Dynamic and flexible H3K9me3 bridging via HP1β dimerization establishes a plastic state of condensed chromatin

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Histone H3 trimethylation of lysine 9 (H3K9me3) and proteins of the heterochromatin protein 1 (HP1) family are hallmarks of heterochromatin, a state of compacted DNA essential for genome stability and long-term transcriptional silencing. The mechanisms by which H3K9me3 and HP1 contribute to chromatin condensation have been speculative and controversial. Here we demonstrate that human HP1β is a prototypic HP1 protein exemplifying most basal chromatin binding and effects. These are caused by dimeric and dynamic interaction with highly enriched H3K9me3 and are modulated by various electrostatic interfaces. HP1β bridges condensed chromatin, which we postulate stabilizes the compacted state. In agreement, HP1β genome-wide localization follows H3K9me3-enrichment and artificial bridging of chromatin fibres is sufficient for maintaining cellular heterochromatic conformation. Overall, our findings define a fundamental mechanism for chromatin higher order structural changes caused by HP1 proteins, which might contribute to the plastic nature of condensed chromatin.

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Heterochromatin is important for genome stability and transcriptional silencing through folding of chromatin into a condensed higher order structure. Methylation of lysine 9 within the histone H3 N-terminal tail is a crucial determinant of heterochromatin formation. The trimethylated form of this modification (H3K9me3) can be found at pericentric heterochromatin in virtually all higher eukaryotes and is viewed as a hallmark of transcriptionally silenced chromatin.\(^2\)\(^,\)\(^3\)

Multiple evidence from genetic and cell biology studies points to an important involvement of heterochromatin protein 1 (HP1) factors, a family of non-histone chromatin proteins found in different isoforms in diverse organisms from S. pombe (Swi6) to human (HP1\(\alpha,\) \(\beta\) and \(\gamma\)) in establishing and maintaining heterochromatic states.\(^2\)\(^,\)\(^3\)

HP1 proteins generally contain two conserved globular domains, a chromo domain (CD) and a chromoshadow domain (CSD), which are linked by a less conserved, flexible hinge region (HR).\(^3\) Depending on the species and isoforms, additional less-conserved regions are found at the N and C termini of the proteins (NT and CT, respectively; Fig. 1a). The CD mediates interaction with H3K9me3 histone tail peptides with relatively low (micromolar) affinity.\(^7\)\(^,\)\(^8\) Work on isolated chromatin components (peptides, histones, DNA) has suggested that the CD/H3K9me3 interaction might not be sufficient for chromatin targeting of HP1 proteins. Contacts via the HR or CSD might also be required. The issue is further complicated by self-dimerization of the CSD, which provides an interaction platform for a plethora of other proteins.\(^9\)\(^,\)\(^10\)

Several recent studies on human HP1\(\alpha\) (hHP1\(\alpha\)) using in vitro reconstituted ‘designer chromatin’ containing defined histone modifications have reported conflicting results regarding binding specificity, chromatin effects as well as dependency on different domains of the protein.\(^11\)\(^–\)\(^14\)

Here we investigated the molecular parameters and consequences of interaction between hHP1\(\beta\) and H3K9me3-containing oligonucleosomes. We focused on this isoform as it is the only essential mammalian HP1 protein, whose knockout in mice leads to perinatal lethality and severe genomic instability.\(^15\)

Our results demonstrate that mammalian HP1\(\beta\) is a prototypic HP1 protein, whose specific interaction with H3K9me3 chromatin only requires dimerization and spacing of the CD as well as general stabilization of this interaction by the NT. We suggest a general mechanism of dynamic higher order organization mediated by HP1 proteins that might be the basis of the plasticity of condensed chromatin.

**Results**

**hHP1\(\beta\) binds H3K9me3 chromatin with high specificity.** To obtain molecular insights into the interaction of HP1 proteins with chromatin, we generated uniformly K9-methylated histone H3 protein by native chemical ligation (synthetic H3 (aa 1–20) tail peptides fused to recombinant H3 (Δ1–20, A21C)) or via...

