition of 5 nM BET-647 and detection of FP signal change. Data fitted to the Hill-Langmuir equation (1).

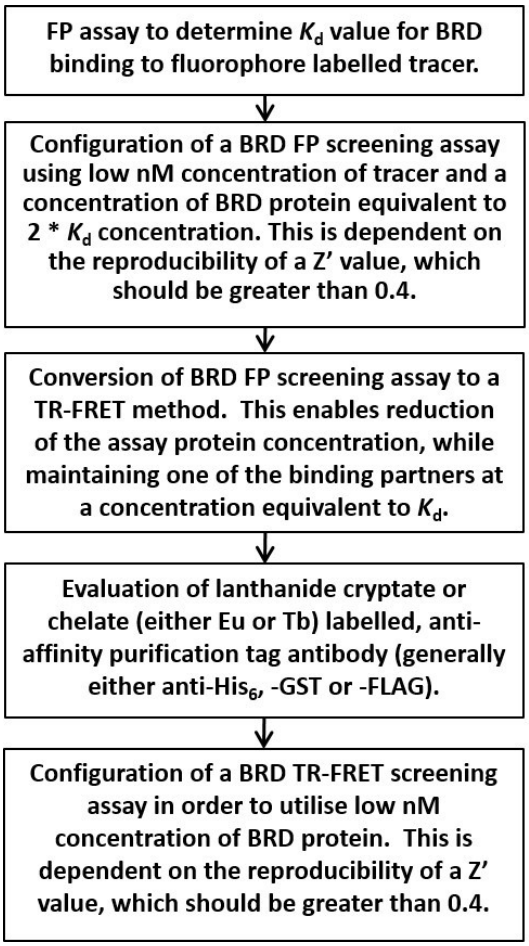

Figure S4. Assay development workflow utilised for purified BRD protein fluorescence binding approaches.

Schematic depicting the work flow applied at GSK to first determine the K_d value for the interaction between the purified BRD truncate protein and the fluorophore labelled tracer, prior to the subsequent steps taken to optimise a FP assay and then convert this binding approach to a TR-FRET assay.

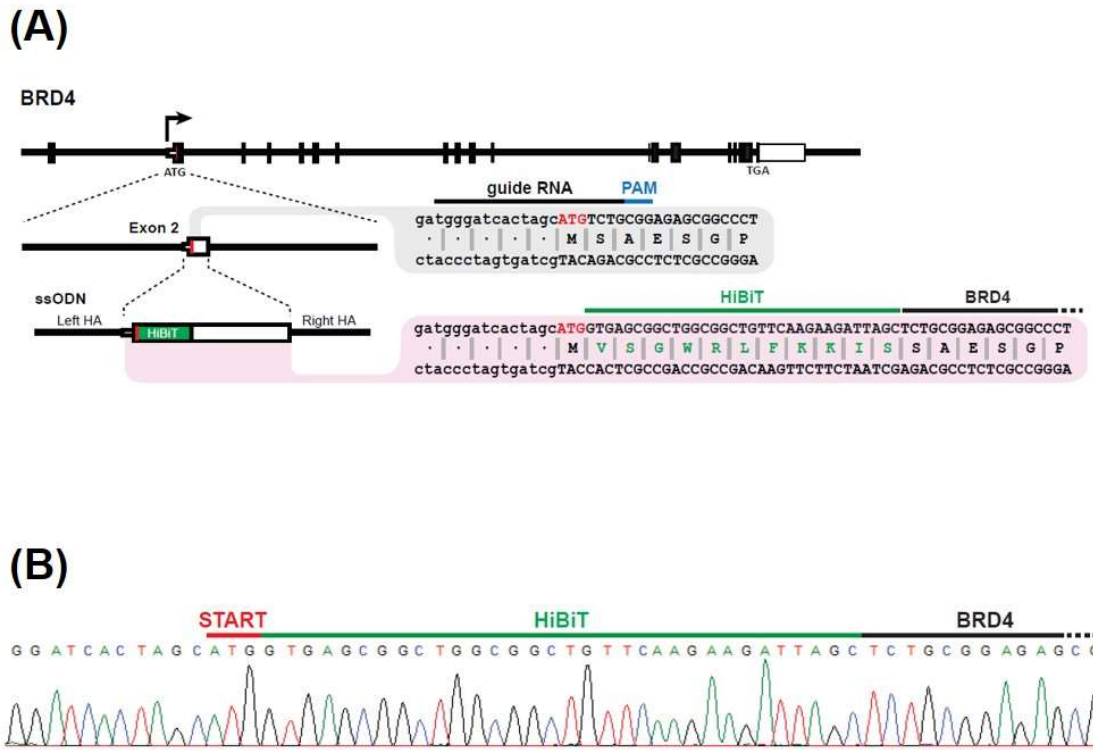

Figure S5. Approaches taken to validate the insertion of a HiBiT tag to the N-terminus of the BRD4 gene and subsequent QC of the modification.

