Supporting Information for

Engineered Reader Proteins for Enhanced Detection of Methylated Lysine on Histones

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Materials and Methods

Protein Sequences

HP1α wild-type Protein sequence:
MKKHHHHHHAEEEEEEYAVEKIIDRRVRKGMVEYYLKWKGYPETENTWEPENNLDCQDLIQQ
YEASRKD

HP1α A25T/K46A/E52D/D62F Protein sequence:
MKKHHHHHHAEEEEEEY**T**VEKIIDRRVRKGMVEYYLKW**K**GYPET**D**NTWEPENNL**F**CQDLIQQ
YEASRKD

CBX1 wild-type protein sequence:
MGSSHHHHHHSSGLVPRGSHMEEEEEEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEP
EENLDCPDLIAEFLQSQKTLEHHHHHH

CBX1 K43A/D59F protein sequence:
MGSSHHHHHHSSGLVPRGSHMEEEEEEYVVEKVLDRRVVKGKVEYLLKW**A**GFSDEDNTWEP
EENL**F**CPDLIAEFLQSQKTLEHHHHHH

(CBX1 wild-type)$_3$ protein sequence:
MBP-
MEEEEEEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEPEENLDCPDLIAEFLQSQKT*GG
GGSGGGGS*EEEEEEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEPEENLDCPDLIAEFL
QSQKT*GGGGSGGGGS*EEEEEEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEPEENLDC
PDLIAEFLQSQKT

(CBX1 K43A/D59F)$_3$ protein sequence:
MBP-
MEEEEEEYVVEKVLDRRVVKGKVEYLLKW**A**GFSDEDNTWEPEENL**F**CPDLIAEFLQSQKT*GG
GGSGGGGS*EEEEEEYVVEKVLDRRVVKGKVEYLLKW**A**GFSDEDNTWEPEENL**F**CPDLIAEFL
QSQKT*GGGGSGGGGS*EEEEEEYVVEKVLDRRVVKGKVEYLLKW**A**GFSDEDNTWEPEENL**F**C
PDLIAEFLQSQKT

CBX3 wild-type protein sequence:
MGSSHHHHHHSSGLVPRGSHMAEPEEFVVEKVLDRRVVNGKVEYFLKWKGFTDADNTWEPE
ENLDCPELIEAFLNSQKLEHHHHHH

CBX3 K58A/D74F protein sequence:
MGSSHHHHHHSSGLVPRGSHMAEPEEFVVEKVLDRRVVNGKVEYFLKW**A**GFTDADNTWEPE
ENL**F**CPELIEAFLNSQKLEHHHHHH

CBX5 wild-type protein sequence:
MGSSHHHHHHSSGLVPRGSHMSEDEEEYVVEKVLDRRMVKGQVEYLLKWKGFSEEHNTWE
PEKNLDCPELISEFMKKYKKMKELEHHHHHH

CBX5 K26A/E31D/D42F protein sequence:
MGSSHHHHHHSSGLVPRGSHMSEDEEEYVVEKVLDRRMVKGQVEYLLKW**A**GFSEE**D**NTWE
PEKNL**F**CPELISEFMKKYKKMKELEHHHHHH

Peptide Synthesis and Purification

Residues 1-15 of H3 with trimethylated lysine at position 9 (Sequence: ARTKQTARKme3STGGKAY, furthermore denoted as H3K9me3) was synthesized on a CEM Liberty Blue peptide synthesizer using Fmoc protected amino acids and Rink Amide AM resin (CEM). A tyrosine residue was placed at the C-terminus for concentration determination. The amino acid residues were activated with Oxyma (Ethyl cyanohydroxyiminoacetate) and DIC (N,N'-Diisopropycarbodiimide). 5 equivalents of the amino acid, Oxyma, and DIC were used for each coupling step. Two coupling cycles of 4 minutes were performed at 90ºC in DMF (dimethylformamide) for each residue. Deprotections of Fmoc were carried out in 20% piperidine in DMF, twice for 1 minute each. The resin was washed with DMF before every deprotection and coupling cycle.

Trimethyllysine was incorporated into the H3 peptide by first double coupling 2 equivalents of Fmoc-Lys(Me)2-OH·HCl (from P3 BioSystems) for 4 minutes at 90ºC in DMF with Oxyma/DIC activation. Immediately after coupling, the resin was washed with DMF, and the residue was further methylated in presence of 7-methyl-1,5,7-triaza-bicyclo[4.4.0]dec-5-ene (MTDB, 1.2 eq) and methyl iodide (10 eq) in DMF for 4 minutes. The resin was washed with DMF and peptide synthesis was continued with aforementioned conditions.

Peptides were cleaved from the resin with 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropylsilane for 3 hours. The TFA was evaporated and products were precipitated with cold diethyl ether. The resulting peptides were extracted with water and lyophilized. Crude peptide material were purified by reversed phase HPLC using a C18 XBridge 5 µM column (Waters) and a gradient of 0 to 20% B in 60 minutes, where solvent A was 95:5 water:acetonitrile, 0.1% TFA and solvent B was 95:5 acetonitrile:water, 0.1% TFA. The purified peptides were lyophilized, and identity was confirmed by ESI-LCMS **(Figure S2)**.

Circular dichroism (CD) of wild-type and engineered HP1α and CBX chromodomains

CD experiments were performed using an Applied Photophysics Chirascan. Spectra were obtained with 25 µM chromodomain in 10 mM sodium phosphate buffer, pH 7.4 at 20°C. All scans were corrected with buffer subtraction. The mean residue ellipticity (MRE) was calculated using eq 1, where θ is MRE, signal is CD signal, l is path length, c is protein concentration, and r is the number of amino acid residues. All spectra were measured using a quartz cuvette with a path length of 0.1 cm **(Figure S3A, Figure S5A)**.

$$\theta = \frac{signal}{10 \cdot l \cdot c \cdot r} \quad (1)$$

Thermal denaturation experiments were performed using the same buffer and concentrations as described above. Measurements were taken between 20 and 80°C. The melting curves were normalized to show fraction folded (α), calculated using eq 2, where θ is the observed MRE, θ_D is the MRE for the fully denatured protein, and θ_F is the MRE for the fully folded protein **(Figure S3B, Figure S5B)**.

$$\alpha = {(\theta - \theta_D)}\Big/{(\theta_F - \theta_D)} \quad (2)$$

Histone peptide microarrays

Histone peptide microarrays were performed as described in Petell et al., 2019.[1] In brief, 500 nM of MBP-tagged protein in 1 X PBST (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.6, 0.1% Tween-20) with 1% non-fat milk was incubated with the array overnight in a humidity chamber at 4ºC, 1000 rpm shaking. The next day the arrays were briefly washed in 1 X PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.6), then cross-linked

by incubating with 1 X PBS containing 0.1% formaldehyde for 15 seconds with inversions. To quench the arrays were placed into 1 X PBS with 1M glycine for 60 seconds while performing inversions and then washed in 1x PBS by inverting five times. After cross-linking, the arrays were then incubated with 1 X PBS with high salt (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 497 mM NaCl, pH 7.6) three times for five mutes each. The array was then incubated with 1:1000 anti-MBP antibody (ThermoFisher, PA1-989) in 1 X PBST with 1% milk for one hour at 4 ºC, 1000 rpm shaking. Next, the arrays were washed three times for five minutes with 1 X PBS, then incubated in the dark with 1:20000 anti-rabbit AlexaFluor 647 (Invitrogen, A21244) in 1 X PBST with 1% milk for 30 minutes with 1000 rpm shaking at 4 ºC. Lastly, the arrays were again washed three times for five minutes with 1 X PBS and then rinsed by submerging into 0.1 X PBS followed by blowing dry with air. The arrays were then imaged using a Typhoon imaging system and quantified with ImageQuant TL. The representative images were linearly adjusted for brightness and background were similar for visual comparison.

