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Structural basis of HP1/PXVXL motif peptide interactions and HP1 localisation to heterochromatin

Abarna Thiru[1], Daniel Nietlispach[1], Helen R Mott[1], Mitsuru Okuwaki[2], Debbie Lyon[2], Peter R Nielsen[1], Miriam Hirshberg[1], Alain Verreault[2], Natalia V Murzina[1,3,*] and Ernest D Laue[1,3,*]

[1]Department of Biochemistry, Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge, UK and [2]Clare Hall Laboratories, London Research Institute, Cancer Research UK, South Mimms, UK

HP1 family proteins are adaptor molecules, containing two related chromo domains that are required for chromatin packaging and gene silencing. Here we present the structure of the chromo shadow domain from mouse HP1β bound to a peptide containing a consensus PXVXL motif found in many HP1 binding partners. The shadow domain exhibits a novel mode of peptide recognition, where the peptide binds across the dimer interface, sandwiched in a β-sheet between strands from each monomer. The structure allows us to predict which other shadow domains bind similar PXVXL motif-containing peptides and provides a framework for predicting the sequence specificity of the others. We show that targeting of HP1β to heterochromatin requires shadow domain interactions with PXVXL-containing proteins in addition to chromo domain recognition of Lys-9-methylated histone H3. Interestingly, it also appears to require the simultaneous recognition of two Lys-9-methylated histone H3 molecules. This finding implies a further complexity to the histone code for regulation of chromatin structure and suggests how binding of HP1 family proteins may lead to its condensation.

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Introduction

Heterochromatin protein 1 (HP1) was originally identified in *Drosophila melanogaster* as an abundant nonhistone chromosomal protein that predominantly localises to pericentric heterochromatin (James and Elgin, 1986). It is highly conserved, and several organisms have more than one HP1 family member, which may have slightly different functions. For example, mammalian cells contain three HP1 isoforms, named HP1α, HP1β (or MOD1 and M31) and HP1γ (or MOD2 and M32) (reviewed in Eissenberg and Elgin, 2000; Li *et al*, 2002). In mouse and human cells, HP1α is found predominantly in centromeres, HP1β is distributed widely on the chromosome, and HP1γ localises mostly to euchromatin (Minc *et al*, 1999, 2000, 2001).

HP1 proteins play a widespread role in gene silencing, both through formation of heterochromatin and at euchromatic promoters (reviewed in Li *et al*, 2002). In contrast to its widespread role in gene silencing, HP1 is also required for the normal expression of some heterochromatic and euchromatic genes (Lu *et al*, 2000; Piacentini *et al*, 2003). In fact, mRNA expression analyses have shown that several hundred *D. melanogaster* genes are upregulated upon mutation of HP1, while several hundred more are downregulated (Li *et al*, 2002).

Sequence comparison of different HP1 proteins identified the presence of an N-terminal 'chromatin organisation modifier' (chromo) domain that is connected to a slightly larger, homologous domain termed the chromo shadow domain (hereafter, shadow domain or HP1C) that is unique to the HP1 family (Paro and Hogness, 1991; Epstein *et al*, 1992; Aasland and Stewart, 1995; Koonin *et al*, 1995). A striking difference between the two domains is that while the chromo domain is monomeric (Ball *et al*, 1997), the shadow domain forms a tightly associated symmetrical dimer (Brasher *et al*, 2000; Cowieson *et al*, 2000). The hinge region linking the two domains is unstructured in solution, enabling the chromo and shadow domains to move independently of each other in the intact protein (Brasher *et al*, 2000). The construction of chimaeric proteins, consisting of either the HP1 protein with its chromo domain replaced by that from Polycomb (Pc), or the Pc protein with an HP1 chromo domain, has shown that both the chromo and shadow domains are important for correct chromatin localisation (Platero *et al*, 1995).

The methylation of histone H3 at Lys-9 (H3-K9) by the SUV39 methyltransferase and its homologues (e.g. Clr4 in *Schizosaccharomyces pombe*) constitutes part of the epigenetic histone code for gene regulation (Rea *et al*, 2000; Strahl and Allis, 2000; Bannister *et al*, 2001; Lachner *et al*, 2001). This modification is specifically recognised and bound by the HP1 chromo domain, resulting in the regulation of chromatin structure and HP1-mediated gene repression (Bannister *et al*, 2001; Lachner *et al*, 2001). For example, mutation of *D. melanogaster* Su(var)3-9 leads to elevated expression of HP1-regulated genes (Hwang *et al*, 2001), and H3-K9 methylation by Clr4 directly recruits the *S. pombe* HP1 homologue Swi6 to silent mating type loci to establish heterochromatic silencing (Noma *et al*, 2001). Recent studies have shown that double-stranded RNA arising from centro-

*Corresponding authors. Department of Biochemistry, Cambridge Centre for Molecular Recognition, University of Cambridge, 80 Tennis Court Road, Cambridge CB1 1GA, UK. Tel.: +44 1223 333 677; Fax: +44 1223 766002; E-mail: e.d.laue@bioc.cam.ac.uk or nm@mole.bio.cam.ac.uk
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meric repeats, together with components of the RNA interference (RNAi) machinery, are required for the establishment of H3-K9 methylation and the formation of heterochromatin. Once established, the maintenance of this heterochromatic state and its inheritance through subsequent mitotic and meiotic cell divisions depends on HP1/Swi6 alone (Hall *et al*, 2002; Jenuwein, 2002; Volpe *et al*, 2002).

The C-terminal shadow domain of HP1 interacts with numerous proteins (reviewed in Li *et al*, 2002). Many of these interactions play an important role in directing heterochromatin formation and/or gene silencing. A large number of the shadow domain interaction partners contain a PXVXL pentapeptide motif, including: TIF1α, TIF1β and $TAF_{II}130$, which regulate transcription; IDN3, which may play a role in chromosome segregation; and the large (p150) subunit of chromatin assembly factor 1 (CAF-1), which contributes to nucleosome assembly during DNA replication and repair (LeDouarin *et al*, 1996; Murzina *et al*, 1999; Vassallo and Tanese, 2002). The function of many of these proteins relies on their interaction with the shadow domain, linking them to H3-K9 methylation and the establishment/maintenance of gene silencing. For example, TIF1β-mediated gene repression relies on the presence of HP1 and the H3-K9 methyltransferase SET1DB (Schultz *et al*, 2002). Finally, the hinge region that connects the chromo and shadow domains is important for nuclear targeting (Smothers and Henikoff, 2001) and, in mouse HP1α/γ, RNA binding (Muchardt *et al*, 2002).

