Engineering an acetyllysine reader with photocrosslinking amino acid for interactome profiling

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aEqual contribution

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1. General materials, methods and equipment

All the plasmids for bacterial expression are obtained as gifts from individual laboratories or purchased from addgene. Details of these constructs are given in sections below. Mutagenic primers are obtained from Sigma-Aldrich (Table S1). Commercially available competent bacterial cells were used for protein expression and mutagenesis. HeLa cells, 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. 4-benzoyl-L-phenylalanine (BzF, 1) was purchased from BLD Pharmatech Ltd (cat# BD135722), and 4-azido-L-phenylalanine (AzF, 8) is a kind gift from Prof. Kabirul Islam lab, University of Pittsburgh, was synthesized and characterized as reported earlier.1 All histone peptides 2-7 were synthesized by GL Biochem (Shanghai) Ltd., and S. Biochem Ltd., and purified by HPLC to 98% purity (Table S3). The unlabeled peptide 6 concentration was determined based on the observation that 1 mg/ml peptide generates an absorbance value (A_{205}) of 30 at 205 nm. The concentration of TAMRA-labeled peptides 2-5 and 7 was determined by measuring the absorbance at a wavelength of 555 nm with an extinction coefficient of 65000 L^{-1}cm^{-1}M^{-1}. The integrity of the purified peptides was confirmed by MALDI mass spectrometry.

2. Protein-peptide docking studies

The crystal structure of ATAD2B bromodomain (PDB ID: 3LXJ) was obtained from protein data bank (RCSB). For docking studies, the receptor structure was prepared by adding the polar hydrogen atoms using PyMoL software. The histone H4 coordinates were retrieved from the RCSB protein data bank and the selected lysine residues were covalently modified using the PyTM plugin of PyMoL (version 1.8.4.0) software. The protein and histone peptide input structures were prepared, such as adding charges, assigning the atom types, and detecting the root for the ligand molecules were done by using the AutoDock tools. The grid box dimensions were set to 50 X 48 X 42 Å along the X, Y, and Z axes with a default grid spacing of 0.375 Å, which covered the entire protein. Molecular docking of covalently modified peptide 3 with ATAD2B bromodomain was performed using AutoDockVina 1.1.2. Molecular interaction between peptide and bromodomain was analyzed using PyMOL (version 2.3.4) software package.
3. Molecular modeling and molecular dynamics (MD) simulations

The crystal structure of the ATAD2B bromodomain was obtained from the protein data bank (ID: 3LXJ) to model the bromodomain with a photocrosslinking amino acid BzF, 1. The targeted amino acids of bromodomain were replaced with BzF (SwissSidechain ID: PBF, 4-benzoyl-phenylalanine) one at a time using a PyMOL plugin (https://www.swisssidechain.ch) software package. All the BzF mutants were subjected to MD simulations with GROMACS (2021.1) software package using CHARMM27 all-atom force field modified according to the SwissSidechain. Then the BzF modeled mutant proteins were solvated in a dodecahedron box with a distance of 1 nm between the protein and edge of the solvated box. The solvated system was neutralized by adding sodium in the simulation. To ensure the complex's steric clashes or geometry, the system was energy minimized using the LINCS constraints and steepest descent algorithms (5000 steps and force < 1000 KJ mol^{-1} nm^{-1}) followed by system equilibration under NVT and NPT ensembles. After the energy minimization and equilibration phase, the system was subjected to production MD for 100 ns by following the same time steps described above.

The final MD trajectories were analyzed to calculate the RMSD (root mean square deviation), RMSF (root mean square fluctuation) and visual inspection of trajectory analysis using the standard GROMACS functions and VMD software package. To analyze the effect of BzF incorporation, RMSF calculation made for each alpha carbon of the protein instead of using time average atom position. RMSF of the wild-type protein was used to normalize the RMSF values of BzF mutants to show the difference induced by the substitution. Mean RMSF identifies the best mutant by comparing it with the wild type protein. All plotting’s are done in Xmgrace and GraphPad Prism software packages.