Quantification was done as described previously.[1] The values for each set of triplicate peptide spots on a subarray were averaged and scaled linearly between 0 – 1 using the minimum and maximum values for that subarray as limits. These values for the subarrays were then averaged and sorted based on the modifications listed in the figures and supplemental datasets. See Supporting Information for the average and standard deviations of individual peptides.

In solution peptide pulldowns

For each pulldown, 5 pmol of protein was incubated with 500 pmol of the indicated peptide for one hour at 4 ºC with rotation in peptide pulldown buffer (50 mM Tris, 0.1% NP-40, 0.5% BSA, 300 mM NaCl, pH 8.0). After this, 5 µL of streptavidin coated magnetic beads (Thermofisher, 88816) were blocked with peptide pulldown buffer and transferred to the protein and peptide mixture, then incubated for one hour more as before. Next, the beads were washed three times for five minutes with peptide pulldown buffer, rotating at 4ºC. Protein was then eluted from the beads by aspirating all buffer and adding 50 µL of 1 X Laemmli's loading buffer and boiling for ten minutes at 95 ºC prior to loading into a 10% SDS-PAGE. For the input controls, 1% of input was loaded onto the gel; for the beads only control, no peptide was included in the pulldown. After running the SDS-PAGE, a Western blot was performed according to standard molecular biology protocols. The primary antibody used was anti-MBP, 1:1000 (ThermoFisher, PA1-989) and the secondary antibody was anti-Rabbit-HRP at 1:20,000 (GE, NA934V).

Nucleosome binding blots

Nucleosome binding tests were performed using purified HeLa mononucleosomes (Epicypher, 16-0002) resolved by 15% SDS-PAGE, transferred, and probed similar to a Western blot with modifications. For each lane, 1 µg of nucleosomes was run and after transfer, lanes were separated for individual incubation with the indicated reader domain or antibody. After blocking with 5% non-fat milk in 1 X TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6) for 15 minutes, a single lane was incubated with the indicated concentration of chromodomain in blocking buffer (or blocking buffer alone for the blots used for the commercial antibody) for one hour at room temperature with shaking. The strip blots were then washed three times for five minutes with 1 X TBST and subsequently incubated with 1:1000 anti-MBP antibody (ThermoFisher, PA1-989) or the indicated concentration of anti-H3K9me3 antibody (Active Motif, 39161) for one hour at room temperature with shaking. The blots were again washed three times for five minutes with 1 X TBST, after which they were incubated with anti-Rabbit-HRP at 1:20,000 (GE, NA934V) in blocking buffer for one hour, with shaking, at room temperature. Last, the blots were washed three times for five minutes and then imaged after incubation with a chemiluminescent substrate according to the manufacturer (GE, RPN2232).

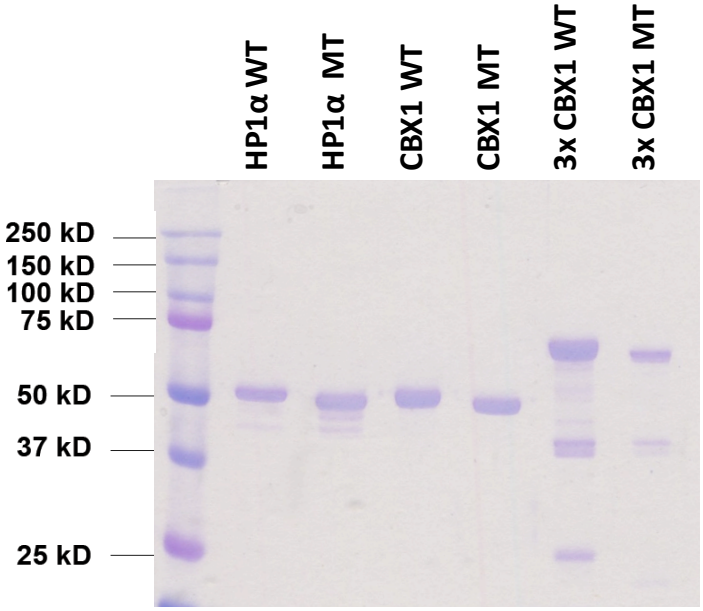


Figure S1: SDS-PAGE of HP1α and CBX chromodomains

H3K9me3: ARTKQTARKme3STGGKAY-NH_2

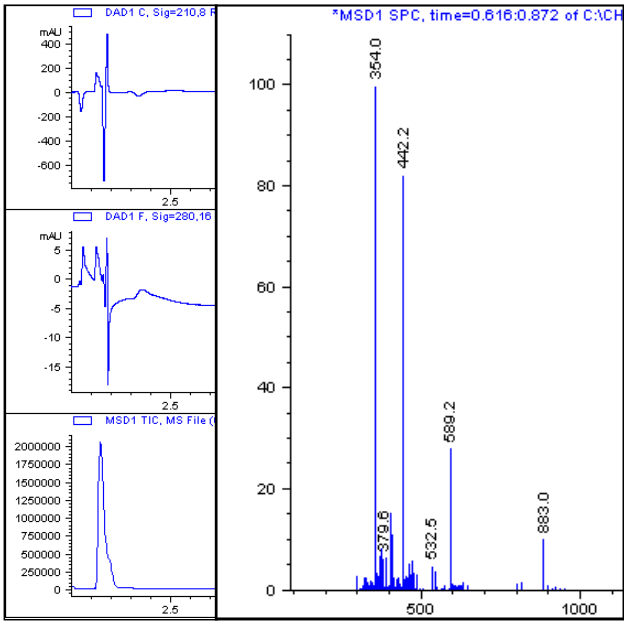
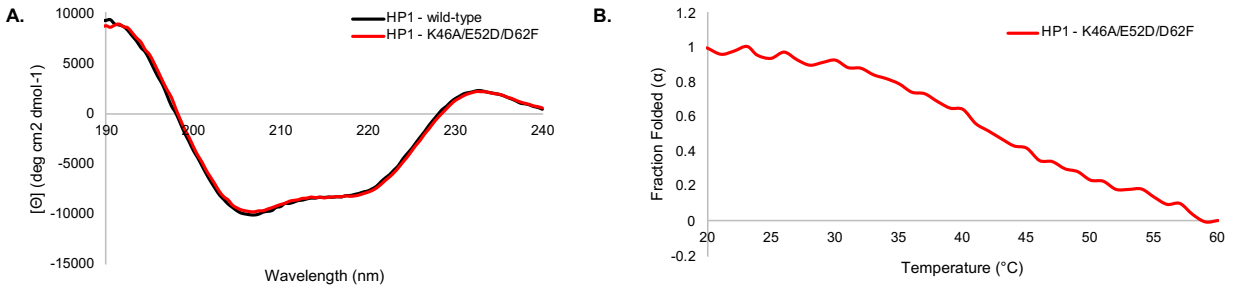


