Hydrophobic Cavity Directed Azide-Acetyllysine Photochemistry for Profiling Non-Histone Interacting Partners of Bromodomain Protein 1

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1. General materials, methods, and equipment

Chemicals: All chemicals used were purchased from established vendors (e.g., Sigma-Aldrich, Acros Organics) and used without further purification. HPLC Grade Acetonitrile was purchased from Fisher Chemical and used for HPLC purification of peptides. Synthesis and purification of azido phenylalanine was performed as previously described.[1] Preparative HPLC was performed using an Agilent 1220 Infinity HPLC with diode array detector. MALDI mass spectra were collected at ultraFlextreme (Bruker) and the data was analyzed using flexAnalysis software. The ESI-MS were recorded on a Q-ExactiveTM Thermo Scientific LC-MS with electron spray ionization (ESI) probe.

Plasmids, mutagenic primers, cell lines and antibodies: All the plasmids for bacterial expression are obtained as gifts from individual laboratories or purchased from Addgene. Mutagenic primers are obtained from Integrated DNA Technologies. Competent bacterial cells used for protein expression and mutagenesis are mentioned in section 3. Human embryonic kidney 293T (HEK293T) cells are obtained from the American Type Culture Collection (ATCC) and used in the current study following manufacturer's protocol. All the antibodies used in the current study are purchased from established vendors and used following manufacturer's protocol unless otherwise noted.

2. Peptide synthesis and purification

Peptide **3** was synthesized by the University of Pittsburgh Peptide Synthesis Facility; Crude peptides were purified using preparative reversed-phase HPLC (XBridge C18, 5 μm, 10 x 250 mm column) eluting with a flow rate of 4.00 mL/min and a gradient of acetonitrile starting from 0% v/v to 50% v/v in 12 min and then to 100% v/v by 18 min in aqueous trifluoroacetic acid (0.1% v/v). Purified peptides were concentrated by SpeedVac concentrator then lyophilized. Dried peptides were resuspended in water and stored at -80 °C before use. Concentrations of peptides with no aromatic amino acids were determined based on the observation that 1 mg/ml peptide generates an absorbance value (A205) of 30 at 205 nm. Concentrations of peptides with aromatic amino acids were determined based on the absorbance and extinction coefficient for the aromatic amino acid at lambda max, using molar ratio of amino acid to peptide to extrapolate. The integrity of the purified peptides was confirmed by MALDI mass spectrometry.

Peptides **2**, **4, 6** and **7** were synthesized on solid support NovaPEG Rink Amide Resin (Sigma Aldrich #8550470005) in 5 mL fritted syringes. Amino acids were purchased from Sigma and used without further purification (Fmoc-Ala-OH #852003, Fmoc-Arg(Pbf)-OH #852067, Fmoc-Asp(Otbu)-OH #852005, Fmoc-Glu(Otbu)-OH #852009, Fmoc-Gly-OH #852001, Fmoc-Ile-OH # 852010, Fmoc-Leu-OH #852011, Fmoc-Lys(Boc)-OH #852012, Fmoc-Lys(Ac)-OH #852042, Fmoc-Phe-OH #852016, Fmoc-Pro-OH #852017, Fmoc-Ser(Tbu)-OH #852019, Fmoc-Thr(tBu)-OH #852000, Fmoc-Val-OH #852021). Resin was swelled for a minimum of 20 minutes with N,N-Dimethylformamide (DMF) (Acros #348430025) prior to addition of C-terminal amino acid. Peptides were prepared at a 0.05 mmol scale. Four equivalents of amino acid, four equivalents of O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) (Sigma #8.51012), and six equivalents of N,N-Diisopropylethylamine (Sigma #496219) were first dissolved in 1-Methyl-2-pyrrolidinone (Sigma #443778) via sonication with an Ultrasonic Cleaner water bath (VWR #97043-968). The amino acid mixture was added to the resin and stirred under atmospheric conditions at 250rpm for 45-60 minutes. Resin was then washed with DMF three times prior to addition of 20% 4-methylpiperidine in DMF (Sigma #M73206). Deprotection solution was mixed with resin for 10 minutes at 250 rpm two times. Resin was then washed with DMF three times, and the next amino acid was added. Once the N-terminal amino acid was deprotected, the resin was washed three times each with DMF and dichloromethane (DCM) (Acros #348465000). The syringe and resin were dried in HighVac dessicator for at least 30 minutes before proceeding. Cleavage cocktail containing 91.5% TFA (Alfa Aesar L063374), 2.5% thioanisole (Acros T28002), 2.5% phenol (Alfa Aesar #J64011), 2.5% deionized water, and 1% triisopropylsilane (Acros Organics # 214520100) was added to the resin. The sealed fritted syringe was rocked for 2-4 hours at room temperature using a fixed angle rocker (Fisher 07202202). After cleavage, the solvent was ejected from the syringe into a 50mL Falcon tube, leaving behind the resin in the syringe. The solution was condensed to remove roughly 50% TFA prior to precipitation via -80°C diethyl ether (Acros #326860010). Precipitate was then pelleted via centrifugation (Sorvall Legend XTR) at 3000 rpm for 5 mins. Supernatant was decanted. Crude peptide was then resuspended in 0.1% TFA prior to purification and characterization as described for peptides from the peptide facility. The integrity of the purified peptides was confirmed by MALDI mass spectrometry.

