SUPPLEMENTAL MATERIAL

Structure-guided drug design identifies a BRD4-selective small molecule that suppresses HIV

Qingli Niu1,2, #, Zhiqing Liu3, #, Edrous Alamer1,2, Xiuzhen Fan1,2, Haiying Chen3, Janice Endsley1,2, Benjamin B. Gelman4, Bing Tian5, Jerome H. Kim6, Nelson L. Michael7,8, Merlin L. Robb7,8, Jintanat Ananworanich7,8,9, Jia Zhou3*, Haitao Hu1,2 *†

SUPPLEMENTAL METHODS

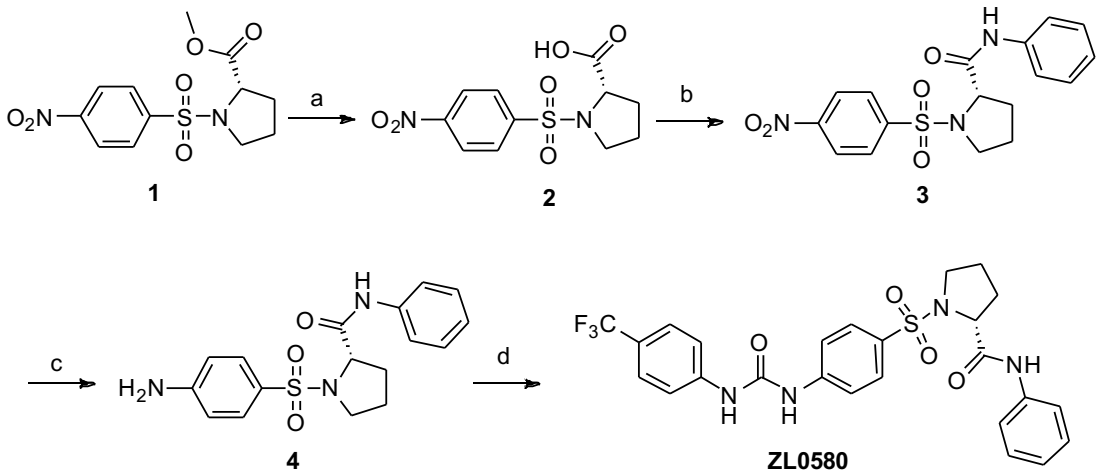
Compound Design

Using structure-based drug design, we started from analyzing the crystal structures of available BRD4 modulators with BRD4 BD1 domain. Most inhibitors occupy the central hydrophobic cavity mimicking acetyl-lysine, and anchored by hydrogen bonds with Asn140 directly and Tyr97 indirectly via a water molecule. A pharmacophore model was summarized as two key aromatic rings attached with a proper linker. We utilized a deconstruction approach and obtained various drug-like fragments from available BET family inhibitors and drug libraries as the critical head and tail moieties as well as the linker scaffold. Using fragment-based drug design method, we reconstructed the fragments to generate a batch of new compounds through fragment merging and elaboration. Among them, compound ZL0580 displayed potent BRD4 binding activity with IC_{50} = 163 nM against BRD4 BD1 with 6.6-fold selectivity over BRD4 BD2. Besides, ZL0580 displayed 6~11 folds selectivity over its close BET family members BRD2, BRD3 and BRDT (IC_{50} values ranges from 0.9~1.9 μM) as well as over 60-fold selectivity over non-BET bromodomain protein CBP (IC_{50} >10 μM).

ZL0580 Synthesis

(4-Nitrophenyl) sulfonyl)-*L*-proline (2).

To a solution of methyl ((4-nitrophenyl) sulfonyl)-*L*-prolinate (**1**) (500 mg, 1.6 mmol) in 16 mL CH_3OH, $LiOH{\cdot}H_2O$ (334 mg, 8.0 mmol) in 8 mL H_2O was added. The mixture was allowed to stir at room temperature for 4 hours. After concentration, the solution was acidified by 10% HCl to pH = 3 and extracted by dichloromethane (DCM) to obtain **2** (540 mg, quant.) as a white solid. The crude product was used directly in the next step.



Reagents and conditions: (a) LiOH, CH_3OH/H_2O, rt., 4 h, quant.; (b) aniline, HBTU, DIEA, DCM, rt., 66%; (c) Zn, NH_4Cl, $EtOH/H_2O$, reflux; (d) 1-isocyanato-4-(trifluoromethyl)benzene, DCM, rt., 66% for two steps.

(*S*)-1-((4-Nitrophenyl) sulfonyl)-*N*-phenylpyrrolidine-2-carboxamide (3).

To a solution of **2** (100 mg, 0.33 mmol) and aniline (31 mg, 0.33 mmol) in 5 mL of DCM, HBTU (321 mg, 1.0 mmol) and DIPEA (295 μL, 1.67 mmol) were added. After stirring at rt. overnight, the mixture was extracted with DCM (20 mL × 3). The organic layer was washed with 1 N $NaHSO_4$ (aq.), saturated $NaHCO_3$ (aq.), brine and dried over anhydrous Na_2SO_4. The resulting solution was evaporated, and the residue was purified by PTLC (DCM/MeOH = 70:1)

to give the desired product **3** (81 mg, 66%) as a pale-yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.35 (d, J = 8.7 Hz, 2H), 8.05 (d, J = 8.7 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.37 – 7.25 (m, 2H), 7.11 (t, J = 7.4 Hz, 1H), 4.32 – 4.18 (m, 1H), 3.68 – 3.56 (m, 1H), 3.29 (dd, J = 16.8, 7.8 Hz, 1H), 2.31 – 2.18 (m, 1H), 1.98 – 1.86 (m, 1H), 1.84 – 1.68 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.94, 150.48, 141.93, 137.12, 129.04, 128.97, 124.85, 124.55, 120.20, 120.10, 62.91, 49.85, 30.36, 24.49.

(*S*)-1-((4-Aminophenyl) sulfonyl)-*N*-phenylpyrrolidine-2-carboxamide (4).

To a solution of **3** (70 mg, 0.19 mmol) in 10 mL CH_3CH_2OH, NH_4Cl (50 mg, 0.93 mmol) in 3 mL H_2O and Zn dust (123 mg, 1.9 mmol) were added. The solution was allowed to reflux for 0.5 hour. Then it was filtered, and the filtrate was extracted by DCM for three times. The organic layer was dried and concentrated to give **4** as a white foam which was used directly in the next step.

(*R*)-*N*-Phenyl-1-((4-(3-(4-(trifluoromethyl) phenyl) ureido)phenyl)sulfonyl)pyrrolidine-2-carboxamide (ZL0580).