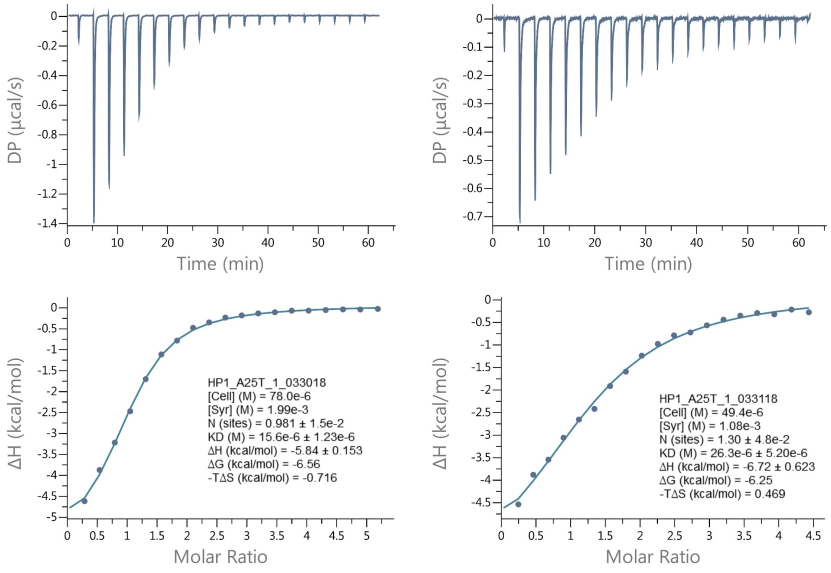
Figure S2: UV-Vis absorbance at 280 nm, TIC, and low resolution mass spectrum of H3K9me3. MS calculated: 1766.06 [M+H], 883.53 [M+2H]. MS observed: 883.0 [M+2H], 589.2 [M+3H], 442.2 [M+4H], 354.0 [M+5H].



C.

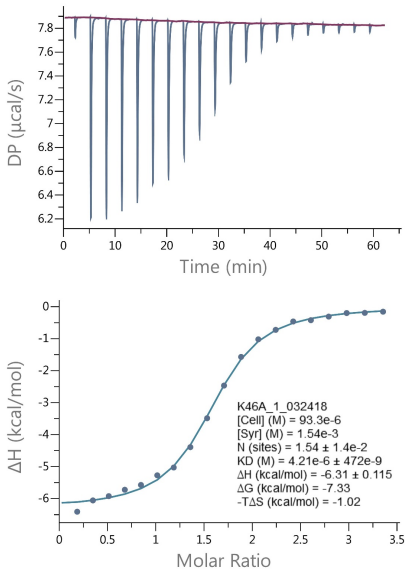
	T_m (°C)	
Protein	Wild-type	Mutant
HP1	47	42

Figure S3: Circular dichroism of HP1α wild-type and engineered chromodomain. (A) CD spectra of HP1α chromodomains in 10 mM sodium phosphate, pH 7.4. (B) Thermal melt (T_m) curve of *e*Reader HP1α in 10 mM sodium phosphate, pH 7.4. (C) T_m of wild-type HP1α[2] and engineered chromodomains; the mutations do not significantly affect the overall stability of the chromodomain.

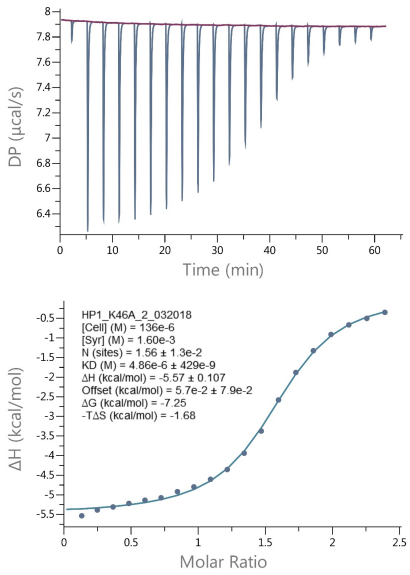


HP1 A25T – 1
[Protein] = 78.0 µM
[Peptide] = 1.99 mM
K_d = 15.6 µM
N = 0.98
C = 5

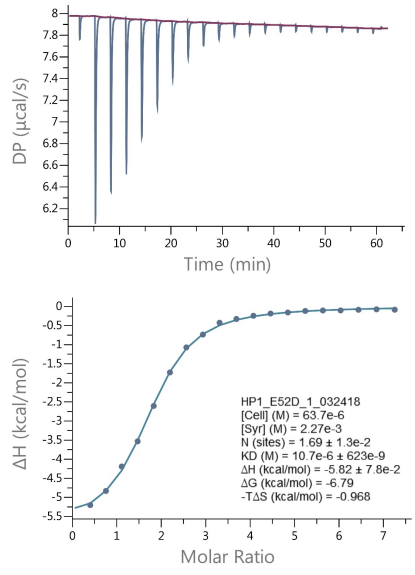
HP1 A25T – 2
[Protein] = 49.4 µM
[Peptide] = 1.08 mM
K_d = 26.3 µM
N = 1.30
C = 2



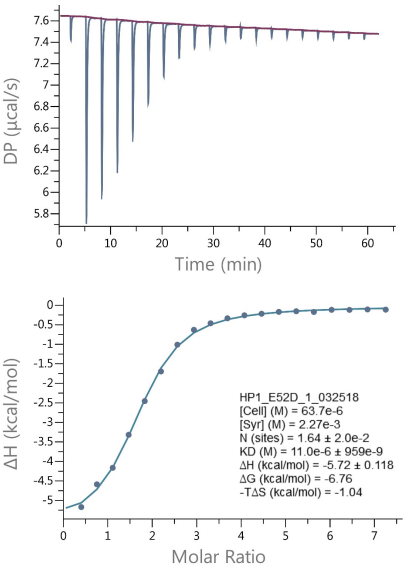
HP1 K46A – 1
[Protein] = 93.3 µM
[Peptide] = 1.54 mM
K_d = 4.21 µM
N = 1.54
C = 22



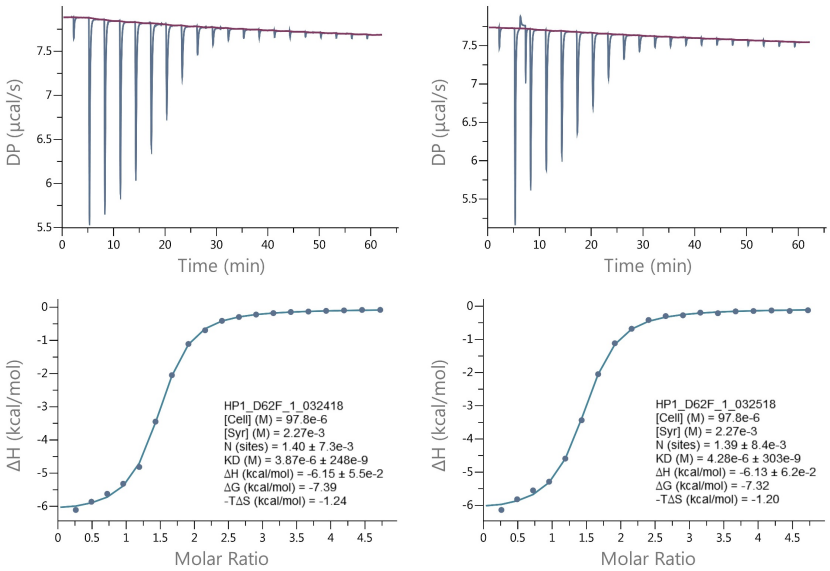
HP1 K46A – 2
[Protein] = 136.0 µM
[Peptide] = 1.60 mM
K_d = 4.86 µM
N = 1.56
C = 28