4. Mutagenesis, expression and purification of ATAD2B bromodomain

The N-terminal His6-tagged wild-type ATAD2B bromodomain plasmid (a gift from Nicola-Burgess-Brown, addgene ID: 39046) was transformed into One Shot BL21 star (DE3) E. coli competent cells (Invitrogen cat# C601003) using pNIC28-Bsa4 kanamycin-resistant vectors. A single colony was picked up and grown overnight at 37°C in 10 mL of Luria-Bertani (LB) broth in the presence of 50 μg mL^{-1} kanamycin. The culture was diluted 100-fold and allowed to grow at 37°C to an optical density (OD600) of 1.0. Protein expression was induced overnight at 17°C with 0.5 mM IPTG in an Innova 44 Incubator shaker (New Brunswick Scientific). Proteins were purified as follows: Harvested cells were resuspended in 15 mL lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol, 20 mM
imidazole, Lysozyme, DNase, and 1:200 (v/v) Protease Inhibitor Cocktail III (Calbiochem). The cells were lysed by pulsed sonication and centrifuged at 13000 rpm for 40 min at 4°C. According to the manufacturer's instructions, the soluble extracts were subject to Ni-NTA agarose resin (QIAGEN cat# 30210). After passing 20 volumes of washing buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol, and 20 mM imidazole), proteins were eluted with a buffer containing 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol, and 300 mM imidazole. Proteins were further purified by gel filtration chromatography (Superdex-75) using AKTA pure FPLC system (GE Healthcare) with a buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, and 5% glycerol. Purified proteins were concentrated using Amicon Ultra-10k centrifugal filter device (Merck Millipore Ltd.), and the concentration was determined using Bradford assay kit (Bio-Rad Laboratories) with BSA as a standard. The proteins were aliquoted and stored at -80°C before use.

The ATAD2B bromodomain mutants N981TAG, I982TAG, V992TAG (amber codon) were generated using QuikChange Lightning site-directed mutagenesis kit (Agilent cat# 210519), and the resulting mutant plasmids were confirmed by DNA sequencing. To express the ATAD2B bromodomain variants carrying the p-benzoyl-l-phenylalanine (BzF, 1) at specific positions, ATAD2B amber variants (N981TAG, I982TAG, V992TAG), and pEVOL-pBpF (a kind gift from Prof. Kabirul Islam, University of Pittsburgh, addgene, plasmid #31190) were co-transformed into One Shot BL21 Star (DE3) E. coli competent cells. After transformation, cells were recovered in 200 μL SOC medium and incubated at 37°C shaker for 1 hour with 225 rpm speed before plating on a dual-antibiotic LB Miller agar plate containing 50 μg mL⁻¹ kanamycin and 35 μg mL⁻¹ chloramphenicol. A single colony was picked up and inoculated into 5 mL of Luria Bertani (LB) Miller broth in the presence of 50 μg mL⁻¹ kanamycin and 35 μg mL⁻¹ chloramphenicol and grown overnight at 37°C shaker with 225 rpm speed. This overnight culture was centrifuged (Eppendorf Centrifuge 5910 R, S-4xUniversal swing-bucket rotor) at 1000 x g (2158 rpm) for 10 min at room temperature, and the supernatant (LB Miller broth) was discarded. The collected cell pellet was then resuspended in 1 mL of M9 medium and used to inoculate 500 mL of GMML medium (Farrell et al., 2005) supplemented with 50 μg mL⁻¹ kanamycin and 35 μg mL⁻¹ chloramphenicol. Cells were allowed to grow at 37°C in an incubator shaker (225 rpm) to an optical density (OD₆₀₀) of 1.0. The unnatural amino acid BzF (BLD Pharmatech Ltd, cat# BD135722) was prepared by adding 1 mL of 1M NaOH to 135 mg and adding it to the bacterial culture at a final concentration of 1 mM. Then the cells were moved to 17°C and allowed to grow for 30
minutes; at this stage, the BzF-specific aminoacyl-tRNA synthetase expression was induced with 0.05% w/v arabinose and allowed to shake an additional 30 minutes at 17°C. Finally, ATAD2B protein expression was induced by the addition of 0.5 mM IPTG and allowed to grow for 20 hours at 17°C. The ATAD2B bromodomain mutants were purified as described above while minimizing the exposure to ambient light. The BzF incorporation was confirmed by MALDI-TOF massspectrometry on the linear positive mode (Bruker-autoflex TOF/TOF) by spotting 1 µL of the mixture of the protein sample and matrix (Sinapic acid) on MALDI plate.