**Figure 1** | hHP1\(\beta\)–chromatin interaction is dependent on H3K9me3. (a) Domain structure of hHP1\(\beta\); boundaries of domains are indicated by respective amino acid positions. For more details see Supplementary Fig. 2. (b) Scheme of pull-down experiments in c using different H3K9me templates immobilized on streptavidin-coated magnetic beads via C-terminal incorporation of a biotinylated lysine (peptide) or ligation of 5’-biotinylated oligonucleotides to DNA templates used in chromatin reconstitution (mono- and oligonucleosomes). (c) Immobilized H3K9me templates according to the pull-down experimental schemes in b were incubated with recombinant hHP1\(\beta\) WT. Material recovered after washing was analysed by western blotting. The indicated salt concentrations were used throughout the experiment. (d) Scheme of chromatin coprecipitation assay; factors bound to oligonucleosomes are precipitated with the template when clustering is induced by addition of Mg\(^{2+}\) ions. (e,f) Chromatin coprecipitation of hHP1\(\beta\) (e) and hHP1\(\alpha\) (f) proteins with oligonucleosomes. Precipitated material was run on SDS-PAGE and stained with Coomassie blue. Input, 10%. (g) The indicated recombinant proteins were incubated with a DNA fragment of 150 bp at 500: 1 and 1,000: 1 molar ratio. Complexes were separated by PAGE. DNA was stained with SYBR Gold.
Very low

Fig. 1a,b). At saturation rates of 11 ± using DNA containing 601 positioning sequences (Supplementary strategy. H3 with different modification status was incorporated chromatin that were not available by the native chemical ligation exclusively for experiments requiring large amounts of designer compared with methylated lysine (Table 1)17, we used H3KC9me aminoalkylation of H3 with cysteine at position 9 instead of lysine (methyl lysine analogue, H3KC9me) using our previously estab-

- Table 1 | Interaction parameters of hHP1β WT and mutant proteins with the H3K9me3-modification in different biochemical environment as deduced by different methods.

|                | Peptide          | Mononucleosome | Oligonucleosome |
|----------------|------------------|----------------|-----------------|
|                | H3K9me3 WT       | H3K9me3 WT     | H3K9me3 WT      |
| 20 mM NaCl     |                  |                |                 |
| $K_d$ (µM)     | 1.4 ± 0.6        | 59 ± 8         | 223 ± 30        |
| $N$            | 0.9 ± 0.0        | 2.1 ± 0.3      | 1.1 ± 0.4       |
| $K_d$ (µM)     | 3.3 ± 0.2        | 22 ± 8         | 58 ± 17         |
| $N$            | 1.0 ± 0.0        | 1.4 ± 0.3      | 1.2 ± 0.3       |
| 150 mM NaCl    |                  |                |                 |
| $K_d$ (µM)     | 0.7 ± 0.1*       | 0.3 ± 2        | 0.2 ± 1         |
| $N$            | 1.0 ± 0.0        | 1.0 ± 1        | 0.9 ± 0.3       |
| 150 mM NaCl    |                  |                |                 |
| $K_d$ (µM)     | 7.0 ± 0.4        | 8 ± 0.3        | 6.3 ± 0.9       |
| $N$            | 6.3 ± 0.9        | 5.2 ± 0.6      | 5.2 ± 0.6       |

*Based on fitting of titration data to a one site-specific binding model (Supplementary Note 1).

-a, not binding; ITC, isothermal titration calorimetry; N, stoichiometry of interaction; $K_d$, apparent dissociation constant; SPR, surface plasmon resonance; WT, wild type.

Figure 2 | Dimerization is necessary and sufficient for hHP1β binding of H3K9me3 chromatin. (a) Schematic representation of hHP1β mutant proteins. White bars indicate positions of W42 and I161, respectively. (b) Chromatin coprecipitation of WT and mutant hHP1β proteins with oligonucleosomes. Precipitated material was analysed by western blotting using antibodies that recognize the CD of hHP1β. Ponceau staining of the region of the western blot membrane containing histones is shown as loading control. Input, 10%. (c) The indicated proteins were incubated with a biotinylated H3K9me3 peptide immobilized on magnetic streptavidin beads. Material recovered after washing was run on SDS–PAGE and stained with Coomassie Blue. Input, 10%. (d) SPR analysis of hHP1β WT interaction with a biotinylated H3K9me3 peptide immobilized at different density on the chip surface (very low, 5 RU; low, 24 RU; very high, 950 RU). (e) Experiment as in b using hHP1β CSD, but analyzed by SDS–PAGE and staining with Coomassie Blue.

