Engineering a Methyllysine Reader with Photoactive Amino Acid in Mammalian Cells

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1. General materials, methods and equipment

Chemicals: All chemicals were purchased from established vendors (e.g. Sigma-Aldrich, Acros Organics) and used without purification unless otherwise noted. Optima grade acetonitrile was obtained from Fisher Scientific and degassed under vacuum prior to HPLC purification. All reactions to prepare 4-azido phenylalanine were carried out in round bottom flasks and stirred with Teflon®-coated magnetic stir bars under inert atmosphere when needed. Analytical thin layer chromatography (TLC) was performed using EMD 250 micron flexible aluminum backed, UV F_{254} pre-coated silica gel plates and visualized under UV light (254 nm) or by staining with phosphomolybdic acid, ninhydrin or anisaldehyde. Reaction solvents were removed by a Büchi rotary evaporator equipped with a dry ice-acetone condenser. Analytic and preparative HPLC was carried out on an Agilent 1220 Infinity HPLC with diode array detector. Concentration and lyophilization of aqueous samples were performed using Savant Sc210A SpeedVac Concentrator (Thermo), followed by Labconco Freeze-Dryer system. Proton nuclear magnetic resonance spectra (1H NMR) were recorded on Bruker Ultrashield™ Plus 600/500/400/300 MHz instruments at 24°C. Chemical shifts of ^{1}H and ^{13}C NMR spectra are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ 0.0) or residual solvent signals: chloroform-d (δ 7.26, singlet), methanol-d_4 (δ 3.30, quintet), and deuterium oxide-d_2 (δ 4.80, singlet). Coupling constants are expressed in Hz. MALDI mass spectra were collected at ultraFlextreme (Bruker) and the data was analyzed using flexAnalysis software. The ESI-MS were recorded on a Q-Exactive™ 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. Details of these constructs are given in sections below. Mutagenic primers are obtained from Integrated DNA Technologies (Table S3). Commercially available competent bacterial cells were used for protein expression and mutagenesis. Human embryonic kidney 293T (HEK293T) 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.

2. Synthesis and purification of AzF 1 and peptides 2 - 5

4-Azido-L-phenylalanine (AzF) **1** was synthesized and characterized as reported earlier.[1] H3 peptides **2** - **5** were synthesized at the University of Pittsburgh Peptide Synthesis facility. Analytical scale separation was performed using Zorbax reversed phase C18 (5µm, 4.6 x 250 mm) column with UV detection at 220 nm and 550 nm (for TAMRA labelled peptide). The column was equilibrated with 0.1% aqueous trifluoroacetic acidic (TFA) and the peptide was separated with the linear gradient of acetonitrile to 10% in 15 min and then to 70% in 5 min with a flow rate of 1 mL/min. The crude peptides were purified using semi-preparative reversed phase HPLC column Xbridge C18 (5µm, 10 x 250 mm) eluting with a flow rate of 5 mL/min and a acetonitrile gradient from 0% to 90% in 15 mins and then to 100 % in 18 mins in 0.1% aqueous TFA. The purified peptides were concentrated on speedvac and dried in the lyophilizer. The dried peptides were resuspended in 0.1% aqueous TFA and the concentration was measured based on the observation that 1 mg/mL of peptide generates an absorbance of 30 at a wavelength of 205 nm. The concentration of TAMRA-labeled peptides **2, 4** and **5** was determined by measuring the absorbance at a wavelength of 555nm with an extinction coefficient of 65000 $L^{-1}cm^{-1}M^{-1}$. The integrity of the peptides was confirmed by MALDI mass spectrometry.

3. Mutagenesis and expression of wild type CBX1 chromodomain

The human CBX1 chromodomain construct for bacterial expression was purchased from Addgene (Plasmid # 25245). It is in kanamycin resistant pET28-MHL vector.[2] It contains a 6xHis tag on the N-terminal. The construct was missing the N-terminal acidic patch of LEEEEE amino acids. We first inserted the missing sequence using QuikChange Lightning Site Directed Mutagenesis Kit (catalog # 210519) following manufacturers protocol. The PCR amplified DNA was transformed in XL-10 Gold cells. Single colonies were picked for mini-prep using GeneJET Plasmid Mini-Prep Kit (Catalog # K0503) and confirmed by DNA sequencing.

The kanamycin resistant pET28-MHL plasmid carrying wild type CBX1 chromodomain was transformed in *E. coli* One Shot BL21-star (DE3) chemically competent cells (Invitrogen) for expression. A single colony was picked and grown overnight in 10 mL of Luria-Bertani (LB) medium at 37°C in presence of 50 µg/mL of kanamycin. The 10 mL culture was diluted to a 100-fold and the cells were grown at 37°C in presence of 50 µg/mL of kanamycin till it reached an

optical density (O.D) of 0.8. The protein over-expression was induced by adding 0.5 mM of IPTG to cultures and grown at 17ºC overnight. The cells were harvested and resuspended in buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10 % glycerol, 5 mM β-mercaptoethanol, 25 mM imidazole, DNase, lysozyme and protease inhibitor tablets. The cells were lysed by pulsed sonication (Qsonica-Q700) and centrifuged at 13,000 rpm for 50 mins at 4ºC. The soluble extracts were incubated with Ni-NTA agarose resin (Thermo) according to manufacturer's protocol. The beads were washed with 20 column volumes of wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10 % glycerol, 5 mM β-mercaptoethanol and 25 mM imidazole). The protein was eluted with 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol and 400 mM imidazole. Eluted protein was subjected to further purification by gel filtration chromatography (Superdex-75) using AKTA pure FPLC system (GE healthcare) with buffer containing 50 mM Tris pH 8.0, 150 mM NaCl and 10 % glycerol. The purified protein was concentrated using Amicon-Ultra 3K centrifugal filter device (Merck Millipore Ltd.). The concentration of the protein was determined using Bradford assay kit (BioRad Laboratories) with BSA as standard. The concentrated protein was aliquoted and stored at -80ºC.