The results so far thus suggest that the HP1 proteins function as adaptors, bringing together different proteins in multiprotein complexes *via* protein–protein interactions with the chromo and shadow domains. In order to understand further and dissect the mechanisms involved, we have solved the structure of the mouse HP1β shadow domain in a complex with the HP1 binding region of the CAF-1 p150 subunit by NMR spectroscopy. The structure of the complex shows how the HP1β shadow domain specifically recognises and binds to PXVXL motif peptides, explaining many previous biochemical studies of this interaction. It allows us to predict which other shadow domains are likely to recognise similar peptides, and provides a framework for predicting the sequence specificity of those that bind different motifs. Structure-based mutagenesis studies identified mutants that bind selectively to certain shadow domain partners, but not others, and suggests that phosphorylation might regulate interactions of partner proteins with shadow domains. By studying mutants, we also further define the roles of the chromo and shadow domains in the localisation of HP1β to heterochromatin *in vivo*.

Results and discussion

Structure determination

CAF-1, whose function is to deposit newly synthesised and acetylated histones H3/H4 into chromatin during DNA replication and repair, comprises three polypeptide subunits known as p150, p60 and p48 (Smith and Stillman, 1989; Kaufman *et al*, 1995; Gaillard *et al*, 1996). In previous work, we showed that the stable association of CAF-1 p150 with mouse HP1 proteins leads to its retention in heterochromatin outside of S-phase (Murzina *et al*, 1999). Most of the amino-acid similarity between the mouse, human and *D. melanogaster* p150 subunits lies within the C-terminal half, which is essential for nucleosome assembly. Sequence analysis suggests that the HP1 interaction region in CAF-1 p150 lies within the less conserved N-terminal region immediately N-terminal to a PEST region that is likely to be responsible for the rapid degradation of CAF-1 p150 *in vivo*—this sequence is of low complexity and probably unstructured. Analysis of the cDNA clones encoding portions of mouse CAF-1 p150 that bound to HP1β in our two-hybrid screen showed that they have a 66-amino-acid region (residues 204–269) in common (Murzina *et al*, 1999). This fragment, together with one of the larger clones isolated in the two-hybrid screen (residues 176–327), was expressed in *Escherichia coli* as a His-tagged fusion protein, purified, and shown to be unstructured by NMR and CD studies (data not shown). Limited proteolysis of the 66-residue peptide bound to HP1C showed that residues 210–238 of CAF-1 p150 were protected from cleavage in the complex. This latter peptide was therefore used for structural studies. The precise interacting region of CAF-1 p150, residues 217–230, was subsequently determined during the course of the structural work using NMR chemical shift mapping and ^{15}N-relaxation studies (Figure 1).

In a symmetric homodimer, such as the shadow domain, signals in the NMR spectra that arise from the same residue in the two equivalent subunits have identical chemical shifts, that is, they display symmetry-related chemical shift degeneracy. Upon binding the CAF-1 fragment, degeneracy in the NMR spectra of the dimeric HP1C is lost, and separate signals are observed for many residues in the two subunits. Within and close to the CAF-1 binding site, equivalent nuclei in the two monomers have distinct chemical shifts. In these regions, the process of assignment of signals in the NMR spectra to particular nuclei is similar to that of a heterodimer–peptide complex (see Figure 1C). Away from the CAF-1 binding site, corresponding nuclei in the two monomers exhibit either the same or only slightly different chemical shifts. Although the assignment of HP1C backbone nuclei in the complex was performed using standard triple-resonance spectra (Cavanagh *et al*, 1996), it was necessary to use NOE data to assign some of the signals to a particular monomer. Structure calculations were carried out using ambiguous NOE restraints and the ARIA program interfaced to CNS (Brunger *et al*, 1998; Linge *et al*, 2001). Signals in the NMR spectra that did not split into two (i.e. those that maintained symmetry-related chemical shift degeneracy) were assigned to both monomers. (A table of key unambiguous NOEs that define the peptide interaction is provided in Supplementary data.) In order to aid convergence in the structure calculations and to incorporate information in the NMR spectra about residual symmetry in HP1C following complex formation, symmetry restraints were imposed over some C_α atoms. Distance symmetry restraints, which permit the evolution of a symmetry axis, were imposed over the C_α atoms in HP1C that retained symmetry-related chemical shift degeneracy upon peptide binding. Non-crystallographic symmetry (NCS) restraints, which minimise the RMSD between equivalent regions of the two monomers, were applied to HP1C C_α atoms and weighted according to the level of symmetry-related chemical shift degeneracy observed at a particular residue. Residues for which chemical shift degeneracy was lost in both backbone and side-chain resonances did not have symmetry restraints applied to their C_α atoms (see Experimental procedures).