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Dimerization mediates H3K9me3-chromatin binding of hHP1β. To determine the molecular requirements for the highly specific hHP1β/H3K9me3–chromatin interaction, we prepared different point as well as deletion mutant proteins (Fig. 2a and Supplementary Fig. 2a). Mutation of residue W24, which was shown previously to impair binding to H3K9me3 (ref. 7), resulted in the loss of recovery of hHP1β with H3K9me3 oligonucleosomes (Fig. 2b). Under the same conditions neither the monomeric CD alone nor an I161A mutant protein, which is incapable of dimerization8, were significantly recovered with H3K9me3 oligonucleosomes. Since both proteins bound an H3K9me3-peptide as efficiently as hHP1β wild type (WT; Table 1), the results implied that the CD/H3K9me3 interaction is not sufficient for chromatin binding. In agreement, hHP1β I161A was ineffective in competing with hHP1β WT for interaction with H3K9me3 oligonucleosomes (Supplementary Fig. 2b). Also, isothermal titration calorimetry (ITC) measurements deduced a 12-fold reduced interaction of hHP1β WT compared with I161A mutant protein. With the H3K9me0 oligonucleosomes, we investigated the effects of its binding onto chromatin reconstitution (Supplementary Fig. 2c and d). Recovery of hHP1β with aggregated chromatin was independent of the DNA sequence used for chromatin reconstitution (Supplementary Fig. 1h).

When analysing recombinant hHP1β in the chromoprecipitation scheme, the protein displayed much less specificity for H3K9me3 compared with hHP1β (Fig. 1f). In agreement and as observed previously, hHP1β but not hHP1β showed a high level of general DNA binding in gel shift experiments (Fig. 1g)13.

hHP1β induces aggregation of H3K9me3 oligonucleosomes. Having established the interaction parameters of hHP1β with oligonucleosomes, we investigated the effects of its binding onto chromatin conformation. At 150 mM NaCl oligonucleosomes are highly soluble. These are in compacted (zig-zag) state but do not interact with each other (Supplementary Fig. 1e). Under these conditions increasing concentrations of hHP1β WT efficiently aggregated H3K9me3 but not H3K9me0 oligonucleosomes to a degree that the complexes precipitated and could be collected by centrifugation (Fig. 3a). This effect was generally reversible as shown in resolubilization experiments (Supplementary Fig. 3a and b). The hHP1β I161A mutant protein, in contrast, failed to induce the aggregation of oligonucleosomes.

To obtain further insights into the chromatin aggregation induced by hHP1β, we set up fluorescence correlation spectroscopy (FCS) measurements for single-molecule analysis. By labelling the DNA in oligonucleosomes with ATTO 610, this allowed delineating the diffusion behaviour and thereby size of the different chromatin species (Supplementary Fig. 3c and d). In this assay, hHP1β WT induced H3K9me3 oligonucleosomes to form large assemblies that reached a maximum hydrodynamic radius of 450 nm at the highest protein concentration (Fig. 3b). No such complex formation by clustering was seen with the I161A mutant protein. With the H3K9me0 oligonucleosomes larger assemblies only appeared at high hHP1β WT concentrations. We note that the half-maximal effects in the FCS experiment and the chromatin precipitation assay were seen at 3.5 and 0.4 μM hHP1β WT, respectively. Since the binding of hHP1β to peptides is slightly enhanced at lower temperature22, we assume that the difference is explained by the experimental setup of the FCS experiment at 23°C and the sedimentation assay at 4°C. Also, we do not know the minimal hydrodynamic size of aggregates that results in the recovery in the centrifugation assay.