To express the ATAD2B bromodomain variant carrying the 4-azido-L-phenylalanine (AzF, 8) at N981 positions, ATAD2B amber variant (N981TAG) and pEVOL-pAzF (a kind gift from Prof. Kabirul Islam, University of Pittsburgh, addgene, plasmid #31186)8 were co-transformed into One Shot BL21 Star (DE3) E. coli competent cells. A similar expression and purification protocol was followed as described above.

5. Fluorescence polarization (FP)

Fluorescence polarization assay was performed to measure the binding affinity of the wild type-ATAD2B bromodomain and its BzF mutants towards the H4 peptides 2-5 carrying a tetramethylrhodamine (TAMRA) at the C-terminus. The FP experiments were performed in 384 flat well Fluotrac 200 microtiter plate (Greiner, cat# 781076) with 250 nM TAMRA-labeled peptide and varying concentrations of proteins (0.5 to 400 µM) in a buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 0.5 mM Tris (2-carboxyethyl) phosphine (TCEP). After 30 min of incubation at room temperature, FP was measured in a Synergy H1MF hybrid multi-mode microplate reader (BioTek) using its FP module and excitation/emission wavelengths 530 and 590 nm, respectively. For the dissociation constant (Kd) determinations, the background-corrected polarization values were plotted against the protein concentrations. The data were fitted to a single-site binding equation \( Y = B_{max} X / (K_d + X) \), where \( Y \) is the specific binding, \( B_{max} \) is the maximal binding, and \( X \) is the concentration of the ligand using the GraphPad Prism software.

6. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry was carried out on a MicroCal PEAQ-ITC instrument (Malvern). Experiments were conducted at 15°C while stirring at 750 rpm. Buffers of protein and peptides were matched to 50 mM HEPES pH 7.5, 200 mM NaCl, 1 mM TCEP, and 5% glycerol. Each titration comprised one initial injection of 0.4 µL lasting 0.8s, followed by 18
injections of 2 μL lasting 4s each at 2.5 min intervals. The initial injection was discarded during data analysis. The microsyringe (40 μL) was loaded with a peptide sample solution at a concentration of 1 mM. It was injected into the cell (200 μL), occupied by a protein concentration of 40-50 μM. All the data was fitted to a single binding site model using the MicroCal PEAQ-ITC analysis software to calculate the stoichiometry (N), the binding constant (K_D), enthalpy (ΔH), and entropy (ΔS) of the interaction. The final titration figures were prepared using OriginPro 2020 software (OriginLab).

7. Circular dichroism spectroscopy
Circular Dichroism (CD) spectra were recorded on a JASCO J-815 CD Spectropolarimeter (JASCO, Japan) at 20°C using a quartz cell with a path length of 10 mm. Two scans were accumulated at a scan speed of 100 nm min⁻¹, with data being collected at every nm from 200 to 270 nm. The ATAD2B wild-type and its mutant proteins were diluted to 2-5 μM concentration in CD buffer containing 100 mM NaH₂PO₄ at pH 7.0. The ellipticity data was converted into molar ellipticity using the below equation.

\[ [\Theta] = m^0M/(10*L*C) \]

Where \([\Theta]\) is the molar ellipticity, \(m^0\) is ellipticity of the sample measured, \(M\) is average molecular weight (g/mol), \(L\) is path length of the cell (cm), and \(C\) is a concentration in g/L. The protein secondary structure determination was done using K2D3 structure prediction software.

8. Thermal shift assay
The thermal shift assay is a rapid and inexpensive biochemical method often used to determine the thermal stability of protein in different in vitro conditions by monitoring the unfolding of the protein at increasing temperatures. The SYPRO orange fluorescent dye (Invitrogen, cat# S6650) was used to calculate the melting temperature (T_m) of the wild-type ATAD2B bromodomain and its mutant proteins. The assay was performed in a 96-well clear low-profile plate (Bio-Rad, #MLL9601) using CFX96 touch Real-Time PCR detection system. A total 25 μL assay containing 10 μg ATAD2B and its mutants were premixed with 5x SYPRO orange dye in 50 mM HEPES, 150 mM NaCl, and pH 7.5. Wells containing only buffer with 5x dye were used for baseline correction. The plate was sealed with an optically clear adhesive film (Bio-Rad, #MSB1001) to prevent sample loss during heating. The temperature was gradually ramped up from 25 to 95 °C while monitoring the change in fluorescence intensity of the dye.
GraphPad Prism software was used to analyze the data and calculate the melting point of the protein sample by applying nonlinear regression using the melting Boltzmann equation:

\[ Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + \exp(Tm - X/slope)) \]