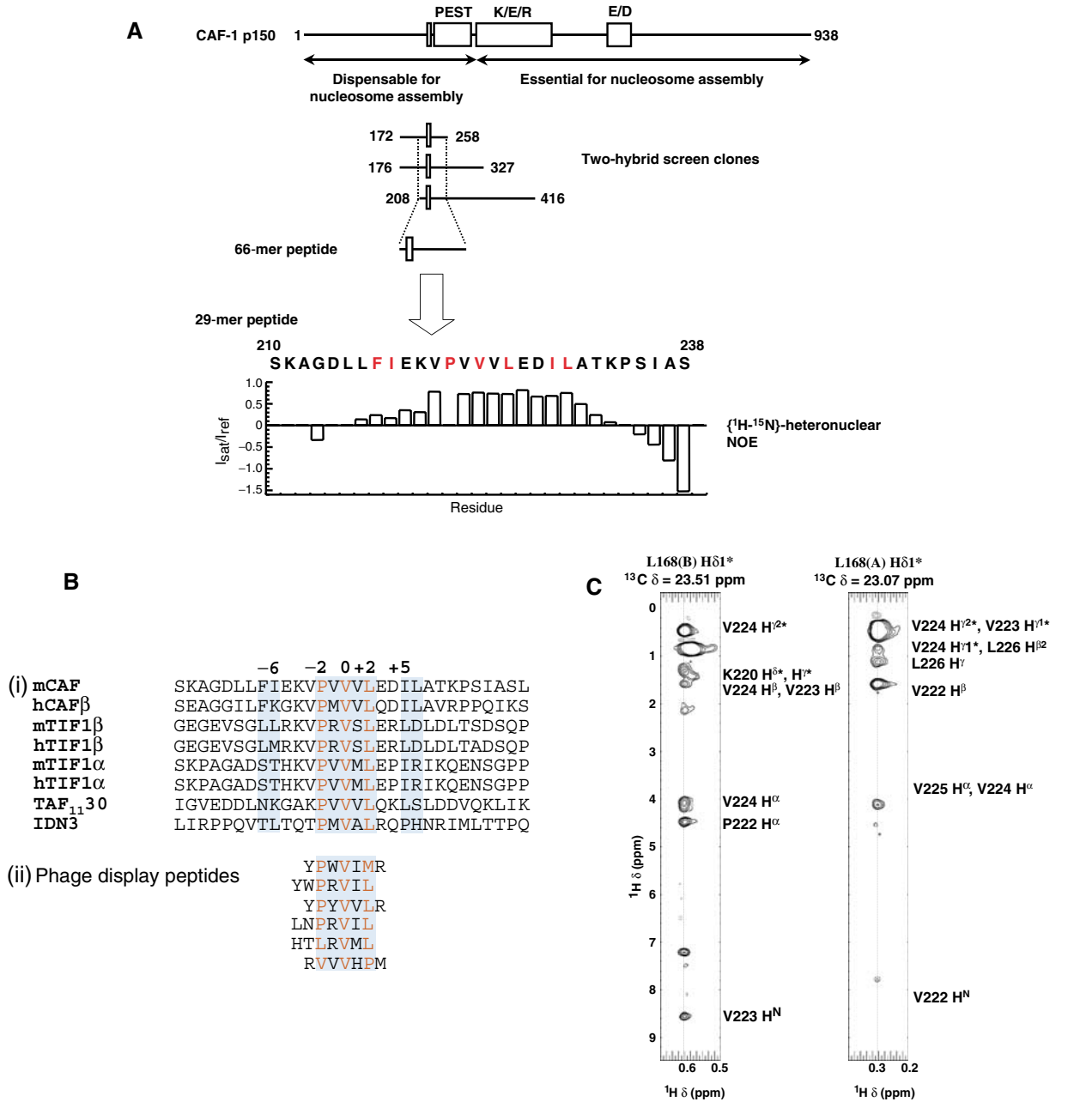

Figure 1 The HP1 interaction region, located within the N-terminal domain of CAF-1 p150, binds to the shadow domain of mouse HP1β. (**A**) A number of overlapping cDNA clones encoding portions of mouse CAF-1 p150 were isolated through a two-hybrid screen using HP1β as bait (Murzina *et al*, 1999). These clones had a 66-residue sequence in common that was expressed and used in limited proteolysis experiments with HP1C to define the interacting fragment. This was subsequently refined using NMR chemical shift mapping and ^{15}N NMR relaxation experiments. The residues coloured red are involved in hydrophobic interactions with the HP1β shadow domain. (**B**) Sequence alignment of (i) proteins that contain the PXVXL motif (conserved residues coloured red) and (ii) peptides identified in phage display experiments (Smothers and Henikoff, 2000). The positions of residues involved in the interaction with CAF-1 are highlighted in blue. (**C**) Example regions of the ^{13}C, ^{15}N X-filtered NOESY spectrum illustrating the different intermolecular NOEs observed to the CAF-1 peptide from L168 Hδ1 in the two HP1C monomers (A, B).

Structure of the HP1C/CAF-1 complex

The CAF-1 peptide, which is unstructured in its free form, binds in an extended conformation across the symmetry axis of the HP1C dimer, ordering the C-terminal tails and burying ~2750 Å^2 in surface area (Figure 2). The PXVXL motif forms a parallel β-sheet with the C-terminal tail of monomer A and an antiparallel β-sheet with monomer B. Additional contacts are made by residues on either side of this motif, which mediate interactions with equivalent hydrophobic patches on the β-sheets of the two monomers. Upon binding, the C-terminal helices of the two HP1C monomers rotate apart to accommodate the CAF-1 peptide (Figure 2B).

The structure of the shadow domain exhibits a novel mode of peptide recognition, in which the interface of a symmetrical dimer (Brasher *et al*, 2000) is required for recognition of a conserved peptide motif found in numerous proteins. In other known protein interaction modules, for example, SH2 domains (reviewed in Pawson *et al*, 2001), peptides typically bind in a hydrophobic groove formed by residues from a single monomeric domain. Remarkably, this particular mode

Table 1 Experimental restraints and structural statistics

Number of experimental restraints	*Unambiguous*	*Ambiguous*
Distance restraints from NOEs		
Intramolecular (HP1β)	1194	2215
Intramolecular (peptide)	335	161
Intermolecular	195	327
Dihedral restraints (HP1β)	133	
Coordinate precision[a]	$\langle$SA$\rangle$	$\langle$SA$\rangle_c$
RMSD of backbone atoms (Å)	0.65±0.11	0.41
RMSD of all heavy atoms (Å)	1.06±0.11	0.92
RMS deviations		
From the experimental restraints		
NOE distances (Å)	0.018±0.0030	0.018
Dihedral angles (deg)	0.494±0.077	0.501
From idealized geometry		
Bonds (Å)	0.0018±9.19e−5	0.0017
Angles (deg)	0.343±0.0090	0.328
Impropers (deg)	0.232±0.0123	0.206
Final energy, E^c_{L-J} (kJ/mol)[b]	−1340.56±15	−1325.76
Ramachandran analysis		
Residues in most favoured regions	67.2%	69.9%
Residues in additionally allowed regions	25.2%	24.2%
Residues in generously allowed regions	5.4%	3.9%
Residues in disallowed regions	2.2%	2.0%

$\langle$SA$\rangle$ represents the average RMS deviations for the ensemble.
$\langle$SA$\rangle_c$ represents values for the structure that is closest to the mean.
[a]Computed over residues 112–172 of HP1β and 12–23 of the CAF-1 peptide.
[b]The Lennard–Jones potential was not used at any stage in the refinement.

of interaction, where the bound PXVXL peptide becomes sandwiched in an intermolecular β-sheet in the shadow domain, is uniquely shared with the chromo domain/histone H3 interaction (Jacobs and Khorasanizadeh, 2002; Nielsen *et al*, 2002). The two interactions differ, however, in that the chromo domain is monomeric and the two strands from the same domain form an antiparallel instead of a mixed β-sheet upon interaction with the peptide (Figure 2B).