(A) Schematic representation of the strategy for integration of the HiBiT tag to the N-terminus of the BRD4 gene. Black boxes, exons of BRD4; ssODN, single-stranded oligonucleotide repair template; HA (homology arms), region of homology to the BRD4 genomic sequence; Red letters, start codon. The black underlined sequence indicates the genomic target sequence of the CRISPR/Cas9 guide RNA. (B) Sanger sequence chromatogram illustrating accurate integration of the HiBiT tag to the N-terminus of the BRD4 gene in a HEK293-wt cell clone.

Supporting Materials and Methods

Data Analysis

All data handling was initially carried out in Microsoft Excel 2016. All further data analysis was carried out in Graphpad Prism 6.0 or Grafit 7.0.2. All high throughput data analysis was carried out in IDBS ActivityBase XE. Saturation binding curves were fitted to the Langmuir-Hill equation, eq. 1,

$$\theta = \frac{[L]n}{Kd + [L]n} \quad (1)$$

where θ is the fraction of the protein bound to the tracer, [L] is the free, unbound ligand concentration, K_d is the apparent dissociation constant and n is the Hill coefficient. Compound inhibition and NanoBRET tracer data binding were fitted using the following four parameter logistic fit was used, eq. 2,

$$y = \frac{a-d}{1+(x/c)^b} + d \quad (2)$$

where 'a' is the minimum, 'b' is the Hill slope, 'c' is the pIC_{50} and 'd' is the maximum. Assay data was normalized to microplate controls using the following equation, eq. 3,

$$x = \frac{100-(100(a-b))}{c-b} \quad (3)$$

where 'a' is the raw compound data, 'b' is the low control raw data and 'c' is the high control raw data. Data generated using TR-FRET assays was normalized to the lanthanide donor fluorescence using the following equation, eq. 4,

$$x = \frac{a}{d} \quad (4)$$

where 'x*'* is the TR-FRET ratio, '*a'* is the acceptor fluorescence and '*d*' is the donor fluorescence. Specific wavelengths are included in the methods. Fluorescence intensities, measured for tracer binding experiments, in the parallel and perpendicular planes were converted to a FP ratio using the following equation, eq. 5,

$$x = \frac{a-b}{a+b} \quad (5)$$

where '*x*' is the FP ratio (millipolarizations, mP), 'a' is parallel fluorescence and 'b' is the perpendicular fluorescence. BRET data was converted to milliBRET units (mBu) by the following equation, eq. 6,

$$x = (\frac{a}{d}) * 1000 \quad (6)$$

where '*x'* is milliBRET units (mBu), '*a*' is the acceptor fluorescence at 610 nm and '*d'* is the donor fluorescence at 480 nm.

Materials

Liquid Handling

All liquid handling was performed using a Thermo Scientific Multidrop unless otherwise stated.

Instruments

TR-FRET, FP and NanoBRET experiments were all read on an Envision Multilabel Reader 2101 (Perkin Elmer, Waltham, MA**).** For NanoBRET experiments, donor emission was measured with a 610 nM long pass filter and acceptor emission with a 450+/-20 filter. The readings were converted to mBu (milliBRET units) using the equation (6). Cytokine release assays were read on an Intellicyt iQue Screener Plus (Sartorius, Royston, UK) or Mirrorball (TTP Labtech, Melbourn UK). Unless otherwise stated all centrifugations were performed using an Eppendorf 5810R (Hamburg, Germany) benchtop centrifuge.

Reagents

All NanoBRET reagents were purchased from Promega (Madison, WI, USA), while all fluorophores, unless otherwise stated were purchased from Fisher Scientific (Waltham, MA, USA)**.** All fluorophore labelled ligands and compounds were synthesised at GSK. MCP-1 cytokine detection, human MCP-1 flex set purchased from BD Bioscience (NJ, USA). Phosphate buffered saline (PBS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), NaCl, bovine serum albumin (BSA), Dulbeccos Modified Eagle's Medium (DMEM), DL-dithiothreitol (DTT), glycerol, DMSO, lipopolysaccharide **(**LPS) (L-6386), imidazole, protease inhibitor cocktail and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were all purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated. Glutamax, Australian foetal bovine serum (AUS FBS), TrypLE cell dissociation reagent and OptiMEM w/o phenol red were all purchased from Gibco (Thermo Fisher Scientific, MA, USA) unless otherwise stated. Europium chelate labelled anti-6-His antibody was purchased from Perkin Elmer (Waltham, MA, USA). All general consumables were purchased from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise stated.