9. Photo-crosslinking experiment with histone peptides and in-gel fluorescence
For photo-crosslinking experiments, 5 \( \mu M \) H4 peptides 2-5 and 7 carrying a tetramethylrhodamine (TAMRA) at the C-terminus were preincubated with 50 \( \mu M \) wild type ATAD2B-BRD or its variants in 100 \( \mu L \) binding buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.001% Tween 20, and 1 mM TCEP for 30 min at 4\(^{\circ}\)C on a rotator. After incubation, the samples were split into two 50 \( \mu L \) volumes in clear PCR tubes (Axygen). The sample containing PCR tubes were placed into semi-micro visible cuvettes (300 nm – 900 nm, Eppendorf cat# 0030079353) filled with ice cubes to maintain the temperature and irradiated with ultraviolet light (365 nm, 8 W lamps, Model: ECX-F20.L V1, Vilber Lourmat, France) for 3 x 10 min at 4\(^{\circ}\)C. Negative samples were not subjected to UV irradiation and stored in the dark at 4\(^{\circ}\)C. After irradiation, the samples were transferred into 1.5 mL tubes having 50-60 \( \mu L \) of Ni-NTA agarose beads (QIAGEN) pre-equilibrated with the buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.001% Tween-20, and 1 mM TCEP), and the samples were incubated for 1 hr. at 4\(^{\circ}\)C on a rotator. The uncrosslinked histone peptides were removed by washing the samples with wash buffer (50 mM HEPES pH 7.5, 400 mM KCl, 1% Triton X-100) for 5 times at room temperature. The proteins were eluted with 30 \( \mu L \) elution buffer containing 50 mM HEPES pH 7.5, 500 mM imidazole and incubated for 5 min at room temperature with intermittent mixing. The eluted proteins were mixed with 10 \( \mu L \) 1 x TruPAGE LDS sample buffer (Sigma cat# PCG3009) and heat-denatured at 100 \(^{\circ}\)C for 10 min. Finally, the crosslinked proteins were separated on a 15% SDS-PAGE gel and imaged on a G: BOX Chemi XRQ gel doc system (Syngene) using TAMRA fluorophore excitation wavelength. The gel was subsequently stained with Coomassie brilliant blue R-250 staining solution to confirm the presence of proteins in all the samples.

10. Mammalian cell culture
HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (Penicillin-Streptomycin cocktail) in a humidified atmosphere containing 5% CO\(_2\). Cells at ~90% confluence stage were treated with 2 \( \mu M \) of histone deacetylase inhibitor Trichostatin A (TSA, cat# T8552, Sigma) dissolved in DMSO to generate hyperacetylated histones.
Twenty hours post-treatment, media was removed and rinsed the cells with cold PBS buffer. The harvested cells were rewarshed with cold PBS buffer and frozen as a dry pellet at -80 °C.

11. Acid extraction of histones
Histones were extracted from HeLa cells using the acid-extraction protocol as previously described.² Briefly, frozen cell pellets from T75 flask of HeLa cells were resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) and incubated for 30 min on rotator at 4°C. Samples were then centrifuged (10,000 x g, 10 min at 4°C), and the supernatant was discarded entirely. The nuclei resuspended in 800 μL of 0.4 N H₂SO₄, vortexed intermittently for 5 min, and further incubated at 4°C on a nutator for overnight. The nuclear debris was pelleted by centrifugation (16000 x g, 10 min at 4°C), and the supernatant containing histones were collected. The histones were precipitated by adding 264 μL TCA (Trichloroacetic acid) drop by drop to histone solution and invert the tube several times to mix the solutions and incubated the samples on ice for 30 min. Finally, histones were pelleted by centrifugation (16000 x g, 10 min at 4°C), and the supernatant was discarded. The histones pellet was washed twice with ice-cold acetone, followed by centrifugation (16000 x g, 5 min at 4°C), and carefully removed the supernatant. The histone pellet was air-dried for 20 min at room temperature and subsequently dissolved in an appropriate volume of ddH₂O and transferred into a fresh tube. The aliquoted histones were stored at -80°C before use.