Recognition of the CAF-1 peptide by the shadow domain

The structure identifies key residues that recognise the CAF-1 peptide. Val-224, which lies at the centre of the PXVXL motif (position 0), binds in a hydrophobic pocket at the dimer interface, with its methyl groups packing against Tyr-164 and Leu-168 from both shadow domain monomers (Figures 2C and D). Larger side chains (e.g. Ile or Leu) cannot fit into this binding pocket, while shorter side chains (e.g. Ala) cannot make optimal contacts. This explains previous data, which showed that Val at position 0 is required for specific binding to HP1C (LeDouarin *et al*, 1996; Murzina *et al*, 1999; Smothers and Henikoff, 2000; Vassallo and Tanese, 2002). Residues at the −2 and +2 positions (Pro-222 and Leu-226, respectively) are also conserved in shadow domain-binding proteins (Figure 1B) and are important for specificity (LeDouarin *et al*, 1996; Murzina *et al*, 1999; Smothers and Henikoff, 2000; Vassallo and Tanese, 2002). Pro-222 binds in a hydrophobic cleft formed by Ala-125, Phe-163, Arg-167, Leu-168 and Trp-170, while Leu-226 binds in the symmetry-related cleft (Figures 2C and E). The structural differences between the two sites upon binding CAF-1, which arise from the rigidity of the −2 Pro, reflect the plasticity of these binding sites and predict that other large hydrophobic groups may bind at +2/−2. This is consistent with phage display experiments that showed that peptides with Leu in the −2 position or Met and Pro in the +2 position can bind to the shadow domain of *D. melanogaster* HP1 (Figure 1B) (Smothers and Henikoff, 2000).

Although the PXVXL motif is sufficient for binding, the structure of the HP1C/CAF-1 complex demonstrates that recognition of additional flanking residues is also important for the interaction. This was not anticipated from previous sequence comparisons or phage display experiments (Murzina *et al*, 1999; Smothers and Henikoff, 2000). Residues 227–231 in CAF-1 form a turn, which is stabilised by the formation of both intra- and intermolecular hydrogen bonds, burying Ile-229 and, to a lesser extent, Leu-230 (+5 and +6 positions) in a patch of hydrophobic residues (Thr-126, Leu-135 and Leu-146) on the β-sheet of monomer B (Figures 2C and E). Many of the shadow domain interaction partners contain Leu, Ile or Pro at the +5 position (Figure 1B), which could interact in a similar way if the surrounding residues have a high propensity to form a turn. Due to the bulge in the polypeptide backbone around Pro-222, it is the side chains of Phe-217 and Ile-218 (−7/−6, as opposed to the symmetry-related −6/−5 positions) that interact with the equivalent β-sheet on monomer A (Figures 2C and E). The structure suggests that, for proteins with a Pro in the −2 position, binding will be strengthened by the presence of hydrophobic side chains in the −7/−6 positions, as in CAF-1 and TIF1β (Figure 1B), whereas proteins having a different hydrophobic residue at −2 will bind more tightly if they contain large hydrophobic residues at the −5/−6 positions.

Analysis of the structure suggests that three groups of HP1C residues confer specificity for recognition of the CAF-1 peptide: Leu-168, which interacts with the central Val;