HP1 E52D – 1
[Protein] = 63.7 µM
[Peptide] = 2.27 mM
K_d = 10.7 µM
N = 1.69
C = 6

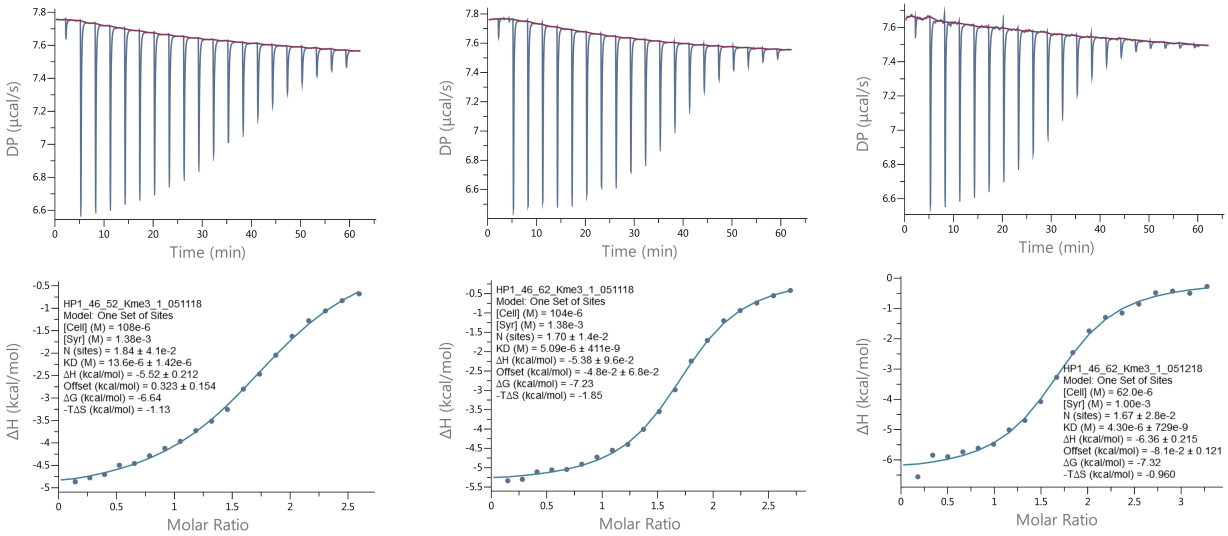


HP1 E52D – 2
[Protein] = 63.7 µM
[Peptide] = 2.27 mM
K_d = 11.0 µM
N = 1.64
C = 6



HP1 D62F – 1
[Protein] = 97.8 µM
[Peptide] = 2.27 mM
K_d = 3.87 µM
N = 1.40
C = 25

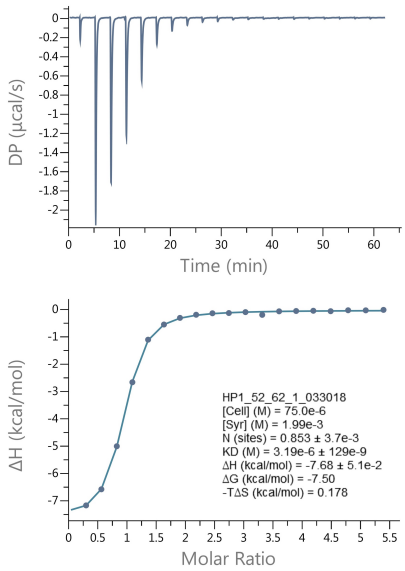
HP1 D62F – 2
[Protein] = 97.8 µM
[Peptide] = 2.27 mM
K_d = 4.28 µM
N = 1.39
C = 23



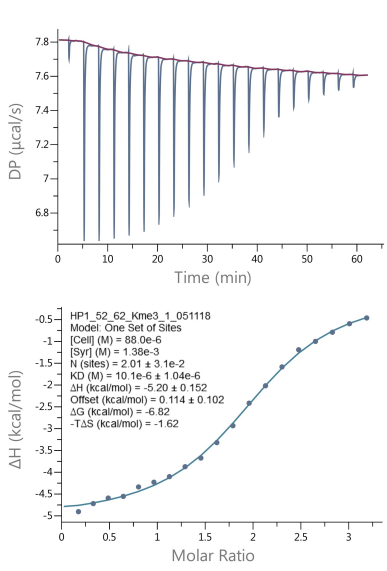
HP1 K46A/E52D – 1
[Protein] = 108.0 µM
[Peptide] = 1.38 mM
K_d = 13.6 µM
N = 1.84
C = 8

HP1 K46A/D62F – 1
[Protein] = 104.0 µM
[Peptide] = 1.38 mM
K_d = 5.09 µM
N = 1.70
C = 20

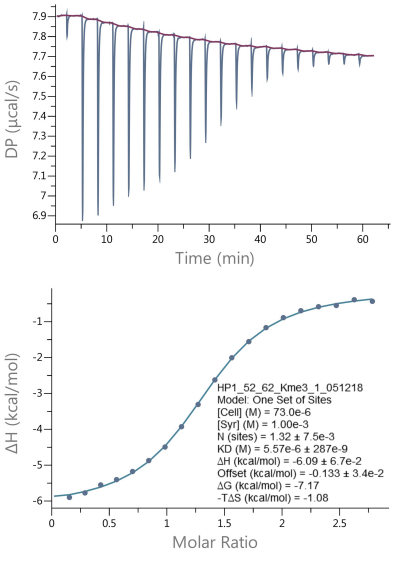
HP1 K46A/D62F – 2
[Protein] = 62.0 µM
[Peptide] = 1.00 mM
K_d = 4.30 µM
N = 1.67
C = 14



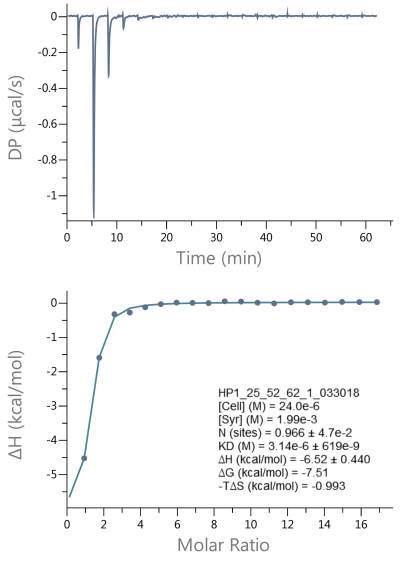
HP1 E52D/D62F – 1
[Protein] = 75.0 µM
[Peptide] = 1.99 mM
K_d = 3.19 µM
N = 0.853
C = 24