To a solution of **4** (0.187 mmol) in 5 mL DCM, 1-isocyanato-4-(trifluoromethyl) benzene (37 mg, 0.2 mmol) was added. After stirring at rt. overnight, the mixture was concentrated and purified by PTLC (DCM/CH_3OH = 50 :1) to give **ZL0580** (65 mg, 66% for two steps) as a white solid. HPLC purity 99.8% (t_R = 19.7 min). ^{1}H NMR (300 MHz, MeOD) δ 7.87 (d, J = 8.8 Hz, 2H), 7.74 (d, J = 8.6 Hz, 2H), 7.68 (d, J = 8.7 Hz, 2H), 7.64 – 7.55 (m, 4H), 7.35 (t, J = 7.7 Hz, 2H), 7.14 (t, J = 7.3 Hz, 1H), 4.24 (dd, J = 7.7, 4.2 Hz, 1H), 3.70 – 3.57 (m, 1H), 3.38 (d, J = 7.6 Hz, 1H), 2.13 – 1.91 (m, 3H), 1.69 (d, J = 5.3 Hz, 1H). ^{13}C NMR (75 MHz, MeOD) δ 171.43, 152.67, 144.19, 142.52, 137.74, 129.56, 128.81, 128.42, 125.75, 125.70, 124.32, 120.44, 118.35,

118.21, 62.61, 49.37, 30.83, 24.31. HR ESI-MS $(M + Na)^+$ m/z = 555.1282 (calcd for $C_{25}H_{24}F_3N_4O_4SNa$: 555.1290).

Compound binding analysis (TR-FRET)

384 well plate-based commercial TR-FRET Assay kits (Cayman Chemical, Ann Arbor, Michigan) were used to determine the binding affinity of ZL0580 to the bromodomains (BDs) of BRD4, BRD2, BRD3, and BRDT as well as CBP by time-resolved fluorescence energy transfer (TR-FRET) assays. A series of concentrations of the compound from 0.01 nM to 100 μM were added into a 384 well test plate and mixed with other reaction components based on the instructions from vendor followed by incubation 1h at room temperature. The commercially available BET inhibitor JQ1 was used as the control. The plates were read in time-resolved format by exciting the sample at 340 nm and reading emissions at 620 and 670 nm, using a 100 μs delay and a 500 μs window at a Tecan M1000 pro reader. A plot of the TR-FRET ratio (670 nm emission/620 nm emission versus inhibitor concentration on semi-log axes results in a sigmoidal dose-response curve typical of competitive assays. These data were further calculated out with the IC_{50} values of the tested compounds to the bromodomains. IC_{50} values are reported as the mean derived from three independent measurements. Each one is generated from at least 8 different concentrations.

PCR quantification of cell-associated HIV DNA and RNA

To quantify cell-associated HIV DNA in normal PBMCs, Total DNA was extracted from cells using Quick-DNA Miniprep Kit (Zymo) according to the manufacturer's instructions. PCR reactions (20ul) contained 10μM of Forward and Reverse primers, 100ng of total DNA,

1×standard *Taq* Reaction Buffer, 200 nM of dNTPs, Taq DNA Polymerase (1.25 units, New England BioLabs) and molecular grade water. PCR cycling conditions were as follows: an initial denaturation (95°C for 30 s), followed by 35 cycles of amplification (95°C for 30 s, 55°C for 30 s, and 68°C for 30 s), and a final elongation step (68°C for 5 min). To quantify HIV mRNA (Gag and 3'-LTR) in HIV-infected PBMCs or J-Lat cells, total RNA was extracted from cells using the Quick-RNA MicroPrep Kit (Zymo) according to the manufacturer's instructions. cDNA was synthesized from RNA using the iScript^{TM} Reverse Transcription Supermix for RT-qPCR (Bio-Rad). HIV Gag or 3'-LTR RNA was quantified by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR reactions (20 µl) contained 10 µM primers, 90ng of cDNA, 10 µl iTaq universal SYBR Green supermix (2X) (Bio-Rad) and molecular grade water. PCR cycling conditions were: 95˚C for 3 min, 45 cycles of 95˚C for 5 sec, 60˚C for 30 sec. For each PCR reaction, GAPDH was also quantified and used for normalization. Primer sequences used in PCR (HIV Gag, 3'-LTR and cellular GPAPDH) were shown in **Table S5**. PCR reactions were performed in duplicate. Mean Ct values were used. HIV Gag and 3'-LTR copy numbers were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

Establishment of Ultra-sensitive nested PCR for HIV production in supernatants

Ultrasensitive two-step nested PCR was established using a similar protocol as described (*1*). First, to generate an HIV Gag standard curve, Gag gene was cloned into plasmid. Viral RNA was extracted from HIV-1 and cDNA was synthesized. Gag gene was amplified using the same PCR conditions as described above and the Gag-Out-F/R primers (**Table S5**). Gag PCR products were gel purified using a Zymoclean Gel DNA Recovery Kit (Zymo) and cloned into pGEM-T Easy

Vector Systems (Promega). Cloned plasmids were prepared and the concentrations were calculated as copy/µl by using an online tool (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr). Plasmids were serially diluted ten-fold from 10^6~10^0 copies and subjected to the first round PCR for amplification (16 cycles), followed by a second-round of nested PCR for 40 cycles by using Gag-F/R primers and the CFX Connect Real-Time PCR Detection System (Bio-Rad). Primer sequences for the first-round and second-round PCRs were shown in **Table S5**. A standard curve was generated based on Cq values of the nested PCR and plasmid copies.

CRISPR/Cas9 knockout of BRD4 and BRD2

Guide RNA sequences were cloned into lentiCRISPR v2 vector (a gift from Dr. F. Zhang; Addgene plasmid number 52961). The constructed plasmids or empty lentiCRISPR V2 plasmid together with lentivirus packaging plasmids, psPAX2 (a gift from D. Trono; Addgene plasmid number 12260) and pCMV-VSV-G (a gift from B. Weinberg; Addgene plasmid number 8454), were used to co-transfect 293T cells for production of lentivirus. Packaged lentiviruses were then used to transduce J-Lat full cells. Target gene KO and the KO control (empty vector) J-Lat cells were selected and maintained in complete RPMI 1640 medium supplemented with 10 µg/ml puromycin. To validate the efficacy of CRISPR/Cas9-mediated gene KO, BRD4 and BRD2 protein expression was measured by western blotting in gene-KO and control J-Lat cells.

