

RESEARCH COMMUNICATION

Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27

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The chromodomain of *Drosophila* Polycomb protein is essential for maintaining the silencing state of homeotic genes during development. Recent studies suggest that Polycomb mediates the assembly of repressive higher-order chromatin structures in conjunction with the methylation of Lys 27 of histone H3 by a Polycomb group repressor complex. A similar mechanism in heterochromatin assembly is mediated by HP1, a chromodomain protein that binds to histone H3 methylated at Lys 9. To understand the molecular mechanism of the methyl-Lys 27 histone code recognition, we have determined a 1.4-Å-resolution structure of the chromodomain of Polycomb in complex with a histone H3 peptide trimethylated at Lys 27. The structure reveals a conserved mode of methyl-lysine binding and identifies Polycomb-specific interactions with histone H3. The structure also reveals a dPC dimer in the crystal lattice that is mediated by residues specifically conserved in the Polycomb family of chromodomains. The dimerization of dPC can effectively account for the histone-binding specificity and provides new mechanistic insights into the function of Polycomb. We propose that self-association is functionally important for Polycomb.

Received April 22, 2003; revised version accepted June 5, 2003.

Assembly of higher-order chromatin structures plays an important role in eukaryotic gene regulation. A histone code hypothesis, linking posttranslational modifications of histones to chromatin-mediated biological processes, has been the subject of many recent studies (Strahl and Allis 2000; Turner 2000). In particular, histone methylation has been shown to mediate diverse biological processes such as heterochromatin formation, X chromosome inactivation, and transcriptional regulation (Grewal and Elgin 2002). Several SET-domain histone lysine methyltransferases have been identified (Zhang and

Reinberg 2001; Lachner and Jenuwein 2002): They methylate various lysine residues located at the flexible N termini of histones H3 and H4. Methylation of different lysine residues has differential biological effects. To decipher the histone code generated by lysine methylation, it is important to understand the mechanism by which the various methyl-lysine signals are differentially recognized by the cellular machinery.

Two well-studied chromatin-regulated biological processes are position effect variegation (PEV) and homeotic gene silencing in *Drosophila*. Heterochromatin formation in PEV is associated with methylation of Lys 9 of histone H3 by SU(VAR)3-9 and its homologs (Rea et al. 2000). Methylation of Lys 27 of histone H3 by a multi-protein complex containing Enhancer of Zeste, E(z), and Extra Sex Combs (ESC), or their human counterparts EZH2 and EED, are associated with the repression of homeotic genes (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002) as well as with the inactive X chromosome (Plath et al. 2003; Silva et al. 2003). Interestingly, Lys 9 methylation has also been reported as an early marker on the inactive X chromosome (Heard et al. 2001). Methylation of Lys 9 and Lys 27 of histone H3 creates binding sites for the chromodomains of HP1 and Polycomb (PC) proteins, respectively (Banister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001; Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). Chromodomains were originally identified as a conserved sequence motif between PC and HP1 (Paro and Hogness 1991), although the chromodomain protein family has expanded considerably since the original identification (Eissenberg 2001). The chromodomain of PC alone is fully functional in nuclear localization and chromosome binding (Messmer et al. 1992). Despite the high sequence homology between the two chromodomains, PC and HP1 bind to distinct chromosomal loci (James et al. 1989; Zink and Paro 1989), and the molecular mechanisms of locus specific recruitment of PC and HP1 remain poorly understood.

The structure of the HP1 chromodomain in complex with a methyl-Lys 9 histone H3 peptide has been determined recently (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002). The structure greatly advanced our understanding of methylated histone tail-chromodomain interactions. However, the HP1 structure offered no insights into the molecular mechanism of specific binding of the highly homologous PC chromodomain to histone H3 tails methylated at Lys 27. Here we report a 1.4-Å-resolution structure of the *Drosophila* Polycomb (dPC) chromodomain in complex with a histone H3 peptide trimethylated at Lys 27 (m₃K27). The structure shows a conserved mode of methyl-lysine recognition and reveals key elements in histone H3 and dPC that can account for the binding specificity. In particular, crystal packing of two dPC molecules via evolutionarily conserved residues gives rise to an interesting model that the chromodomain of dPC functions as a dimer. This model agrees well with in vitro chemical cross-linking and in vivo domain swap results; it predicts that histone-histone interactions are important for the selective binding of the histone H3 tail to dPC, and dPC facilitates homeotic gene silencing by mediating interactions of nucleosomes methylated at Lys 27.