hHP1β clusters chromatin exposing high density of H3K9me3. In analysing the interaction of hHP1β WT with H3K9me3 in different biochemical context, we noticed a significant difference in binding strength at low and high-salt concentrations. While the protein bound better to free H3K9me3 peptide at 20 mM NaCl (Kₐ = 1.4 ± 0.6 μM) compared with 150 mM NaCl (Kₐ = 3.3 ± 0.2 μM), this trend was reversed in the context of chromatin. H3K9me3 mononucleosomes were bound 2.7-fold better at the higher compared with the lower salt concentration. At 20 mM NaCl we could not detect any binding of hHP1β WT to H3K9me3 oligonucleosomes but deduced a Kₐ of 11 ± 2 μM for binding to the same template at 150 mM NaCl (Table 1 and Supplementary Fig. 4). Obviously, two counteracting effects determine binding of hHP1β to H3K9me3 chromatin. On one end, the CD/H3K9me3 interaction is sensitive to higher salt conditions. On the other end, at low salt concentration, the H3 tails in chromatin are not fully available, likely due to electrostatic...
**Figure 3 | hHP1β clusters oligonucleosomal arrays in dependence of H3K9me3.** (a) Oligonucleosomes (2.5 nM) were incubated with increasing concentrations of hHP1β WT or I161A at 150 mM NaCl. DNA remaining in the supernatant after centrifugation was incubated with EtBr and measured by fluorescence reading. Data were normalized to amounts in absence of added protein. Averages of three independent experiments are plotted; error bars represent s.d.; n = 3. (b) Analysis of the hydrodynamic radius of oligonucleosomes (10 nM; DNA labelled with ATTO 610) in the presence of increasing concentrations of hHP1β WT or I161A by FCS at 150 mM NaCl. For details on experimental setup see Supplementary Fig. 3. Error bars represent s.d.; n = 3. (c) Oligonucleosomes were reconstituted after mixing H3K9me0 and H3K9me3 octamers at different ratio. Chromatin precipitation analysis at 150 mM NaCl was carried out with hHP1β WT at saturating concentration. Data are presented as in a. (d) Precipitation behaviour of oligonucleosomes (2.5 nM) was analysed at different concentrations of NaCl with hHP1β WT at saturating concentration. Data are presented as in a. (e) H3K9me3-containing chromatin was reconstituted using wild-type core histones or tailless H2A, H2B or H4. Oligonucleosomes (2.5 nM) were incubated with increasing concentrations of hHP1β WT at 150 mM NaCl. Data are presented as in a. (f) Chromatin coprecipitation of hHP1β with H3K9me3-containing oligonucleosomes reconstituted with wild-type or tailless core histones at the indicated concentrations of MgCl2. Precipitated material was analysed by western blotting. The region of the western blot membrane of the experiment at 7.5 mM MgCl2 containing histones was stained with Ponceau. (g) Oligonucleosomes at 50 mM NaCl were incubated with hHP1β WT or I161A. Complexes were fixed with 0.05% (v/v) glutaraldehyde, spotted on mica surfaces and analysed by scanning force microscopy. Control, no protein added; scale bar, 100 nm. (h) Quantification of results representatively shown in g according to the classification on the top: A, extended; B, partially condensed; C, fully condensed; D, aggregates; n > 50 for each condition.
binding to DNA\textsuperscript{18,22}. Conditions of higher salt increase the availability of the H3 tails and induce chromatin condensation. The high density of H3K9me3 in this situation provides a favourable binding platform for hHP1\textbeta interaction, which in turn promotes clustering of chromatin.

If this interpretation were correct, we would expect the effect of hHP1\textbeta chromatin clustering to be dependent on the level of H3K9me3 in the oligonucleosomes. Indeed, when mixing H3K9me0 and H3K9me3 at different ratios for oligonucleosomal reconstitution, we observed that a content of more than 50% H3K9me3 was required for hHP1\textbeta to induce efficient chromatin clustering (Fig. 3c).

To further test the idea that hHP1\textbeta preferentially binds to condensed chromatin and mediates clustering, we performed salt titration of H3K9me0 and H3K9me3 oligonucleosomes in presence of the protein. We found a transition of hHP1\textbeta inducing clustering of H3K9me3 oligonucleosomes at salt concentrations above 50 mM NaCl, which are known to promote intrafiber condensation (Fig. 3d). No clustering of H3K9me0 chromatin was seen in the presence of hHP1\textbeta even at 150 mM NaCl.

Mg\textsuperscript{2+}-induced chromatin aggregation requires minimally the N-terminal tails of H3 or H4. In contrast, the tails of H2A and H2B are not sufficient for this process\textsuperscript{23,24}. To further analyse hHP1\textbeta-dependent chromatin clustering, we set up H3K9me3 oligonucleosomal templates containing tailless H2A, H2B or H4. Compared with oligonucleosomes containing WT histones these required higher MgCl\textsubscript{2} concentration for inducing intrafiber aggregation and precipitation (Supplementary Fig. 5), as was shown previously\textsuperscript{24}. We determined the Mg50 value for WT H3K9me3 oligonucleosomes at around 2 mM, whereas the Mg50 for H3K9me3 oligonucleosomes containing tailless H2A or H2B were around 3 mM and that for H3K9me3 oligonucleosomes containing tailless H4 was around 4 mM. While hHP1\textbeta clustered H3K9me3 oligonucleosomes containing tailless H2A or H2B as efficiently as H3K9me3-oligonucleosomes containing WT core histones, the effect on H3K9me3 oligonucleosomes containing tailless H4 was impaired (Fig. 3e). Also and in agreement with the altered aggregation behaviour (Supplementary Fig. 5), chromatin coprecipitation showed reduced recovery of hHP1\textbeta with H3K9me3 oligonucleosomes in the absence of the H4 N-terminal tail at 3.5 mM MgCl\textsubscript{2}, whereas recovery at 7.0 mM...
MgCl₂ was comparable to the level obtained with H3K9me3 oligonucleosomes containing WT core histones (Fig. 3f).