3. Mutagenesis, expression, and purification of Proteins

The N-terminal 6xHis-tagged human BRD1 Bromodomain bacterial expression construct PNIC28-Bsa4 was obtained from Addgene (39095). Wild type plasmids were transformed into *E. coli* BL21 (DE3) Star competent cells (Invitrogen). A single colony was added to 10 mL Luria-Bertani (LB) Miller broth with 50 μg/mL kanamycin sulfate and grown overnight at 37°C. Overnight cultures were diluted 100-fold the following morning and allowed to grow at 37°C to an optical density (OD600) of 0.8, at which time 0.6 mM IPTG was added to induce protein expression overnight at 17 °C with in an Innova 44® Incubator shaker (New Brunswick Scientific). Protein purification was performed as follows: Cells pelleted by centrifugation for 20 minutes at 4000 rpm, and supernatant was discarded. Cell pellets were resuspended in 15 mL Lysis Buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% v/v glycerol, 25 mM imidazole, 1 mg/mL Lysozyme, 10μg/mL or 20U/mL DNase, and Roche protease inhibitor cocktail) and lysed by pulsed sonication (Qsonica-Q700, Fisherbrand Replaceable Microtip 1/8" Probe, pulsed 10 seconds on, 10 seconds off, for 5 minutes processing time, 60Amps; repeated sonication a second time if solution was still opaque and viscous) at 13000 rpm, 19632.4 x g for 40 min at 4 °C. Soluble extracts were added to an equilibrated column containing Ni-NTA agarose resin (Thermo) according to manufacturer's instructions. Bound protein was washed with 20 volumes of Wash Buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% v/v glycerol, and 25 mM imidazole). Proteins were then eluted with Elution Buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% v/v glycerol, and 400 mM imidazole). Eluted proteins were then further purified by size exclusion chromatography (Superdex-75 or Superdex-200) using AKTA pure FPLC system (GE healthcare) with FPLC buffer (50 mM HEPES pH 7.5 and 150 mM NaCl). Purified protein was concentrated using Amicon Ultra-10k centrifugal filter device (Merck Millipore Ltd.), at which time protein concentration was determined using Bradford assay kit (BioRad Laboratories) with BSA as a standard. Concentrated proteins were stored at -80°C before use.

TAG variants were generated using QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The resulting mutant plasmids were confirmed by DNA sequencing. To express variants, BL21 Star (DE3) cells were co-transformed with pEVOL-based *M. jannaschii* TyrRS-tRNACUATyr pair for AzF (addgene ID: 31186).[1-2] Cells were recovered for 1-2 hours in 200 μL