Cell culture

HEK293-wt cells (CRL-1573) were purchased from ATCC. All cells were cultured in DMEM, supplemented with 10% AUS FBS, 5 mM Glutamax and 5 mM HEPES, referred to in text as M1 media, passaged using TrypLE cell dissociation reagent, and maintained under 5% CO_2 and 37 °C conditions. Human whole blood was obtained fresh from donors at GSK on the day of assay and treated with sodium heparin (1 ml/100 ml of blood) (Thermo Fisher Scientific**,** MA, USA). Isolation and preservation of Human Peripheral Blood Mononuclear Cells (PBMCs) was performed as described in Dunne *et al.,* 2009[51]. PBMCs were subsequently cultured in RPMI-1640, 10% Foetal Calf Serum, 1% Penicillin/Streptomycin, 1% L-Glutamine 200 mM.

BET protein expression and purification

A recombinant truncate of human bromodomain containing BET proteins were expressed in *E. coli* cells using a pET15b vector with a 6-His tag at the *N*-terminal. The construct lengths used are detailed in Table 1. along with the bromodomain binding site residues changed using site directed mutagenesis (SDM), which were made to each of the constructs to either enable monitoring of binding to specific domains or to prevent binding to each of the domains.

The following expression and purification strategy were utilized for all BET constructs detailed in Table S8. Protein expression was carried out in *E. coli* BL21(DE3) cells, followed by ultracentrifugation, resuspension of the subsequent cell pellet in 50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM imidazole and 1 µl/mL protease inhibitor cocktail and lysis using sonication. Purification was carried out using a nickel sepharose high performance affinity column (GE Healthcare), including an initial wash step and then a subsequent elution using a linear gradient, over 20 column volumes, of 0–500 mM imidazole, which was added in a buffer comprised of 50 mM HEPES (pH 7.5), 150 mM NaCl, 500 mM imidazole. A further chromatography step was then used to purify the BET proteins, which was carried out using a Superdex 200 prep grade size exclusion column. Purified protein was stored at −80 °C in 20 mM HEPES (pH 7.5) and 100 mM NaCl. Protein identity was confirmed by peptide mass fingerprinting and predicted molecular weight confirmed by electrospray, liquid chromatography mass spectrometry (ESI-LC-MS).

BRD4 Fluorescence Polarization assays

All assay components were diluted in an assay buffer composed of 50 mM HEPES (pH 7.4), 150 mM NaCl and 0.5 mM CHAPS. The BET truncated protein constructs were titrated in assay buffer from stock concentrations of either 2 or 10µM, in black, small volume 384-well microplates (Greiner Bio-one 784076, Frickenhausen, Germany). Subsequently, equal volumes of solutions containing 10 nM of a known BET binding tracer were added to the 384-well microplates containing titrations of each of the BET proteins. The BET binding tracer (BET-647) used was an Alexa Fluor 647 labelled derivative of a proprietary GSK BET inhibitor, which has previously been disclosed by Chung, *et al.*[27]. Alternatively, in parallel experiments using only BRD4, an Alexa Fluor 647 labelled derivative of the bromosporine compound, referred to as BSP-647, was utilized. The test 384-well microplates were then centrifuged for 1 minute at 1000 RPM in an Eppendorf 5810R (Hamburg, Germany) benchtop centrifuge, prior to incubation under a microplate cover (Greiner Bio-one 656191, Frickenhausen, Germany), in light restricted conditions at room temperature for 15 mins. Fluorescence polarization (FP) was subsequently detected on a Perkin Elmer Envision multimode microplate reader using the following settings; excitation = 620 nm; emission 1 = 688 nm P polarized; emission 2 = 688 nm S polarized; Dichroic mirror module = 658 nm). Fluorescence polarization ratio was calculated using equation 5. The mP data were then plotted against the concentration of BET protein tested and subsequently fitted to equation (1).