Since the salt titration had shown that at 50 mM NaCl discriminatory effects of hHP1β onto H3K9me0 and H3K9me3 oligonucleosomes become apparent (Fig. 3d), we selected this condition to visualize the chromatin complexes formed by hHP1β using scanning force microscopy (Fig. 3g). To rule out the clear dose response (Fig. 3a,b), we reasoned that at this intermediate condition different states of hHP1β-dependent chromatin clustering could be observed at different concentrations of the protein. At 0.5 μM hHP1β WT, we mostly observed structures whose size was consistent with individual but highly condensed H3K9me3 chromatin species. At 5 μM hHP1β WT larger clusters that must contain multiple oligonucleosomes were visible. Similar to the ‘in solution’ assays, the frequency of condensed chromatin fibres and large chromatin clusters was much lower for H3K9me0 chromatin with hHP1β WT or for H3K9me3 chromatin with I161A mutant protein (Fig. 3h).

hHP1β bridges H3K9me3 of different chromatin fibres. We next investigated whether dimerization is sufficient for the effect of the hHP1β CSD in chromatin clustering. At 150 mM NaCl 5 μM hHP1β WT are sufficient for inducing maximal chromatin clustering (Fig. 3a). Under these conditions, neither the I161A mutant, nor the isolated CD or CSD had any effect on modified or unmodified chromatin (Fig. 4a). As expected, mutation of W42A that abolishes interaction with H3K9me3 also failed to cluster oligonucleosomes. However, artificial dimerization of the NT, CD and HR domains of hHP1β together with anti-His6 and anti-H3 antibodies identified three major clusters (Fig. 4b). Since the loading control of the experiment), western blotting using scanning force microscopy (Fig. 3g). To detect a wider range of effects, we refined the chromatin clustering assay using the mutant hHP1β proteins at nonsaturating level and lowering the NaCl concentration to 100 mM. Randomizing the sequence of the HR (hHP1β HR(scramble)) had no effect on the ability of hHP1β to cluster chromatin. Since work on HP1z proteins has implied this region in DNA/RNA binding9-18, we also eliminated all the negative (hHP1β HR (all E, D to A)) or positively charged residues (hHP1β HR (all K, R to A)) separately as well as altogether (hHP1β HR (all E,D,K,R to A); Supplementary Table 2). In agreement with strong, unspecific DNA binding (Fig. 5c) the hHP1β HR (all E, D to A) mutant was recovered much more efficiently on H3K9me3 chromatin compared with the WT protein but also showed high recovery on the H3K9me0 template (Fig. 5d). It had very strong chromatin clustering activity and precipitated H3K9me0 and H3K9me3 oligonucleosomes equally well (Fig. 5e). In stark contrast, the hHP1β HR (all K, R to A) mutant protein, which did not bind DNA, was less recovered with the H3K9me3 template and lost its ability to cluster chromatin. Neutralizing all charges of the HR in the hHP1β HR (all E, D, K, R to A) mutant not only recapitulated the lack of DNA binding of the WT protein but also fully restored its activity in chromatin coprecipitation and clustering.

To further investigate these findings, we established a series of hHP1β proteins mutated within the NT and HR (Fig. 5b). None of these mutant proteins differed significantly from hHP1β WT in their binding to a free H3K9me3 tail peptide (Supplementary Fig. 7b). To detect a wider range of effects, we refined the chromatin clustering assay using the mutant hHP1β proteins at nonsaturating level and lowering the NaCl concentration to 100 mM. Randomizing the sequence of the HR (hHP1β HR(scramble)) had no effect on the ability of hHP1β to cluster chromatin. Since work on HP1z proteins has implied this region in DNA/RNA binding9-18, we also eliminated all the negative (hHP1β HR (all E, D to A)) or positively charged residues (hHP1β HR (all K, R to A)) separately as well as altogether (hHP1β HR (all E,D,K,R to A); Supplementary Table 2). In agreement with strong, unspecific DNA binding (Fig. 5c) the hHP1β HR (all E, D to A) mutant was recovered much more efficiently on H3K9me3 chromatin compared with the WT protein but also showed high recovery on the H3K9me0 template (Fig. 5d). It had very strong chromatin clustering activity and precipitated H3K9me0 and H3K9me3 oligonucleosomes equally well (Fig. 5e). In stark contrast, the hHP1β HR (all K, R to A) mutant protein, which did not bind DNA, was less recovered with the H3K9me3 template and lost its ability to cluster chromatin. Neutralizing all charges of the HR in the hHP1β HR (all E, D, K, R to A) mutant not only recapitulated the lack of DNA binding of the WT protein but also fully restored its activity in chromatin coprecipitation and clustering.