SOC medium in a 37°C shaker prior to plating on an LB Miller agar plate containing 50 μg/mL kanamycin sulfate and 35 μg/mL chloramphenicol. Single colonies were picked and added to each of four inoculates containing 10 mL of Luria-Bertani (LB) Miller broth in presence of appropriate antibiotics (35 μg/mL chloramphenicol and 50 μg/mL kanamycin sulfate. Overnight cultures were centrifuged (ThermoScientific Sorvall Legend XTR Centrifuge, TX-1000 rotor, 4°C) for 10 min at 1000 x g, 2100 rpm. Supernatant was removed, and cell pellets were resuspended in ~1mL M9 media and used to inoculate 1L of GMML medium (M9 minimal media supplemented with 1% v/v glycerol, 300 μM leucine, 1 mM $MgSO_4$, 0.1 mM CaCl2, 50 μg/mL kanamycin sulfate, and 35 μg/mL chloramphenicol, and trace amounts of Na_2MoO_4, $CoCl_2$, $CuSO_4$, $MnSO_4$, $MgSO_4$, $FeCl_2$, $CaCl_2$, and H_3BO_3). Cells were allowed to grow at 37 °C to an optical density (OD600) of 0.8. AzF was prepared by diluting in 20mL sterilized deionized water and added aseptically to a final concentration of 1mM. Cells were allowed to shake an additional 30 minutes at 17 °C, at which time the synthetase expression was induced with 0.05% w/v arabinose and allowed to shake an additional 30 minutes at 17 °C. Finally, 0.6 mM IPTG was added to induce BET protein expression while shaking overnight at 17 °C with an Innova 44® Incubator shaker (New Brunswick Scientific). Protein purification, concentration, and storage were performed as previously described.

Expression and chemical acetylation of cysteinylated histone H4 was carried out as previously described.[1, 3]

4. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed with an ITC_{200} instrument (MicroCal, Malvern) at 15 °C while stirring at 750 rpm. Buffers were matched to 50mM HEPES pH 7.5 and 150 mM NaCl. Each titration was performed as follows: one initial injection of 0.4 μL for 0.8 seconds, followed by 19 injections of 2.0 μL for 4 seconds, with ≥2 mins between each injection. The initial injection was discarded prior to data analysis. The microsyringe (40μL) containing 2-5mM peptide was injected into the cell (200μL) containing 100 to 200 μM BRD1 or variant. Data was fitted to a single binding site model using Microcal ITC_{200} Software with Origin Lab 7 to yield enthalpies of binding (ΔH), entropies of binding (ΔS), and binding constants (Ka). Further thermodynamic parameters (changes in free energy ΔG, and dissociation constants (K_d)) were calculated from these values. The thermodynamic parameters are provided in Table S2.

5. Photo-crosslinking experiment with peptides and in-gel fluorescence

For peptide photo-crosslinking, 1 μM TAMRA-labeled tetra-acetylated H4 peptide was preincubated with 25 μM of wild type BRD1 or variant in buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 0.5 mM TCEP) for 30 minutes at room temperature in darkness. Samples were then kept 3 inches beneath a UV lamp (MAXIMA ML-3500S UV, 50,000 μW/cm2, 350nm-385nm 50% irradiated, 365nm 100% irradiated) for 30 minutes at 4 °C on ice. Negative controls were kept in a drawer on ice for the full 30 minutes. 10uL of sample was mixed with 10uL 4X Laemmli dye (Biorad #1610747) and separated on a 4-12 % Criterion XT precast gel (Bio-Rad Laboratories). Gels were imaged using a ChemiDoc MP Imaging at the TAMRA fluorophore excitation wavelength (Emission filter 605/50, Light: green Epi illumination). The gel was then stained with Coomassie brilliant blue R-250 to confirm the presence of proteins in all the samples and again imaged with the ChemiDoc.

6. Full length histone crosslinking and Western blotting

Photo-crosslinking experiments were conducted as follows: 20μM acetylated histone $H4K_C5acK_C8ac$[1] and 50μM BRD1 wildtype or variant were diluted to 20μL with buffer (10mM Tris HCl pH 7.5, 150mM NaCl, 0.05% w/v TWEEN 20, and 0.5mM TCEP), vortexed (Fisher Scientific Mini Vortexer 120V), centrifuged (Fisherbrand Sprout), and split into two 10μL aliquots. Crosslinking and gel electrophoresis were carried out as described for peptide crosslinking.