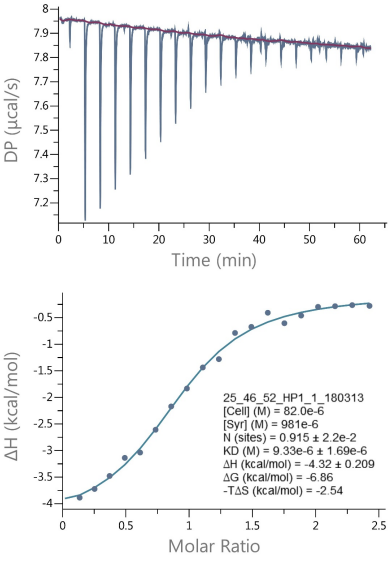
HP1 E52D/D62F – 2
[Protein] = 88.0 µM
[Peptide] = 1.38 mM
K_d = 10.1 µM
N = 2.01
C = 9



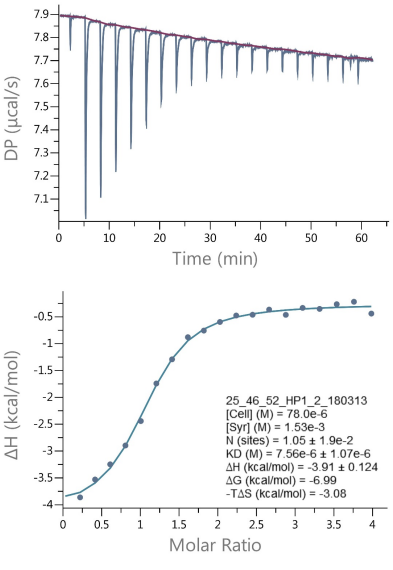
HP1 E52D/D62F – 3
[Protein] = 73.0 µM
[Peptide] = 1.00 mM
K_d = 5.57 µM
N = 1.32
C = 13



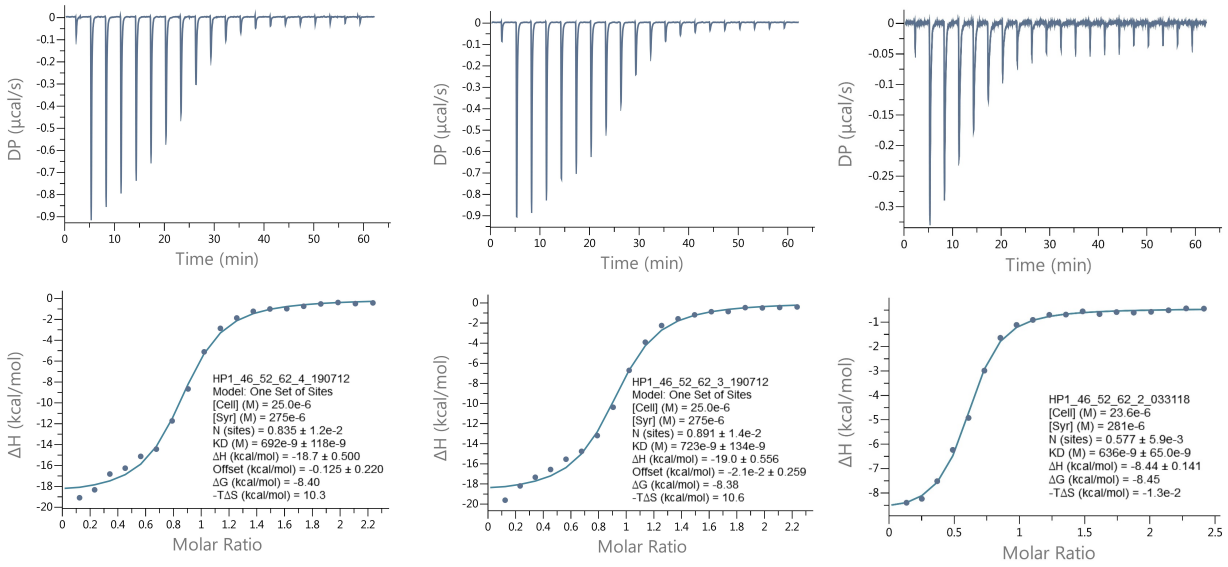
HP1 A25T/E52D/D62F – 1
[Protein] = 24.0 µM
[Peptide] = 1.99 mM
K_d = 3.14 µM
N = 0.966
C = 8



HP1 A25T/K46A/E52D – 1
[Protein] = 82.0 µM
[Peptide] = 0.981 mM
K_d = 9.33 µM
N = 0.915
C = 8



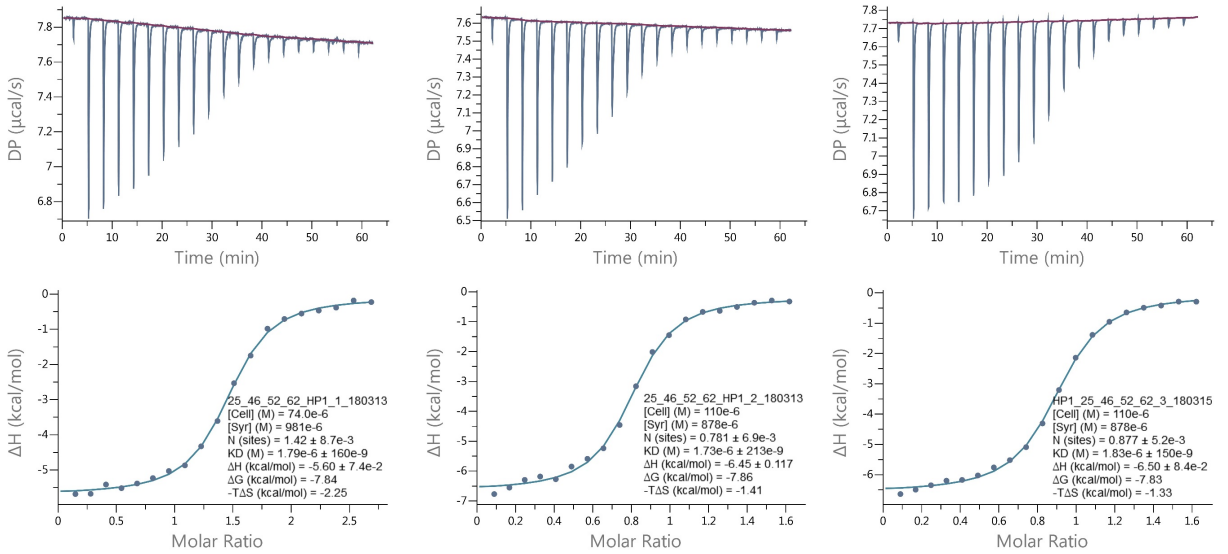
HP1 A25T/K46A/E52D – 2
[Protein] = 78.0 µM
[Peptide] = 1.53 mM
K_d = 7.56 µM
N = 1.05
C = 5



HP1 K46A/E52D/D62F – 1
[Protein] = 25.0 µM
[Peptide] = 0.275 mM
K_d = 0.692 µM
N = 0.835
C = 36

HP1 K46A/E52D/D62F – 2
[Protein] = 25.0 µM
[Peptide] = 0.275 mM
K_d = 0.723 µM
N = 0.891
C = 34

HP1 K46A/E52D/D62F – 3
[Protein] = 23.6 µM
[Peptide] = 0.281 mM
K_d = 0.636 µM
N = 0.577
C = 37