Western Blotting (WB)

Protein expression in WT or Gene-KO J-Lat cells was measured by WB. Briefly, following experimental treatments, cells were harvested, washed and then lysed in NP-40 cell lysis buffer (Thermo Fisher Scientific) that contains 20 mM Tris-HCl, 0.15mM of NaCl, 0.2mM EDTA, 1%

NP40, protease inhibitor cocktail (Sigma p8340), and 1mM PMSF. Cells were lysed in the lysis buffer by rotation at RT for 1 hour. Cell lysates were centrifuged at 4°C and the supernatants were collected, followed by measurement of total protein concentration using Microplate BCA Protein Assay Kit (Pierce™, Thermo Fisher Scientific). 20 μg proteins were separated using SDS-PAGE gel and then transferred to an Immuno-Blot PVDF Membrane. The membrane was blocked (5% skim milk powder in TBST) and then incubated with individual protein-specific primary antibodies as described in the **Methods** of the paper. Primary antibody incubation was conducted overnight at 4°C. After washing three times in TBST, the membrane was incubated with secondary anti-rabbit or anti-mouse IgG-HRP (Thermo Fisher), depending on the primary antibody used. The membrane was incubated in Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher) and the results were captured. Molecular weights of target proteins were assessed by comparison with molecular weight markers.

Chromatin immunoprecipitation (ChIP)-qPCR

About 15×10^6 J-Lat cells were transferred to 6-well plate and treated accordingly as described in the Main Text. Cells were harvested and washed. Cell pellets were re-suspended in 10ml PBS buffer and fixed with 37% formaldehyde to cross-link DNA and proteins. The fixation reaction was stopped by adding 1.1ml of 10x Glycine for 5 min at RT. Cells were centrifuged and the supernatants were removed, followed by re-suspension of cells in ice-cold Lysis Buffer on ice for 30 min. Cells were centrifuged at 5,000 rpm for 10 min to pellet the nuclei. Nuclei pellets were re-suspended in 700μl shearing buffer plus 3.2μl of PIC and 3.2μl of PMSF, and were then sonicated using the determined conditions. Sheared chromatin samples were used to set up ChIP reactions by adding the magnetic beads and 5 μg of anti-Tat (1mg/ml) (MA1-71509; Thermo

Fisher), Anti-BRD4 (E2A7X) (13440, Cell Signaling) or control mouse IgG (2.5mg/ml) (Cell Signaling) for overnight rotation at 4°C. Following multiple washes with ChIP buffers, the chromatin was reverse cross-linked and treated with proteinase K. The eluted DNA was further purified by phenol-chloroform extraction and used in performance of real time PCR. The primer sequences used in CHIP-qPCR for HIV 5'-LTR and GAPDH promoter are shown in **Table S5**. The results were analyzed by the fold enrichment method with the formula % enrichment = 2− (CT IP − CT mock).

MNase Nucleosomal Mapping

Following treatments, J-Lat cells were cross-linked using the same method as described in the CHIP assay. Cells were then washed with 1ml buffer B (0.25% Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Herpes, pH 7.6) and 1ml buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Herpes, pH 7.6). Following one wash in cold PBS, ~15 x 10^6 cross-linked cells were suspended in 1ml buffer A (300 mM sucrose, 2 mM Mg acetate, 3 mM Cacl2, 10 mM Tris pH 8.0, 0.1% Triton X-100, 0.5 mM DTT), incubated on ice for 5 min, and then dounced with 2ml dounce grinders (Tight Pestle, Kontes) for 20 times. Nuclei were collected by centrifuging at 4°C, 750 x g for 5 min. Pellets were washed twice in 1ml buffer D (25% glycerol, 5 mM Mg acetate, 50 mM Tris pH 8.0, 0.1 mM EDTA, 5 mMDTT) at 15 x 10^6 nuclei/ml. Chromatin was collected by centrifuging at 4°C, 750xg for 5 min. The pellets were suspended in 1ml buffer MN (60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 0.25 mM sucrose, 1.0 mM $CaCl_2$) at 1.5 x 10^7 nuclei/ml. 150µl aliquots (2.25 x 10^6 nuclei) were treated with 0, 0.5, 5, 20, 50, or 500 U/ml of MNase (USB) for 30 min at 37°C. Reactions were stopped by addition of EDTA (12.5 mM) and SDS (0.5%). After 4 hours of proteinase K digestion at 37°C, each

reaction was processed similar to that in ChIP assay from the point of DNA elution. After measuring concentrations, digested and undigested DNA samples were diluted to the same concentration (5 ng/µl) and used for real-time qPCR analysis. Fold change was calculated using delta CT method and the ratio of the amount of digested DNA to the undigested DNA for each primer was calculated.

SUPPLEMENTAL FIGURES

Figure S1

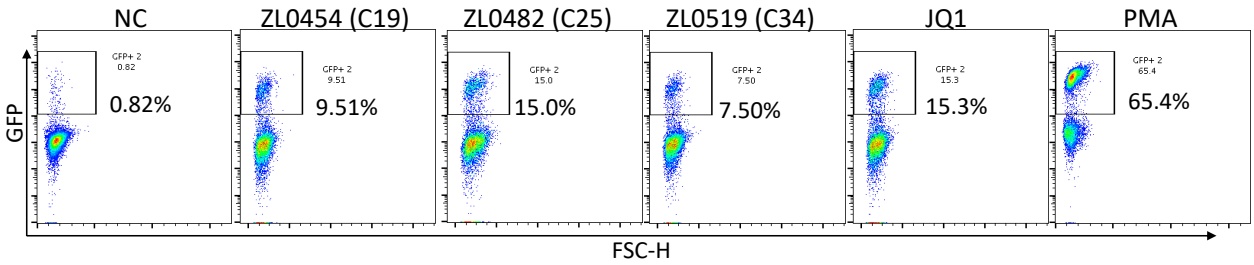


Figure S1. Ability of compounds to activate latent HIV in J-Lat cells. J-Lat cells (full-length; 10.6) were treated with individual compounds (C1-C62) (10 μM) for 24 hours. Cells treated with DMSO (NC), JQ1 (10 μM) or PMA (100ng/ml) were included as controls. HIV activation was expressed as % GFP+ in J-Lat cells, which was measured by flow cytometry. Among the 62 compounds, ZL0454 (C19), ZL0482 (C25) and ZL0519 (C34) were identified to modestly activate latent HIV in this model. Results of these three compounds, together with NC, JQ1 and PMA, for activation of GFP expression in J-Lat cells were shown.

Figure S2

		Cq (3'-LTR)		Cq (GAPDH)	
Days	**Treatment**	**Replicate 1**	**Replicate 2**	**Replicate 1**	**Replicate 2**
2	**NC**	27.04	26.46	18.08	18.02
	ZL0580	26.19	25.23	17.57	17.73
	JQ1	21.69	21.18	17.56	17.88
7	**NC**	28.38	27.94	19.57	19.26
	ZL0580	31.08	31.92	19.57	19.26
	JQ1	24.08	24.10	19.79	19.52
14	**NC**	26.67	26.40	18.34	18.13
	ZL0580	31.12	31.23	18.55	18.69
	JQ1	25.40	25.42	18.92	19.08
No cDNA-Template Control		0	0	0	0

Figure S2. qPCR raw Cq values for HIV 3'-LTR and cellular GAPDH RNAs in unstimulated/resting J-Lat cells. J-Lat cells were treated with NC, ZL0580 and JQ1 as

described in Figure 1G of main text. qPCR for HIV 3'-LTR and cellular GAPDH RNAs was performed on Day 2, 7 and Day 14 following treatment. The raw data indicates that basal HIV transcription can be readily and reliably detectable in un-stimulated J-Lat cells.

