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Investigation of the β-Sheet Interactions between *d*HP1 Chromodomain and Histone 3

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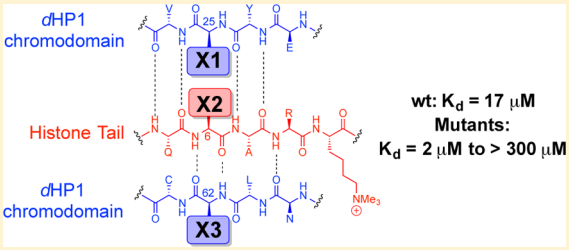
Supporting Information

ABSTRACT: Methylated lysine 9 on the histone 3 (H3) tail recruits heterochromatin protein 1 from *Drosophila* (*d*HP1) via its chromodomain and results in gene silencing. The *dHP1* chromodomain binds H3 K9Me$_3$ with an aromatic cage surrounding the trimethyllysine. The sequence selectivity of binding comes from insertion of the histone tail between two β-strands of the chromodomain to form a three-stranded β-sheet. Herein, we investigated the sequence selectivity provided by the β-sheet interactions and how those interactions compare to other model systems. Residue Thr6 of the histone tail forms cross-strand interactions with Ala25 and Asp62 of the chromodomain. Each of these three residues was substituted for amino acids known to have high β-sheet propensities and/or to form favorable side chain−side chain (SC−SC) interactions in β-sheets, including hydrophobic, H-bonding, and aromatic interactions. We found that about 50% of the chromodomain mutants resulted in equal or tighter binding to the histone tail and about 25% of the histone tail mutants provided tighter binding compared to that of the native histone tail sequence. These studies provide novel insights into the sequence selectivity of the *d*HP1 chromodomain for the histone tail and relates the information gleaned from model systems and statistical studies to β-sheet-mediated protein−protein interactions. Moreover, this work suggests that the development of designer histone−chromodomain pairs for chemical biology applications is feasible.



Post-translational modifications of histone proteins play critical roles in controlling gene expression. Methylation of the H3 histone protein at various lysine residues is among the most important and complex modifications.[1] For example, trimethyllysine 9 of histone protein H3 (H3 K9Me$_3$) is a modification that recruits the effector protein, heterochromatin protein 1 (HP1), via the highly conserved chromodomain.[2] Once recruited to the nucleosome, HP1 is believed to prevent gene expression by condensing DNA into tightly packed and genetically silent heterochromatin by oligomerizing with other HP1 proteins.[3,4] Moreover, there are a number of other effector proteins containing chromodomains that recognize unique methyllysine marks, and each has a distinct effect on gene expression.[5] These protein−protein interactions (PPIs) have become potential medicinal targets, with several small molecule and peptide-based inhibitors reported recently.[6−10] Thus, understanding the factors that control sequence selectivity in the recognition of methyllysine marks is important for gaining a biophysical understanding of this type of cellular signaling and developing inhibitors for this class of interaction.

Crystallography and NMR studies of the chromodomain from *Drosophila*, *d*HP1, bound to the H3 K9Me$_3$ tail have been used to understand the nature of the interactions between the chromodomain and trimethylated histone tail.[2,5,11] The binding of H3 K9Me$_3$ by the chromodomain is mediated by two modes of recognition. The first is a cation−π interaction between the trimethyllysine 9 of the H3 histone tail and a three-member aromatic cage of the chromodomain. The second is the formation of an antiparallel three-stranded β-sheet motif with Thr6 through Ser10 of the H3 histone tail (TAR(KMe$_3$)S) as the central strand and two β-strands of the chromodomain as the edge strands (Figure 1). van der Waals contacts, electrostatic interactions between side chains, and H-bonds between the backbone amides of the chromodomain and histone tail are key for selectivity and for stabilizing the complex.[12] However, while insight has been gained into how these proteins select for mono-, di-, or trimethyllysine, few studies have been performed beyond Ala scans and crystallographic studies that investigate the nature of the sequence selectivity.[2,13]

General insights into β-sheet structure come from statistical analyses of protein structures, which have established what amino acids have the highest propensity in β-sheets, and mutation studies in model proteins, which have measured those amino acids and side chain−side chain (SC−SC) interactions that are the most stabilizing to β-sheets (Table S1). Statistical analyses of the PDB found that amino acids with β-branched or aromatic side chains were shown to have the highest β-sheet propensity, whereas small, conformationally restrictive (proline) or charged amino acids have the lowest propensity.[14,15] Wouters and Curmi also performed statistical correlation

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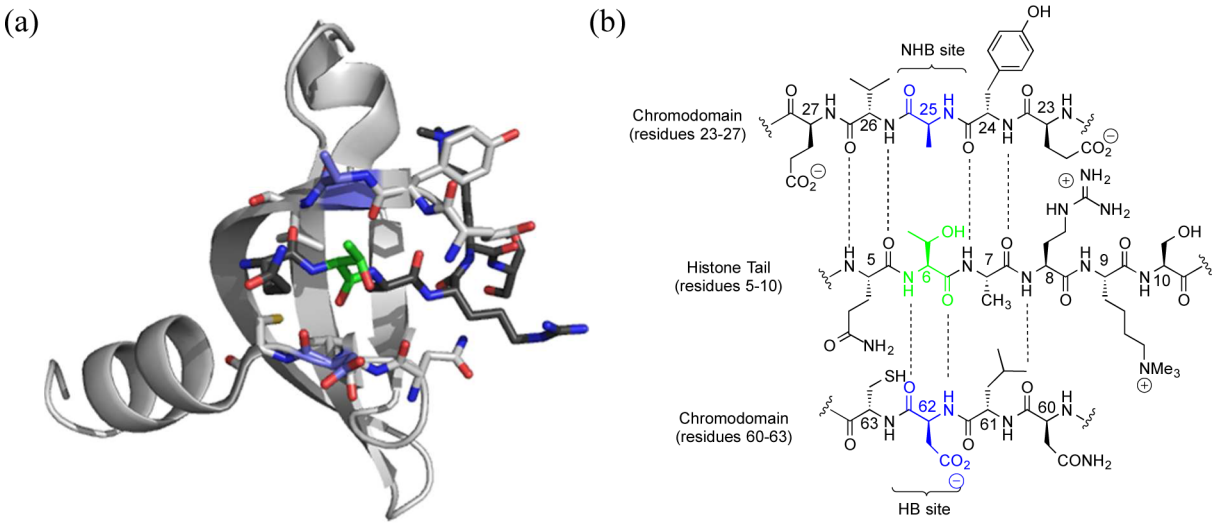