On the basis of these results, we concluded that the HR of hHP1β does not directly contribute to chromatin binding and clustering but provides a flexible and spacing linker. Increase of the net negative charge of this region results in repelling of DNA, whereas increase of the net positive charge causes unspecific
chromatin binding. In agreement with this interpretation, deletion of the HR resulted not only in significant reduction of the chromatin clustering activity of hHP1β but also of the NT, CD(hHP1β)-GST hybrid protein (Supplementary Fig. 7d and e). Furthermore, exchange of the hHP1β HR with the corresponding regions of hHP1δ and Swi6, which contain a surplus of positive charges (Supplementary Fig. 2a and Supplementary Table 2), caused strong DNA binding concomitant with strong chromatin clustering of both, H3K9me0 and H3K9me3 oligonucleosomes (Supplementary Fig. 7f and g).

The NT region of hHP1β is also highly enriched in charged amino acids (Supplementary Fig. 2a). In agreement with the manifold contacts with the H3 tail detected in the cross-linking experiments, deletion of this region of hHP1β ΔNT) caused loss of recovery with precipitated H3K9me3 chromatin (Fig. 5d) as well as failure of chromatin clustering (Fig. 5e). Mutating the negatively charged residues (hHP1β NT (all E to A)) enhanced DNA binding and resulted in strong, unspecfic chromatin clustering. Mutating the positively charged residues (hHP1β NT (all K to A) had no effect on DNA binding but caused enhanced chromatin clustering of H3K9me0, while the effect on H3K9me3 oligonucleosomes was similar to hHP1β WT. Together with the results from the cross-linking, we concluded that the NT of hHP1β balances unspecific binding to DNA and the H3 tail. Indeed, opposing double mutation of charged residues in the NT and HR regions neutralized each other’s chromatin effects (hHP1β NT (all E to A) HR (all K,R to A), Fig. 5d,e).

**CD of hHP1β only functions in mediating H3K9me3 interaction.**

In contrast to what has been described for Swi6 (ref. 29), we failed we found Swi6 recovered to a higher degree in H3K9me3 activity of the HR of Swi6 (Supplementary Fig. 9b). Interestingly, in mouse embryonic stem cells (mESC) knock out of essential H3K9me3 methyltransferases exist33.

Previous genome-wide analysis mainly focused on correlations between mHP1z and H3K9me3 at repetitive elements33. Given the general high abundance of H3K9me3 at these regions34, this does not allow obtaining quantitative estimation of HP1 dependency on genome-wide H3K9me3 levels. To address this, we performed genome-wide antibody-based chromatin immunoprecipitation-sequencing (ChIP-seq) to measure mHP1β binding and compared the results with H3K9me3 enrichment previously obtained in the same mESC line35. The mouse genome was partitioned into 1 kb-sized tiles, and mHP1β and H3K9me3 enrichments above input were calculated for each of these tiles. mHP1β and H3K9me3 were found highly correlated along the entire genome (Fig. 6a, Pearson’s R = 0.77), and this was also evident at single loci (Fig. 6b). Importantly, an independent data set consisting of green fluorescent protein (GFP)-tagged mHP1β that was generated in a separate mESC line confirmed these findings (Supplementary Fig. 10a and b). Furthermore, by measuring genome-wide changes in H3K9me3 after conditional SETDB1 deletion36, we observed that the strongest loss of H3K9me3 occurred at sites enriched for mHP1β in WT cells (Fig. 6c).
hHP1β dimerization maintains compacted chromatin states.

To test whether dimerization is essential for the chromatin functions of HP1β, we first determined the subnuclear distribution of WT and mutant proteins in mouse fibroblasts. GFP-hHP1β largely localized to characteristic areas of pericentromeric heterochromatin enriched in H3K9me3. As deduced from our in vitro observations, the W42A, I161A or isolated CD mutant proteins, in contrast, showed diffuse distribution. However, heterologous dimerization of the NT–CD–HR region of hHP1β via GST (NT, CD, HR(hHP1β)-GST) was sufficient to fully restore heterochromatin localization. In agreement with a contributing role of the HR, the NT, CD(hHP1β)-GST hybrid protein, did not completely copy the appearance of the WT factor (Supplementary Fig. 11a and b).

We then adopted a previously described ‘chromatin reporter system’ that contains repetitive DNA arrays of LacO coupled to a regulatory transcription cassette (Fig. 7a). Integrated into the genome of U2OS cells these can be visualized after expression of mCherry-LacI as singular spot of heterochromatin37,38.