4. Mutagenesis and expression of CBX1 variants carrying AzF

Site-directed amber (TAG) mutagenesis was performed using QuickChange Lightening site-directed mutagenesis kit (Agilent Technologies) on the wild type CBX1 chromodomain construct described above. PCR protocol was 18 cycles of 20 seconds denaturing at 95ºC, annealing of primers at 60ºC for 10 seconds, and polymerization at 68ºC for 5minutes. The PCR amplified gene was transformed in BL21 XL10 Gold cells and colonies are grown in presence of antibiotics (Kan-50 ug/μL). The mutant plasmids were extracted using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and confirmed by sequencing.

The mutated plasmids were co-transformed with evolved *Methanococcus jannaschii* p-Azido-L-phenylalanine RS (2copies + tRNA) expression vector (Addgene ID: 31186) in BL21 star (DE3) cells (Invitrogen).[3] 15μL of cells were incubated with 10 ng of both the plasmids on ice for 30 minutes followed by heat-shock at 42ºC for 25-30 seconds and immediately cooling to 4ºC for 2 minutes. The cells were first grown in SOC media for an hour at 37ºC and then plated on LB agar plates containing Kanamycin (50 μg/mL) and Chloramphenicol (35 μg/mL) overnight at 37ºC. A

single colony from the plate was grown overnight in 10 mL LB with 50 μg/mL Kanamycin and 35μg/mL Chloramphenicol at 37°C. This culture was centrifuged at 1000 g for 10 minutes and 8 mL of supernatant was removed. The pelleted cells were re-suspended in 2 mL LB to inoculate 1L of GMML medium containing 50 μg/mL Kanamycin and 35 μg/mL Chloramphenicol. The cultures shake at 37°C for 8-9 hours till the OD_{600} was reached 0.7-0.8. The cells were introduced to AzF compound to a final concentration of 1mM and cooled by shaking for 30 minutes at 17°C. After the cultures cooled to 17°C, they were induced with 0.05% arabinose and after half an hour of addition the cells are induced with 0.25 mM IPTG. The cells were agitated at 17°C for 20 h at 225 rpm. The cultures were centrifuged at 4000 rpm for 20 minutes. The pellet was re-suspended in 15 mL lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole, Lysozyme, DNase, and Roche (protease inhibitor cocktail) followed by sonication (Qsonica-Q700) at 60 Amp for 2 minutes with pulse on/off for 10 seconds. The lysed cells were centrifuged at 13000 rpm for 50 minutes. The clear supernatant was loaded on equilibrated Ni-NTA agarose resin (Thermo). The supernatant and the bead mixture were shaken for 45 minutes at 4°C. After 45 minutes of incubation with beads, the supernatant was let to flow-through the column followed by washing with 20 mL of wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol and 25 mM imidazole). The protein was eluted with 5 mL of elution buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol and 400 mM imidazole) in 5X 1mL fractions. The fractions were then loaded on to Superdex75 column in an AKTA pure FPLC system (GE healthcare). The FPLC buffer contained 10% glycerol, 50 mM Tris-HCl pH 8.0 and 150 mM NaCl. The eluted fractions were concentrated using Amicon Ultra-3K centrifugal filter device (Merck Millipore Ltd.). The concentration of protein was measured using Bradford assay kit (BioRad Laboratories) with BSA as standard. The proteins were stored in -80°C as aliquots.

5. Expression and purification of histone H3 K9C mutant

Gene sequence encoding wild type *Xenopus laevis* histone H3 was a kind gift from Prof. Minkui Luo at the Memorial Sloan-Kettering Cancer Center. This construct originally contains C110A mutation. K9C mutation (hereafter called $H3K_C9$) was generated by the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) following manufacturer's protocol. The resulting mutant plasmid was confirmed by DNA sequencing. The plasmid was transformed into

BL21 codon plus (DE3) RIPL competent cells.[4] A single colony was picked up and grown overnight at 37 °C in 10 mL of LB broth with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. The inoculation culture was diluted 1:100 fold in fresh LB medium and cells were grown at 37 °C until OD_{600} reached to ~0.7. Protein expression was induced by the addition of 0.3 mM IPTG followed by growing for an additional 3 h at 37 °C. Cells were harvested by centrifugation at 5000 rpm for 30 min, and then resuspension of the pellet in 5 mL of lysis buffer (10 mM Tris-HCl pH 7.5, 2 M guanidinium hydrochloride (GdnHCl), 5 mM *β*-mercaptoethanol, 10% glycerol, DNase, Lysozyme and Roche protease inhibitor cocktail). The cells were lysed by pulsed sonication and centrifuged at 20,000g for 40 min at 4 °C. Insoluble histone was recovered from inclusion bodies by dissolving in 6 M GdnHCl and 10 mM Tris-HCl pH 7.5, and incubated for 10 min at room temperature followed by centrifugation at 20,000g for 40 min at 4 °C. The soluble histone supernatant was purified by size exclusion chromatography on a Superdex-200 using AKTA pure FPLC system.[5] Fractions were concentrated using Amicon Ultra-4 centrifugal 3K filter and further purified with preparative reverse-phase HPLC (XBridge C18, 5 µm, 10 x 250 mm column) eluting with a flow rate of 4 mL/min starting from 10% acetonitrile to 70 % in 15 min and then to 100 % over 5 min in aqueous trifluoroacetic acid (0.01%). The purified protein was concentrated by SpeedVac followed by lyophilization. The protein was stored at -80°C before use.

6. Chemical trimethylation of H3K$_C$9 (Semisynthetic H3K$_C$9me3)

For the generation of trimethylated H3K$_C$9me$_3$, 1 mg of lyophilized histone was dissolved in 98 µl of alkylation buffer (4 M Guanidine-Hydrochloride, 1 M HEPES pH 7.8, 10 mM D/L-methionine).[5,6] Once histone was fully dissolved, 2 µl of 1 M DTT (prepared fresh) was added to the histone solution followed by 1 h incubation at 37 °C. The solution was then added to 10 mg of (2-bromoethyl) trimethyl ammonium bromide (Sigma, cat# 117196) and protected from light. After incubating for 2.5 h at 50 °C (gently agitating every 30 min), 1 µl of 1 M DTT was added to quench the reaction. The solution incubated at 50 °C for an additional 2.5 h. The reaction was quenched by adding 5 µl of β-mercaptoethanol (β-ME). A PD10 (GE, cat# 17-0851-01) column was used to desalt the solution and the histones were eluted with 2 mM β-ME. SDS-PAGE was performed to determine presence of the histone protein. The samples were lyophilized and then resuspended in 50 mM Tris pH 8.0. Protein concentration was determined by Bradford assay and

the molecular weight was verified by LC-MS. Protein was stored in -80 °C until used in photo-crosslinking assay.