Figure 1. (a) Crystal structure of the *d*HP1 chromodomain (light gray) in complex with the H3 K9Me$_3$ histone tail (dark gray). A25 and D62 of the *d*HP1 chromodomain are shown in blue, and T6 of the histone tail is shown in green (PDB code: 1KNE). (b) β-Strands of the chromodomain and histone tail with the hydrogen bonds between the backbones. Color coding of A25, D62, and T6 are the same as that in (a).

analysis to determine which cross-strand amino acids are preferred in the hydrogen-bonded (HB) or non-hydrogen-bonded (NHB) sites. These studies found that β- and γ-branched amino acids are most favored in NHB sites.[16]

Smith and Regan measured interaction energies between cross-strand amino acid pairs by making pairwise mutations to the B1 domain of streptococcal protein G (GB1) in a solvent-exposed HB site.[17] Their findings corroborated the initial statistical analysis, showing that pairs composed of β-branched and aromatic residues had the most favorable interaction energy and that charge−charge interactions between side chain pairs were the least stabilizing.[17] Other studies involving β-hairpins have shown that edge−face interactions between two cross-strand aromatic residues, such as two Trp[18] or two Phe residues,[19] are highly stabilizing. The cation−π interaction of Trp or Phe with Lys or Arg is another favorable cross-strand interaction.[20]

While much work has been performed to understand β-sheet structure, very little has been done to understand β-sheets in the context of protein−protein interactions, such as the recognition of histone tails by chromodomains. Moreover, β-sheet-mediated PPIs are poorly understood relative to α-helix-mediated PPIs.[21] Herein, we report a mutation study to investigate the β-sheet interaction between the *d*HP1 chromodomain and H3 histone tail (Figure 1). We substituted naturally occurring amino acids at the D62 and A25 positions of the *d*HP1 chromodomain and in the T6 position of H3 K9Me3 for those with higher β-sheet propensity. We also incorporated known favorable cross-strand pairs into the D62 and A25 sites. The purpose of this research is 3-fold: first, to evaluate the selectivity of the recognition domain for H3 K9Me$_3$; second, to determine whether binding can be optimized or selectivity can be varied by mutating residues within the chromodomain and histone tail peptide; and third, to measure how previous studies from β-sheet models and statistical studies compare in the context of a PPI. The *d*HP1 chromodomain−H3 K9Me3 complex provides a model to meet this end with significant biological relevance. We find that the chromodomain is quite plastic, with more than 50% of the mutants resulting in equal or tighter binding to the wild-type histone sequence. In contrast, only two out of the eight histone tail mutants provided equal or better binding than the native sequence. Lastly, measurements of mutant histone peptides with mutant chromodomains suggest that orthogonal histone−chromodomain pairs are possible.

EXPERIMENTAL SECTION

Peptide Synthesis. H3 histone tail peptides (residues 1−16) were synthesized by solid-phase peptide synthesis using a Tetras synthesizer. Amino acids with Fmoc-protecting groups purchased from Creosalus were used with Wang resin from Anaspec on a 0.06 mmol scale with 4 equiv of amino acids per step. Amino acids were activated with 4 equiv of HOBt and HBTU with DIPEA in NMP using two cycles of 30 min. The Fmoc protecting groups were removed using 2% piperidine and 2% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in DMF using two washes for 15 min. Trimethyllysine was incorporated into the peptides using Fmoc-Lys(Me)$_2$-OH from Anaspec. Prior to deprotection of the final Fmoc group, the dimethylated lysine was reacted with MTBD (10.8 μL, 0.075 mmol) and iodomethane (37.4 μL, 0.60 mmol) in 5 mL of DMF for 5 h while bubbling with N_2 in a peptide flask with a vented septum to generate the trimethyllysine. After methylation and deprotection of the N-terminal Fmoc, 5(6)-carboxyfluorescein was reacted using 2 equiv of the fluorophore, HOBt, HBTU, and DIPEA overnight with bubbling N_2. The resin was washed twice with DMF and then once with DCM for 5 min. The DCM was removed from the resin by vacuum for 5 min, and the peptide was cleaved using 10 mL of 95% TFA/2.5% H_2O/2.5% triisopropylsilane for 3 h. The TFA was evaporated, and the products were precipitated using cold diethyl ether. Peptide products were extracted into water and lyophilized. Reverse-phase HPLC with a Vydac C-18 semiprep column was used to purify the peptides with a gradient of 0−100% B over 60 min (A was 95:5 water/acetonitrile with 0.1% TFA, and B was 95:5 acetonitrile/water with 0.1% TFA). The peptide was lyophilized and analyzed by ESI-TOF mass spectroscopy.