BRD4 TR-FRET assays

All assay components were diluted in buffer composition of 50 mM HEPES (pH 7.4), 150 mM NaCl, 5% glycerol, 1 mM DTT and 1 mM CHAPS. The final concentration of BRD4 bromodomain protein used in the TR-FRET assays described herein was 10 nM. BET-647 was used as the competing ligand at a concentration equivalent to the K_d determined using FP assays. BRD4 protein and tracer were premixed and 5 µl of this assay mixture was then added to wells containing 50 nl of various concentrations of test compound or DMSO vehicle (0.5% DMSO final) in black, small volume 384-well microplates. Subsequently, the test 384-well microplates were then centrifuged for 1 minute at 1000 RPM in a benchtop centrifuge, prior to incubation under a microplate cover (Greiner Bio-one, 656191 Frickenhausen, Germany), in light restricted conditions at room temperature for 15 minutes. The BRD4 protein::tracer interaction was detected using TR-FRET following a 5 µl addition of 1.5 nM europium chelate labelled anti-6-His antibody in assay buffer. Time resolved fluorescence resonance energy transfer (TR-FRET) was then detected on a TRF laser equipped Perkin Elmer Envision multimode microplate reader (excitation = 337 nm; emission 1 = 615 nm; emission 2 = 665 nm; dual wavelength bias dichroic = 400 nm, 630 nm). TR-FRET ratio was calculated using equation (4) and was normalized, using equation (3), to a mean of 16 replicates per microtiter plate of both 10µM I-BET151 and 1% DMSO controls. IC_{50} values were determined for each of the compounds tested by collating the TR-FRET ratio data with the test compound concentrations and subsequently fitting these data to equation (2).

CECR2 protein expression and purification

A recombinant truncate of human bromodomain containing CECR2 protein were expressed in *E. coli* cells using a pET28a vector with 6-His and FLAG affinity purification tags at the *N*-terminal. The minimal construct length of CECR2 residues 424-543 was sufficient to cover the bromodomain containing module of the full-length protein (Entrez gene ID 27443), which was predicted to result in a protein with an expected molecular weight of 17.3kDa.

Protein expression was carried out in *E. coli* BL21(DE3) cells, followed by ultracentrifugation, resuspension of the subsequent cell pellet in 50 mM HEPES (pH 7.5), 500 mM NaCl, 5 mM imidazole, 5% glycerol, 1 mM DTT and 1 µl/mL protease inhibitor cocktail and lysis using sonication. Purification was carried out using a nickel sepharose high performance affinity column (GE Healthcare, IL, USA), including an initial wash step and then subsequent elution using dual imidazole gradients. The first gradient was over 10 column volumes, from 0 to 150 mM imidazole, followed by a second gradient, over 8 column volumes from 150 to 300 mM imidazole. The elution buffer comprised of 50 mM HEPES (pH 7.5), 500 mM NaCl, 5% glycerol, 1 mM DTT and 500 mM imidazole. A further chromatography step was then used to purify the CECR2, which was carried out using a Superdex 200 prep grade size exclusion column. Purified protein was stored at −80 °C in 50 mM HEPES (pH 7.5), 500 mM NaCl, 5% glycerol and 1 mM DTT. Protein identity was confirmed by peptide mass fingerprinting and predicted molecular weight confirmed by ESI-LC-MS (data not shown).

CECR2 Fluorescence Polarization assays

All assay components were diluted in an assay buffer composed of 50 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM DTT and 0.5 mM CHAPS. The CECR2 protein was titrated in assay

buffer from stock concentrations of either 5 or 10μM, in black, small volume 384-well microplates (Greiner Bio-one 784076, Frickenhausen, Germany). Subsequently, equal volumes of solutions containing 10 nM of a known CECR2 binding tracer, were added to the 384-well microplates containing titrations of each of CECR2 protein. Two CECR2 binding tracers were used both labelled with AlexaFluor647, one based on a proprietary GSK small molecule and another derived from bromosporine (BSP-647)[33]. The test 384-well microplates were then centrifuged for 1 minute at 1000 RPM in a benchtop centrifuge, prior to incubation under a microplate cover (Greiner Bio-one 656191, Frickenhausen, Germany), in light restricted conditions at room temperature for 15 minutes. Detection of FP signal change and subsequent K_d determination was carried out as detailed previously for the BET proteins.