HP1 A25T/K46A/E52D/D62F – 1
[Protein] = 74.0 µM
[Peptide] = 0.981 mM
K_d = 1.79 µM
N = 1.42
C = 34

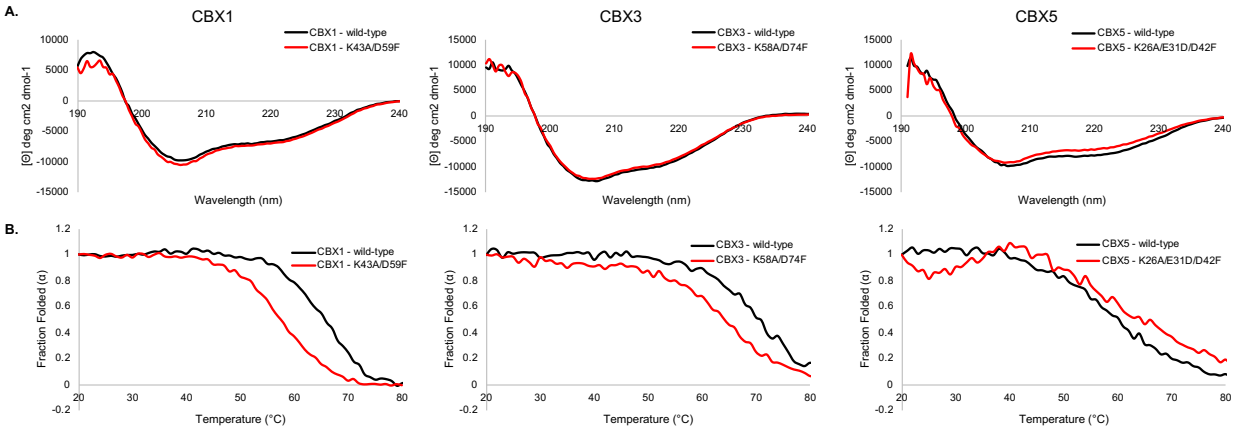
HP1 A25T/K46A/E52D/D62F – 2
[Protein] = 110.0 µM
[Peptide] = 0.878 mM
K_d = 1.73 µM
N = 0.781
C = 51

HP1 A25T/K46A/E52D/D62F – 3
[Protein] = 110.0 µM
[Peptide] = 0.878 mM
K_d = 1.83 µM
N = 0.877
C = 60

Figure S4: ITC curves of H3K9me3 binding to engineered HP1α chromodomains.

Protein	K_d (μM)
WT	14 ± 2
A25T	21 ± 8
K46A	5 ± 1
E52D	11 ± 1
D62F	4 ± 1
46/52*	14 ± 1
46/62	5 ± 1
52/62	6 ± 4
25/52/62*	3 ± 1
25/46/52	8 ± 1
46/52/62	0.7 ± 0.2
25/46/52/62	2 ± 1

Table S1: Binding affinities measured by ITC for *e*Reader HP1α chromodomains. * Denotes single run. Error is calculated from the error in the fit.

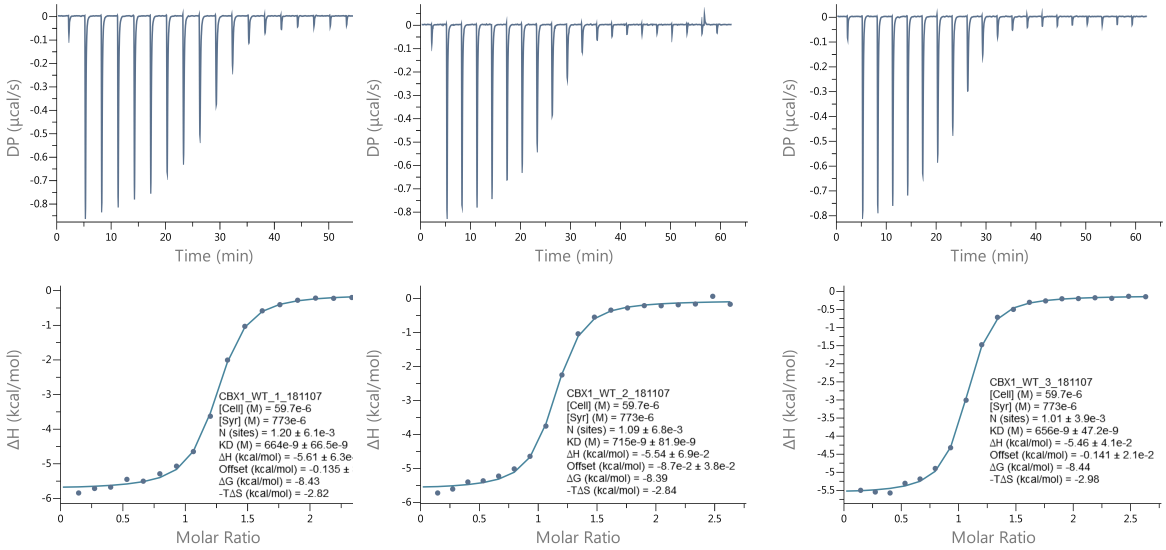


C.

Protein	T_m (°C)	
	Wild-type	Mutant
CBX1	65	57
CBX3	70	64
CBX5	60	63

Figure S5: Circular dichroism of CBX wild-type and engineered chromodomains. (A) CD spectra of CBX chromodomains in 10 mM sodium phosphate, pH 7.4. (B) Thermal melt (T_m) curves of CBX chromodomains in 10 mM sodium phosphate, pH 7.4. (C) T_m of wild-type CBX and

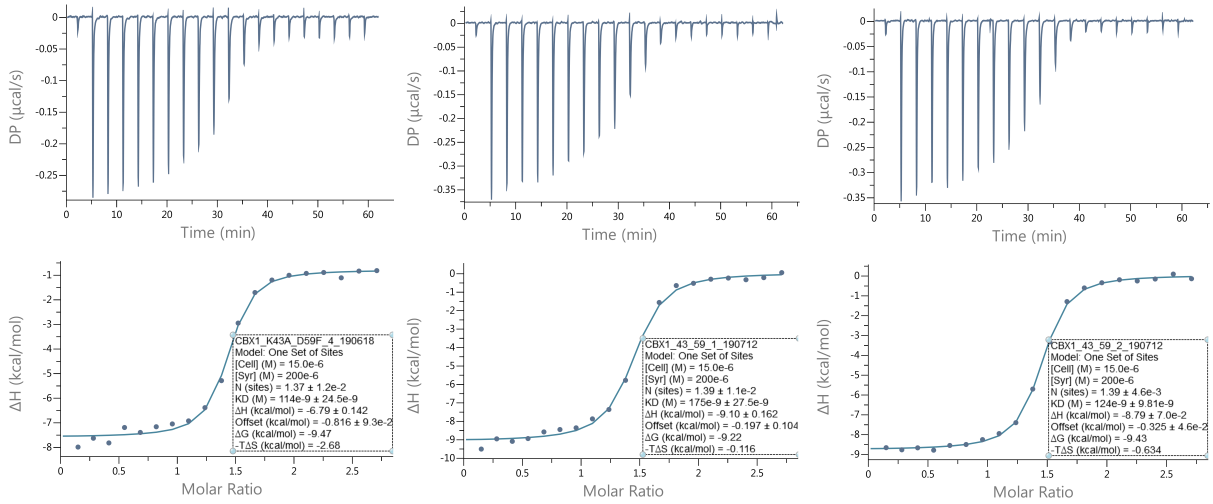
engineered chromodomains; the mutations do not significantly affect the overall stability of the chromodomains.