[**Keywords:** Chromatin; histone code; histone methylation; Polycomb; chromodomain]

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.269603>.

Results and Discussion

The structure of the dPC chromodomain (amino acids 23–77) in complex with a histone H3 peptide (amino acids 19–33) containing m₃K27, termed H3m₃K27 hereafter, was solved by molecular replacement using the structure of the chromodomain of *Drosophila* HP1 as a search model (Fig. 1A). The entire dPC chromodomain and residues 20–30 of the H3m₃K27 peptide are ordered in the structure. The 1.4-Å-resolution structure has excellent stereochemical quality, as 91.5% of the protein main chain ϕ/ψ angles are within the most favored region and none in the disallowed region of the Ramachandran plot, calculated using PROCHECK (Laskowski et al. 1993). Detailed statistics of the crystallographic analysis are shown in Table 1.

Chromodomain–histone interactions

The dPC chromodomain consists of three β -strands (β 1– β 3) that form a twisted antiparallel β -sheet. A helix (α A) located at the C-terminal end is packed against one edge of the β -sheet next to β 1 (Fig. 1B). The histone H3 peptide is bound in a cleft formed between a segment of dPC

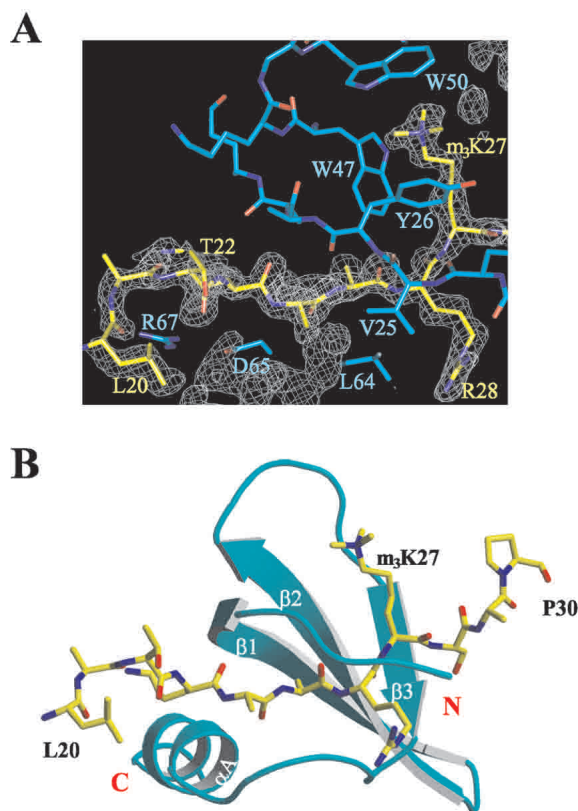


Figure 1. Structure of the dPC chromodomain in complex with the H3m₃K27 peptide. (A) A simulated annealing omit electron density map showing the binding of H3m₃K27. The 1.4 Å ($F_{\text{O}} - F_{\text{C}}$, ϕ_{C}) difference map was calculated with the peptide omitted, and the map is contoured at the 1.0 σ contour level with a 3.0 σ cutoff. The refined structure is superimposed as a bond model: red, oxygen; blue, nitrogen; cyan in dPC, carbon; yellow, carbon in the peptide. Key residues of histone H3 and dPC are labeled as follows: cyan, dPC; yellow, histone H3. (B) Overall structure of the dPC chromodomain–H3m₃K27 peptide complex. dPC is shown in a ribbon representation, and the H3m₃K27 peptide is shown as a bond model.

Table 1. Summary of crystallographic analysis

Diffraction data ^a	
Resolution (Å)	1.40 (1.45–1.40)
Measured reflections	85538
Unique reflections	17050 (811)
Completeness (%)	86.8 (42.3)
Average I/ σ	24.5 (4.1)
R_{merge} (%) ^b	4.2 (19.5)
Refinement	
Resolution range (Å)	50.0–1.40 (1.46–1.40)
R -factor/ R_{free} (%) ^c	19.7/21.6 (29.8/32.0)
Number of protein atoms	559
Number of ions	1 acetate, 2 BME, 2 Cl ⁻
Number of water molecules	147
RMSD	
Bond lengths	0.006 Å
Bond angles	1.29°
Dihedrals	24.12°
Improper	0.70°

^aNumbers enclosed in parentheses are the values of the highest resolution shell.