Chromodomain Expression. A pET11a plasmid containing *Drosophila* HP1 chromodomain (residues 17−76 and an N-terminal His$_6$-tag) was supplied the Khorasanizadeh group. The

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Table 1. Dissociation Constants (μM) and $\Delta G°$ Values (kcal/mol) of wt and Mutant *d*HP1 Chromodomain Binding to wt and Mutant H3 K9Me$_3$[a]

	H3 K9Me$_3$ histone tail				
chromodomain	WT	T6K	T6I	T6F	T6L
WT	17 ± 3 [−6.5 ± 0.1]	**10 ± 1 [−6.82 ± 0.06]**	27 ± 5 [−6.2 ± 0.1]	**20 ± 2 [−6.4 ± 0.1]**	**24 ± 4 [−6.3 ± 0.1]**
A25F	**14 ± 1 [−6.6 ± 0.04]**	**16 ± 1 [−6.50 ± 0.04]**	46 ± 5 [−5.9 ± 0.1]	66 ± 6 [−5.7 ± 0.1]	33 ± 4 [−6.1 ± 0.1]
A25K	77 ± 8 [−5.6 ± 0.1]	253 ± 33 [−4.9 ± 0.1]	180 ± 26 [−5.1 ± 0.1]	203 ± 18 [−5.0 ± 0.05]	376 ± 72 [−4.7 ± 0.1]
A25L	**17 ± 1 [−6.50 ± 0.03]**	**13 ± 3 [−6.7 ± 0.1]**	**19 ± 2 [−6.4 ± 0.1]**	26 ± 4 [−6.2 ± 0.1]	26 ± 6 [−6.2 ± 0.1]
A25T	**9 ± 1 [−6.9 ± 0.1]**	**10 ± 1 [−6.8 ± 0.1]**	47 ± 5 [−5.9 ± 0.1]	64 ± 5 [−5.7 ± 0.04]	44 ± 3 [−5.9 ± 0.04]
D62F	**6 ± 1 [−7.1 ± 0.1]**	**12 ± 2 [−6.7 ± 0.1]**	**24 ± 4 [−6.3 ± 0.1]**	**13 ± 2 [−6.7 ± 0.1]**	28 ± 3 [−6.2 ± 0.1]
D62I	37 ± 3 [−6.0 ± 0.1]	46 ± 5 [−5.9 ± 0.1]	48 ± 4 [−5.90 ± 0.05]	108 ± 20 [−5.7 ± 0.1]	45 ± 13 [−5.9 ± 0.2]
D62K	**9 ± 2 [−6.9 ± 0.1]**	30 ± 3 [−6.2 ± 0.1]	**17 ± 3 [−6.5 ± 0.1]**	**16 ± 2 [−6.5 ± 0.1]**	**10 ± 1 [−6.80 ± 0.09]**
D62T	39 ± 4 [−6.0 ± 0.1]	58 ± 8 [−5.8 ± 0.1]	76 ± 11 [−5.6 ± 0.1]	138 ± 20 [−5.3 ± 0.1]	26 ± 5 [−6.2 ± 0.1]

[a]Values in bold indicate those that exhibit equal or tighter binding than wild-type. The $\Delta G°$ values are given in brackets (kcal/mol) and were calculated using the following equation: $\Delta G° = -RT \ln(K_d)$. The data was measured in 50 mM potassium phosphate buffer, pH 8.0, 25 mM NaCl, and 4 mM DTT at 25 °C.

protein was expressed in BL21(DE3 Gold) *Escherichia coli*. The chromodomain was purified using Ni-affinity chromatography (Qiagen) and dialyzed in 50 mM sodium phophate, pH 8, 25 mM NaCl. Mutants were generated using the QuikChange (Stratagene) protocol.

Fluorescence Anisotropy. The concentrations of *d*HP1 chromodomain (ε_{280} = 18 450 M^{-1} cm^{-1}) and 5(6)-carboxyfluorescein-labeled peptides were determined by UV/vis spectroscopy (ε_{492} = 78 000 M^{-1} cm^{-1}).

Fluorescence anisotropy measurements were performed using a BMG Labtech Polarstar Omega microplate reader. Binding assays were performed in 50 mM potassium phosphate, pH 8, 25 mM NaCl, and 4 mM DTT with 1 μM fluorescein-labeled peptide. Samples were incubated at room temperature for 30 min before the measurements were taken at 25 °C (λ_{ex} = 485 nm; λ_{em} = 520 nm). Fluorescence anisotropy (A) values were fit to eq 1 using Kaleidagraph, where A_f and A_b are anisotropy values of free and bound peptide, respectively, P_t is total peptide, and [CD] is the chromodomain concentration.

$$A = A_f + (A_b - A_f) \times \frac{(P_t + K_d + [CD]) - \sqrt{(P_t - K_d - [CD])^2 - 4P_t[CD]}}{2P_t} \quad (1)$$

Circular Dichroism. CD measurements were performed on an Aviv 62DS Circular Dichroism Spectrometer. CD data was obtained for the chromodomain using 33.3 μM protein in 10 mM Na_2HPO_4, pH 7.4, 2 mM dithiothreitol (DTT) at 25 °C. Wavelength scans were performed in triplicate and averaged. All scans were corrected by subtracting the spectrum of the buffer used in the experiment. The signal was converted mean residue ellipticity (MRE) using eq 2, where Θ is MRE, signal is CD signal, l is path length (mm), c is protein concentration, and r is the number of amino acid residues.

$$\Theta = \frac{\text{signal}}{10 \cdot l \cdot c} \cdot \frac{1}{r} \quad (2)$$

Thermal denaturation experiments were preformed using the same buffer and concentrations as described above, and measurements were taken between 3 and 93 °C. The melting curves were normalized to show the fraction folded using eq 3, 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.

$$\text{fraction folded} = (\Theta - \Theta_D)/(\Theta_F - \Theta_D) \quad (3)$$

RESULTS

Experimental Design. The β-sheet interactions between H3 K9Me$_3$ and *d*HP1 chromodomain were explored using a number of trimethyllysine histone 3 (H3) tail peptides with mutations to the Thr6 position and *d*HP1 chromodomain proteins with mutations to either the Ala25 or Asp62 positions. The Thr6 residue of the histone tail was chosen because alanine scans have demonstrated its significance in binding and sequence selectivity.[2] The threonine is also solvent-exposed and does not make significant contacts with the interior of the protein. The Ala25 and Asp62 positions were chosen due to their cross-strand location from Thr6. These two residues in the chromodomain were also ideal because they are not known to have high β-sheet propensity or to form particularly favorable cross-strand interactions with Thr.