12. Photocrosslinking with hyperacetylated histone H4 and Western blotting
20-25 μg hyperacetylated histones (extracted from HeLa cells) were incubated with 50 μM ATAD2B-N981BzF mutant in 50 μL binding buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.001% Tween 20, and 1 mM TCEP for 30 min at 4°C on a rotator. After incubation, the samples were crosslinked as described above. Finally, the crosslinked proteins were separated on a 12%-SDS-PAGE gel and transferred onto a 0.45 μm PVDF membrane at a constant voltage of 80V for 1.5 hr. at 4°C. The membrane was rinsed in TBST buffer (50 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and blocked for an hour at room temperature (RT) in 5% milk buffer prepared in TBST. Immunoblotting was performed with the following primary antibodies: anti-H4K5ac (Invitrogen cat# MA532009), anti-H4K8ac (Invitrogen cat# MA5-33386), anti-H4K12ac (Invitrogen cat# MA533388), anti-H4K16ac (Invitrogen cat# MA527794), and anti-6xHis (Invitrogen cat# MA121315) overnight at 4°C. The membranes were washed with TBST buffer thrice at RT for five minutes each. The blots were then
incubated with the HRP conjugated secondary antibodies Goat anti-Rabbit IgG (Invitrogen cat# 31466) or Goat anti-mouse IgG (Invitrogen cat# 31431) with 5% nonfat dry milk, dilution 1:5000 in TBST. The membranes were rewashed with TBST buffer thrice at RT for five minutes each. Protein bands were visualized by chemiluminescence using SuperSignal West Pico PLUS substrate (Invitrogen cat# 34577) following the manufacturer’s protocol.

13. Preparation of HeLa cell lysate
Twenty hours post-treatment with deacetylase inhibitor (TSA), media was removed and rinsed the cells with cold PBS buffer. The harvested cells were resuspended in ice cold lysis buffer containing 25 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol (Pierce IP lysis buffer, Cat# 87787), and supplemented with 1X Pierce protease inhibitor cocktail and incubated on ice for 5 min with periodic mixing. Cell lysate was centrifuged at 13,000 x g for 10 min at 4 °C to pellet the cell debris. The supernatant was transferred into fresh microcentrifuge tube and the protein concentration was determined by Bradford assay (Bio-Rad Laboratories). The supernatant containing cell lysate was used for subsequent photocrosslinking and Western blotting experiments.

14. Photocrosslinking with HeLa cell lysate and Western blotting
For photo-crosslinking studies, ~0.5 mg of HeLa cell lysate was incubated with 50 μM of ATAD2B bromodomain analogues (N981BzF, V992BzF and N981AzF) one at a time in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 0.001% Tween 20, and 1 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 1h at 4 °C with gentle rotation. To remove un-crosslinked proteins, present in cell lysates, samples were washed 5 times with washing buffer (50 mM HEPES pH 7.5, 400 mM KCl, 5% Triton X-100). The crosslinked proteins were eluted in 30 μL elution buffer containing 50 mM HEPES pH 7.5, 500 mM imidazole and incubated for 5 min at room temperature with intermittent mixing. The eluted proteins were separated on a 4-15% Mini-PROTEAN TGX precast SDS-PAGE gel (Bio-Rad, #4561085) and transferred onto a 0.45 μm PVDF membrane at a constant voltage of 80V for 1.5 hr. at 4°C. The membrane was rinsed in TBST buffer (50 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and blocked for an hour at room temperature (RT) in 5% milk buffer prepared in TBST. Immunoblotting was anti-6x-His tag antibody (Invitrogen cat# MA121315) overnight at 4° C. The membrane was washed with TBST buffer thrice at RT for five minutes.
each. The blots were then incubated with the HRP conjugated secondary antibody Goat anti-mouse IgG (Invitrogen cat# 31431) with 5% nonfat dry milk, dilution 1:5000 in TBST. The membrane was rewashed with TBST buffer thrice at RT for five minutes each. Protein bands were visualized by chemiluminescence using SuperSignal West Pico PLUS substrate (Invitrogen cat# 34577) following the manufacturer’s protocol.