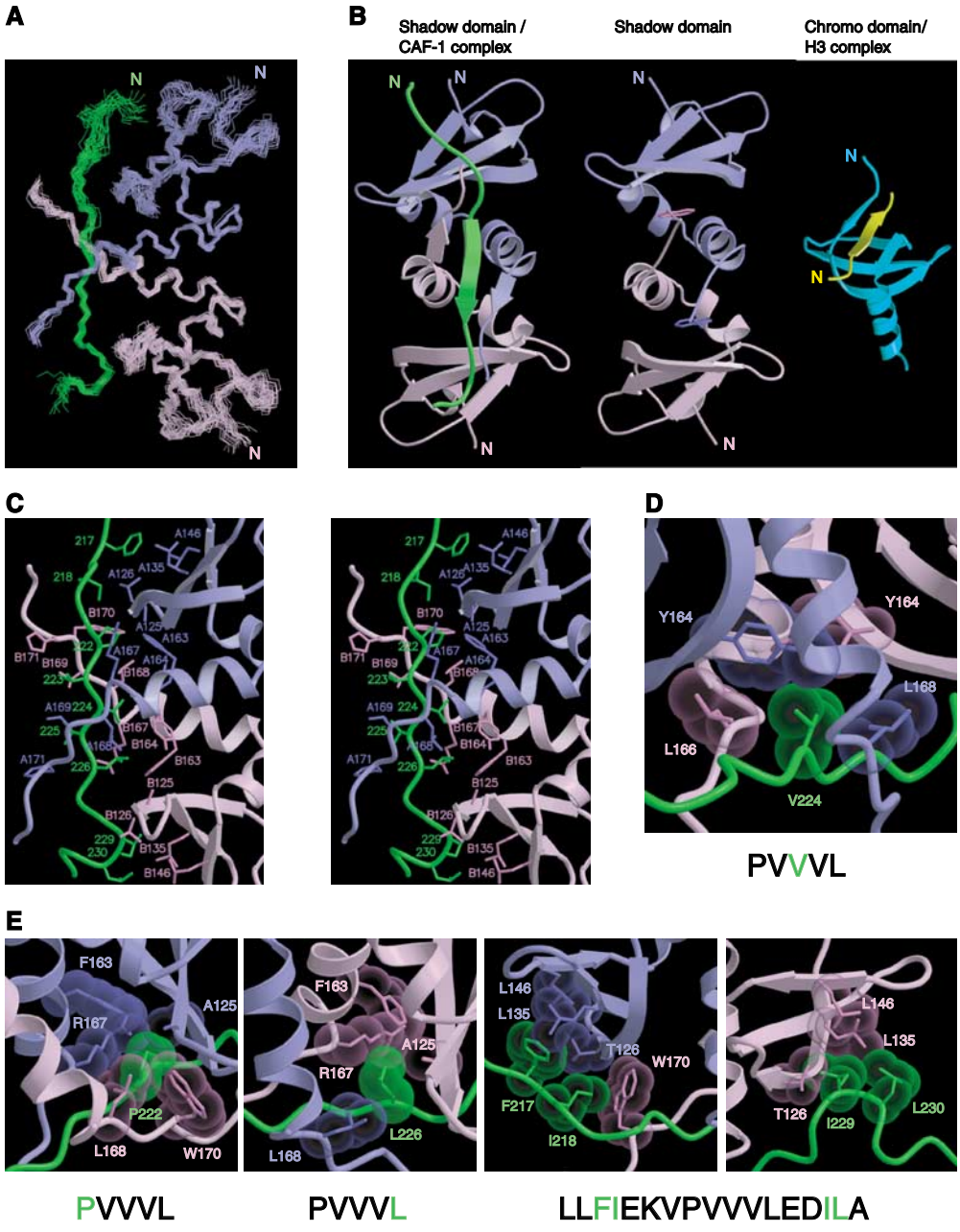

Figure 2 Structure of the HP1β shadow domain/CAF-1 complex. (**A**) The backbone of residues 110–172 of HP1β and 214–232 of CAF-1 from the 25 lowest energy structures (out of 72 that converged from the 100 computed). The structure has good covalent geometry and nonbonded contacts (Table 1). The peptide backbone around residues Asp-214–Glu-219 of CAF-1 is not well defined in the structure because only the Phe-217 and Ile-218 side chains interact with the shadow domain. ^{15}N relaxation studies show that the peptide backbone in this region has increased mobility within the complex when compared to residues in the PXVXL motif (Figure 1A). (**B**) The structure closest to the mean, rotated by 90° about the *z*-axis, compared to the orientation shown in (A). This structure is compared with that of the free shadow domain (PDB code: 1DZ1; Brasher *et al*, 2000) and the chromo domain/histone H3 complex (PDB code: 1GUW; Nielsen *et al*, 2002). In the structure of the free shadow domain, the side chains of Trp-170, which stabilise the position of the C-terminal tails, are shown. (**C**) Stereoview of the structure closest to the mean showing side chains of residues involved in the HP1β/CAF-1 peptide interface. (**D**, **E**) Close-up views of the interactions made by (D) Val-224 (position 0) and (E) Pro-222 and Leu-226 (positions −2/+2), Phe-217/Ile-218 (−7/−6 positions) and Ile-229/Leu-230 (+5/+6 positions). In (A–D), the HP1β monomers are coloured blue and magenta and the CAF-1 peptide is coloured green. (Note: Trp-170 is not shown on monomer A because it does not interact with Leu-226 at the +2 position.)

Ala-125 and (to a lesser extent) Phe-163, Arg-167, Leu-168 and Trp-170, which interact with the hydrophobic residues at +2/−2; and Thr-126, Leu-135 and Leu-146, which interact with the flanking hydrophobic residues. Shadow domains can thus be divided into different groups based on the conservation of these residues: (i) those that contain these residues and are likely to interact with a PXVXL motif peptide (as in CAF-1); (ii) those that have an altered specificity at the +2/−2 positions; and (iii) a third group that have altered specificity at position 0 (Figure 3). In addition, some proteins have charged residues in the hydrophobic patches on the β-sheet, predicting an altered interaction with the flanking residues. Consistent with the predictions in Figure 3, it seems likely that the *D. melanogaster* HP1C shadow domain

has a different specificity because it is responsible for localisation of this isoform to euchromatic sites (Smothers and Henikoff, 2001). Similarly, the shadow domain of mammalian HP1β cannot functionally replace that of Swi6 (Wang *et al*, 2000). In some cases, it is possible to predict the altered specificity. For example, Chp2 from *S. pombe*, which has an Ile instead of Leu at position 168 (HP1β numbering), might interact with peptides that have an Ala at the central position. Conversely, HP1C in *D. melanogaster*, which has Ala at position 168, might instead recognise peptides with Ile instead of Val at the centre of the PXVXL motif.

Design of mutants that selectively interact with particular PXVXL motif proteins

Although the shadow domain has relatively loose sequence specificity, we were interested to see if we could use the structure to design mutants in HP1β that might selectively disrupt interactions with particular partner proteins. Such mutants should be of value in dissecting further the respective biological roles of the different PXVXL-containing partner proteins. The +1 and −1 positions of the PXVXL motif are less conserved in the different interacting proteins (Figure 1B), suggesting that mutation of HP1 residues that interact with these positions might specifically disrupt some interactions while leaving others intact. Thr-169, which interacts with CAF-1 residues in the +1 and −1 positions (Figure 2C), was therefore mutated to either Asp or Lys. GST pull-down assays showed that the T169D mutation, which positions the negatively charged Asp acid close to Val side chains at the −1 and +1 positions in CAF1 and IDN3, selectively reduces the affinity of HP1β for both these proteins, but not for TIF1β. By contrast, mutation of Thr-169 to Lys selectively abolished the interaction with TIF1β (see Supplementary Figure 1), possibly due to charge repulsion of Arg at the −1 position in TIF1β (Figure 1B). Many HP1 interacting proteins have Val residues at either the +1 or −1 position (Figure 1B), and their interactions are likely to be reduced by the T169D mutation. The T169 K mutant, however, may be specifically disrupted in its interactions with TIF1β because only that protein has an Arg at the –1 position. Because TIF1β and other PXVXL motif-binding proteins show loss of function or mislocalisation when binding to HP1 is disrupted (Murzina *et al*, 1999; Lechner *et al*, 2000; Vassallo and Tanese, 2002; Cammas *et al*, 2002), these mutants should be useful for dissecting further the biological role of different PXVXL motif proteins in functional assays.

In *D. melanogaster*, casein kinase II (CKII) phosphorylation of at least two distinct regions of HP1 affects targeting to heterochromatin: Ser-15 in the N-terminal region as well as Ser-169 and Ser-172 at the C-terminus of the shadow domain (all HP1β numbering). Ser-172 is within a consensus CKII site (S/T, X, X, D/E), and it is thought that Ser-169 becomes a consensus CKII site following phosphorylation of Ser-172 (Zhao and Eissenberg, 1999). In mouse HP1β, there is potential for three such interdependent phosphorylation sites at Thr-169, Ser-172 and Ser-175. The effect of the T169D mutation suggests that phosphorylation of Thr-169 in HP1 may regulate the interactions of some PXVXL motif proteins (including CAF-1 and IDN3) with the shadow domain.