^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I and $\langle I \rangle$ are the measured and averaged intensities of multiple measurements of the same reflection. The summation is over all the observed reflections.

^c R -factor = $\sum ||F_{\text{O}}| - |F_{\text{C}}|| / \sum |F_{\text{O}}|$, where F_{O} denotes the observed structure factor amplitude and F_{C} denotes the structure factor calculated from the model.

N-terminal to β 1 and the loop connecting β 3 and α A. Consistent with the high sequence homology of PC and HP1 chromodomains (Fig. 2A), the overall structures of the dPC and HP1 chromodomains are very similar (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002). The C $_{\alpha}$ positions of dPC and HP1 chromodomains can be aligned with a root-mean-squared deviation (RMSD) of 1.1 Å, and the two peptides (amino acids 5–10 and 23–28 of histone H3) can be aligned with an RMSD of 0.45 Å. Many features of the interactions between dPC and the H3m₃K27 peptide are also similar to that between HP1 and H3m₃K9 peptides. First, m₃K27 is bound in a hydrophobic pocket formed by three aromatic residues, Tyr 26, Trp 47, and Trp 50 (Fig. 1A). Second, the main-chain carbonyl and amino groups of Lys 23, Ala 24, Ala 25, and Arg 26 of the H3m₃K27 peptide are involved in β -sheet-like hydrogen bonding with dPC residues 24–28 located at the N terminus and residues 62–65 located in the loop connecting β 3 and α A (Fig. 2B). Third, the hydroxyl group of Ser 28 of histone H3 makes hydrogen bonds to Glu 58 and Asn 62 (Fig. 2B). Fourth, Ala 25 of histone H3 is buried in a shallow hydrophobic pocket surrounded by Ala 28, Trp 47, Ile 63, and Leu 68.

One noticeable difference between the structures of dPC and HP1 occurs in the methyl-lysine binding pocket, in which m₃K9 or m₃K27 interact with three aromatic residues via hydrophobic and cation- π interactions. In the HP1 structure, the residue corresponding to Trp 50 of dPC is a tyrosine or phenylalanine (Fig. 2A). The conformation of Trp 50 is stabilized by the presence of Tyr 54, which packs against Trp 50 with its ring approximately perpendicular to that of Trp 50. The side chain of Tyr 54 does not interact with m₃K27 directly or via water molecules, whereas the corresponding residue in HP1 interacts with m₃K9 either directly or via a water molecule (Jacobs and Khorasanizadeh 2002; Nielsen et