Side chain−side chain (SC−SC) interactions also stabilize β-sheets, but the preference for different SC−SC interactions depends on whether residues are in the hydrogen-bonded (HB) or non-hydrogen bonded (NHB) positions (Figure 1).[15,16] In the NHB site, the amide backbone of two cross-strand amino acids are not involved in H-bonds. The NHB and HB sites have significant differences that influence which amino acids are favored in those sites. First, the α-carbons in HB sites are separated by 5.5 Å, whereas α-carbons in NHB are separated by only 4.5 Å. Second, rotamer conformations differ significantly in the two sites, which influences the preference that residues have for either NHB or HB sites.[16,22] Since the Ala25−Thr6 and Thr6−Asp62 pairs occur in NHB and HB positions, respectively, the histone tail−chromodomain complex allows for exploration of the interactions that are preferred in each position. Additionally, they are solvent-exposed residues that appear to form minimal contacts with other amino acids within the chromodomain or histone tail. Therefore, mutations were also incorporated to include amino acid residues known to form favorable SC−SC interactions, including hydrophobic packing, hydrogen bonding, cation−π, and π−π interactions.[23] By incorporating amino acids known to stabilize β-sheet structure into the *d*HP1 chromodomain and histone, the β-sheet-mediated interaction between the two molecules should be stabilized.

Structural Characterization. Circular dichroism (CD) was used to investigate the effect that various mutations have

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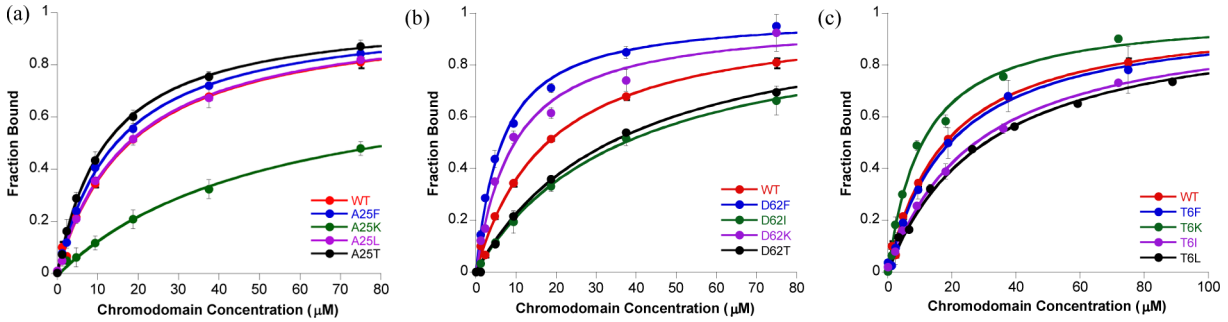

Figure 2. (a) Fluorescence anisotropy binding experiments of 1 μM 5(6)-FAM H3 K9Me$_3$ histone tail with A25 mutants. (b) Fluorescence anisotropy binding experiments of 1 μM 5(6)-FAM H3 K9Me$_3$ histone tail with D62 mutants. (c) Fluorescence anisotropy binding experiments of 1 μM 5(6)-FAM H3 K9Me$_3$ T6 mutants with wt chromodomain. The experiments were performed in 50 mM potassium phosphate buffer, pH 8.0, 25 mM NaCl, and 4 mM DTT at 25 °C. Each curve is an average of three runs.

Table 2. $\Delta\Delta G$ Values of A25 Chromodomain Mutants Relative to wt Chromodomain Binding to 5(6)-FAM H3 K9Me$_3$ Histone Tail,[a] Relative β-Sheet Propensities Referenced to wt, and Cross-Strand Pairing Preferences

	relative β-sheet propensity				pairing preference		
A25 mutant	stat[b]	exp[c]	cross-strand pair (A25X−Thr6)	$\Delta\Delta G$	stat[d]	stat[e]	agreement[f]
A			Ala−Thr	0	NS	N	
F	>Ala	≫Ala	Phe−Thr	−0.1 ± 0.1	U	U	C
K	~Ala	>Ala	Lys−Thr	+0.9 ± 0.1	F	F	no
L	>Ala	>Ala	Leu−Thr	0 ± 0.1	U	U	C
T	>Ala	≫Ala	Thr−Thr	−0.4 ± 0.1	F	F	yes

[a] $\Delta\Delta G = \Delta G(\text{A25X}) - \Delta G(\text{A25})$. [b] Summary of β-sheet propensity relative to Ala as determined by statistical analysis.[15] [c] Summary of experimental β-sheet propensity of amino acids relative to Ala.[23] > indicates between 0 and 1 kcal/mol more stable, and ≫ indicates greater than 1 kcal/mol more stable. [d] Summary of pairing preferences taken from ref 11. U indicates an unfavorable interaction (with a pair correlation between 0 and 1); F indicates a favorable interaction (with a pair correlation greater than 1); NS indicates not statistically significant, with a confidence level below 90%.[16] [e] Summary of pairing preferences taken from ref 17. F indicates a favorable interaction, U indicates an unfavorable interaction, N indicates a null hypothesis.[22] [f] Agreement between binding affinities reported in this work and predictions based on β-sheet propensities and pairing preferences. C indicates conflicting expectations based on β-sheet propensity and pairing preference.

on the global structure of chromodomain. The CD spectra for the wild-type (wt) protein exhibits minima at 208 and 222 nm, corresponding to the α-helix, which masks the weaker signal from the β-sheet.[24] The wt chromodomain also exhibits a maximum at 232 nm, which is likely due to exciton coupling of the aromatic residues in the aromatic cage.[25] The CD spectra of the Ala25 chromodomain mutants are similar (Figure S1A). The global structures of the Asp62 mutants deviate more from the wt chromodomain, but they still have the strong minima at 208 and 222 nm and the exciton coupling peak at 232 nm (Figure S1B). Thus, there appears to be no significant perturbation to the global structure due to the mutations.

The melting temperatures of the *d*HP1 chromodomain and the mutants were measured by CD at 222 nm to determine how the different point mutations affect the stability of the chromodomain. The melting temperature of the wt protein was measured to be approximately 47 °C (Figure S2 and Table S3). Each of the mutants had a melting temperature between 45 and 49 °C, indicating that the various point mutations resulted in an insignificant effect on global stability. This experiment also indicates that wt and mutant chromodomains are thermally stable at 25 °C, the temperature at which all binding experiments were carried out.