Figure S3.

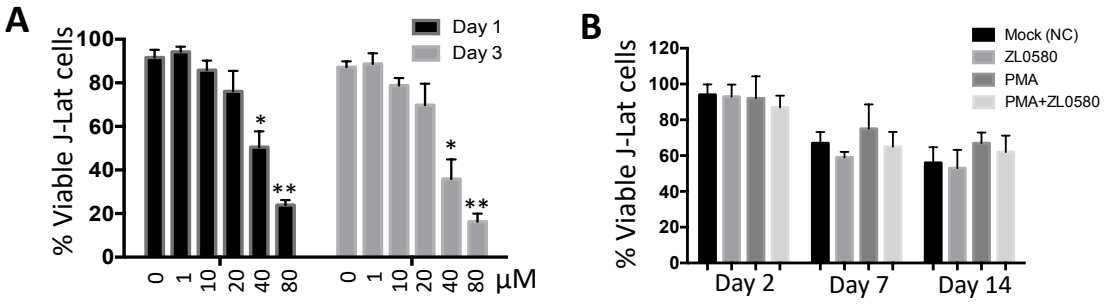


Figure S3. J-Lat cell viability after ZL0580 treatments. (A) Viability of PMA-activated J-Lat cells after treatment with various concentrations of ZL0580 (0-80µM). Cells were stimulated with PMA and treated with ZL0580 at indicated concentrations. Day 1 and Day 3 after treatments, cells viability was measured by flow cytometry based on Aqua blue staining. **(B)** Viability of un-stimulated and PMA-activated J-Lat cells after ZL0580 treatment (10µM). Cells were mock treated (NC), or treated with ZL0580 alone (10µM), PMA alone, or PMA+ZL0580 (10µM). Cell viability was measured on Day 2, Day 7 and Day 14 after treatments. * $p<0.05$; ** $p<0.005$ (comparison with 0µM).

Figure S4.

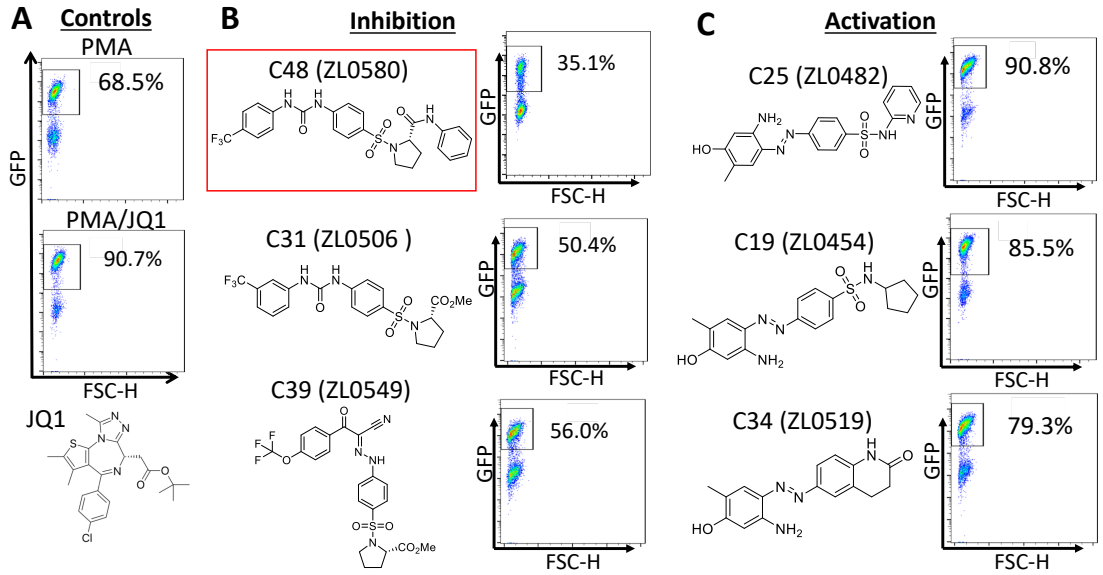


Figure S4. **Specificity of ZL0580 in suppressing HIV in J-Lat: a preliminary structure-activity relationship.** (**A**) Structure of JQ1 and its synergistic effect on enhancing PMA-stimulated HIV activation were shown as control. (**B**) In addition to ZL0580, another two compounds (ZL0506 & ZL0549) were identified to suppress HIV but at weaker levels. **(C)** Three other compounds in this library (ZL0482, ZL0454, & ZL0519) manifest an effect similar to JQ1 and enhance PMA-induced HIV activation. The chemical structures of these compounds and their regulatory effects on PMA-stimulated HIV activation were shown in (**B** & **C**).

Figure S5

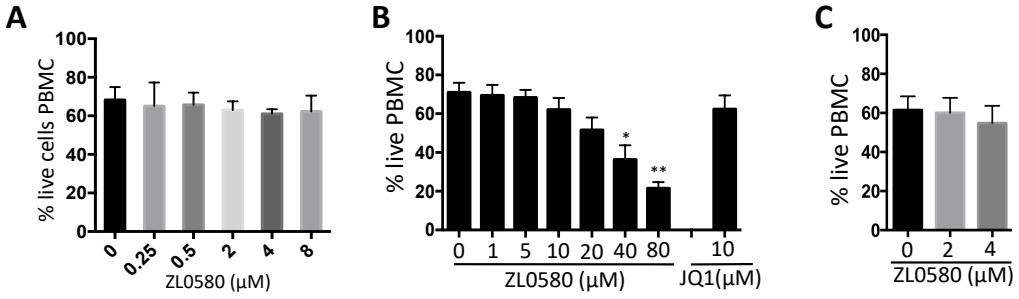


Figure S5. Cellular toxicity of ZL0580 on activated or resting human PBMCs. (**A-B**) PBMCs (n=3) were activated with PHA for 2 days and treated with low-range (0-8 μM) (A) or high-range (0-80 μM) concentrations of ZL0580. JQ (10 μM) treatment was included as a control (B). 3 days after treatment, cells were stained with aqua blue and cell viability was measured by flow cytometry. (C) Cell viability of resting PBMCs (n=3) that were presented in Fig. 2E-F. Resting PBMCs (infected with HIV for 24 hours) were treated with ZL0580 (0, 2, 4 μM). Cell viability was measured by flow cytometry as described above on Day 6 after treatment (on the day when intracellular p24 was examined by FACS in Fig. 2E-F). * $p<0.05$; ** $p<0.005$.