Separated protein bands were transferred to a 0.2 μm nitrocellulose membrane (BIORAD cat #1620112) at constant amplitude of 5.5 mA/cm^2 on a Trans-Blot SD Semi-Dry Transfer Cell (BioRad 1703940). The membrane was washed briefly with TBST (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.01% w/v Tween-20) then blocked with 5% w/v milk in TBST buffer for 1 hour. Immunoblotting was performed overnight at 4 °C with 1:500 to 1:300 diluted H4 primary antibody (Histone-H4 mAb, cat# 61521, Active Motif). Antibody solutions were removed, then membranes were washed with TBST three times. Secondary antibody was added to membranes at a dilution of 1:5000 antimouse (HRP Goat anti-Mouse IgG, cat #15014, Active Motif) and incubated at room temperature for ≥1 hour. Membranes were then washed three times with TBST, and then visualized using VISIGLO HRP Chemiluminescent substrates A and B (cat# N252-120ML and N253-120ML, aMReSCO) following manufacturer's protocol.

7. Mammalian cell culture and cell lysis

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 in a T150 flask. At 60-70% confluence stage, cells were treated with 5 μM of histone deacetylase inhibitor suberanilohydroxamic acid (SAHA, cat#10009929, Cayman chemical company) dissolved in DMSO to generate hyperacetylated histones.[4] Twelve hours post treatment, cells were harvested and lysed with 600 μL of cold RIPA buffer (Sigma) supplemented with 1X Roche protease inhibitor cocktail and 5 mM TCEP by sonicating for 15 min at amplitude of 60 with a repeating 20 sec pulse cycle. Cell lysates were centrifuged at 21 000*g* for 30 min at 4 °C to remove cell debris. The supernatant was then passed through the detergent removal spin column (Pierce, cat. no. 87778) and eluted with Tris buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 2 mM TCEP, 1X Roche protease inhibitor cocktail) following manufacturer's protocol. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories). This stock solution was used for subsequent photo-crosslinking and Western blotting experiments.

8. Photo-crosslinking with HEK293T cell lysate

For photo-crosslinking studies, ~1 mg of HEK293T cell lysates were incubated with 50 μM of BRD4-L92AzF in a buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 0.5 mM TCEP. After 1 h of incubation at room temperature, the samples were subjected to UV irradiation at 365 nm for 30 min at 4 °C. Negative controls were not subjected to UV exposure. Samples were then bound to Ni-NTA agarose resin and incubated for 1 hr. at 4 °C with gentle rotation. To remove un-crosslinked proteins present in cell lysates, samples were washed with washing buffer (50 mM Tris-HCl pH 8.0, 400 mM KCl, 5% Triton X-100). During each washing step samples were incubated at 60 °C for 5 min. Finally, the proteins were eluted with a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, and 400 mM imidazole. The eluted proteins were separated on a 4-12% Criterion XT precast SDS-PAGE gel (Bio-Rad Laboratories), stained with Coomassie Blue and analyzed by tandem mass spectrometry.

9. LC-MS/MS analysis[5]

In gel trypsin digestion. In gel trypsin digestion was carried out as previously described.[22] Excised gel bands were washed with HPLC water and destained with 50% acetonitrile (ACN)/25mM ammonium bicarbonate until no visible staining. Gel pieces were dehydrated with 100% ACN, reduced with 10mM dithiothreitol (DTT) at 56°C for 1 hour, followed by alkylation with 55mM iodoacetamide (IAA) at room temperature for 45min in the dark. Gel pieces were then again dehydrated with 100% ACN to remove excess DTT and IAA, and rehydrated with 20ng/µl trypsin/25mM ammonium bicarbonate and digested overnight at 37°C. The resultant tryptic peptides were extracted with 70% ACN/5% formic acid, vacuum dried and re-constituted in18µl 0.1% formic acid.