Binding Studies. Binding affinities of the *dHP1* chromodomain and its mutants to a FAM-labeled histone H3 peptide containing K9Me$_3$ (H3 K9Me$_3$) and its mutants were measured by fluorescence anisotropy (Table 1). Binding affinities were compared to that of the wt *d*HP1 chromodomain and the histone tail, which was found to have a dissociation constant of 17 μM under the conditions used in this study.[2]

Single Mutants at Ala25 of the *d*HP1 Chromodomain. Four mutations at the Ala25 position were studied, which include A25F, A25K, A25L, and A25T (Figure 2a). These mutations were selected for their high β-sheet propensities[14,15,23] or their ability to form favorable cross-strand interactions (Table 2).[17]

Inspection of the binding curves in Figure 2a indicates that binding to wt H3 KMe3 is not very sensitive to the amino acid at position 25. A25F, A25L, and A25T all have similar binding affinities to the wt histone tail as that of the native chromodomain, with A25T providing slightly tighter binding, consistent with both its β-sheet propensity and pairing preferences (Table 1). This mutant provides a cross-strand Thr−Thr interaction in a NHB position, which has been shown in numerous studies to be a highly favorable interaction in this position.[16] A25F and A25L have conflicting β-sheet propensities and pairing preferences, making a prediction difficult.[14,23] In contrast, the A25K mutant is significantly destabilizing by nearly 1 kcal/mol (Table 2), which is in conflict with both its β-sheet propensity and pairing preference. Indeed, we found that the A25K mutant was universally destabilizing with all of the histone tails investigated (Table 1). Several possible explanations can account for the decrease in binding affinity of A25K. First, the incorporation of a basic residue into the chromodomain may result in weakened binding to an already basic histone tail peptide due to charge−charge repulsion. A second possibility is that the A25K mutant changes the

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Table 3. ΔΔG Values (kcal/mol) of D62 Chromodomain Mutants Relative to wt Chromodomain Binding to 5(6)-FAM H3 K9Me$_3$ Histone Tail[a] and Pairing Preferences

	relative β-sheet propensity				pairing preference			
D62 mutant	stat[b]	exp[c]	cross-strand pair	ΔΔG	stat[d]	stat[e]	exp[f]	agreement[g]
D			Thr−Asp	0	NS	N	NR	
F	≫Asp	≫Asp	Thr−Phe	−0.6 ± 0.1	NS	N	F (−0.19)	yes
I	≫Asp	≫Asp	Thr−Ile	+0.5 ± 0.1	NS	N	F (−0.19)	no
K	>Asp	>Asp	Thr−Lys	−0.4 ± 0.1	F	N	NR	yes
T	≫Asp	≫Asp	Thr−Thr	+0.5 ± 0.1	F	U	U (0.21)	C

[a] $\Delta\Delta G = \Delta G(\text{D62X}) - \Delta G(\text{D62})$. [b] Summary of β-sheet propensity relative to Asp as determined by statistical analysis.[15] [c] Summary of experimental β-sheet propensity of amino acids relative to Asp.[23] > indicates between 0 and 1 kcal/mol more stable, and ≫ indicates greater than 1 kcal/mol more stable. [d] Summary of pairing preferences taken from ref 11. U indicates an unfavorable interaction (with a pair correlation between 0 and 1); F indicates a favorable interaction (with a pair correlation greater than 1); NS indicates not statistically significant, with a confidence level below 90%.[16] [e] Summary of pairing preferences taken from ref 17. F indicates a favorable interaction; U indicates an unfavorable interaction; N indicates a null hypothesis.[22] [f] Values represent interaction energies in kcal/mol, as determined from a double mutant cycle from mutations of protein GB1. NR = not reported.[17] [g] Agreement between binding affinities reported in this work and predictions based on β-sheet propensities and pairing preferences. C indicates conflicting expectations based on β-sheet propensity and pairing preference.

Table 4. ΔΔG Values (kcal/mol) of T6 H3 KM9Me$_3$ Mutants Relative to wt H3 KM9Me$_3$ Binding to the *d*HP1 Chromodomain[a] and Pairing Preferences with A25 and D62

	relative β-sheet propensities				A25 pairing preference		D62 pairing preference		
T6 mutant	stat[b]	exp[c]	cross-strand interactions	ΔΔG	stat[d]	stat[e]	stat[d]	stat[e]	agreement[f]
T			Ala−Thr−Asp	0	NS	N	NS	N	
F	>Thr	<Thr	Ala−Phe−Asp	+0.1 ± 0.1	NS	F	U	N	C
I	>Thr	∼Thr	Ala−Ile−Asp	+0.3 ± 0.1	F	N	NS[e]	U	C
K	<Thr	≪Thr	Ala−Lys−Asp	−0.3 ± 0.1	NS	U	F	N	C
L	∼Thr	≪Thr	Ala−Leu−Asp	+0.2 ± 0.1	NS	N	NS	NS	yes

[a] $\Delta\Delta G = \Delta G(\text{T6B}) - \Delta G(\text{T6})$. [b] Summary of β-sheet propensity relative to Thr as determined by statistical analysis.[15] [c] Summary of experimental β-sheet propensity of amino acids relative to Thr.[23] > indicates between 0 and 1 kcal/mol more stable, and ≫ indicates greater than 1 kcal/mol more stable. [d] Summary of pairing preferences taken from ref 11. U indicates an unfavorable interaction (with a pair correlation between 0 and 1); F indicates a favorable interaction (with a pair correlation greater than 1); NS indicates not statistically significant, with a confidence level below 90%.[16] [e] Summary of pairing preferences taken from ref 17 (Supporting Information). F indicates a favorable interaction; U indicates an unfavorable interaction; N indicates a null hypothesis.[22] [f] Agreement between binding affinities reported in this work and predictions based on β-sheet propensities and pairing preferences. C indicates conflicting expectations based on β-sheet propensity and pairing preference.

conformation of the neighboring Y24, which makes up part of the KMe3 binding pocket. Third, A25K may introduce a charge−charge repulsion with the neighboring K46, which could influence the binding pocket. A double mutant cycle was performed to rule out this third possibility (see the Supporting Information), but the first two possible explanations cannot be excluded.