7. Preparation of histone extracts

Histone extraction was carried out following protocol the protocol by D. Shechter et al.[7] The HEK293T cells are grown in 10% FBS and DMEM media to a 100% confluency and collected by trypsinization. The cells are washed twice with PBS buffer and incubated in hypotonic solution (10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and 1X protease inhibitor) for 30 mins at 4°C. The intact nuclei were pelleted by centrifuging at 4°C, 10,000 g for 10 mins. The supernatant was discarded, and the nuclei was resuspended in 400 µL of 0.4 N H_2SO_4, overnight. The samples were the centrifuged at 16000 g, 4°C for 10 mins to remove nuclear debris. The supernatant was transferred to a fresh 1.5 mL centrifuge tube, 132 µL of TCA was added dropwise to precipitate the histones and incubated on ice for 30 mins. The histones were pelleted by centrifuging at 16,000 g, 4°C for 4 mins. The supernatant was removed, and the pelleted histones were washed thrice with ice-cold acetone, centrifuging at 16,000g, 4°C for 5 mins after every wash. Finally, the histone pellet was air dried for 20 mins and re-suspended in deionized H_2O. 1 µL of histone extracts was loaded on a 15% gel with the BSA standards to quantify the amount of H3 for the assay.

8. Fluorescence polarization (FP) assay

Fluorescence polarization assay was employed to screen the binding affinity between the CBX1 mutants expressing the unnatural amino acid and TAMRA-attached H3K9me3 peptide **2**.[2, 8] The assay was performed in a 384 well small volume black/clear microtiter plates (Falcon) with 200 nM TAMRA labelled peptides and varying concentrations of protein (0.3-750 µM) in 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20 and 0.5 mM of Tris (2-carboxyethyl) phosphine. The plate was centrifuged at 3000 rpm for 3 mins and the polarization was read for each well on TECAN M 1000 plate reader with an excitation at 530 nm and emission at 570 nm. For determination of dissociation constants (K_d), the background corrected fluorescence polarization was plotted against the concentrations in µM. The data was fitted to 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 ligand, using SigmaPlot software. The mP values for various concentrations were then divided by the highest mP value to get fraction bound as plotted in the graph.

9. Isothermal titration calorimetric measurements

Isothermal titration calorimetric (ITC) measurements were carried out at 25°C on an ITC_{200} (Microcal, Malvern).[8] Both protein and peptide **3** were dissolved in 50 mM Tris-HCl pH 8.0, 200 mM NaCl and 10% glycerol. The sample cell contained the protein and the syringe had a 10-fold higher peptide concentration. Each titration comprised of an initial injection of 0.4 µL lasting 0.8s, followed by 19 injections of 2 µL lasting 4s each at 2 min intervals with a stirring speed of 750 rpm. The initial injection was discarded during data analysis. The micro syringe (40 µL) was loaded with a solution of the peptide sample at a concentration of 2-4 mM and it was injected into the cell (200 µL), occupied by a protein at a concentration of 150-250 µM. The data points were fitted to a single binding site model using the Microcal ITC_{200} Origin data analysis software to yield enthalpies of binding (ΔH) and binding constants (K_a). Further thermodynamic parameters i.e. change in entropy (ΔS), change in free energy (ΔG) and dissociation constants (K_d) were calculated from these values.

10. Photo-crosslinking with peptide, semisynthetic histone $H3K_C9me3$ and histone extracts

1µM of TAMRA-labeled H3K9me3 peptides **2, 4** and **5**, one at a time, was incubated with 25 µM CBX1-WT or mutants in binding buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 0.5 mM TCEP) for 30 mins at 4°C. The samples were irradiated with UV light at 365nm for 30 mins at 4°C using Transilluminator 2040 EV (Stratagene). Subsequently samples were heated at 95°C for ten minutes with the 4x laemmeli sample buffer (Bio-rad), pre-mixed with β-mercaptoethanol and proteins were separated on a 15% SDS-PAGE gel. The protein bands were visualized on ChemiDoc MP Imaging System using excitation filter 605/50 for TAMRA unit.

10 µg of synthetic H3K9cMe3 or histone H3 from extracted histones was incubated with 45 µM of CBX1 mutants in the binding buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 0.5 mM TCEP). The samples were subjected to UV irradiation at 365 nm for 30 min at 4°C. Negative controls were kept in the dark. The samples were separated on 15% SDS-PAGE gel and transferred onto 0.45 µm PVDF membrane at a constant voltage of 80V for an hour at 4°C.

The membrane was washed once in TBST buffer (50 mM Tris pH 7.4, 200 mM NaCl, and 0.01% Tween) for 5mins at room temperature and blocked for an hour at room temperature in 5% milk buffer prepared in TBST. Immunoblotting was performed with primary antibodies with dilutions as per manufacturer's protocol for Anti-6X His tag® antibody - ChIP Grade (cat #ab9108, Abcam) and H3 C-term (Invitrogen cat# 701517) overnight at 4ºC. The membranes were washed with TBST buffer thrice at room temperature for five minutes each. The blots were then incubated with the HRP conjugated secondary antibodies Goat anti-Rabbit IgG (Active Motif cat# 15015) with 5% nonfat dry milk, dilution 1:5000 in TBST. The membranes were washed again with TBST buffer thrice at RT for five minutes each. Protein bands were visualized by chemiluminescence using VISIGLO HRP Chemiluminescent substrates A and B (cat# N252-120ML and N253-120ML, aMReSCO) following manufacturer's protocol.

11. Expression of CBX1-Y26AzF mutant in HEK293T cells

Wild type full-length human CBX1 with N-term HA tag in ampicillin resistant pcDNA 3.1 vector was purchased from Addgene (Addgene #24079). We introduced a Strep affinity tag in the C-terminal followed by amber stop codon at Y26 using QuickChange Lightening site-directed mutagenesis kit (Agilent Technologies) on the wild type CBX1 construct following manufacturer's protocol. The plasmids were confirmed by sequencing.