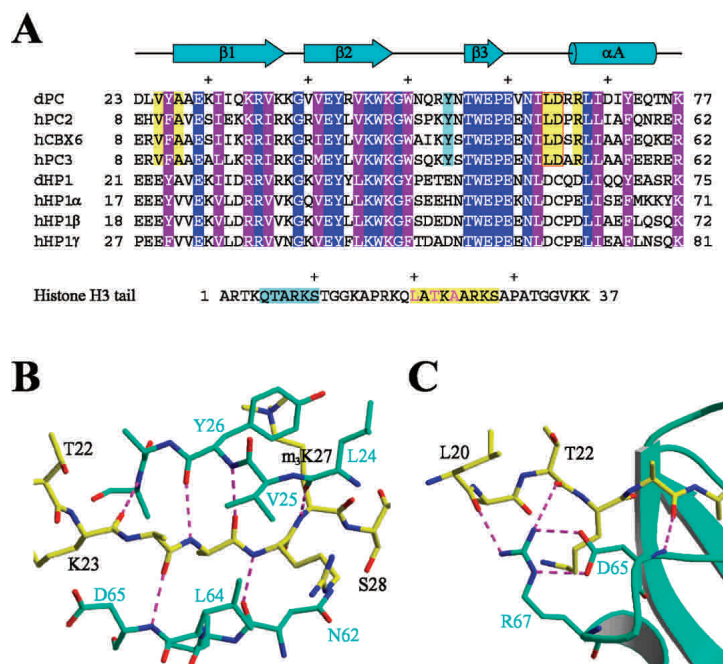


Figure 2. Chromodomain–histone interactions. (A) Alignment of chromodomain sequences of *Drosophila* and human PC and HP1 proteins. dPC, *Drosophila* Polycomb (SWISS-PROT database accession code P26017); hPC2, human Polycomb 2 homolog (SWISS-PROT O00257); hCBX6, human chromobox homolog 6 (SWISS-PROT O95503); hPC3, human Polycomb 3 homolog (SWISS-PROT Q9HC52); dHP1, *Drosophila* HP1 (SWISS-PROT P05205). hHP1 α , hHP1 β , and hHP1 γ are human HP1 homologs with the SWISS-PROT accession codes P45973, P23197, and Q13185, respectively. Identical residues among these proteins are shown in white letters over a blue background, and similar residues are shown in white letters over a purple background. Amino acids specifically conserved in the PC family are highlighted: Yellow indicates residues involved in PC-specific interactions with histone H3, and the residues enclosed in a red box are involved in the formation of a dPC dimer. Tyr 54 highlighted in cyan is located at the methyl-lysine binding pocket. Every 10 residues are indicated with a “+” above the sequence. Secondary structural elements and their nomenclatures are shown above the sequences. The sequence of the N-terminal tail of histone H3 is also shown. HP1-interacting residues are highlighted in cyan, and the residues interacting with the chromodomain of dPC are highlighted in yellow. Residues shown in red letters are important for specific binding to dPC. (B) A bond model showing extensive hydrogen bonding between main-chain atoms of dPC and the H3m₃K27 peptide. Hydrogen bonds are drawn in magenta broken lines, and the same color code as in Figure 1 is used for dPC and H3m₃K27. (C) Unique interactions between dPC and H3m₃K27 occur at the N-terminal end of the peptide. The H3m₃K27 peptide and Arg 67 and Asp 65 of dPC are shown in a bond model superimposed with the ribbon diagram of dPC.

al. 2002). In the dPC structure, the C _{ϵ} –N _{ζ} bond of m₃K27 points to the center of the six-carbon ring of Trp 50, and the distance between the N _{ζ} atom of m₃K27 and the center of the six-carbon ring is 4.1 Å (Fig. 1A). This geometric arrangement enables a favorable cation- π interaction between the methylammonium group of m₃K27 and Trp 50. In the HP1 structures, the C _{ϵ} –N _{ζ} bond of m₃K9 is not lined up with the center of the phenyl ring (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002), which results in a less-than-optimal cation- π interaction between m₃K9 and the tyrosine or phenylalanine.

Leu 20 and Thr 22 of histone H3 are involved in unique interactions with Arg 67 of dPC. The carbonyl groups of Leu 20 and Thr 22 form hydrogen bonds with the side chain NH₂ groups of Arg 67 (Fig. 2C). The conformation of Arg 67 is stabilized by two hydrogen bonds

with the side chain of Asp 65, which itself interacts with the H3m₃K27 peptide via main-chain hydrogen bonding as described earlier. Although both Arg 67 and Asp 65 are specifically conserved in PC chromodomains (Fig. 2A), and the observed interactions are not present in the HP1–H3m₃K9 complex, the interactions between Arg 67 and Leu 20 and Thr 22 of H3m₃K27 cannot account for the binding specificity as only the main-chain atoms of histone H3 are involved in the interactions.

Polycomb binding specificity

It is puzzling that the PC-specific interactions identified above are through the main-chain atoms of the histone H3 peptide, as they provide no clear answers to the apparent in vitro binding preferences of dPC and HP1 (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002). A careful examination of the structure identifies a potential chromodomain dimer that can account for the binding specificity of dPC (Fig. 3). The dimer is formed across the crystal lattice, which at first sight might be discounted as a crystal-packing artifact. However, the following reasons prompt us to examine the dimeric interaction in detail: (1) The residues involved in the protein–protein interaction are specifically conserved in the Polycomb family of proteins. (2) There are very few solvent molecules at the interface of the two dPC complexes. (3) Dynamic light scattering in solution shows that the chromodomain of dPC has an apparent molecular mass of 14.2 kD, which is close to twice the 6.8 kD calculated mass of a monomer. (4) A dPC chromodomain dimer is consistent with previous in vivo domain-swap (Platero et al. 1995) and in vitro chemical cross-linking studies (Cowell and Austin 1997).