Figure S6

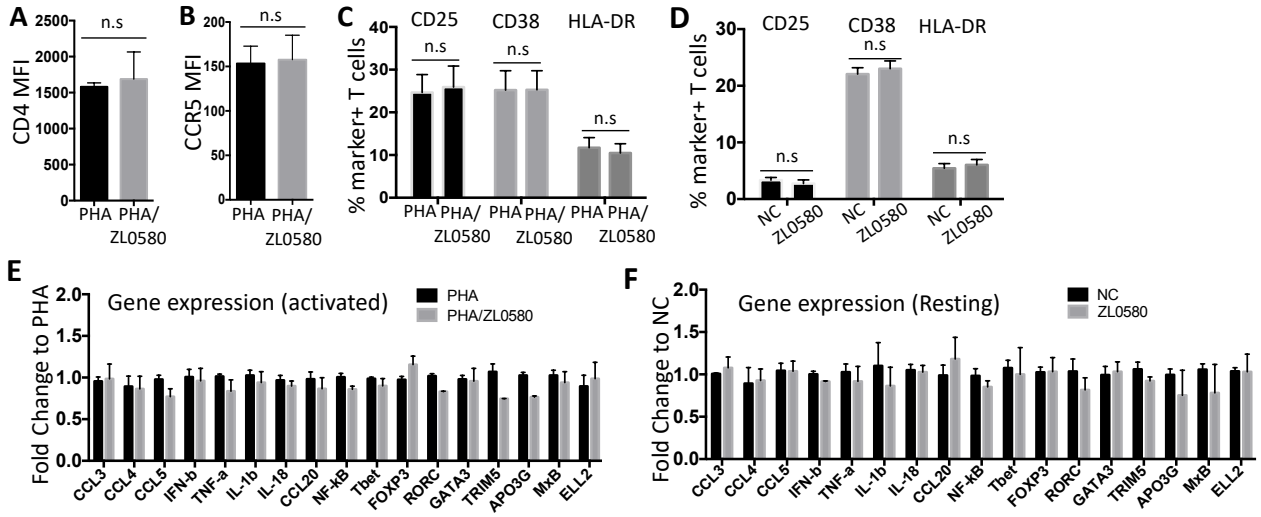


Figure S6. Effects of ZL0580 on T cell phenotypes and gene expression of PBMCs.

(A-C) Normal human PBMCs (n=3) were activated with PHA for 2 days and treated with ZL0580 (8μM) (PHA/ZL0580) or not (PHA alone) for 3 days (as described in Figure 2A). Surface expression of CD4 (A), CCR5 (B), and activation markers (CD25, CD38 and HLA-DR) (C) on activated T cells were examined by flow cytometry and compared between PHA and PHA/ZL0580. **(D)** Effect of ZL0580 on T-cell activation markers in un-stimulated PBMCs.

PBMCs (n=3) (without PHA activation) were treated with ZL0580 (8μM) or not (NC) for 3 days. Expression of activation markers (CD25, CD38 and HLA-DR) on T cells was measured and compared between NC and ZL0580. Error bars represent standard deviations of the 3 PBMC donors. **(E-F)** Impact of ZL0580 treatment on gene-expression profile in PHA-activated (E) or resting (F) PBMCs. PHA-activated or un-stimulated human PBMCs (n=2) were treated with ZL0580 (8μM) or NC as described above. Two days after treatment, cellular RNAs were extracted and the expression of a wide range of genes associated with T-cell phenotype, lineage, differentiation and functions were measured by quantitative PCR. The data were compared between PHA and PHA/ZL0580 for activated PBMCs (E) or between NC and ZL0580 for un-stimulated PBMCs (F). Error bars represent standard deviations of PCR replicate. n.s. non-significant.

Figure S7

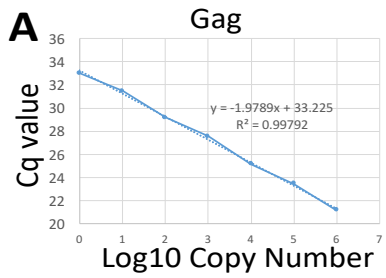


Figure S7. Generation of standard curve for quantification of HIV RNA copies by nested PCR. To generate Gag-expressing plasmid, HIV RNA was extracted from the virus and cDNA was synthesized. Gag gene was amplified, gel purified and cloned into pGEM®-T Easy Vector Systems. The standard plasmid was 10 times diluted from 10^6~10^0 and subjected to the first round PCR of 16 cycles of amplification with the primers Gag-out-F/R (**Table S5**), followed by

the second round ultra-sensitive nested PCR for 40 cycles using the primers Gag-F/R (**Table S5**). The standard curve was generated according to the Cq values of the nested real-time PCR.

Figure S8

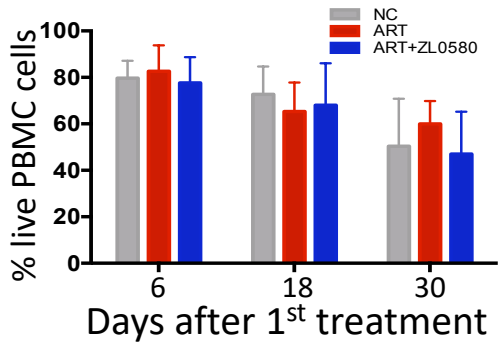


Figure S8. Cell viability of RV254 PBMCs at various days after treatments. PBMC cell viability was determined based on Aqua Blue staining and flow cytometric analysis as described earlier.

Figure S9

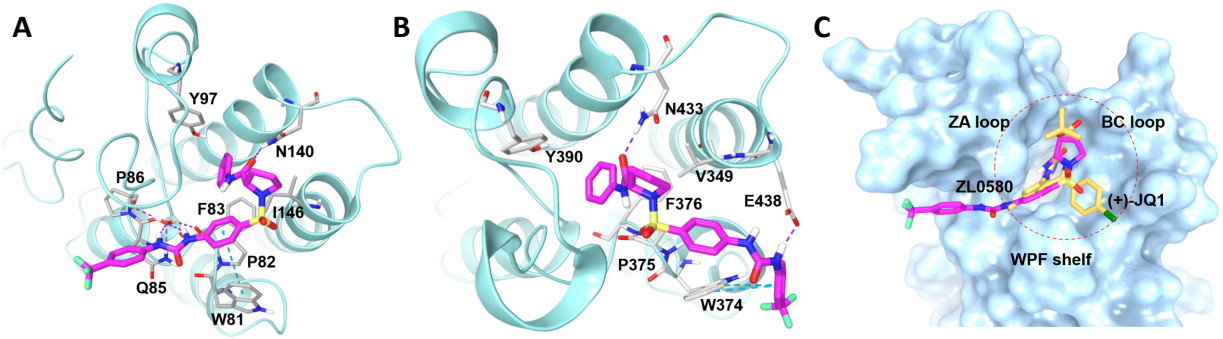


Figure S9. Docking analysis of ZL0580 binding to BD1 and BD2 domains of BRD4.