Maintain HEK293T cells in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 ^{0}C in a 5% CO_2 atmosphere. Transfect the cells grown to 60–80% confluence using Lipofectamine 2000. To 500µL DMEM, added 5µg of CBX1-Y26TAG mutant, 5µg of suppressor tRNA cDNA [RS-V1(AzF)-pCDNA 3.1] and 0.5 µg of mutant amino-acyl tRNA synthetase cDNA for AzF [RS pSVB-YAM-pUC backbone] and incubated at room temperature for 15 min. The evolved tRNA and synthetase pair was a kind gift from Prof. Thomas Sakmar from the Rockefeller University.[9] We also performed a similar transfection using the wild type CBX1 and empty pCDNA vector to serve as negative controls. Add this mixture to 500µL of DMEM with 21 µL Lipofectamine. After equilibrating 15 min at room temperature, bring the total volume to 4 mL. Apply transfection mixture to cells, and return to 37 ^{0}C in 5%CO_2 atmosphere. After 4–6 h, supplement the cells with 4 mL DMEM containing 20% FBS and 1 mM AzF. On the next day, replace the growth media with DMEM containing 10% FBS and 0.5 mM AzF. Harvest cells 48 h post-transfection, to analyze expression.

The cells were harvested by trypsinization and, washed twice with ice-cold 10 mM phosphate-buffered saline (PBS) pH 7.2. Pelleted cells were re-suspended in nuclei isolation buffer [NIB; 15 mM Tris–HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 250 mM sucrose, and 1X protease inhibitor mixture]. An equal volume of NIB buffer containing 0.6% NP-40 was added to the cells, the suspension was gently mixed and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 2000 *g* for 5 min at 4 °C and supernatant was discarded. The nuclei pellets were suspended in nuclei extraction buffer (NEB; 25 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40, and 1X protease inhibitor mixture). Samples were sonicated and centrifuged at 2000 *g* for 5 min at 4 °C. The nuclear extracts were separated on a 4-12% Criterion XT precast SDS-PAGE gel (Bio-Rad Laboratories) and analyzed for expression of CBX1 by Western blotting as described below. 100 µg nuclear extract were separated on 4-12%-SDS-PAGE and transferred onto a 0.2 µm supported nitrocellulose membrane (Bio-Rad Laboratories) at a constant current of 80 V for 1 hr. at 4 °C. Membranes were blocked with 5 mL of TBST buffer (50 mM Tris HCl pH 7.4, 200 mM NaCl, 0.01% Tween-20) with 5% nonfat dry milk for 1 hr at room temperature with gentle shaking. The blocking buffer was then removed, and membranes were rinsed with 5 mL of TBST buffer. Immunoblotting was performed with 1:500 diluted primary antibody (HA-tag mAb, Invitrogen Catalog#26183) for overnight at 4 °C. The antibody solutions were removed, and membranes were washed three times with TBST buffer. The blots were then incubated with HRP-conjugated secondary antibody Goat anti-Mouse IgG (cat# 15014, Active motif) with 5% nonfat dry milk (1:5000 dilution) in TBST for 1.5 h at room temperature. After similar washing, protein bands were visualized by chemiluminescence using VISIGLO HRP Chemiluminescent substrates A and B (cat# N252-120ML and N253- 120ML, aMReSCO) following manufacturer's protocol.

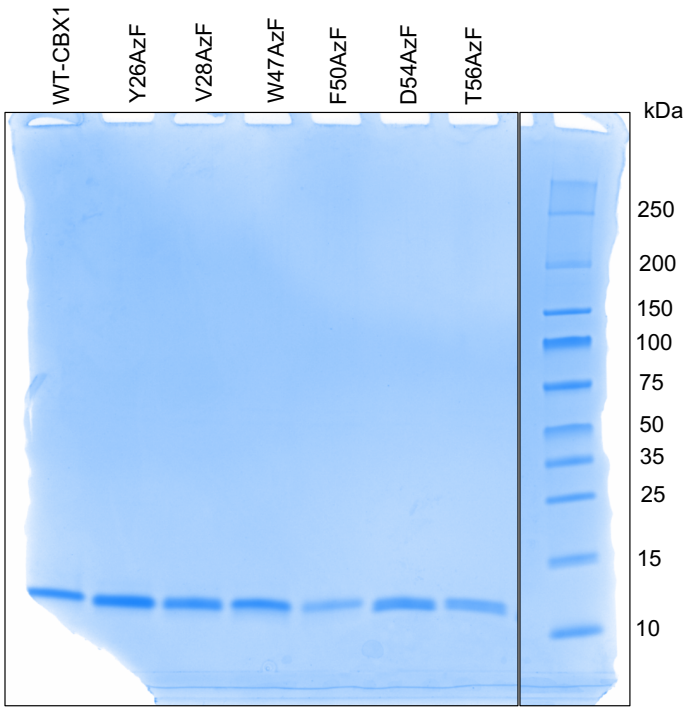
12. In-cell crosslinking, nuclear extraction and affinity tag-based pull-down

After growing the cells for 48 hours post transfection with AzF on 10 cm plates as described above, the cells were washed twice with PBS. In 5mL of cold PBS the cells were either exposed to U.V. light at 365nm for 30 minutes, at 4 °C (in cold room) or harvested directly.