It is interesting to note that the HP1 chromodomain apparently exists as a monomer (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002), although the full-length protein is known to oligomerize via the C-terminal chromo shadow domain (Brasher et al. 2000; Cowieson et al. 2000). In the crystal structure of the chromodomain of dPC, the two monomers are located across two adjacent asymmetric units. The two chromodomains of dPC in the dimer interact via intermolecular hydrogen bonds between the main-chain atoms of Leu 64 and Arg 66 (Fig. 3A). Additionally, the N _{η 1} atom of Arg 66 makes one hydrogen bond with the carbonyl of Val 61. Leu 64 is specifically conserved in the PC family of proteins, whereas Arg 66 and Val 61 are not. However, the two main-chain hydrogen bonds between Leu 64 and Arg 66 appear to be specific to the PC family of proteins. This is because the side chain of Cys 63 of HP1 (*Drosophila* HP1 numbering) is packed in a hydrophobic core, whereas the corresponding residue in dPC, Asp 65, is excluded from a similar hydrophobic environment. The packing of Cys 63 of HP1 causes an alteration of the main-chain conformation that prevents the formation of similar intermolecular hydrogen bonding. Both Asp 65 of dPC and Cys 63 of HP1 are specifically conserved in their respective chromodomain subfamilies (Fig. 2A).

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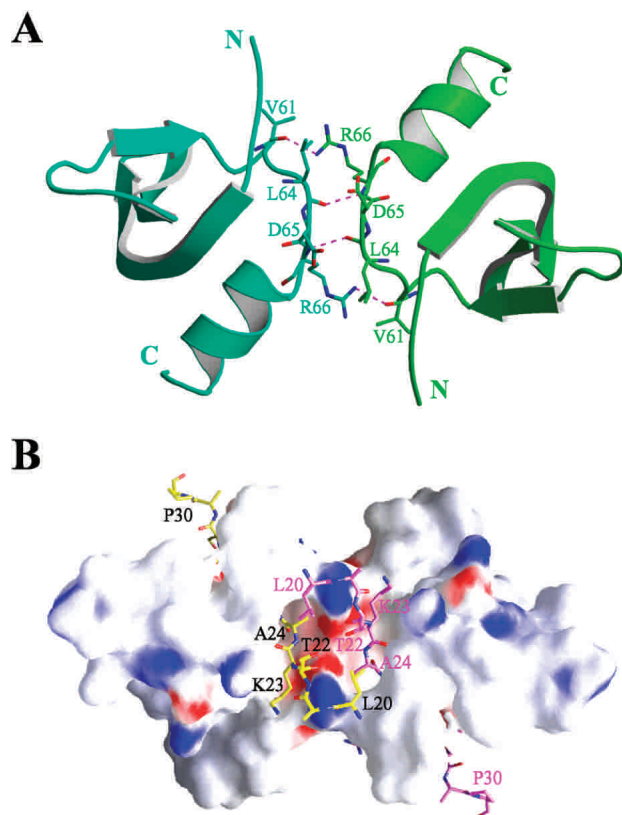


Figure 3. The dPC chromodomain dimer. (A) Two dPC chromodomain monomers are shown in a ribbon representation, colored cyan and green, respectively. Key residues involved in dimerization are shown in a bond model. Hydrogen bonds involving these residues are indicated with magenta broken lines. (B) The dPC chromodomain dimer juxtaposes the two binding sites of H3m₃K27. The dPC chromodomain dimer is shown in a surface representation. The dimer is viewed from a similar direction as in A. Surface areas colored blue or red indicate positive or negative electric potentials. Two bound H3m₃K27 peptides are shown in a bond model. Carbon atoms in the second peptide are colored magenta.

The dPC chromodomain dimer juxtaposes the two H3-binding clefts in an antiparallel fashion and results in histone–histone interactions involving Leu 20, Thr 22, and Ala 24 of histone H3 (Figs. 3, 4). In the structure, Arg 67 of dPC stabilizes the N-terminal region of the histone H3 peptide through hydrogen bonding of its N_η atoms with the histone H3 carbonyl groups of Leu 20 and Thr 22 (Fig. 2C). These interactions position the side chain of Leu 20 in a mostly hydrophobic pocket formed by the side chains of Val 25, Tyr 26, and Ala 27 of another PC molecule in the dimer, and the side chains of Thr 22 and Ala 24 of the other histone H3 peptide (Fig. 4). This recognition mode can effectively exclude the binding of a histone H3 peptide encompassing methylated Lys 9, as the residues corresponding to Leu 20, Thr 22, and Ala 24 of histone H3 would be Arg 2, Lys 4, and Thr 5, respectively. Figure 4 shows that replacing Leu 20 and Thr 22 with an arginine and a lysine, respectively, will introduce steric conflicts and is strongly disfavored from an energetic standpoint. Thus, both the histone H3 sequence at positions 20, 22, 24, and the dimerization of the chromodomain of dPC are key determinants for the recognition of the histone H3 methyl-Lys 27 code.