Single Mutants at D62 of the *d*HP1 Chromodomain. The mutations made to the D62 position of the chromodomain include D62F, D62I, D62K, and D62T (Figure 2c and Table 1). This position is in an edge strand and provides a cross-strand interaction with Thr6 in an HB site. These mutants exhibit a greater effect on binding affinity than the mutants at position A25. D62F and D62K exhibit measurably tighter binding, whereas D62I and D62T exhibit weaker binding (Table 3).

D62F provides the greatest increase in binding affinity of any mutant, corresponding to a 3-fold improvement in K_d. Although statistical analyses of the Protein Data Bank (PDB) show no preference for a Thr−Phe pair at an HB position, double mutant studies in GB1 show that the interaction is weakly stabilizing when the Phe is in an edge strand. In contrast, the native Thr−Asp pair is not predicted to be favorable based on statistical studies. Thus, the more favorable binding affinity of D62F may be a result of a combination of a greater β-sheet propensity in combination with a more favorable SC−SC interaction relative to wt. Additionally, in the chromodomain−histone tail complex, D62F is in a diagonal cross-strand position from R8 of the histone tail and may allow for a stabilizing cation−π interaction.[20,26] Unfortunately, a double mutant cycle to determine the interaction energy with D62F and R8 of the histone tail was not possible due to the importance of R8 for binding.

The D62K mutation improves binding to the native histone tail by 2-fold compared to that of the wt chromodomain, which is consistent with statistical studies showing a significant pairwise correlation for Thr−Lys pairs in HB sites (Table 3).[16] The finding that D62T destabilizes the binding event correlates with pairwise preferences found in experimental studies and at least one statistical analysis, indicating that Thr does not pair well with Ile or Thr in HB positions despite their high β-sheet propensities (Table 3). The destabilizing effect of the D62I mutation, in contrast, is not predicted by β-sheet propensities or pairing preferences.

Single Mutants at T6 of the Histone Tail. Jacobs et al. previously reported alanine scans of H3 K9Me$_3$ and found that T6A hindered binding by 7-fold, which was proposed to be due to loss of complementary interactions between the larger Thr6 residue and residues Ala25 and Asp62 in the chromodomain.[2] However, no other mutations were explored to examine the scope of the selectivity between the chromodomain and H3 K9Me$_3$. In this study, T6 was replaced with four other amino

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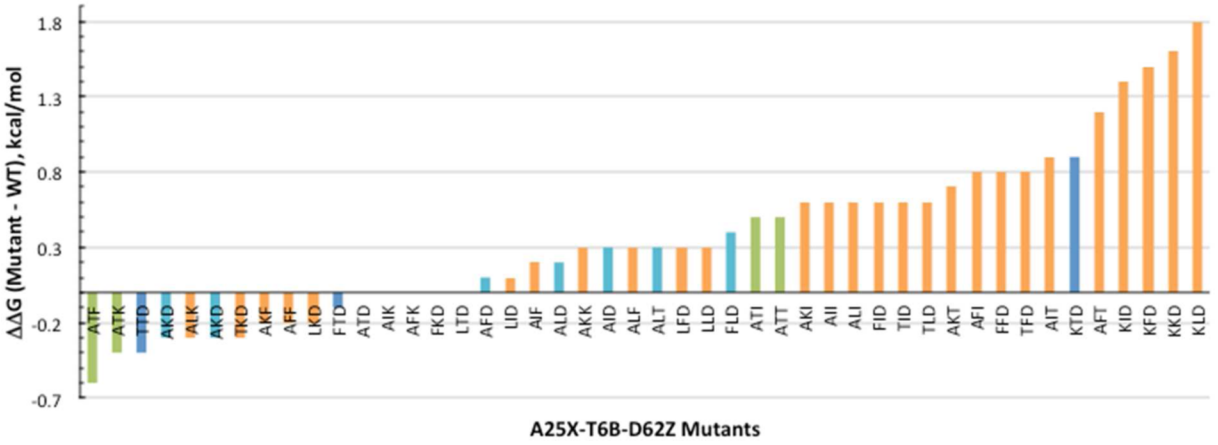

Figure 3. Difference in binding energy of mutant chromodomain–histone pairs relative to wt for A25X–T6B–D62Z single and double mutants. Error is ±0.1 kcal/mol. Blue indicates single A25X mutants, green indicates single mutants at D62, cyan indicates single mutants at T6, and orange indicates double mutants at T6 and either A25 or D62.

acids known to have high β-sheet propensity, including Ile, Lys, Leu, and Phe (Figure 2c and Table 1). Since T6 is in a central position in the β-sheet, the impact of mutations at this site must be considered with respect to interaction with both A25 and D62.

Changing Thr6 to Ile, Leu, and Phe had a slightly negative effect on binding to the wt chromodomain (Table 1). Previous statistical studies do not provide significant insight, as most pairs did not provide statistically significant data (Table 4).[16,22] Nonetheless, the small impact of these mutations is surprising given the sequence-selective nature of histone–chromodomain interactions and suggests that T6 plays only a minor role in controlling sequence selectivity.

The T6K histone tail mutant is the only mutant at this position that results in improved binding to the native chromodomain (a K_d of 10 μM versus 17 μM, respectively, $\Delta\Delta G = -0.3$ kcal/mol; Table 4). This introduces both an Ala–Lys interaction in the NHB position and a Lys–Asp interaction in the HB position. Complementary charged residues in a β-sheet are known to have a favorable side chain interaction in the HB site of a β-sheet,[17] although Ala–Lys interactions have been found to be unfavorable in the NHB site (Table 4).[16,17] In this case, having a basic lysine cross-strand from aspartic acid, which is an HB site, may result in a favorable electrostatic interaction that overcomes the unfavorable Ala–Lys pair.