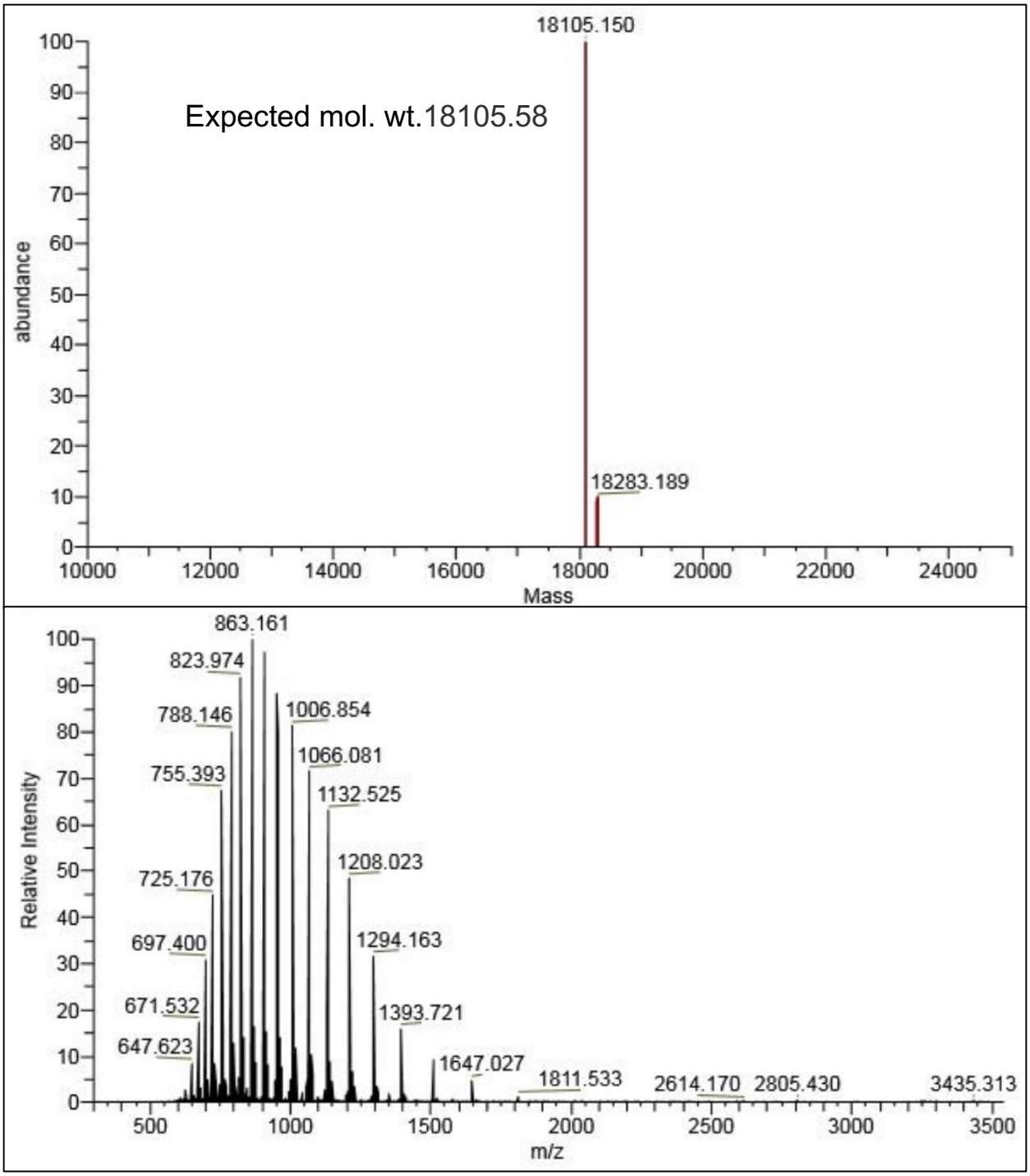
Tandem mass spectrometry. Proteolytic peptides from in gel trypsin digestion were analyzed by a nanoflow reverse-phased liquid chromatography tandem mass spectrometry (LC-MS/MS). Tryptic peptides were loaded onto a C18 column (PicoChip™column packed with 10.5cm Reprosil C18 3µm120Å chromatography media with a 75µm ID column and a 15µm tip, New Objective, Inc., Woburn, MA) using a Dionex HPLC system (Dionex Ultimate 3000, ThermoFisher Scientific, San Jose, CA) operated with a double-split system (Personal communication with Dr. Steve Gygi from Department of Cell Biology, Harvard Medical School) to provide an in-column nano-flow rate (~300nl/min). Mobile phases used were 0.1% formic acid for A and 0.1% formic acid in acetonitrile for B. Peptides were eluted off the column using a 52 minute gradient (2-40% B in 42 min, 40-95% B in 1min, 95% B for 1 min, 2% B for 8 min) and injected into a linear ion trap MS (LTQ-XL,ThermoFisher Scientific) through electrospray.