Localisation of HP1β to heterochromatin

Domain-swap studies have shown that the shadow domain, like the chromo domain, is required for the localisation of HP1 proteins to heterochromatin (Platero *et al*, 1995; Smothers and Henikoff, 2001). To investigate further the

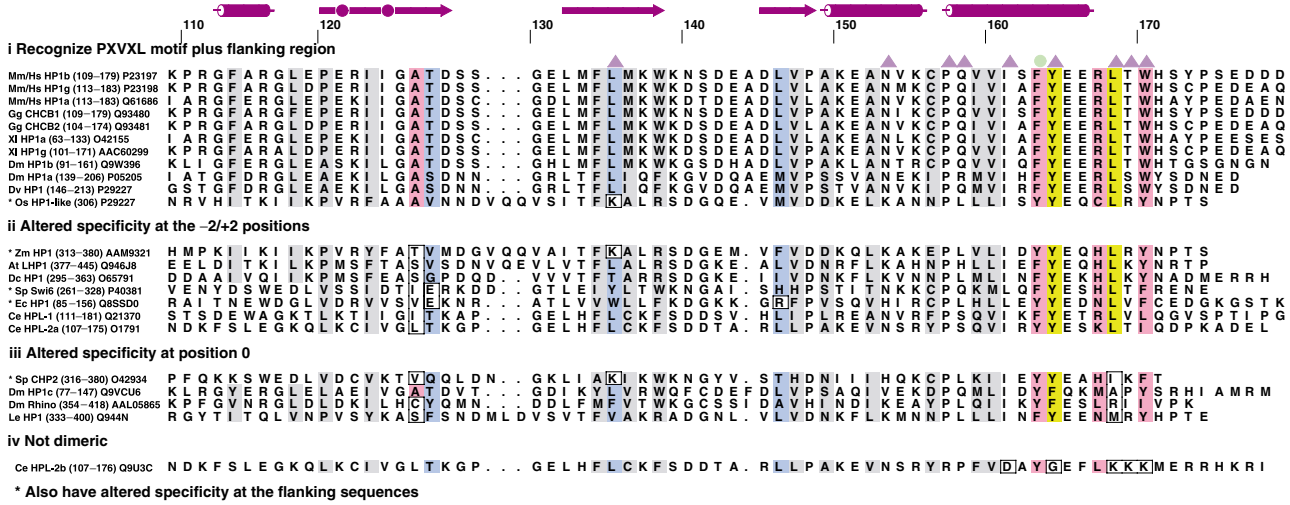

Figure 3 Shadow domain alignment in which the residues involved in complex formation are highlighted (numbering is for mouse HP1β). Residues important for recognition of the PXVXL motif are highlighted in yellow (Val-224) and red (Pro-222/Leu-226 at the −2/+2 positions). Residues that form the hydrophobic patch that interacts with the flanking N- and C-terminal sequences (Phe-217/Ile-218 and Ile-229/Leu-230 at the −6/−7 and +5/+6 positions, respectively) are highlighted in blue. Residues that are not conserved and are predicted to alter specificity are boxed. Conserved residues that define the fold of the shadow domain are highlighted in grey, while residues important for dimerisation are indicated by blue triangles. Phe-163 (indicated by a green dot) is important for both the structure of the shadow domain and peptide binding. The positions of secondary structure elements in mouse HP1β are indicated by purple arrows (β-strands) and cylinders (α-helices)—the dots indicate the positions of the conserved bulges in the first strand. Sequences are labelled by species name (Mm—*Mus musculus*, Hs—*Homo sapiens*, Gg—*Gallus gallus*, Xl—*Xenopus laevis*, Dm—*Drosophila melanogaster*, Dv—*Drosophila virilis*, Os—*Oryza sativa*, Zm—*Zea mays*, At—*Arabidopsis thaliana*, Dc—*Daucus carota*, Sp—*Schizosaccharomyces pombe*, Ec—*Encephalitozoon cuniculi*, Ce—*Caenorhabditis elegans*, Le—*Lycopersicon esculentum*).

relative importance of the two domains for the localisation of mouse HP1β, we used the HP1β/CAF-1 structure to understand the effect of previously identified mutations. We then studied the localisation of HP1β to heterochromatin using full-length mutants that selectively abrogate (i) interactions of the shadow domain with PXVXL-containing partners (W170A (Brasher *et al*, 2000) and L168 H (Lechner *et al*, 2000)), (ii) interactions of the chromo domain with histone H3 (W42L (Jacobs and Khorasanizadeh, 2002)) and (iii) dimerisation of the shadow domain (I161E (Brasher *et al*, 2000)).

Appropriate GFP fusion proteins were expressed in mouse L cells and their localisation to heterochromatin was examined by immunofluorescence microscopy. The stability of binding and retention of fusion proteins in heterochromatin after Triton extraction were then assessed using both immunofluorescence microscopy and Western blotting of the supernatant and pellet fractions. Expression of GFP-chromo or GFP-shadow domain fusion proteins showed that neither domain was by itself sufficient for localisation to heterochromatin (although weak staining is seen in heterochromatin with the isolated chromo domain). In addition, unlike the full-length protein, neither domain was tightly bound nor retained in heterochromatin following permeabilisation of the cells and washing with Triton (Figures 4A and E). Similar results were obtained with C-terminal GFP fusions (data not shown). Note the fact that the isolated GFP-shadow domain protein is not localised to heterochromatin directly demonstrates that dimerisation with endogenous HP1 is not a major factor driving localisation in the experiments we describe.

Previous studies have shown that the L168 H (Lechner *et al*, 2000) and W170A (Brasher *et al*, 2000) mutants do not interact with many PXVXL motif proteins. The structure of the HP1β/CAF-1 complex now allows us to understand how these mutants disrupt the interaction. Leu-168 plays a key role in interacting with the central Val (see above), whereas Trp-170 appears to stabilise the position of the C-terminal tails thereby facilitating formation of the antiparallel β-sheet with the peptide (Figure 2B). Importantly, the structure therefore suggests that no PXVXL motif proteins are likely to interact with either mutant. These results gave us the confidence to study these mutants further by examining their heterochromatin localisation. Surprisingly, these mutations did not affect localisation of HP1β to heterochromatic foci (Figure 4B). However, in the absence of interactions with PXVXL motif proteins, HP1β does not bind as tightly to its cognate sites as the wild-type protein, as shown by its lower resistance to Triton extraction (Figures 4B and E). These results show that interactions between the shadow domain and PXVXL motif protein(s) are required for stable binding of HP1β to heterochromatin. Consistent with this, fluorescence recovery after photobleaching (FRAP) experiments have shown that deletion of the shadow domain weakens (or destabilises) the association of HP1 proteins with heterochromatin (Cheutin *et al*, 2003; A Verreault, unpublished data). Interestingly, however, the T169D and T169 K mutants, whose affinity of binding to a subset of the PXVXL motif proteins is reduced (see above), localise to heterochromatin and bind with similar affinity to the wild-type protein (Figure 4B). These results suggest that the retention of HP1β in heterochromatin is mediated by interactions with proteins other than CAF-1 and IDN3 (interactions reduced in T169D) or TIF1β (interaction reduced in T169 K) (Supplementary Figure 1).