Double Mutants. There are some general trends between mutant chromodomain and mutant histone tails (Figure 3). The range of interaction energies over all mutants varies by about 2.2 kcal/mol upon mutation of a cross-strand pair. Of these, five of the seven most destabilizing cross-strand pairs are attributable to A25K mutants. Fifteen mutants are equally or more stable than the wt histone–chromodomain pair. Of those, eight are double mutants, and five of those retain Ala in the 25 position (Table 5).

There are several double mutants that result in equivalent or improved binding affinity relative to the wt protein–protein interaction (Figure 3). At the A25 position, Thr, Leu, and Phe are tolerated only when paired with T6 (Table 2) or T6K. All of these A25 mutants have higher β-sheet propensities than Ala, but only the A25T–T6 and A25T–T6K pairs are predicted to give a favorable interaction in the NHB site, such as in this case.[16,22]

Table 5. $\Delta\Delta G$ Values (kcal/mol) of Double Mutants and Pairing Preferences of T6B with A25X and D62Z

				A25X pairing preference		D62Z pairing preference	
A25 mutant	T6 mutant	D62 mutant	$\Delta\Delta G$	stat[a]	stat[b]	stat[a]	stat[b]
A	**L**	**K**	−0.3 ± 0.1	NS	N	NS	N
T	**K**	D	−0.3 ± 0.1	**F**	**F**	**F**	N
A	**K**	**F**	−0.2 ± 0.1	NS	U	U	N
A	**F**	**F**	−0.2 ± 0.1	NS	**F**	**F**	N
L	**K**	D	−0.2 ± 0.1	U	U	F	N
A	**I**	**K**	0	**F**	N	U	N
A	**F**	**K**	0	NS	**F**	U	N
F	**K**	D	0	U	N	**F**	N

[a]Summary of pairing preferences taken from ref 11. U indicates an unfavorable interaction (with a pair correlation between 0 and 1); F indicates a favorable interaction (with a pair correlation greater than 1); NS indicates not statistically significant, with a confidence level below 90%.[16] [b]Summary of pairing preferences taken from ref 17. F indicates a favorable interaction; U indicates an unfavorable interaction; N indicates a null hypothesis.[22]

At the T6 position, the Phe, Ile, Lys, and Leu variants all result in more favorable interactions, depending on the flanking residues in the chromodomain. T6F is paired favorably only with Ala at position 25 and with Asp, Phe, or Lys at position 62 and is disfavored in the case of the D62I and D62T chromodomain mutants. Thus, β-branched amino acids cross-strand from T6F appear to be poorly tolerated. Favorable binding in the case of T6F–D62F is consistent with statistical and mutation studies that have shown that Phe–Phe interactions are favorable in a HB site.[16,22] T6F–D62K may introduce a favorable cation–π interaction, although this is not supported by pairwise preferences.

Five of the nine T6K binding pairs have binding affinities that are equal to or more favorable than the wt protein–peptide interaction. These include binding of T6K to the wt chromodomain and A25F, A25L, A25T, and D62F mutations. None of these mutants are predicted to be more stable based on pairwise interactions alone.[16] The A25 mutants may have favorable binding to T6K due to β-sheet propensity at position 25 in combination with a favorable salt bridge between T6K

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and D62. In the case of the D62F mutant (like the T6F−D62K double mutant), a favorable cation−π interaction may be introduced.[20]

At position 62, Asp, Lys, and Phe result in equivalent or more favorable interactions than the wt chromdomain−histone tail pair. D62K is tolerated with T6, T6L, T6I, and T6F, suggesting that D62K is not particularly selective. D62F is paired with T6, T6K, and T6F, of which T6−D62F and T6F−D62F are predicted to give favorable SC−SC interactions.[17]

Selectivity for Histone Sequences. Interestingly, the wt chromodomain does not exhibit significant selectivity for T6 over some of the other T6B mutants, with a variation in binding affinity of about a factor of 3 (K_d of 10−27 μM, $\Delta\Delta G$ = 0.6 kcal/mol) between the best and worst histone tails (Table 1). In some ways, this is not surprising because the side chain is on the solvent-exposed surface of the β-sheet. However, inspection of the mutant chromodomains indicates that better selectivity can be achieved. The A25T mutant has the best selectivity across the five histone sequences investigated, exhibiting a variation in binding affinity of a factor of 7 in K_d (9−64 μM, with tightest binding to the wt histone tail), amounting to an energetic difference of 1.2 kcal/mol (Table 1). Even so, the A25T chromodomain mutant does not differentiate between wt H3 and the T6K mutant.

Comparison of H-Bonded and Non-H-bonded Sites. Differences in cross-strand pair preferences in HB and NHB have been found in statistical correlation studies.[16] Therefore, comparisons were made between the NHB and HB sites of the chromodomain−histone tail complex (A25X−T6B and T6B−D62Z, respectively). In this case, we chose to make comparisons in which the central T6B position is constant in each pair. Thus, we compared A25L−T6K to T6K−D62L. In this way, the interaction between T6B and the unvaried position is constant. As shown in Figure S23, significant variations of the impact of position were observed, ranging from as little as 0.1 kcal/mol to as much as 2 kcal/mol. Only a few key examples will be discussed in detail. Because A25K has been found to be universally disruptive, pairs with that mutation (the first four entries in Figure S23) will not be discussed.