CECR2 TR-FRET assay

All assay components were diluted in buffer composition of 50 mM HEPES (pH 7.4), 150 mM NaCl, 5% glycerol, 1 mM DTT and 1 mM CHAPS. The TR-FRET assay was configured with a concentration of 10 nM CECR2 bromodomain protein and a concentration of GSK tracer equivalent to the K_d determined using the previously described FP assay. CECR2 protein and tracer were premixed and 5 μl of this assay mixture was then added to wells containing 50 nl of various concentrations of test compound or DMSO vehicle (0.5% DMSO final) in black, small volume 384-well microplates. Subsequently, the test 384-well microplates were then centrifuged for 1 minute at 1000 RPM in a benchtop centrifuge, prior to incubation under a microplate cover (Greiner Bio-one 656191, Frickenhausen, Germany), in light restricted conditions at room temperature for 15 minutes. The CECR2 protein::tracer interaction was detected using TR-FRET following a 5 μl addition of 1.5 nM europium chelate labelled anti-6-His antibody in assay buffer. TR-FRET was detected, and subsequent data analysis carried out as described in the BET TR-FRET assay methodology section, with the exception of the identity of the 100% inhibition control test compound, which was changed from 10μM I-BET151 to 10μM bromosporine.

Generation of CRISPR/Cas9 edited HEK293 HiBiT cell line

Cell culture

HEK293-wt cells (CRL-1573) were purchased from ATCC. All cells were cultured in EMEM (Eagle's Minimum Essential Media; Gibco) supplemented with 10% FBS (fetal bovine serum) and 2 mM Glutamax, passaged using TrypLE cell dissociation reagent, and maintained under 5% CO_2 and 37 °C conditions.

CRISPR/Cas9 guide RNA design and genome editing

To generate a guide RNA duplex targeting the N-terminus of the BRD4 gene, Alt-R crRNA (genomic target sequence: tgggatcactagcatgtctg) and tracrRNA were resuspended to 100**μ**M with nuclease-free duplex buffer (Integrated DNA Technologies, Coralville, IA) and mixed at equimolar concentration. Oligos were annealed by heating to 95 °C for 5 minutes in a PCR thermocycler and allowed to slowly cool to room temperature. The resulting crRNA-tracrRNA duplexes were combined with *S. pyogenes* Cas9 (120pmol and 100pmol, respectively;

Genscript), at room temperature, and allowed to form Cas9 ribonucleoprotein (RNP) complexes by incubation for 10-20 minutes.

For nucleofection, 2e5 HEK293-wt cells were resuspended in 20 µl nucleofection solution (SF Kit; Lonza, Basel, Switzerland) and the resulting suspension combined with 10 µl RNP and 1 µl of the 100µM single-stranded oligonucleotide (ssODN) template (IDT ultramer) bearing the HiBiT tag:

(sequence:catctgctgactgatatctcacgggggctcttctcttcctttgtagagtgcctggtgaagaatgtgatgggatcactagc ATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCtctgcggagagcggccctgggacgagatt gagaaatctgccagtaatgggggatggactagaaacttcccaaatgtctac; HiBiT sequence in uppercase)

The aforementioned was gently mixed with a pipette. The suspension was transferred to a 4D nucleofector multi-well strip cuvette and pulsed with program CM-130. After pulsing, 80 µl of cell culture media was added to the cuvette and the resulting reaction transferred to a single well of a 6-well microplate, containing pre-warmed media, for culture and expansion.

Clonal derivation and validation

Single cell clones were derived by dilution cloning in 96-well microplates and HiBiT-positive clones were identified by luminescence using the Nano-Glo HiBiT Lytic Detection System as per manufacturer's protocol. In brief, NanoGlo Luciferase Assay reagent was prepared by combining one volume (20 µl) of NanoGlo substrate to 50 volumes (1 ml) of NanoGlo Buffer. One half volume (10 µl) of purified LgBiT was added to the solution, mixed, and allowed to equilibrate to room temperature. 100 µl of prepared Assay Reagent was added to each well (containing each single cell clone) containing an equal volume (100 µl) of culture media, mixed by shaking for 5 minutes, and transferred to a white-bottom 96-well microplate for luminescence imaging on a ViewLux microplate imager (Perkin Elmer, MA, USA).

Cell clones positive for luminescence were subjected to Sanger sequencing for verification of accurate HiBiT integration. A PureLink® Genomic DNA Kit (Invitrogen, Thermo Fisher Scientific, MA, USA) was used to isolate and purify the genomic DNA from cell clones as per manufacturer's protocol and eluted in 50mL PureLink® Genomic Elution Buffer. PCR amplicons were generated and subjected to Sanger sequence analysis using primers (IDT; PCR-FOR: CTTGGAGACCACAGCCAGAG, PCR-REV: TGCCTCTTGGGCTTGTTAGG, SEQ1: GGTTGGTGCTGGCTGCGTTG, and SEQ2: CTTGGAGACCACAGCCAGAG).

Supporting References

All references in the Supporting Information can be found in the References section of the main manuscript.