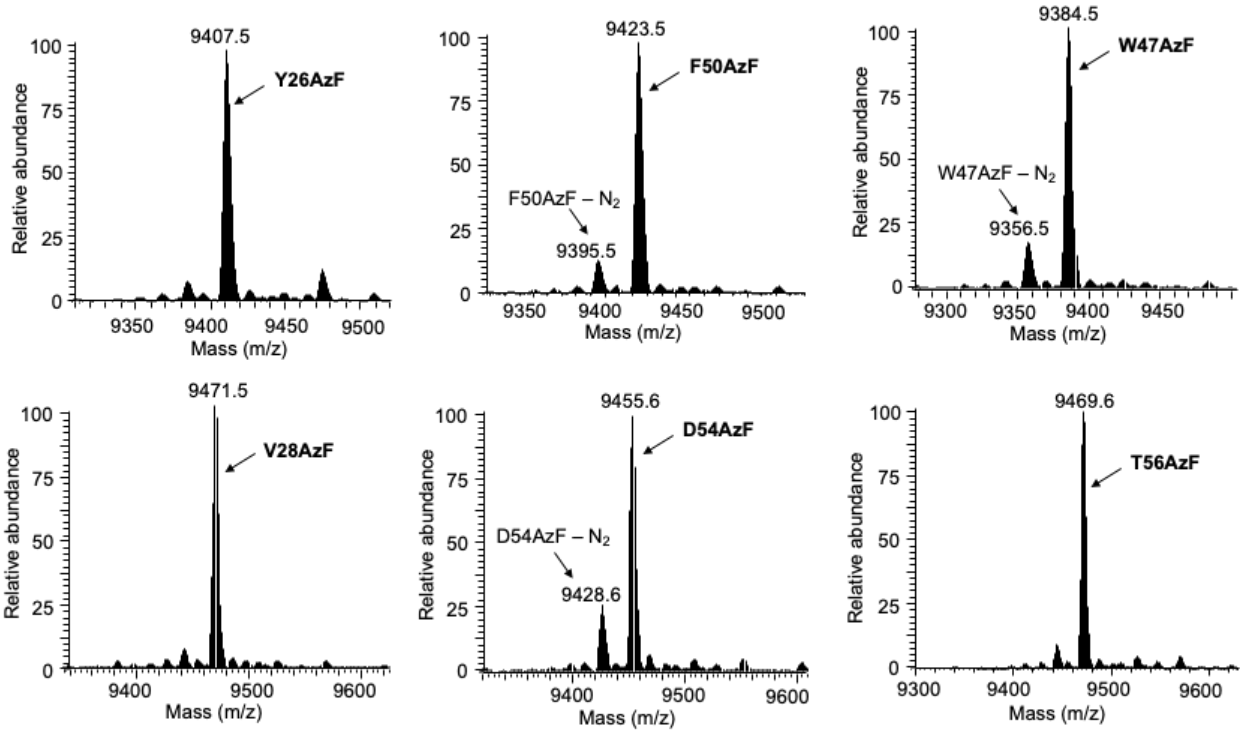
The cells were then uplifted with trypsin to isolate intact nuclei. Nuclei was isolated by incubating the HEK293T cells with 15 mM Tris pH 8.0, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1mM $CaCl_2$, 1mM DTT, 2 mM sodium orthovanadate, 250 mM sucrose, 1x Protease inhibitor, and 0.4%

NP-40 buffer, at 4° C for 5 mins. After centrifugation at 2000 g for 5 mins at 4° C the nuclei pellet was resuspended in 50 mM Tris pH 8.0, 150 mM NaCl and 1x protease inhibitor. Streptactin beads (Strep-Tactin Superflow Plus (2 mL) from Qiagen, Catalog #30002) were washed with ddI H_2O twice and equilibrated with 50 mM Tris pH 8.0 at room temperature. 5µg of nuclei was incubated with the washed beads for an hour at room temperature with gentle shaking. The beads were then washed thrice to remove non-specific interactions with 200 mM NaCl, 50 mM Tris pH 8.0, 0.1% Triton X-100 and 1xprotease inhibitor tablet. Samples were eluted with 4x Dye and heating at 95° C for 5 mins. The supernatant was loaded on 4-12% Criterion XT precast gel (Bio-Rad Laboratories) and stained with Coomassie brilliant blue R-250 staining solution to detect enrichment of crosslinked proteins.