The LTQ XL was operated in a date-dependent MS/MS mode in which each full MS spectrum [acquired at 30000 automatic gain control (AGC) target, 50ms maximum ion accumulation time, precursor ion selection range of m/z 300 to1800] was followed by MS/MS scans of the 5 most abundant molecular ions determined from full MS scan (acquired based on the setting of 1000 signal threshold, 10000 AGC target, 100ms maximum accumulation time, 2.0 Da isolation width, 30ms activation time and 35% normalized collision energy). Dynamic exclusion was enabled to minimize redundant selection of peptides previously selected for CID.

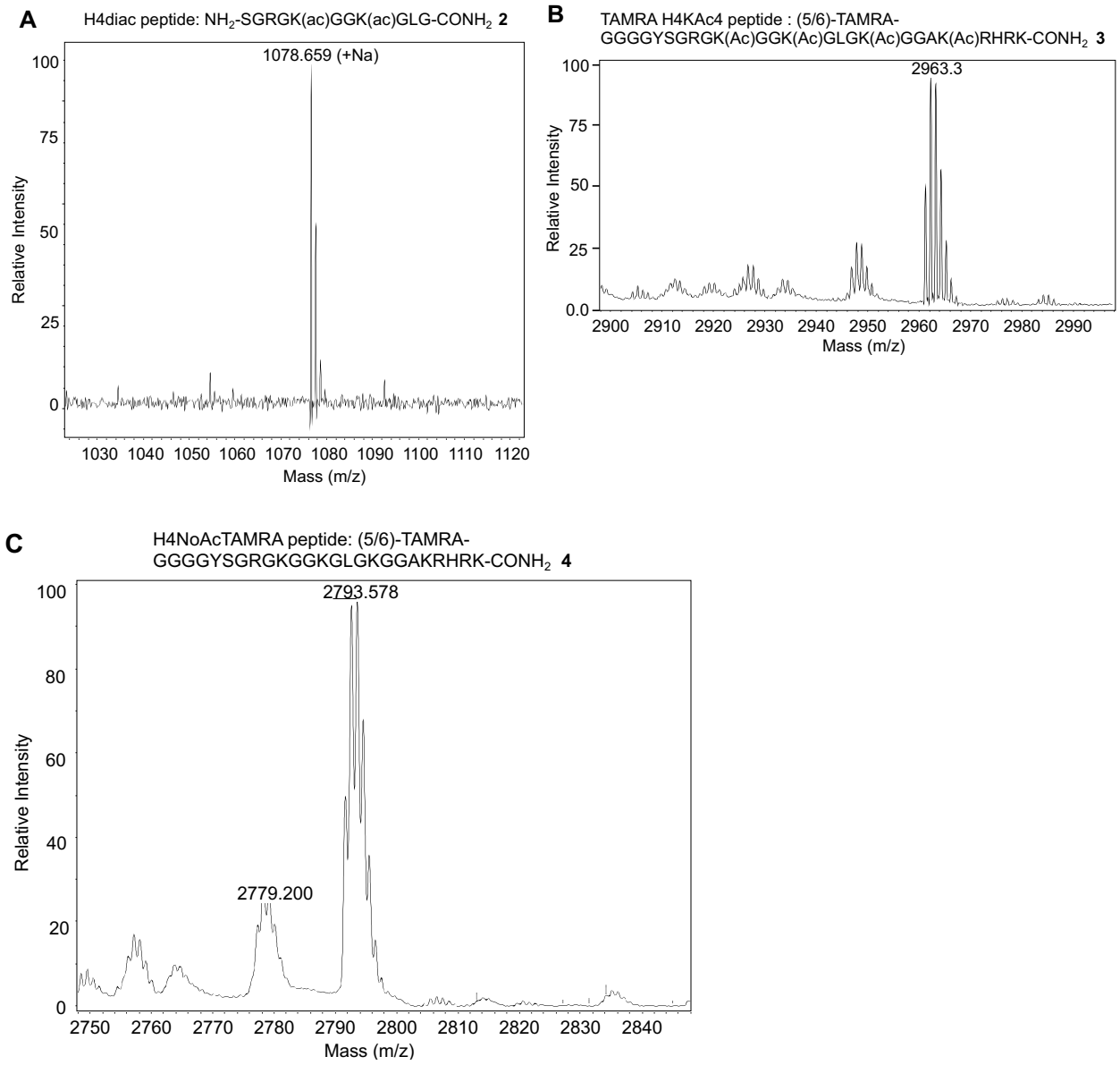
Peptide identification by database search. MS/MS spectra were searched using MASCOT search engine (Version 2.4.0, Matrix Science Ltd) against the UniProt human proteome database. The