CBX1 wild-type – 1
[Protein] = 59.7 µM
[Peptide] = 0.773 mM
K_d = 0.664 µM
N = 1.20
C = 90

CBX1 wild-type – 2
[Protein] = 59.7 µM
[Peptide] = 0.773 mM
K_d = 0.715 µM
N = 1.09
C = 83

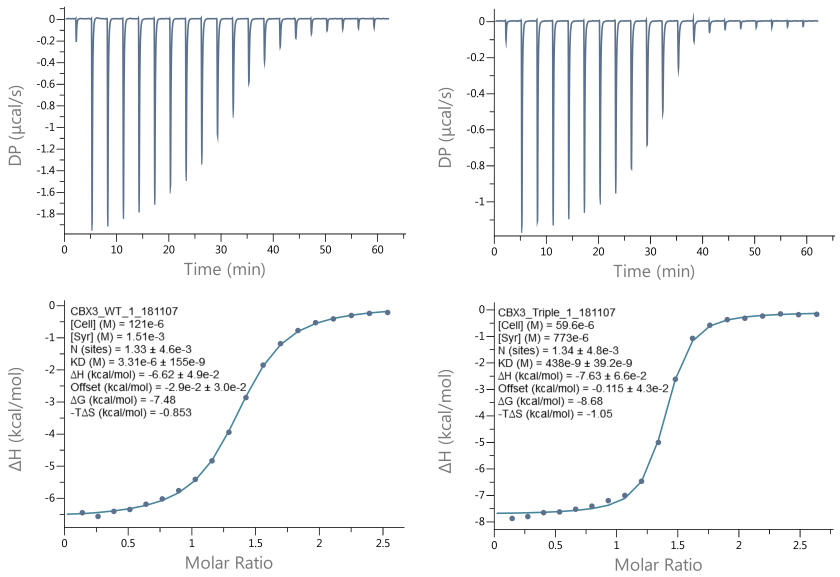
CBX1 wild-type – 3
[Protein] = 59.7 µM
[Peptide] = 0.773 mM
K_d = 0.656 µM
N = 1.01
C = 91



CBX1 K43A/D59F – 1
[Protein] = 15.0 µM
[Peptide] = 0.200 mM
K_d = 0.114 µM
N = 1.37
C = 131

CBX1 K43A/D59F – 1
[Protein] = 15.0 µM
[Peptide] = 0.200 mM
K_d = 0.175 µM
N = 1.39
C = 86

CBX1 K43A/D59F – 1
[Protein] = 15.0 µM
[Peptide] = 0.200 mM
K_d = 0.125 µM
N = 1.39
C = 120



CBX3 wild-type – 1
[Protein] = 121.0 µM
[Peptide] = 1.51 mM
K_d = 3.31 µM
N = 1.33
C = 37

CBX3 K58A/D74F – 1
[Protein] = 59.6 µM
[Peptide] = 0.773 mM
K_d = 0.438 µM
N = 1.34
C = 136

Figure S6: ITC curves of H3K9me3 binding to engineered CBX chromodomains.

Protein	K_d (µM)
CBX1 WT	0.6 ± 0.1
CBX1 Mutant	0.13 ± 0.05
CBX3 WT*	3 ± 1
CBX3 Mutant*	0.4 ± 0.1

Table S2: Binding affinities measured by ITC for eReader CBX domains. * Denotes single run. Error is calculated from the error in the fit.

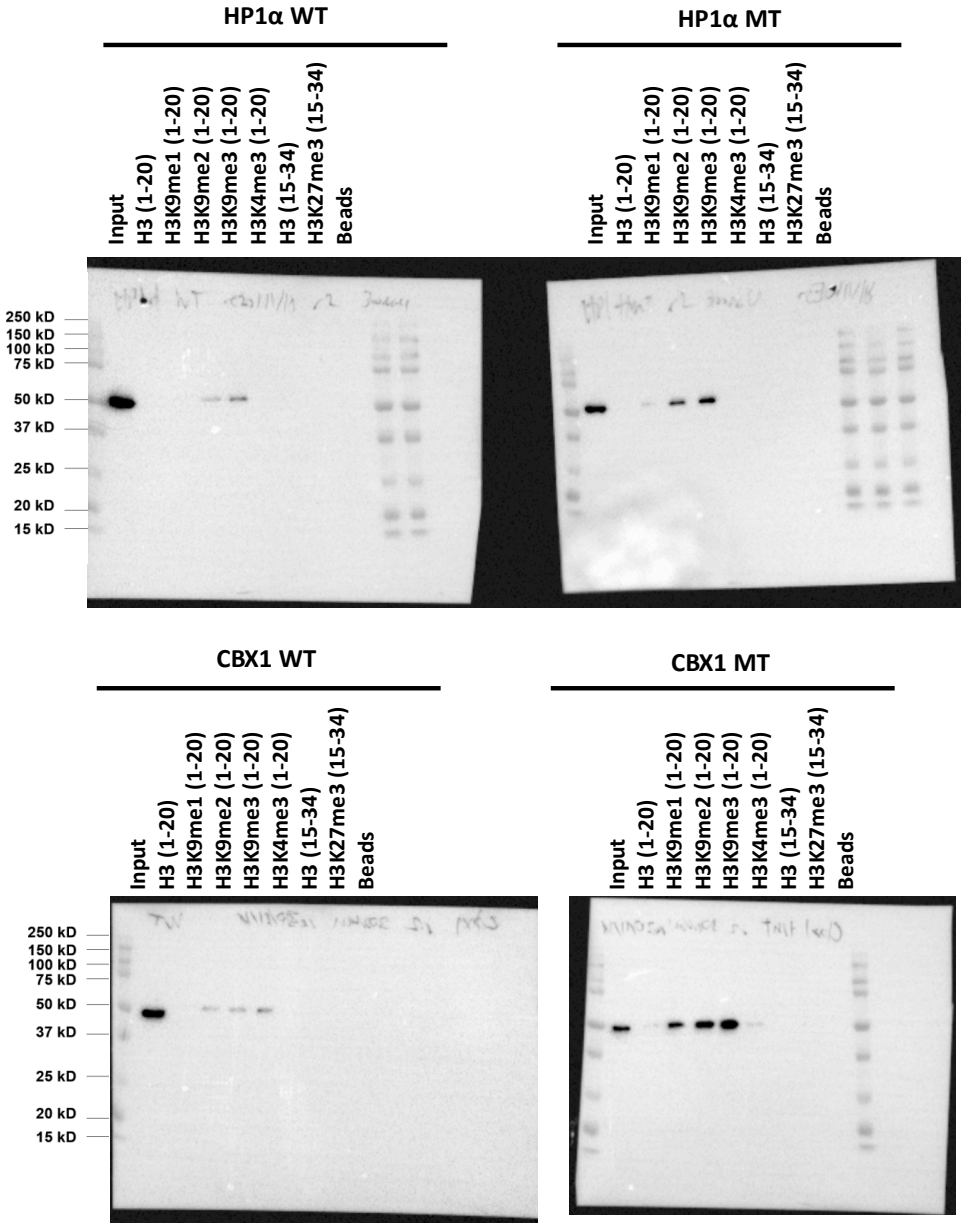


Figure S7: Full images of representative peptide pulldowns.