(A) Docking pose of ZL0580 with BRD4 (BD1) (PDB code: 3MXF) in ribbon representation. ZL0580 is shown in magenta sticks, key residues of N140, Y97, I146, W81, P82, F83, Q85 and P86 in gray sticks and conserved water in red sticks. H-bond is shown in purple dotted lines and

π stacking in blue dotted line. demonstrates ZL0580 can well access the conserved KAc binding pocket of BRD4 (BD1). The amide ketone of ZL0580 forms H-bond with the critical conserved residue Asn140. One terminal phenyl ring interacts with Y97 of ZA loop, and the proline fragment interacts with the gatekeeper I146 through hydrophobic interaction. Both of NH on the urea linker interact with Q85 and P86 with water-mediated H-bonds. The middle phenyl ring forms a π stacking with W81. The phenyl urea sulfonamide fragment extends to the region between WPF shelf and ZA channel, suggesting ZL0580 is further stabilized by hydrophobic interactions with this region of binding site. **(B)** Docking pose of ZL0580 with BRD4(BD2) (PDB code: 4Z93) in ribbon representation. ZL0580 is shown in magenta sticks, key residues of N433, Y390, V349, W374, P375, F376, and E438 in gray sticks. H-bond is shown in purple dotted lines and π stacking in blue dotted line. It shows ZL0580 can access the conserved KAc binding pocket of BRD4 (BD2) in a pose substantially different from that binds at BRD4(BD1). ZL0580 can still form a H-bond with the conserved residue Asn433 (Asn 140 in BRD4(BD1)). However, one NH on the urea linker forms a H-bond with E438, making the phenylurea sulfonamide fragment swinging to the other side of WPF shelf and shifted away from ZA loop, resulting in less interaction with the conserved Y390 (Y97 in BRD4(BD1)) of ZA loop and hydrophobic residues of WPF shelf overall. Such difference on the binding poses may lead to the selectivity of ZL0580 towards BRD4(BD1). **(C)** Surface representation of ZL0580 (magenta) docked pose superimposed with BRD4 (BD1)/(+)-JQ1 complex structure and overlay analysis comparison (PDB code: 3MXF). It depicts ZL0580 can well access into the KAc binding pocket of BRD4(BD1) with partial scaffold extending to an additional region. The proline sulfonamide fragment of ZL0580 can overlap nicely with the crystallographically determined JQ1 binding

mode (circled area), while the phenylurea sulfonamide moiety extends to the region between WPF shelf and ZA channel, which further stabilizes ligand-target interactions.

Figure S10

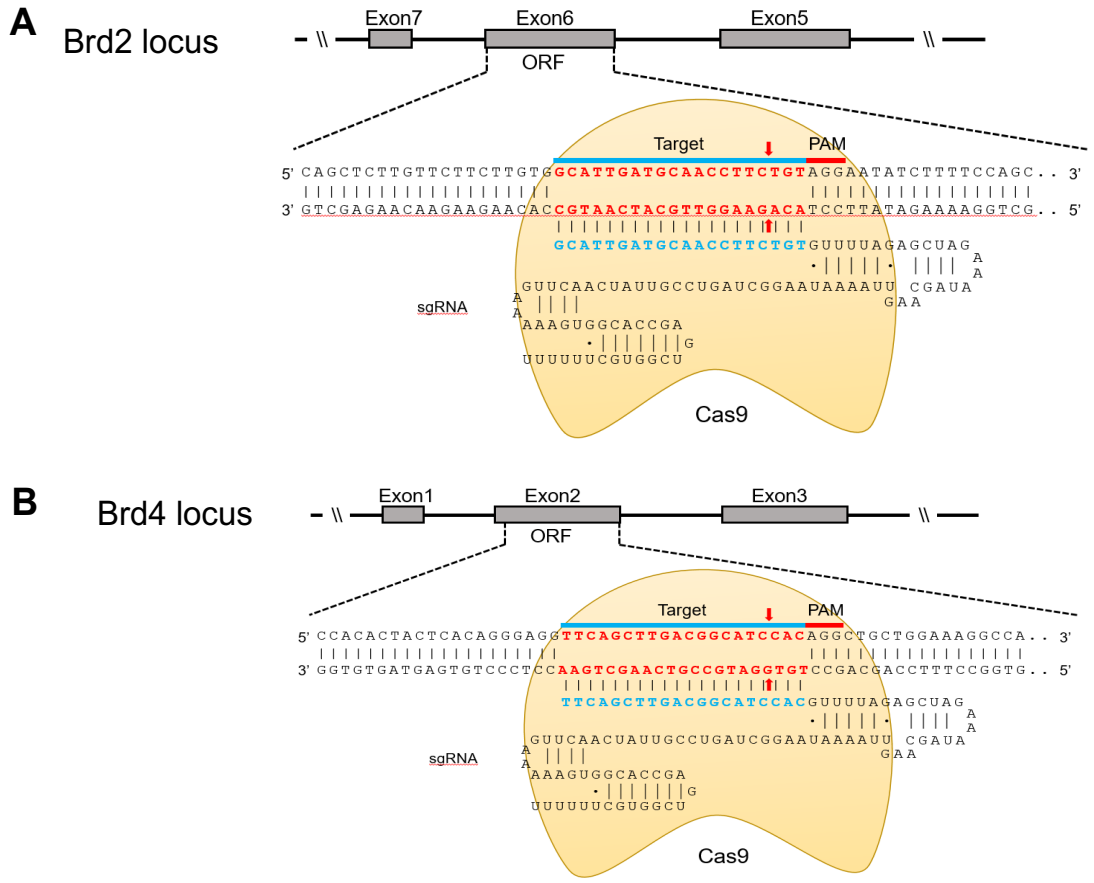


Figure S10. Schematic illustration of CRISPR/Cas9 for generation of BRD2-KO **(A)** or BRD4-KO **(B)** J-Lat cells.