15. Supplementary figures and tables

Supplementary figure S1: (A) The α carbon RMSF of BzF variants of ATAD2B bromodomain are plotted against residue numbers. The top panel represents the standard, unnormalized RMSF, and the bottom panel represents the normalized RMSF, where the value of the wild-type protein RMSF is subtracted from each variant’s RMSF. Note the difference in the y-axis scale between the two panels. The N981BzF, I982BzF, and V992BzF are very similar to the wild-type, but the two other mutants, I1048BzF and R1051BzF, display observable changes in the ZA-loop region.
**Supplementary figure S2:** Molecular dynamics (MD) simulation snapshots of ATAD2B and its mutants. (A-F) Dashed circle depicts a small alpha helical part of ZA-loop. (C-F) The dashed circle indicates distortions in alpha helices which occurred due to the incorporation of benzophenone. (E-F) The red arrows represents the bending in αC helix and which altered the structural fold of the bromodomain.

**Supplementary figure S3:** (A) The RMSD of wild-type ATAD2B and its mutants. (B) The average profile of the RMSD of wild-type ATAD2B and the selected residues substituted with BzF in this study.
**Supplementary figure S4:** Coomassie blue staining showing expression and purity of wild type ATAD2B bromodomain and its BzF containing mutants.
Supplementary Figure S5: HPLC purity traces for the peptide 2.

Supplementary Figure S6: MS spectra for the peptide 2.
Supplementary Figure S7: HPLC purity traces for the peptide 3

Supplementary Figure S8: Mass spectra for the peptide 3.
Supplementary Figure S9: HPLC purity traces for the peptide 4

Supplementary Figure S10: Mass spectra for the peptide 4.
Supplementary Figure S11: HPLC purity traces for the peptide 5.

Supplementary Figure S12: MS spectra for the peptide 5.
Supplementary Figure S13: HPLC purity traces for the peptide 6.

Supplementary Figure S14: Mass spectra for the peptide 6.
Supplementary Figure S15: HPLC purity traces for the peptide 7.

Supplementary Figure S16: Mass spectra for the peptide 7.
Supplementary Figure S17: Dissociation constants of wild-type ATAD2B bromodomain and its mutants towards the TAMRA-labeled (A) unacetylated histone H4 peptide 2 and (B) acetylated histone H4 peptide 3 as determined by background-corrected fluorescence polarization values. Error bars represent standard deviation from two independent measurements. We did not observe saturation in mP values for the weakly bound peptides. For these cases, K_D values are estimated based on the approximate B_max values. (NB: No binding)

Supplementary Figure S18: Dissociation constants of wild-type ATAD2B bromodomain and its mutants towards the TAMRA-labeled acetylated histone H4 peptide 4 as determined by background-corrected fluorescence polarization values. Error bars represent standard deviation from two independent measurements. (NB: No binding)
Supplementary Figure S19: Dissociation constants of wild-type ATAD2B bromodomain and its mutants towards the TAMRA-labeled acetylated histone H4 peptide 5 as determined by background-corrected fluorescence polarization values. Error bars represent standard deviation from two independent measurements. We did not observe saturation in mP values for the weakly bound peptides. For these cases, K\text{D} values are estimated based on the approximate B\text{max} values. (NB: No binding)
Supplementary Figure S20. (A) In-gel fluorescence showing crosslinking of ATAD2B mutants with histone peptides 2-5. (B) Dissociation constant of wild-type ATAD2B bromodomain towards the TAMRA-labeled acetylated histone H4 peptide 7 (Fig. S15-16, Table S3) as determined by background-corrected fluorescence polarization values. Error bars represent standard deviation from two independent measurements. (C) The N981BzF mutant captured transient interacting partner (H4K16ac peptide, 7) of ATAD2B bromodomain. Coomassie staining of the same gel showed the presence of proteins in all the samples.

Supplementary Figure S21. Western blot showing crosslinking of histone H4K16ac isolated from HeLa cells with ATAD2B-N981BzF as confirmed by anti-H4K16ac antibody.
**Supplementary Figure S22:** (A) Chemical structure of 4-azido-L-phenylalanine (AzF). (B) Bacterial expression of ATAD2B-N981TAG mutant using an evolved M. jannaschii tRNA-RS pair in the absence (−) and presence (+) of 1 mM 4-azido-L-phenylalanine (AzF) judged by Coomassie blue staining. Further, the protein is purified by size-exclusion chromatography. (C) MALDI-MS spectra of ATAD2B-WT and its mutant bearing AzF. (D) Dissociation constants ($K_D$) of ATAD2B-N981AzF mutant from peptide 6 as measured by ITC.