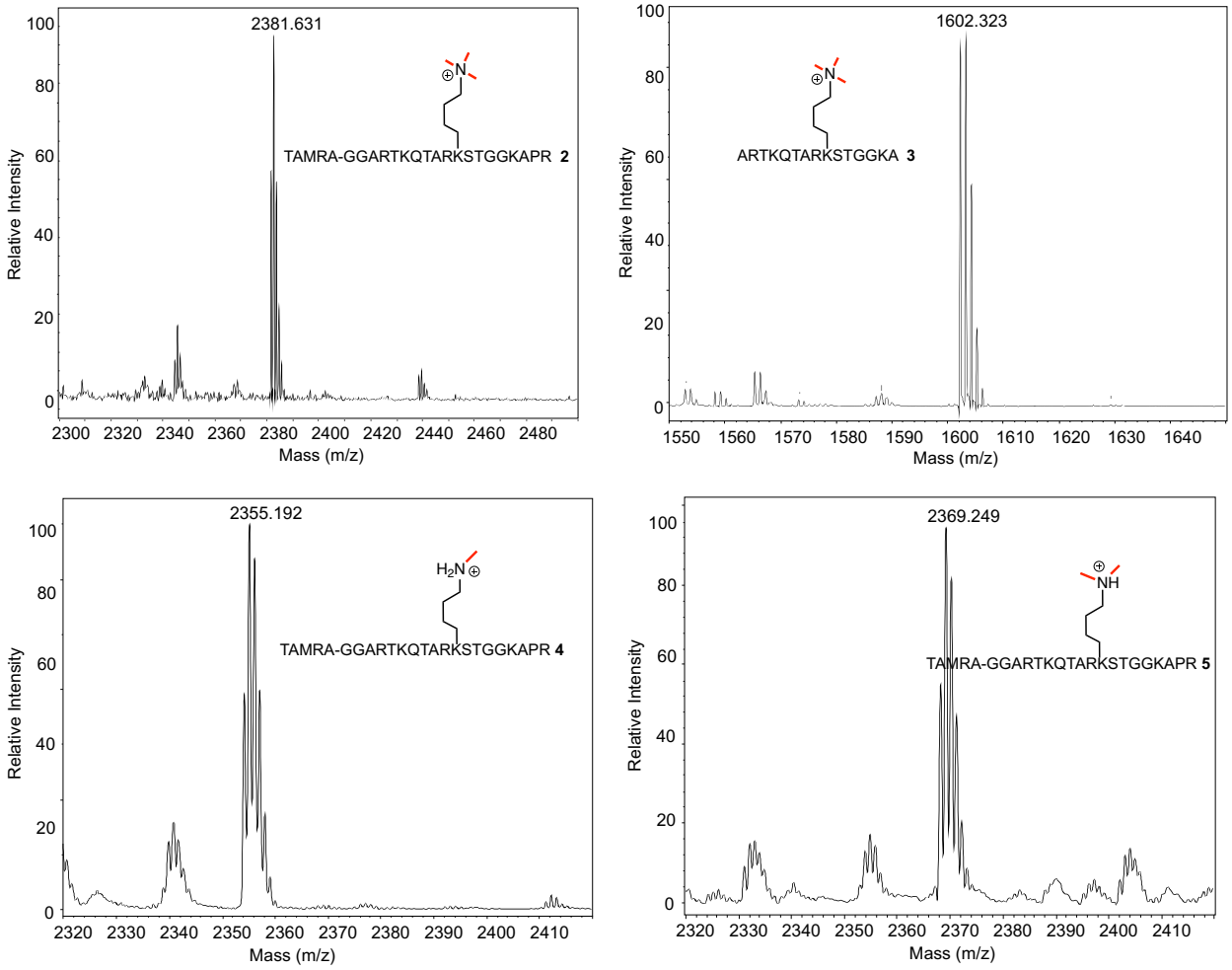
13. Supplementary figures and tables



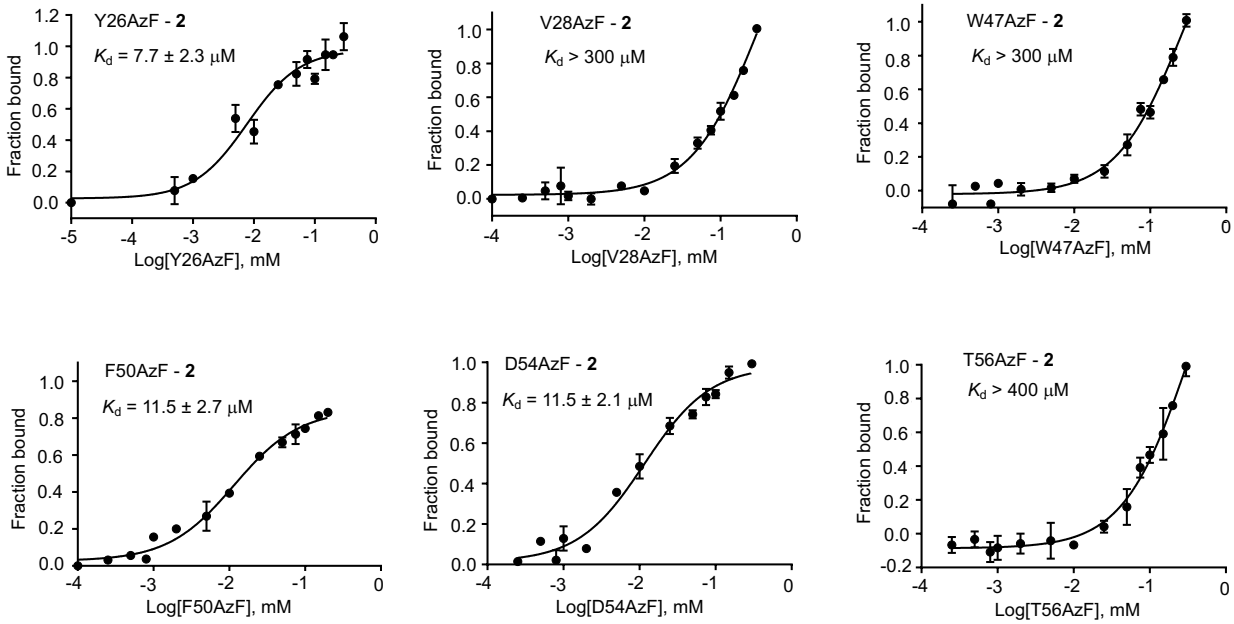
Supplementary Figure S1. Coomassie blue staining showing expression and purity of wild type CBX1 chromodomain and its AzF containing mutants. ESI-MS analyses of intact proteins confirmed the integrity of these proteins (See Supplementary Figure S2 and Table S1).



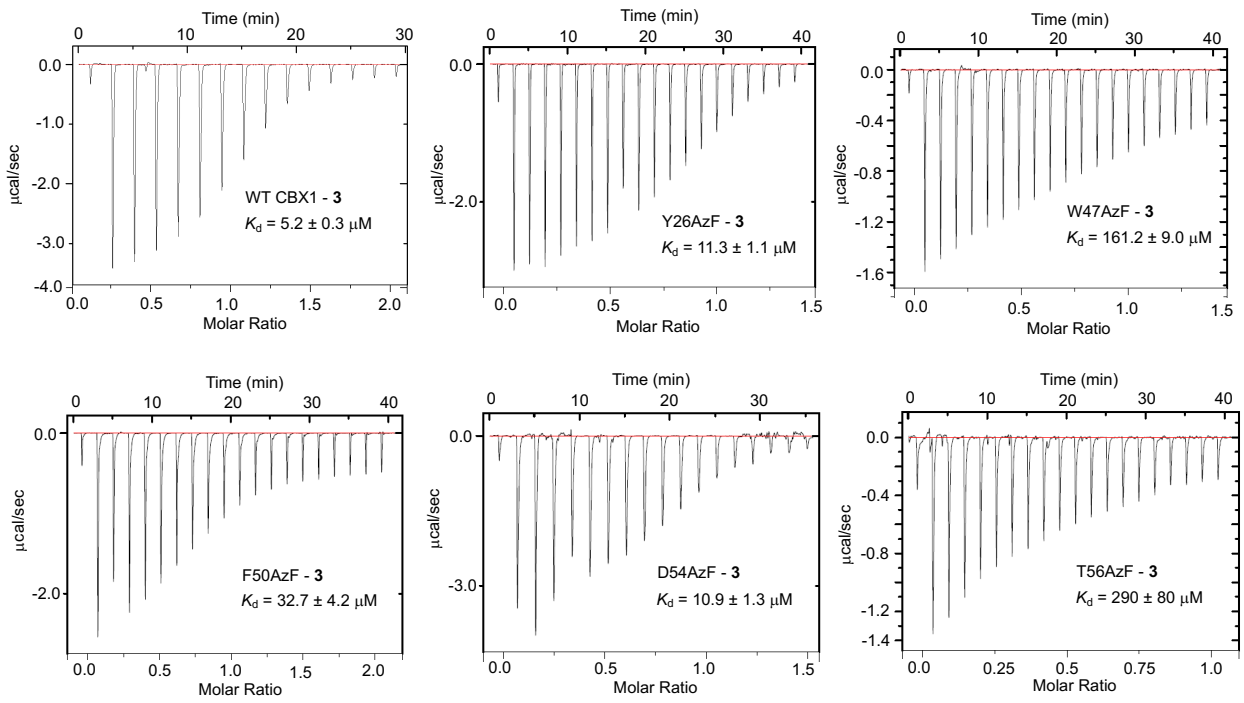
Supplementary Figure S2: ESI-MS spectra of CBX1 chromodomain mutants carrying AzF. The proteins were purified as single species by FPLC (See Figure S1). The observed loss of nitrogen molecule likely during mass acquiring process. The relative percentages of other small impurities are not determined.