following modifications were used: static modification of cysteine (carboxyamidomethylation, +57.05 Da), variable modification of methionine (oxidation, +15.99Da). The mass tolerance was set at 1.4Da for the precursor ions and 0.8 Da for the fragment ions. Peptide identifications were filtered using PeptideProphet™ and ProteinProphet® algorithms with a protein threshold cutoff of 99% and peptide threshold cutoff of 90% implemented in Scaffold™ (Proteome Software, Portland, Oregon, USA).

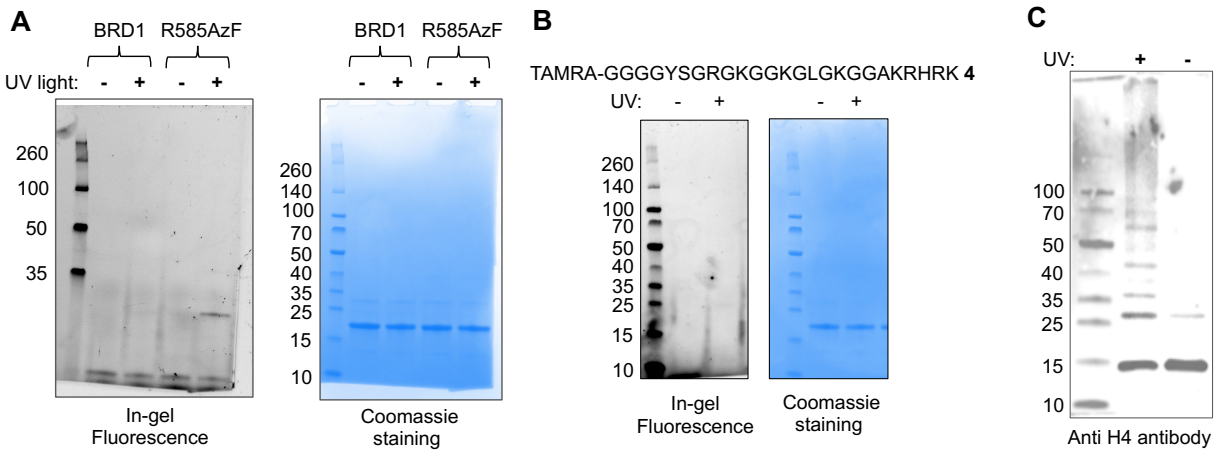
10. Supplementary Figures and Tables



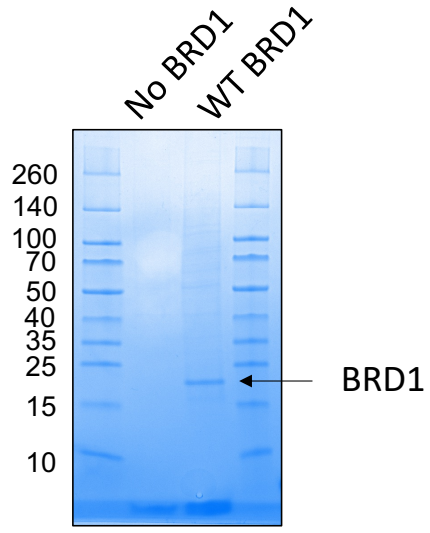
Supplementary Figure S1: HRMS average mass protein deconvolution data for wild type BRD1. m/z at 18283.19 indicates a minor protein carrying α-N-gluconoylation.