Figure S11

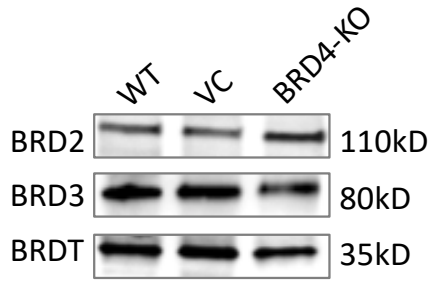


Figure S11. Expression of other BET proteins in BRD4-KO J-Lat cells. Expression of other BET proteins (BRD2, BRD3, BRDT) in WT, VC and BRD4-KO J-Lat cells was measured by WB. Protein sizes are indicated.

Figure S12

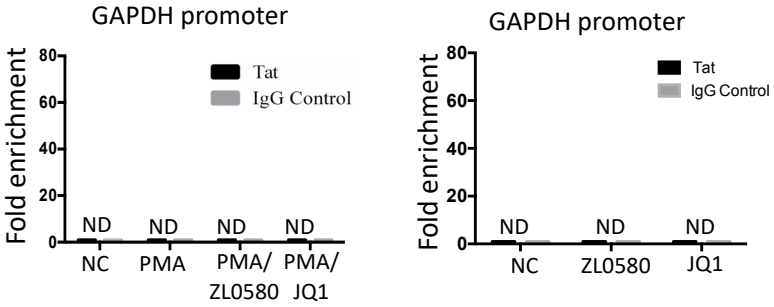


Figure S12. CHIP-qPCR analysis for binding of Tat to GAPDH promoter region. PMA-activated (left) or resting (right) J-Lat cells were treated as indicated. 24 hours after treatment, cells were subjected to CHIP-qPCR analysis using Tat or control IgG antibody to detect the specific binding Tat to GAPDH promoter region. Data were normalized to control IgG as well as to NC and expressed as fold enrichment. ND represents “non-detectable”.

SUPPLEMENTAL TABLES

Table S1. Displacement of radioligand binding by compound **ZL0580** (10 μM) in a broad panel of receptors and transporters [National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (PDSP)]. Receptor binding profiles were generously provided by NIMH PDSP, Contract # HHSN-271-2013-00017-C.

Receptor / transporter	Radioligand	% inhibition (10 μM) [A]	K_i (μM) [B]
5-HT_{1A}	[^{3}H]-8-OH-DPAT	4.1	>10
5-HT_{1B}	[^{3}H]-GR125743	50.2	>10
5-HT_{1D}	[^{3}H]-GR125743	6.1	>10
5-HT_{1E}	[^{3}H]-5-HT	5.2	>10
5-HT_{2A}	[^{3}H]-Ketanserin	37.6	>10
5-HT_{2B}	[^{3}H]-LSD	42.9	>10
5-HT_{2C}	[^{3}H]-Mesulergine	1.5	>10
5-HT_3	[^{3}H]-LY278584	0.8	>10
5-HT_{5A}	[^{3}H]-LSD	15.2	>10
5-HT_6	[^{3}H]-LSD	-3.1	>10
5-HT_7	[^{3}H]-LSD	-17.8	>10
D_1	[^{3}H]-SCH23390	10.4	>10
D_2	[^{3}H]-*N*-Methylspiperone	31.9	>10
D_3	[^{3}H]-*N*-Methylspiperone	-27.2	>10
D_4	[^{3}H]-*N*-Methylspiperone	19.9	>10
D_5	[^{3}H]-SCH23390	19.5	>10
DAT	[^{3}H]-WIN35428	-14.3	>10
SERT	[^{3}H]-Citalopram	25.4	>10
NET	[^{3}H]-Nisoxetine	4.0	>10
α_{1A}	[^{3}H]-Prazosin	-15.3	>10
α_{1B}	[^{3}H]-Prazosin	-7.7	>10
α_{1D}	[^{3}H]-Prazosin	-9.8	>10
α_{2A}	[^{3}H]-Rauwolscine	1.3	>10
α_{2B}	[^{3}H]-Rauwolscine	5.3	>10
α_{2C}	[^{3}H]-Rauwolscine	2.5	>10
β_1	[^{125}I]-Pindolol	22.7	>10
β_2	[^{3}H]-CGP12177	6.0	>10
β_3	[^{3}H]-CGP12177	33.3	>10
δOR	[^{3}H]-DADLE	15.8	>10
κOR	[^{3}H]-U69593	29.9	>10
μOR	[^{3}H]-DAMGO	30.2	>10
$GABA_A$	[^{3}H]-Muscimol	-19.2	>10

H_1	[^{3}H]-Pyrilamine	NT	NT
H_2	[^{3}H]-Tiotidine	12.2	>10
H_3	[^{3}H]-α-methylhistamine	NT	NT
M_1	[^{3}H]-QNB	40.1	>10
M_2	[^{3}H]-QNB	34.0	>10
M_3	[^{3}H]-QNB	7.4	>10
M_4	[^{3}H]-QNB	13.5	>10
M_5	[^{3}H]-QNB	2.9	>10
σ_1	[^{3}H]-Pentazocine (+)	21.4	>10
σ_2	[^{3}H]-DTG	17.1	>10

[A.] Data represent mean % inhibition (n = 4) for compound **ZL0580** assessed for displacement of binding at targets. Inhibition of binding >50% at 10 μM of compound **ZL0580** resulted in determination of K_i (see [B]).

[B.] Data represent K_i (μM) values obtained from non-linear regression of radioligand competition isotherms. K_i values are calculated from best fit IC_{50} values using the Cheng-Prusoff equation. NT = not tested.

Table S2. Characteristics of progressive, viremic HIV-infected subjects in RV21 (No ART)

Participant	ART	Status	CD4%	CD4 Count
1	No	Viremic	20	223
2	No	Viremic	30	561
3	No	Viremic	27	600
4	No	Viremic	35	636
5	No	Viremic	38	869
6	No	Viremic	15	189
7	No	Viremic	14	157
8	No	Viremic	47	564

Table S3. Characteristics of ART-suppressed, aviremic HIV-infected subjects in RV254

Participant	Treatment Regimen	ART Duration	Plasma VL [C]	CD4 Count
1	HAART [A]	6 months	50	644
2	HAART	6 months	50	830
3	Mega HAART [B]	6 months	50	571
4	Mega HAART	6 months	50	540
5	Mega HAART	6 months	50	671
6	HAART	6 months	50	890
7	HAART	6 months	50	568
8	Mega HAART	6 months	50	961
9	HAART	6 months	50	794
10	Mega HAART	6 months	50	853
11	HAART	6 months	50	513

A. HAART: TDF/FTC/EFV

B. Mega HAART: TDF/FTC/EFV/RAL/MVC

C. Post ART (6-months) viral load: limit of detection (50 copies/ml)

Table S4. Target genes and primer sequences for PCR quantification of cellular gene expression in normal PBMCs