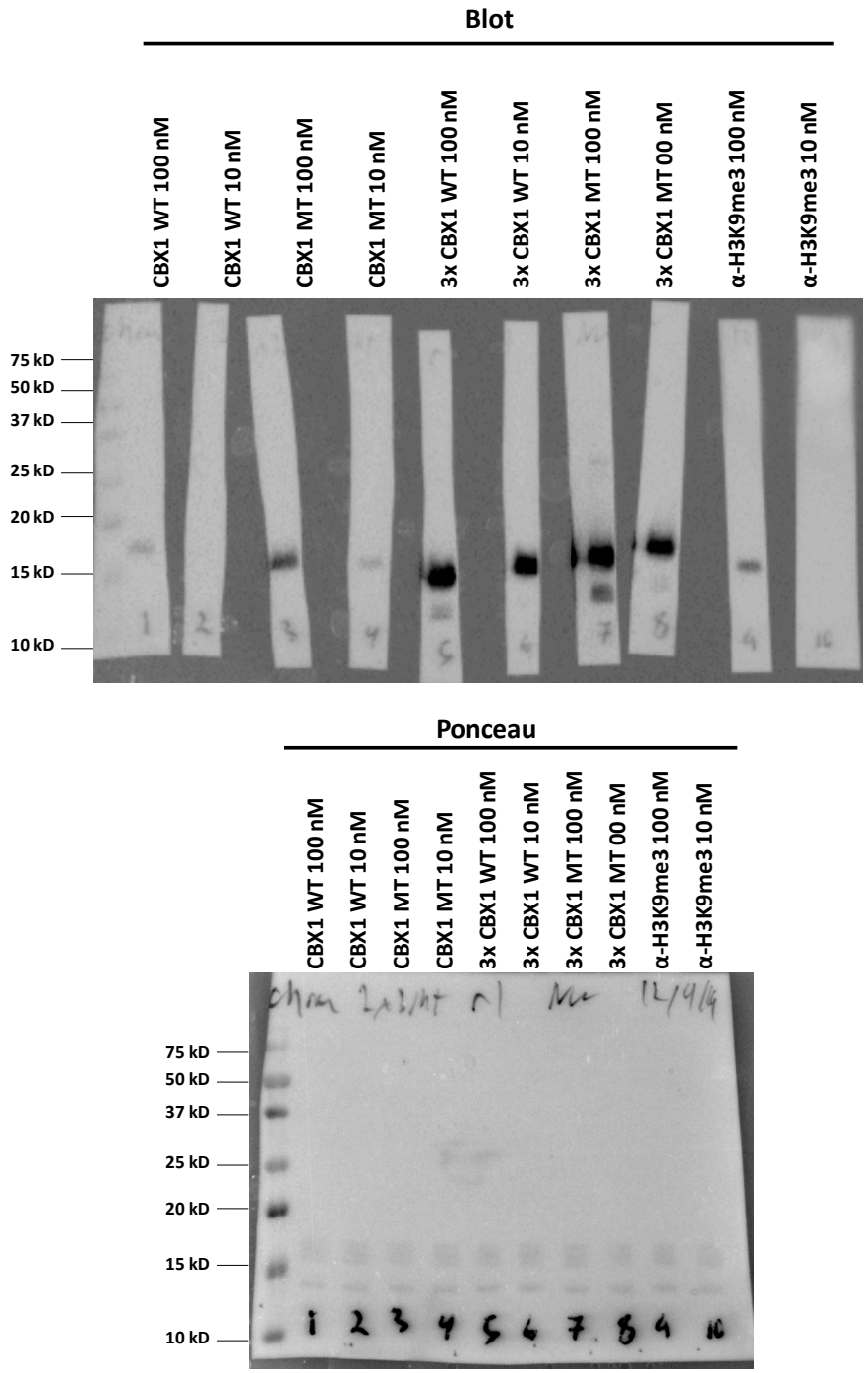


Figure S8: Full images of representative nucleosome blot.

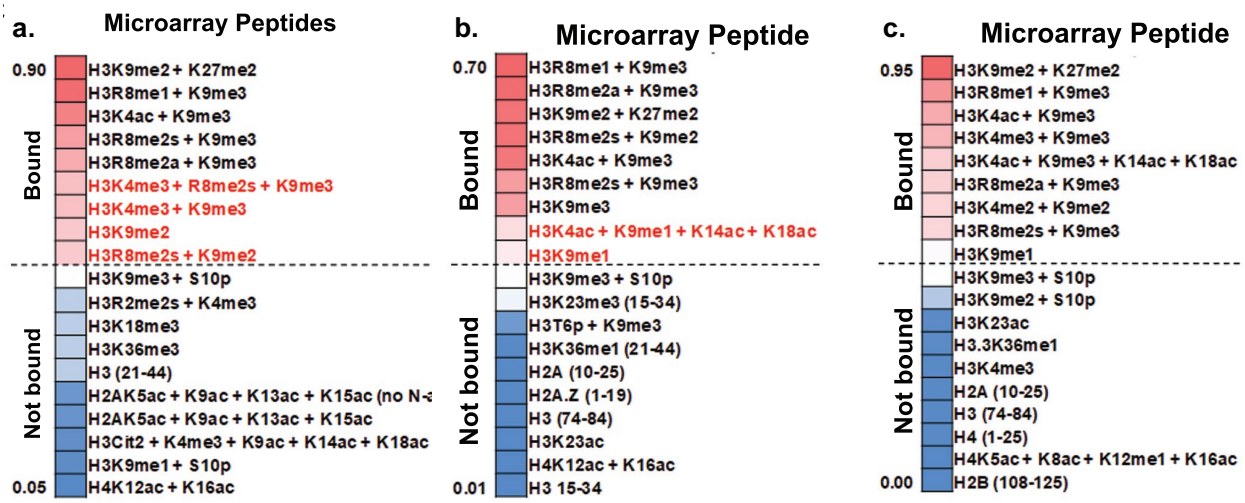


Figure S9: Heat maps generated from microarray experiments. Heat map of representative high, intermediate, and low peptide hits ranked by signal intensity for the **(a)** HP1α chromodomain *e*Reader, associated with Figure 2, **(b)** CBX1 chromodomain *e*Reader, associated with Figure 4, and **(c)** CBX1 3x *e*Reader, associated with Figure 5, as determined by quantification of the array results as described in Methods. The specific peptides bound by the wild-type and engineered domains are broadly similar with the exception of several newly detected peptides that were seen with the engineered (highlighted in red text) and a dashed line between the visible, bound peptides and the unbound.

1 Petell, C. J., Pham, A. T., Skela, J. & Strahl, B. D. Improved methods for the detection of histone interactions with peptide microarrays. *Scientific Reports* **9**, 6265, doi:10.1038/s41598-019-42711-y (2019).

2 Eisert, R. J., Kennedy, S. A. & Waters, M. L. Investigation of the β-sheet interactions between d hp1 chromodomain and histone 3. *Biochemistry* **54**, 2314-2322, doi:10.1021/acs.biochem.5b00024 (2015).