**Supplementary Figure S23.** Crosslinking of ATAD2B-V992BzF mutant with cell lysate isolated from HeLa cells as confirmed by immunoblotting with anti-6xHis antibody.
**Supplementary Table S1:** List of the forward primers designed for site-directed mutagenesis. Reverse primers used are the reverse-complement to the given forward primers.

<table>
<thead>
<tr>
<th>ATAD2B mutants</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>N981TAG</td>
<td>GGCGACCGATAAACGTTTTTAGATTTTTAGCAAACCGGTGG</td>
</tr>
<tr>
<td>I982TAG</td>
<td>GGCGACCGATAAACGTTTTAATTAGTTTAGCAAACCGGTGG</td>
</tr>
<tr>
<td>V992TAG</td>
<td>GGTGGATATTGAAGAATAGACCGATTATCTGGAAGTG</td>
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**Supplementary Table S2:** MALDI-MS characterization of wild-type ATAD2B and its mutants.

<table>
<thead>
<tr>
<th>ATAD2B bromodomain</th>
<th>Calculated Mass (Da)</th>
<th>Observed Mass (Da) (MALDI-MS)</th>
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<tbody>
<tr>
<td>WT</td>
<td>18273.72</td>
<td>18274.49</td>
</tr>
<tr>
<td>N981BzF</td>
<td>18410.90</td>
<td>18411.88</td>
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<tr>
<td>I982BzF</td>
<td>18411.85</td>
<td>18412.83</td>
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<tr>
<td>V992BzF</td>
<td>18425.86</td>
<td>18427.12</td>
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<tr>
<td>N981AzF</td>
<td>18347.80</td>
<td>18345.65</td>
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**Supplementary Table S3:** List of histone peptides 2-7 varying acetylation sites were synthesized and used for this study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide Sequence</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4Kunac (1-20) - 2</td>
<td>SGRGKKGGKLKGGAKHRK-TAMRA</td>
<td>2404.74</td>
</tr>
<tr>
<td>H4K5acK8ac (1-12)</td>
<td>SGRGKacGKacGLG-K TAMRA</td>
<td>1597.42</td>
</tr>
<tr>
<td>H4K12ac (1-20) - 4</td>
<td>SGRGKGGKGLGGAKHRK-TAMRA</td>
<td>2171.47</td>
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<tr>
<td>H4Kac4 (1-20) - 5</td>
<td>SGRGKacGKacGLGKacGHRK-TAMRA</td>
<td>2572.89</td>
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<tr>
<td>H4K5acK8ac (1-11)</td>
<td>SGRGKacGKacGLG</td>
<td>1057.16</td>
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<tr>
<td>H4K16ac (11-20) - 7</td>
<td>GKGGKacHRK-TAMRA</td>
<td>1548.88</td>
</tr>
<tr>
<td>ATAD2B bromodomain</td>
<td>$K_0$ (μM) by ITC</td>
<td>$N$</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>-----</td>
</tr>
<tr>
<td>WT</td>
<td>25 ± 1.3</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>N981BzF</td>
<td>23 ± 2.1</td>
<td>0.97 ± 0.03</td>
</tr>
<tr>
<td>N981AzF</td>
<td>28 ± 1.9</td>
<td>0.98 ± 0.01</td>
</tr>
</tbody>
</table>

**Supplementary Table S4:** Thermodynamic parameters measured by isothermal titration calorimetry (ITC) for the binding of peptide 6 to wild type-ATAD2B bromodomain and its mutants.

<table>
<thead>
<tr>
<th>ATAD2B bromodomain</th>
<th>Molar ellipticity at 222 nm</th>
<th>% $\alpha$-helix</th>
<th>% $\beta$-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-106321.4</td>
<td>91.1%</td>
<td>0.26%</td>
</tr>
<tr>
<td>N981BzF</td>
<td>-95775.3</td>
<td>91.1%</td>
<td>0.26%</td>
</tr>
<tr>
<td>I982BzF</td>
<td>-99417.5</td>
<td>91.1%</td>
<td>0.26%</td>
</tr>
<tr>
<td>V992BzF</td>
<td>-95012.1</td>
<td>91.1%</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

**Supplementary Table S5:** The molar ellipticity at 222 nm and the relative percentage of $\alpha$-helix and $\beta$-sheet composition for the wild-type ATAD2B and its BzF bromodomain mutants, calculated from circular dichroism experiments.

**16. References**