Structural implications

The crystal structure presented here shows that the chromodomains of dPC and HP1 have a common overall structure, and they also bind methyl-lysine-containing substrates similarly. The structure identifies that Arg 67 and Asp 65 of dPC are important for Polycomb-specific interactions with histone H3. Curiously, these residues interact with the main chain of the histone peptide. This is also true in the HP1 structure, where most of the chromodomain–histone interactions are through main-chain atoms. An important question concerning the recognition of the methylated histone tail by Polycomb and HP1 is what determines their binding specificities. The difference in histone-binding affinity of Polycomb and HP1 is not sufficient to account for their substrate specificities. Our crystallographic analyses have identified a dPC chromodomain dimer in the crystal lattice that can account for the binding specificity of dPC, and we have outlined compelling reasons in support of the potential physiological significance of the observed dimeric interactions.

Although dimerization of dPC was never pointed out explicitly before, self-association of the PC chromodomain was noted in several previous studies (Platero et al. 1995; Cowell and Austin 1997). Interestingly, an *in vivo* domain-swap experiment replacing the chromodomain of HP1 with that of dPC showed that the chimeric protein binds to both heterochromatin and dPC binding sites in polytene chromosomes (Platero et al. 1995). Furthermore, some endogenous dPC is misdirected to the heterochromatic center, whereas some endogenous HP1 is mislocalized to the dPC binding sites. These observations not only confirmed earlier observations that the chromodomain of dPC and the C-terminal chromoshadow domain of HP1 possess intrinsic nuclear localization and chromosomal binding properties (Messmer et al. 1992; Powers and Eissenberg 1993), they also indicated that the dPC chromodomain directs the mislocalization of endogenous dPC through protein–protein contacts.

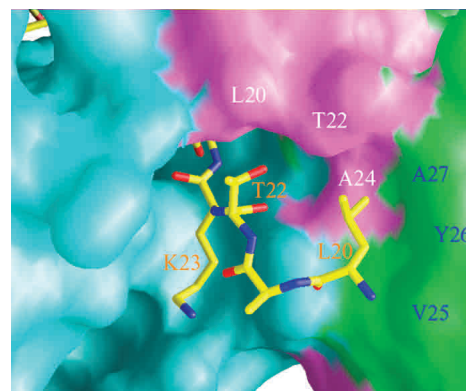


Figure 4. A shallow hydrophobic binding pocket of the N-terminal region of one H3m₃K27 peptide. Two dPC molecules and a second H3m₃K27 are shown in a surface representation. Cyan and green surfaces are that of the two dPC molecules (same coloring scheme as in Fig. 3A), and the magenta surface is that of another H3m₃K27 peptide. The binding site is formed by Val 25, Tyr 26, and Ala 27 of the other dPC chromodomain (green surface; residues labeled in blue letters) and N-terminal residues of a separate H3m₃K27 peptide (magenta surface; residues labeled in white letters) in the dimer.