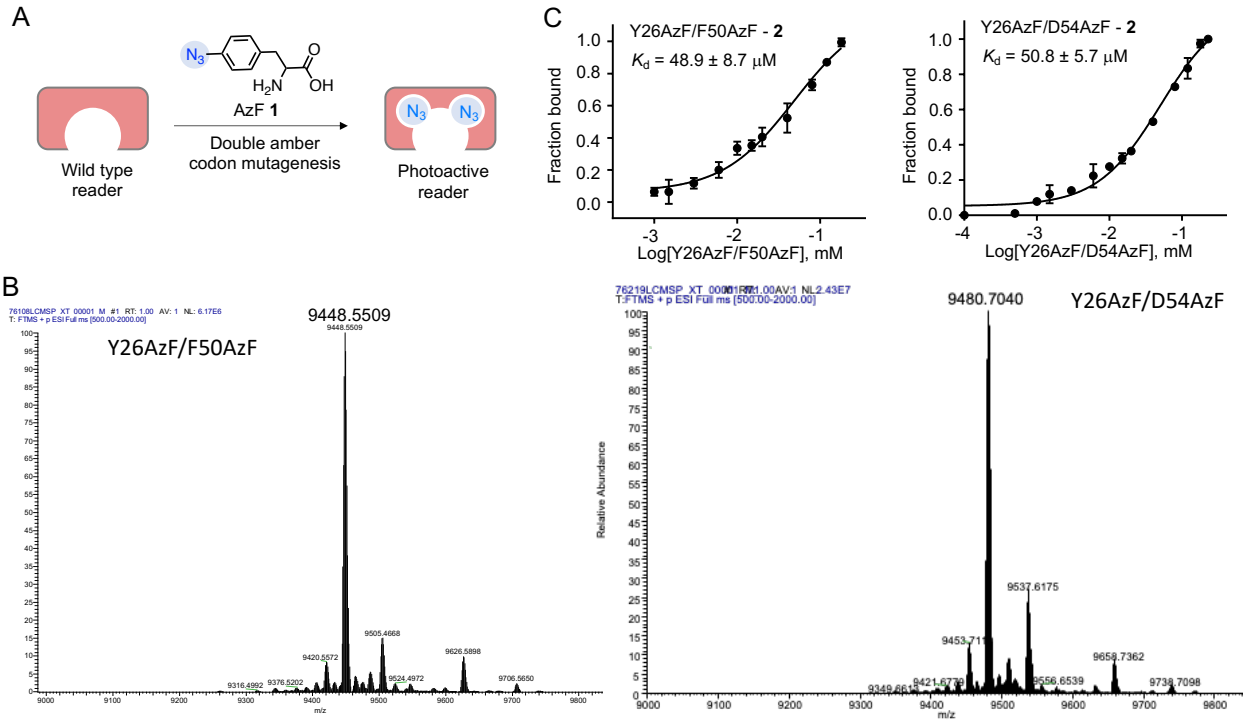
Supplementary Figure S3. MALDI-MS spectra of peptides **2** - **5**. The peptides were purified as single species by HPLC. The relative percentages of the small impurities are not determined.



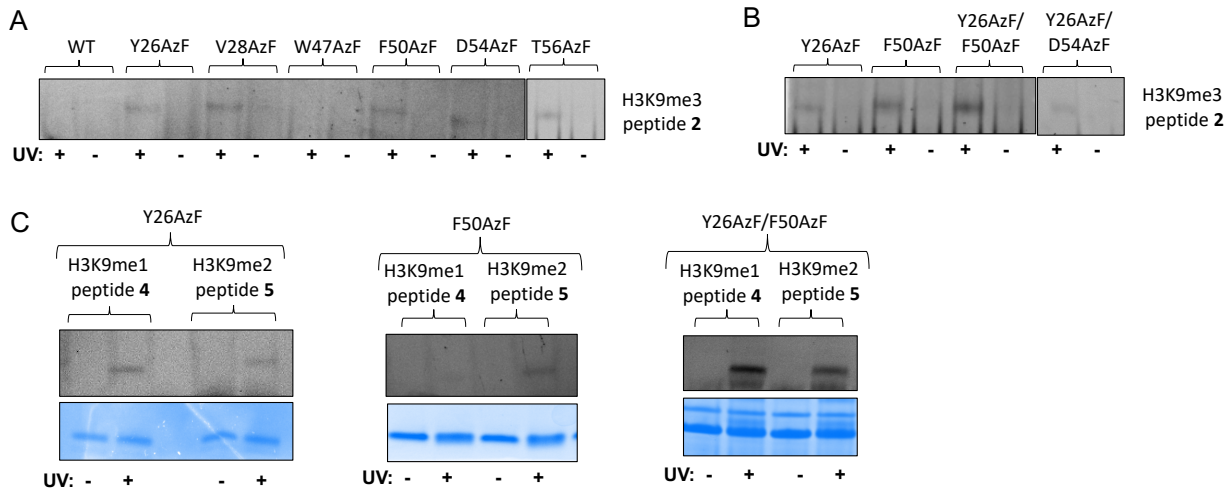
Supplementary Figure S4. Dissociation constants of CBX1 chromodomain mutants towards the TAMRA-labeled methylated histone H3 peptide **2** as determined by background-corrected fluorescence polarization values. Error bars represent standard deviation from two independent measurements. We did not observe saturation in mP values for the weakly bound peptides. For these cases, K_d values are estimated based on the approximate Bmax values.