Gene	Sequence (5'-3')
CCL3	F: 5'-TGCTCAGAATCATGCAGGTC-3' R: 5'-TGATGCAGAGAACTGGTTGC-3'
CCL4	F: 5'-CTTCCTCGCAACTTTGTGGT-3' R: 5'-GCTTGCTTCTTTTGGTTTGG-3'
IFN-β	F: 5'-TTCACCAGGGGAAAACTCAT-3' R: 5'-TCCTTGGCCTTCAGGTAATG-3'
IL-1β	F: 5'-TCCAGGGACAGGATATGGAG-3' R: 5'-TCTTTCAACACGCAGGACAG-3'
TNF-α	F: 5'-CCTGTGAGGAGGACGAACAT-3' R: 5'-GGTTGAGGGTGTCTGAAGGA-3'
IL-18	F: 5'-TTGTCTCCCAGTGCATTTTG-3' R: 5'-GAAGCGATCTGGAAGGTCTG-3'
CCL20	F: 5'-GCGCAAATCCAAAACAGACT-3' R: 5'-TGGGCTATGTCCAATTCCAT-3'
CCL25	F: 5'-CAATCCCATCAGCAGCAGTA-3' R: 5'-TGTAGGGCGACGGTTTTATC-3'
TRIM5	F: 5'-CTGGCATCCTGGGCTCTCAAAGT-3' R: 5'-CATACCCCCAGGATCCAAGCAGTT-3'
APOBEC3G	F: 5'-GGCTCCACA TAAACACGGTTTC-3' R: 5'-AAGGGAATCACGTCCAGGAA-3'
MxB	F: 5'-GCACAGTGATGAGCAAGCAGTAA-3' R: 5'-TCCTATTTTGGCAGATTCTGCTG-3'
SAMHD1	F: 5'-TTTTGGGATTCCGTTTGTGT-3' R: 5'-TCACTGAAAGTTGCCAAGAAAA-3′
NF-kB	F: 5'-CAGTGGTGCCTCACTGCTAA-3' R: 5'-GGACAACGCAGTGGAATTTT-3'
T-bet	F: 5'-CCCTTGGTGTGGACTGAGAT-3' R: 5'-TCACCACTGGAAGGATAGGG-3'
ELL2	F: 5'-GGGGGATCCGCCATGGCGGCGGGGGGGACA-3' R: 5'-GGGACCGGTCTAGGACCATGACTCTGCTTGCT-3'
SIAH1	F: 5'-TGT TTG TAG CAA CTG TCG CC-3' R: 5'-AGC CAC TTT CTC CAT AGC CA-3'
GAPDH	F: 5'-CAATGACCCCTTCATTGACC-3' R: 5'-GACAAGCTTCCCGTTCTCAG-3'

Table S5. Primer sequences for Q-PCR quantification of HIV DNA/RNA and CHIP-qPCR analysis

Primers	Sequence
Gag-F	5′-GGAAGCTGCAGAATGGGATA-3′
Gag-R	5′-GCTATGTCACTTCCCCTTGG-3′
Gag-out-F	5′-GAGCCACCCCACAAGATTTA-3′
Gag-out-R	5′-AGGGTTCCTTTGGTCCTTGT-3′
3’LTR-F	5′-CAGATGCTGCATATAAGCAGCTG-3′
3’LTR-R	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTGAAG-3′
CHIP promotor region	
5' LTR-F	5′-GTTAGACCAGATCTGAGCCT-3′
5' LTR-R	5′-GTGGGTTCCCTAGTTAGCCA-3′
GAPDH-CHIP-F	5′-CCACATCGCTCAGACACCAT-3′
GAPDH-CHIP-R	5′-CCCGCAAGGCTCGTAGAC-3′

Table S6. Guide RNA sequences for BRD4 and BRD2 CRISPR/Cas9 knockout

Primers	Sequence
BRD2-gRNA	5′-GCATTGATGCAACCTTCTGTAGG-3′
BRD4-gRNA	5′-TTCAGCTTGACGGCATCCACAGG-3′

Table S7. Primer sequences for high-resolution MNase mapping

F-1-5'GATCtGTGGATCTACCACAC3'
R-1-5'GCACCATCCAAAGGTCAGTGG3'
F-2-5'CCTGATTGGCAGAACTACACAC3'
R-2-5'TCTACTTGCTCTGGTTCAACTGG3'
F-3-5'CCTTTGGATGGTGCTTCAAGTTAG3'
R-3-5'ATGCTGGCTCATAGGGTGTAAC3'
F-4-5'TAAGGAAGAGAAAGAACAGGCTTG3'
R-4-5'GAAATGCTAGGAGGCTGTCA3'
F-5-5'GAGCCAGCATGGGATGG3'
R-5-5'CTCCGGATGCAGCTCTC3'
F-6-5'TGACAGCCTCCTAGCATTTC3'
R-6-5'CACACCTCCCTGGAAAGTC3'
F-7-5'CACATGGCCCGAGAGCTG3'
R-7-5'CCCAGGCCACACCTCCCTGG3'
F-8-5'TACTACAAAGACTGCTGACATCG3'
R-8-5'TCTGAGGGCTCGCCACTC3'
F-9-5'GGGACTTTCCGCTGGGGAC3'
R-9-5'CCCAGTACAGGCAAAAAGCAGC3'
F-10-5'GGTGTGGCCTGGGCGGGA3'
R-10-5'GTTCCCTAGTTAGCCAGAGAGC3'
F-11-5'AGTGGCGAGCCCTCAGATG3'
R-11-5'AGCAGTGGGTTCCCTAGTTAGC3'
F-12-5'TTTGCCTGTACTGGGTCTCTCTGG3'
R-12-5'CACAACAGACGGGCACACACT3'
F-13-5'GCTCTCTGGCTAACTAGGGAAC3'
R-13-5'AGACGGGCACACACTACTTTG3'
F-14-5'AGCTCTCTGGCTAACTAGGG3'
R-14-5'AAAGGGTCTGAGGGATCTCTAG3'
F-15-5'TCTCTGGCTAACTAGGGAACC3'
R-15-5'AAAGGGTCTGAGGGATCTCTAG3'
F-16-5'AGTGTGTGCCCGTCTGTTGTG3'
R-16-5'CTTTCGCTTTCAAGTCCCTGTTCG3'
F-17-5'GGTAACTAGAGATCCCTCAGAC3'
R-17-5'CTTCAGCAAGCCGAGTCC3'
F-18-5'GTGTGGAAAATCTCTAGCAGTG3'
R-18-5'CTTCAGCAAGCCGAGTCC3'
F-19-5'GCGGAGGCTAGAAGGAGAGAG3'
R-19-5'GCTCCCTGCTTGCCCATAC3'
F-20-5'AGAGATGGGTGCGAGAGC3'
R-20-5'ATTAACTGCGAATCGTTCTAGC3'