The dimerization model predicts that the binding specificity of the dPC chromodomain arises from histone–histone interactions resulting from the close proximity of the two histone-tail-binding sites in the dPC chromodomain dimer. We pointed out earlier that the binding of two methyl-Lys9 histone H3 tails by the dPC chromodomain dimer is disallowed because of potential steric clashes. It is possible that the dPC chromodomain dimer may bind only one histone H3 tail in some instances. In this case, the available structural information cannot exclude the binding of a methyl-Lys9 histone H3 tail to the dPC chromodomain dimer. The observation by several groups that the PC chromodomain binds specifically to methyl-27 histone H3 peptides *in vitro* clearly supports the binding of two histone H3 tails to the dPC dimer (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002). The structure also provides insights into the function of PC proteins in the assembly of repressive higher-order chromatin structure. Because the two histone H3-binding sites are closely juxtaposed, the two histone tails are unlikely to come from the same nucleosome. In the nucleosome core particle structure (Luger et al. 1997), Lys 27 is ordered in one of the H3 tails, whereas the ordered residues start from Pro 38 in the other H3 molecule. The observed distance between the C α atoms of Pro 38 and Lys 27 of the same H3 molecule is 26 Å. The two histone-H3 Pro 38 residues are 73 Å apart (linear distance) in the nucleosome core particle structure, which converts to ~80 Å along the arc of wrapped DNA. The two Lys 27 C α atoms must be within 27 Å to occupy the two binding sites in the dPC chromodomain dimer. Assuming that the two H3 tails N-terminal to Pro 38 can be maximally stretched and free to adopt any conformation, we still cannot model the simultaneous binding of two histone tails from the same nucleosome to the dPC chromodomain dimer without steric clashes. Thus, we believe that the histone tails binding to the dPC chromodomain dimer must come from two separate nucleosomes. The *in vivo* binding of two methyl-Lys 27 histone-H3 tails, from spatially adjacent nucleosomes, will effectively lock the nucleosomes into a more compact configuration. This compaction will lead to a repressive chromatin state associated with the silencing of homeotic genes. A similar function for HP1 in the assembly of heterochromatin has been proposed, although dimerization of HP1 via the C-terminal chromo shadow domain makes the binding of two histone tails from the same nucleosome, as well as separate ones, possible.

Materials and methods

The chromodomain of dPC (amino acids 23–77) was produced in *Escherichia coli* as a GST fusion protein. The GST tag was subsequently removed by thrombin digestion. The eluted protein was further purified on a Superdex-75 gel-filtration column (AP biotech), and eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Highly purified dPC fractions were pooled and concentrated to ~15 mg/mL in a buffer containing 5 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 1 mM DTT. A solution dynamic light-scattering study (at ~5 mg/mL) shows that dPC exists as a homogeneous species with an apparent molecular mass of 14.2 kD. The calculated molecular mass of a dPC (amino acids 23–77) monomer is 6.8 kD. We interpreted that dPC may exist as a dimer in solution under the conditions tested. For cocrystallization, chemically synthesized histone H3 peptide (amino acids 19–33) trimethylated at Lys 27 was mixed with dPC (~15 mg/mL final concentration) in an ~1:1 (histone peptide vs. dPC monomer) molar ratio prior to crystallization. The mixture was incubated on ice for 1 h to allow complex

formation. The crystal of the dPC–H3 peptide complex was grown by hanging-drop vapor diffusion at 16°C in a condition containing 100 mM sodium cacodylate (pH 6.5), 0.2 M ammonium sulfate, 30% PEG-8k, and 10 mM DTT.

X-ray diffraction data were collected at 95°K using a CCD detector (ADSC) at beamline X26C of the National Synchrotron Light Source, Brookhaven National Laboratory. Raw data were processed using the HKL software package (Otwinowski and Minor 1997). The crystal has I212121 symmetry and unit cell dimensions of 32.43 Å × 77.03 Å × 77.27 Å. The structure was solved by molecular replacement using the program AmoRe (Navaza 2001). The crystal structure of the *Drosophila* HP1 chromodomain (Jacobs and Khorasanizadeh 2002) was used as the search model (the methyl-Lys 9 histone H3 peptide was omitted). An initial dPC model based on the molecular replacement solution was subjected to automatic main-chain tracing and side-chain docking using ARP/warp (Perrakis et al. 1999). The H3 peptide structure was built manually. The graphics program O (Jones et al. 1991) was used for model building and visualization. The structure was refined using CNS (Brunger et al. 1998). Detailed statistics of the crystallographic analysis can be found in Table 1. Figures were prepared using PyMol (DeLano 2002), Molscript (Kraulis 1991), Raster3D (Merritt and Bacon 1997), and Grasp (Nicholls et al. 1991).

The PDB accession code is 1PF8.

Acknowledgments

We thank Dieter Schneider and Annie Heroux for technical assistance at the National Synchrotron Light Source, Brookhaven National Laboratory, and Ira Hall, Lee Henry, and Leigh Johnson for comments on the manuscript. R.M.-X. also acknowledges Sepideh Khorasanizadeh for communication of results prior to publication. Y.Z. is a Kimmel Scholar and is supported by grants from the NIH (GM63070) and American Cancer Society (RSG-00-351-01-GMC). R.M.-X. is supported by the W.M. Keck foundation and a grant from the NIH (GM63716).

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Genes Dev. 2003, **17**:

Access the most recent version at doi:[10.1101/gad.269603](https://doi.org/10.1101/gad.269603)

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