The loss of Lys-9-methylated histone H3 binding (the W42L mutant) (Jacobs and Khorasanizadeh, 2002) significantly disrupts both localisation and retention of HP1β in heterochromatin (Figures 4C and E), consistent with previous results (Bannister *et al*, 2001; Lachner *et al*, 2001). Interestingly, an even more severe effect was observed with a mutant (W42L, W170A), in which both Lys-9-methylated histone H3 binding and PXVXL motif protein binding were disrupted. In contrast to the situation with the W42L mutant, none of the W42L, W170A mutant is retained in chromatin upon Triton extraction (Figures 4C and E). Overall, these results suggest that chromo domain interactions with Lys-9-methylated histone H3 are required for localisation of HP1β to heterochromatin, while shadow domain/PXVXL motif protein(s) interactions are required for stable binding and retention.

The binding of the CAF-1 peptide in an extended conformation across the symmetry axis of the HP1C dimer clearly explains why the monomeric I161E shadow domain mutant (Brasher *et al*, 2000) does not bind to PXVXL motif proteins. Surprisingly, this mutation significantly disrupted not just the retention of the protein in heterochromatin but also its localisation, despite the fact that interactions with Lys-9-methylated H3 are unaffected. Although some binding to heterochromatin is observed, the protein can be seen throughout the nucleus and is completely extracted by Triton (Figures 4C and E). If binding to Lys-9-methylated H3 is additionally disrupted (the W42L, I161E mutant), no localisation to heterochromatin is observed (Figures 4C and E).

Comparison of the localisation of the dimeric W170A mutant, in which binding to PXVXL motif proteins is disrupted, with the I161E mutant, in which the ability of the shadow domain to both dimerise and bind to PXVXL motif proteins is disrupted, shows that the latter is localised to heterochromatin less efficiently and is also less tightly bound after Triton permeabilisation (Figures 4B and C). Neither the chromo domain on its own nor the HP1β I161E mutant, which has an intact chromo domain, is properly targeted to heterochromatin and both are completely extracted by Triton (Figures 4A, C and E). These results show that an intact chromo domain within the context of a monomeric protein is not sufficient for stable association of HP1β with heterochromatin. In other words, the results demonstrate that an intact dimer structure is required for efficient localisation of HP1β to heterochromatin via the chromo domain. A similar suggestion has been made based on the structure of the Pc chromo domain/Lys-27-methylated histone H3 complex (Fischle *et al*, 2003; Min *et al*, 2003). Interestingly, however, in this case, dimerisation of the chromo domain itself has been suggested to be important for specific recognition of Lys-27-methylated histone H3 and chromatin compaction (Min *et al*, 2003).

The loose sequence specificity by which the shadow domain recognises a peptide motif allows HP1 family proteins to bind specifically to many different proteins and to recruit different new partners as required in a dynamic chromatin structure. We show here that stable retention of HP1 in heterochromatin requires interactions with PXVXL motif-containing proteins, suggesting a secondary function for this interaction apart from recruitment of diverse complexes to regions of chromatin methylated at Lys-9 of histone