Activation of transcription via doxycycline-induced expression of the rtTA activator resulted in decondensation of the array (Fig. 7b, YFP-LacI). Unfolding of the array on such activation was largely reduced by tethering of LacI-YFP-hHP1β WT but not the dimerization deficient I161A mutant to the locus (Fig. 7b, YFP-LacI). As expected, LacI-YFP-hHP1β W42A behaved very similar to the WT protein, as tethering of the exogenous protein to the locus was achieved by the LacI domain independently of CD/H3K9me3 interaction. Nevertheless, heterologous dimerization of the NT–CD–HR region of hHP1β via GST (NT, CD, HR(hHP1β)-GST) was sufficient to prevent unfolding of the array on activation. In agreement with contribution of the HR, the deletion of this domain resulted in an intermediate effect.

**Discussion**

We postulate that hHP1β exemplifies the fundamental interaction mode of HP1 proteins with chromatin. In our model (Fig. 8a,b) dimerization of the chromatin-binding module (that is, NT–CD) is required for increasing the avidity of the interaction by enabling longer residence time of HP1 on chromatin. While the CD is the main chromatin-binding domain, the charged NT provides additional anchoring via unspecific electrostatic interaction with the H3 tail. Flexibility within the HR allows binding

**Figure 6** Genome-wide enrichment of HP1β correlates with SETDB1-dependent H3K9me3 deposition. (a) Plot of genome-wide correlation of mHP1β and H3K9me3 (ref. 35) as deduced by ChIP-seq in mouse ESC and using 1kb-sized windows. Dashed line indicates the data trend computed by loss regression. (b) Tracks displaying number of library-normalized reads per 100 bp from the indicated ChIP-seq experiments. Data obtained from antibody-based enrichments of endogenous mHP1β (top) and GFP-tagged mHP1β (middle) and the respective input tracks are shown. H3K9me3 data in SETDB1f/− and SETDB1−/− experiments are from ref. 36. Gene models and repetitive elements are indicated at the bottom. (c) Analysis as in a but comparing mHP1β binding to H3K9me3 loss in SETDB1−/− mESC. H3K9me3 loss is shown as the log2-difference between SETDB1f/− and SETDB1−/−.
of the chromatin-binding module to two H3 tails of the same nucleosome and different nucleosomes within the same or distinct chromatin fibres. Since neither the CD within a HP1 dimer nor the H3 tails are in a fixed orientation, the entropic effect of bivalent interaction onto binding strength is relatively mild (Table 1). Altogether these parameters result in highly dynamic and reversible interaction of HP1 with H3K9me3 chromatin that is in agreement with the structural parameters deduced for hHP1β binding to mononucleosomes30, recent single-molecule studies on hHP1α (ref. 14), as well as the highly mobile behaviour of HP1 proteins within heterochromatin of yeast and mammalian cells39–41.

In our model, modulation of the general chromatin-binding mode is brought about by varying sequences in the HR and NT domains as well as possibly their post-translational modification in other HP1 proteins (different variants, different species). We think that within these regions the overall charge but not a particular sequence is mainly dictating the chromatin-binding properties. Consistent with the requirement of a spacing and flexible linker (Fig. 8a), these domains are of relative low-sequence conservation and of varying lengths in different HP1 proteins (for example, HR(hHP1γ), 30 aa; HR(Swi6), 120 aa). In hHP1α the HR contains a surplus in positively charged residues (Supplementary Fig. 2a). Consequently, the protein shows limited discrimination between H3K9me0 and H3K9me3 chromatin due to CD-independent binding of DNA12. Phosphorylation of the NT neutralizes the surplus of positive charges and thereby directs an H3K9me3-specific chromatin-binding mode13. Interestingly, the NT and HR of hHP1α but not of hHP1β are extensively post-translationally modified42.

Swi6 contains an even more positively charged HR, which causes more pronounced binding to unmodified chromatin29, as well as an unusual long NT (Supplementary Fig. 2a). The superstoichiometric recruitment of Swi6 to H3K9me3 chromatin observed by others29 and us has been attributed to ordered oligomerization of the protein via additional, induced CD–CD interaction43. However, we find that the Swi6(CD) is not sufficient for this effect (Fig. 5d,e). Contrariwise, our studies on hHP1α and work on hHP1β (ref. 14) clearly show that these proteins do not multimerize beyond dimerization in chromatin binding. Also, recent modelling approaches deduced an allosterically controlled mode of Swi6 in the absence of CD–CD interaction44. While the functional differences between Swi6 and mammalian HP1 proteins need to be further investigated, we note that this new interpretation of previous data is fully compatible with our general model.