The Phe−Phe pair is favored by −1 kcal/mol in the HB position relative to the NHB position. Phe−Phe cross-strand interactions are common in protein structures and exhibit a strong preference for HB sites in statistical correlations.[16] Wouters and Curmi showed that Phe−Phe pairs often take a gauche−/gauche+ conformation, which results in an offset stacked π−π interaction.[16] In addition, the GB1 mutation studies demonstrated that a Phe−Phe pair in an HB site was stabilizing by about −0.9 kcal/mol, which is quite similar to the magnitude found here.[17]

The Thr−Thr pair is favored in the NHB position by −1 kcal/mol. Statistical analyses have demonstrated that cross-strand Thr−Thr pairs have a preference for the gauche+ rotamer conformation, which favors inter-residue H-bonds between the hydroxyl groups of the threonine side chains in NHB sites.[22] In an HB site, both Thr residues must be in the unfavorable gauche-rotamer conformation to accommodate the H-bond between the hydroxyl groups.[22] Experimental results reported by Smith and Regan in the GB1 domain are consistent with the findings here, showing that Thr−Thr pairs in the HB position have an unfavorable interaction energy.[17]

DISCUSSION AND CONCLUSIONS

Significance to the *d*HP1 Chromodomain−Histone H3 Protein−Protein Interaction. Chromodomains are sequence-selective reader proteins for methyllysine. While insight has been gained into how these proteins select for mono-, di-, or trimethyllysine, minimal studies have been performed that investigate the nature of the sequence selectivity.[13] Previous mutation studies to the TAR(K9Me$_3$)S sequence have shown that the R8A mutation reduces binding to *d*HP1 chromodomain by a factor of 20 and the A7M mutation reduces binding by a factor of 25, whereas the T6A mutation reduces binding by a factor of 7.[2] S10 contributes the least to sequence selectivity, as mutation to Ala reduces binding only by a factor of 3. The solvent-exposed T6 does not contribute as significantly to sequence selectivity as A7, which is buried, or R8, which forms a salt bridge with E23 of the chromodomain. Nonetheless, T6 plays a significant role in differentiating between *d*HP1 chromodomain and the *Drosophila* polycomb (*d*Pc) chromodomain, which binds to K27Me$_3$ within the sequence AAR(K27Me$_3$)S. The *d*HP1 chromodomain differentiates between TAR(K9Me$_3$)S and AAR(K27Me$_3$)S by a factor of 16.[11] The human counterparts of *d*HP1, CBX1, CBX3, and CBX5, also preferentially bind H3 K9Me$_3$ over K27Me$_3$, but the human homologues of *d*Pc are generally less selective.[13] Thus, the small differences in the binding of mutant histone tails to wt *d*HP1 chromodomain (ranging from 10 to 27 μM, a factor of 2.7, $\Delta\Delta G$ = 0.6 kcal/mol) is perhaps surprising, especially when compared to its human homologue, CBX3. On the basis of a dot blot assay, CBX3 binds to only the native TAR(K9Me$_3$)S or VAR(K9Me$_3$)S sequences, exhibiting much higher sequence selectivity at position 6.[13] Structural analysis of CBX3 suggests this is due to a polar clasp around T6 that is not found in the *d*HP1 chromodomain.

The T6K mutation, with a K_d of 10 μM versus 17 μM for T6, may have biological relevance. One study demonstrated all isoforms of the mammalian HP1 chromodomain, which have 70% homology to *d*HP1 chromodomain, recognize dimethyllysine 26 of histone H1.4.[27] The sequence surrounding K26Me$_2$ of H1.4 is **K**AR(KMe$_2$)S, as opposed to **T**AR(KMe$_2$)S, containing a Lys at the i − 3 position instead of Thr. These studies provide evidence that the *d*HP1 chromodomain can interact with other methyllysine marks containing Lys at the i − 3 position *in vitro* and may also be able to do so *in vivo*.

The effect of mutation of the chromodomain on the binding of the wt histone tail is more significant, ranging from 6 to 77 μM (a factor of 12.8, $\Delta\Delta G$ = 1.5 kcal/mol). Perhaps more surprising is the fact that several chromodomain mutants, including A25T, A25F, D62F, and D62T, were found that bind more tightly to the wt histone tail than the native protein. In particular, the D62F mutant binds the wt histone tail 3-fold more tightly. Nonetheless, D62 is completely conserved among *Drosophila* and human HP1 chromodomain isoforms (Figure S23).[13] In contrast, A25 is not completely conserved; Val is found in the human HP1 isoforms, whereas Ala is found in the human polycomb chromodomain isoforms (Figure S23). Lastly, the K46A mutant, which is outside of the β-sheet but neighbors the aromatic cage, results in the tightest binding mutant reported, with a K_d (2 μM) nearly 10-fold stronger than the wt chromodomain (see the Supporting Information). These studies indicate that the *d*HP1 chromodomain is not optimized for the tightest binding to the H3 histone tail; binding must be sufficient for its biological role. This likely is a reflection of the

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fact that the *d*HP1 chromodomain functions as part of a multiprotein complex rather than in isolation. These tighter-binding mutant chromodomains may be useful in biotechnology applications, such as reading histone tails in a microarray format.[28−30]

In conclusion, we have used the *d*HP1 chromodomain and H3 K9Me$_3$ histone tail peptide to investigate how information gleaned from statistical and experimental studies of β-sheet propensity and SC−SC interactions applies to protein−protein recognition. Our studies have shown that changing residues that are disfavored in β-sheet structures (Ala and Asp) to those such as Leu, Thr, and Phe,[23] which have high β-sheet propensities and/or introduce favorable cross-strand interactions, can have a favorable influence on binding affinity. However, this work demonstrates that, while statistical and experimental information from protein structures can be informative, the complexity of β-sheets, with differences in HB and NHB sites, edge versus internal strands, and SC−SC interactions between multiple residues, limit the predictive power of these tools. The higher complexity of β-sheet interactions relative to α-helix mediated protein−protein interactions has made them a much more challenging target, but recent work characterizing hot spots in β-sheet-mediated PPIs has begun to shed light on approaches for inhibiting them.[31]

Additionally, these studies provided insight into the scope of the selectivity of the *d*HP1 chromodomain for its intended target, exhibiting only modest selectivity for the wt histone tail relative to the mutants studied. Moreover, mutation to the *d*HP1 chromodomain demonstrated the ability to tune the affinity for the wt histone tail and selectivity for different histone tail sequences, suggesting the possibility of developing orthogonal mutant pairs for chemical biology applications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental characterization data for peptides, protein mutants, and binding interactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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