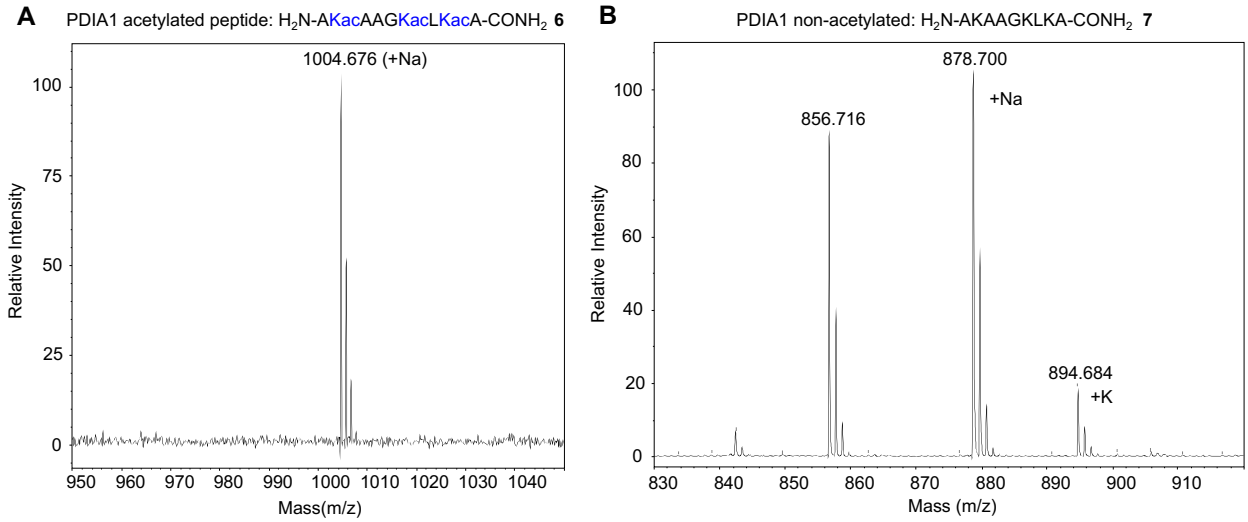
Supplementary Figure S2: MALDI-MS spectra of H4Kac2 peptide **2** (A), TAMRA-H4Kac4 peptide **3** (B) and TAMRA-H4Kac0 peptide **4** (C).



Supplementary Figure S3: (A) Full gel showing in-gel fluorescence confirms crosslinking of **3** with R585AzF but not with wild type BRD1. Coomassie staining used as loading control. This corresponds to Figure 4B in the manuscript. (B) Photo-crosslinking of BRD1-R585AzF mutant with TAMRA-H4Kac0 peptide **4**. No fluorescent band in in-gel fluorescence indicates that the mutant did not crosslink with the non-acetylated peptide **4**. (C) Full gel showing immunoblotting using H4 antibody confirmed crosslinking between R585AzF mutant and H4 only in the presence of UV light. This corresponds to Figure 4D in the manuscript.



Supplementary Figure S4: Coomassie staining of enriched proteins from HEK293T cell extracts treated with or without wild type BRD1-BD. Pulled down was performed using Ni-NTA beads.



Supplementary Figure S5: MALDI-MS spectra of acetylated **6** (A) and non-acetylated **7** PDIA1 Peptides.

Supplementary Table S1: Analyzed proteomic data for 1 R585AzF. This table is provided separately.

Protein	% Glycerol	Peptide	Peptide Sequence	Protein (μM)	Peptide (mM)	K_D(μM)	N	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
BRD1 WT	0	H4Kac2 **2**	SGRGK(Ac)GGK(Ac)GLG	100	2	17±4	1.03 ± 0.05	-2.193	4.092	-6.285
BRD1 R585AzF	0	H4Kac2 **2**	SGRGK(Ac)GGK(Ac)GLG	100	2	110 ± 35	1.2 ± 0.30	-4.923	0.297	-5.220
BRD1 WT	0	PDIA1 **6**	AK(Ac)AAGK(Ac)LK(Ac)A	200	5	25 ± 12	0.80 ± 0.09	-1.818	4.265	-6.083

Supplementary Table S2: Thermodynamic parameters measured by isothermal titration calorimetry (ITC) for the binding of the indicated peptides and the BRD1 bromodomains. Conditions are detailed in the experimental section.

11. References

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