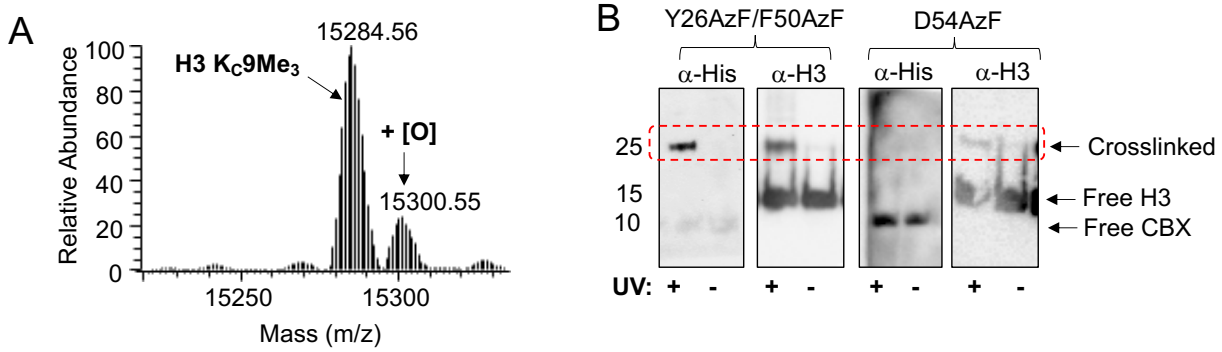
Supplementary Figure S5. Dissociation constants of methylated histone H3 peptide **3** to wild type CBX1 chromodomain and selected mutants as measured by isothermal titration calorimetry. Experimental details and thermodynamic parameters are provided in section 9 and Supplementary Table S2, respectively.



Supplementary Figure S6. (A) Schematic showing site-specific incorporation of two AzF units in CBX1 chromodomain. (B) ESI-MS spectra of the Y26AzF/F50AzF and Y26AzF/D54AzF mutants. (C) Dissociation constant of the double mutants from peptide B as measured by fluorescence anisotropy.



Supplementary Figure S7. (A) In-gel fluorescence showing photo-crosslinking of CBX1 chromodomain (wild type and AzF mutants) towards TAMRA-attached H3K9me3 peptide **2**. While wild type protein did not show any crosslinking, the mutants underwent crosslinking to varied degrees as evident from the fluorescent bands only in the presence of UV light. (B) Comparative crosslinking experiment with peptide **2** demonstrated that Y26AzF/F50AzF mutant was marginally better than the individual single mutants. (C) In-gel fluorescence showing photo-crosslinking of Y26AzF, F50AzF and Y26AzF/F50AzF mutants towards TAMRA-H3K9me1 **4** and TAMRA-H3K9me2 **5** peptides. Consistently, the double mutant showed enhanced crosslinking.



Supplementary Figure S8. (A) ESI-MS spectrum of semisynthetic full-length H3 carrying $K_C$me3 at position 9. The H3 was employed to crosslink CBX1 mutants. See Figure 4A in the manuscript for relevant Western blot data. (B) Western blotting with anti-6xHis and H3 antibodies show crosslinking of Y26AzF/F50AzF and D54AzF mutants with nucleosomal H3. D54AzF mutant did not undergo noticeable crosslinking with the nucleosomal H3.

CBX-Protein	Expected Mol. Wt.	Observed Mol. Wt.
Y26AzF	9408.32	9407.50
V28AzF	9472.36	9471.52
W47AzF	9385.28	9384.54
F50AzF	9424.32	9423.51
D54AzF	9456.41	9455.63
T56AzF	9470.39	9469.61
Y26AzF/D54AzF	9481.41	9480.72
Y26AzF/F50AzF	9449.32	9448.55

Supplementary Table S1: Calculated and experimental molecular weights of CBX1 chromodomain mutants developed in the current study.

CBX-Protein	K_d (μM) by ITC	N	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol/deg)
WT	5.2 ± 0.3	0.985 ± 0.01	-7.20	-7.99 ± 0.07	-2.65
Y26AzF	11.3 ± 1.1	0.905 ± 0.01	-6.78	-7.65 ± 0.10	-3.04
W47AzF	161.2 ± 9.0	0.830 ± 0.02	-5.22	-6.53 ± 0.34	-4.55
F50AzF	32.7 ± 4.2	0.952 ± 0.03	-6.20	-8.73 ± 0.38	-8.77
D54AzF	10.9 ± 1.3	0.806 ± 0.02	-6.84	-9.06 ± 0.19	-7.69
T56AzF	290.0 ± 80.0	0.243 ± 0.174	-5.20	-1.58 ± 1.33	-36.7

Supplementary Table S2. Thermodynamic parameters measured by isothermal titration calorimetry (ITC) for the binding of peptides **3** to Wild type CBX1 chromodomain and its mutants.

CBX1 mutants	Primer Sequences
Y26TAG	GGAGGAGGAGGAA**TAG**GTGGTGGAAAA AGTTCTCG
V28TAG	GAGGAGGAATATGTG**TAG**GAAAAAGTTCTCGACCGT
W47TAG	GGAGTACCTCCTAAAG**TAG**AAGGGATTCTCAGATGAGG
F50TAG	CTAAAGTGGAAGGGA**TAG**TCAGATGAGGACAAC
D54TAG	GGATTCTCAGATGAG**TAG**AACACATGGGAGCCA
T56TAG	CTCAGATGAGGACAAC**TAG**TGGGAGCCAGAAGA
Y26TAG/F50TAG	Used F50TAG primers on fully-sequenced Y26TAG construct
Y26TAG/D54TAG	Used D54TAG primers on fully-sequenced Y26TAG construct
FL-hCBX1-Y26TAG	GAAGAGGAGGAAGAGGAA**TAG**GTGGTGGAAAAAGTTCTC GACCG
FL-hCBX1_Strep	AAAAGATGACAAGAACTGGAGTCACCCGCAGTTCGAAAAGTAACG GCGTCGAGCATGCAT

Supplementary Table S3. List of the forward primers designed for site-directed mutagenesis. Reverse primers used are the reverse-complement to the given forward primers.

14. References

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