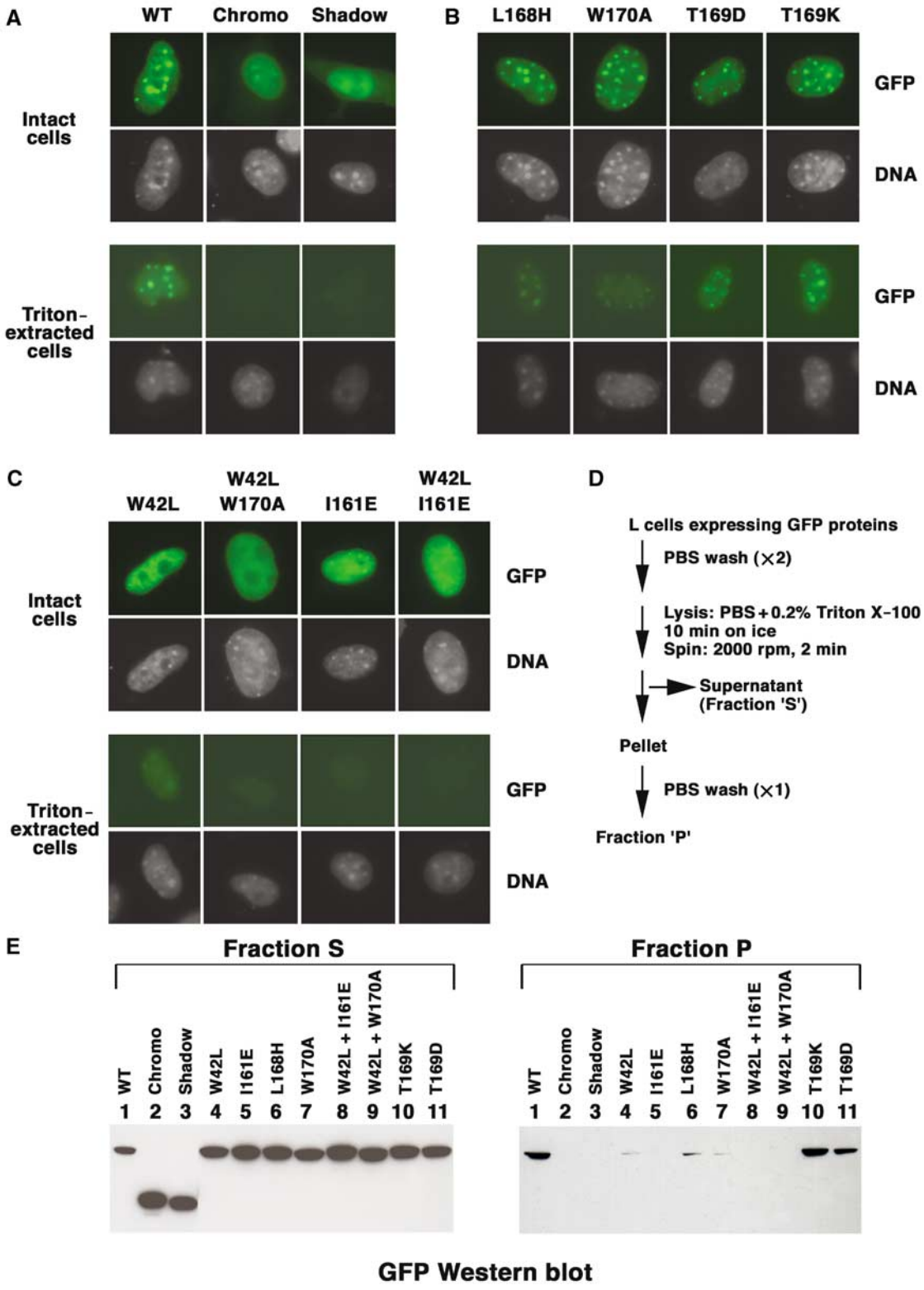

Figure 4 Localisation of HP1β to heterochromatin. Mouse L cells were transfected with constructs expressing full-length GFP-HP1β, GFP-chromo domain (residues 10–80) or GFP-shadow domain (residues 104–185). Heterochromatin targeting was assessed by staining DNA with limiting amounts of Hoechst 33258. (**A**) Localisation of GFP full-length HP1β compared with that of either the chromo or shadow domain alone. (**B**) Localisation of GFP full-length HP1β with point mutations that impair binding to all PXVXL proteins (W170A and L168 H) or affect the charge of Thr-169 and thereby weaken binding to some PXVXL proteins (T169D and T169 K). (**C**) Localisation of GFP full-length HP1β with point mutations that cripple binding to Lys-9-methylated histone H3 (W42L), Lys-9-methylated histone H3 and PXVXL proteins (W42L, W170), dimerisation and binding to PXVXL proteins (I161E), and dimerisation and binding to PXVXL proteins and binding to Lys-9-methylated histone H3 (W42L, I161E). In (A–C), a comparison is shown between intact cells and cells that have been permeabilised with Triton and washed. Exposure was 10 times longer for the Triton-extracted cells. (**D**) Experimental scheme of the Western blot in (E). (**E**) Western blot analysis (using an anti-GFP antibody) of GFP fusion proteins in the supernatant (S) and pellet (P) fractions following Triton permeabilisation and extraction of transfected cells.

H3. Interestingly, the results also suggest that efficient HP1β localisation requires the simultaneous interaction of both chromo domains (in an HP1β dimer mediated by the shadow domain interaction (Brasher *et al*, 2000)) with two separate Lys-9-methylated histone H3 molecules, although we cannot exclude that the dimer structure of HP1β is required for a currently uncharacterised interaction. This conclusion implies a further complexity to the histone code, where up until now post-translational modifications of a single histone protein were thought to define whether particular proteins interact to promote or inhibit diverse downstream processes, for example, gene expression controlled by chromatin structure (Strahl and Allis, 2000). This has some potentially interesting functional consequences, because it suggests that if the two H3 molecules are part of different nucleosomes, this mode of recognition by the HP1 family proteins might lead to a more condensed chromatin structure (Cryderman *et al*, 1998, 1999). In addition, it would provide a plausible explanation for how HP1 family proteins might bring together the DNA sequences needed for activation of heterochromatic genes (Lu *et al*, 2000) or attract distant loci in the vicinity of pericentric heterochromatin to promote their silencing (Brown *et al*, 1999; Francastel *et al*, 1999).

Experimental procedures

Protein expression and purification

HP1β shadow domain (residues 104–176) was expressed and purified as described (Brasher *et al*, 2000). Unlabelled and ^{15}N GST-CAF 1 (residues 210–238) was expressed in *E. coli* BL21 pREP4 cells and purified using standard protocols. After thrombin digestion, the free peptide was purified on a Superdex 75 column (Amersham Biosciences). For pull-down assays, GST-TIF1β (residues 449–567), GST-IDN3 (residues 433–515) and GST–CAF-1 (residues 176–327) were expressed and purified using standard protocols.

Site-directed mutagenesis

Site-directed mutagenesis of the HP1β shadow domain and the full-length GFP fusion HP1β protein was performed using the QuickChange kit (Stratagene).

Fluorescence microscopy

Transfection of mouse cells with GFP expression plasmids and Hoechst staining were performed as described (Murzina *et al*, 1999). Images were acquired using a Zeiss Axiovert 200 M inverted fluorescence microscope equipped with a ×63 Plan Apochromat 1.3NA objective, a GFP filter set (Chroma) and an AxioCam MRm cooled CCD camera controlled using Zeiss AxioVision software.

NMR spectroscopy and structure calculation

NMR spectra were recorded on samples in 150 mM NaCl, 20 mM sodium phosphate (pH 7.4) and 10% D_2O at 25°C on Bruker DRX600 and DRX800 spectrometers. The protein backbone and side-chain resonances were assigned using triple-resonance experiments (Cavanagh *et al*, 1996) using ^{13}C, ^{15}N-labelled HP1C in a complex with 10% excess of unlabelled CAF-1 peptide. Peptide resonances were assigned using ^{13}C, ^{15}N-rejected and *J*-resolved NOESY and TOCSY experiments recorded on the ^{13}C, ^{15}N-labelled sample. NOEs were assigned using 2D ^{1}H, 3D ^{13}C- and 3D ^{15}N-separated NOESY spectra. Intermolecular contacts were obtained from a ^{13}C, ^{15}N X-filtered NOESY spectrum (Zwahlen *et al*, 1997) and a 3D *J*(CH,NH)-resolved NOESY spectrum (D Nietlispach *et al,* unpublished results). Spectra were processed using AZARA (W Boucher, unpublished) and analysed using ANSIG (Kraulis, 1989; Kraulis *et al*, 1994). Structures were calculated using ARIA1.1 and CNS1.0 (Brunger *et al*, 1998; Linge *et al*, 2001), starting from the structure of free HP1C and an extended template for the peptide. NCS restraints were applied to all C_α atoms in HP1C and were weighted according to the loss of symmetry-related chemical shift degeneracy for pairs of equivalent residues in the two monomers. The weights were as follows: 2.0 for residues that retained complete symmetry-related chemical shift degeneracy upon peptide binding, 1.0 for those that differ in $^{1}H^{N}$ and/or ^{15}N chemical shifts, 0.5 for those that differ in $^{1}H^{N}$, ^{15}N and $^{1}H^{\alpha}$ chemical shifts and 0.0 for those that exhibit loss of chemical shift degeneracy in the side-chain chemical shifts as well. In addition, distance symmetry restraints were imposed over the C_α atoms of residues in HP1C where the NMR spectra were unaltered by peptide binding, that is, only over those regions where there is clear NMR-based evidence that the structures of the two monomers remain symmetrical. Seven iterations were calculated, each on 20 structures. Finally, 100 structures were calculated prior to analysis of the 25 lowest in energy. In the final structures, no distance restraints were violated by more than 0.5 Å and no dihedral angle restraints were violated by more than 5°.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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