How does the flexible and dynamic binding mediate the chromatin clustering effects of hHP1β? We think the protein stabilizes condensed structures rather than inducing these de novo. It does not work as a locally directed and static chromatin clamp as suggested on the basis of the alleged binding mode of Swi6 (refs 45,46). Yet, via a large number of transient parameters...
diverse biology ascribed to these proteins in hetero- and euchromatin\textsuperscript{10,47,48}. For example, an intimate relationship exists between heterochromatin and the nuclear periphery in various metazoans\textsuperscript{30}. H3K9 methylation appears to be the major driving force for the localization of genomic regions to the nuclear periphery and these generally overlap with lamina-associated domains\textsuperscript{30}. Although a direct involvement in the recruitment to the nuclear periphery is controversial, the very dense chromatin areas might be clustered and stabilized by HP1. We think the dynamic mode of HP1 binding and actions on H3K9me3 chromatin allows flexible clustering and re-clustering of different regions of chromatin within the same or distinct chromosomes thereby contributing to the establishment and maintenance of heterochromatin. Importantly, such working mechanism is in agreement with an emerging view of high plasticity of nuclear chromatin in the absence of hierarchical organization\textsuperscript{51–53}.

**Methods**

**Plasmids.** Plasmids containing DNA templates for chromatin reconstitution were a gift of Dr Daniela Rhodes (MRC Cambridge, UK). Plasmids for expression of recombinant histones from *Xenopus laevis* were a gift from Dr. Karolin Luger (University of Colorado, Boulder). Complementary DNAs (cDNAs) corresponding to hHP1\textsubscript{b} (GenBank NM_001127228) were amplified by PCR using a 5′ primer that introduces a His\textsubscript{6} affinity tag and cloned into pET11a expression vector. Alternatively, the hHP1\textsubscript{b} (GenBank BC0068221) and hHP1\textsubscript{b} C DNAs were cloned into pCold I (Takara Bio/Clontech, Saint-Germain-en-Laye, France). For point and deletion mutants, site-directed mutagenesis was carried out using the Stratagene QuickChange protocol or a Q5 site-directed mutagenesis kit (New England Biolab, Frankfurt, Germany) according to the manufacturers’ instruction. cDNAs for other chimeric proteins were synthesized by Geneviz (South Plainfield, USA). YFP-LacI-NSL C1 and YFP-LacI C1 expression vectors were kind gifts from Dr Supriya Prasanth (University of Illinois, IL, USA)\textsuperscript{38}. hHP1\textsubscript{a} WT, mutant and chimera cDNAs were amplified from the bacterial expression vectors by PCR and cloned into *SfoII*–*BamHI* sites of the YFP-LacI C1 vector. A detailed list of the plasmids used in this study can be found in Supplementary Table 3.

**Western blotting.** Anti-histone H3 (1/10,000; ab1791, Abcam, Cambridge, UK), swine anti-rabbit IgG (1/2500; P0399; DAKO, Hamburg, Germany), anti-His\textsubscript{6}-peroxidase (1/500; 1965085801; Roche, Mannheim, Germany); anti-hHP1\textsubscript{a} (1/2000; MAR3448, Merck Millipore, Schwabbach, Germany). Unprocessed scans of gels and blots are provided in Supplementary Fig. 2.

**Expression and purification of recombinant HP1 proteins.** For pET11a vectors, proteins were expressed using ZYM-5052 auto-inducing medium overnight at 30°C. For pCold vectors, proteins were expressed in the same cells using standard LB medium overnight at 15°C in the presence of 0.4 mM IPTG. For production of pBpa-containing hHP1\textsubscript{b}, His-tagged hHP1\textsubscript{b} (GenBank NM_001127228) was expressed in the presence of pSUP-BPARS\textsuperscript{36} in *E. coli* BL21 DE3 in standard LB medium supplemented with 2 mM pBpa. Protein expression was induced at OD\textsubscript{600nm} = 1 by the addition of 1 mM IPTG for 4 h. All proteins were purified on Ni-NTA beads (Qiagen, Hilden, Germany) using standard protocols followed by anion exchange chromatography (MonoQ, GE Healthcare, Freiburg, Germany). Alternatively, proteins were purified using His-Pur Cobalt resin (Thermo Scientific, Braunschweig, Germany) with several rounds of high-salt washes (0.8 M NaCl) before elution. Purified proteins were dialyzed against PBS, 10% (v/v) glycerol, 2 mM DTT or a buffer containing 10 mM triethanolamine (pH 7.5), 150 mM NaCl, 1 mM DTT 30% (v/v) glycerol. Aliquots of purified protein were stored at −80 or −20°C, respectively.

**Expression and purification of recombinant histones.** Core histones were expressed in *E. coli* BL21 (DE3) RIL cells using ZYM-5052 auto-inducing medium. Histone proteins were found exclusively in inclusion bodies, which were solubilized in unfolding buffer (7 M deionized urea, 20 mM Tris-HCl (pH 7.5), 10 mM DTT). The material was dialyzed against urea chromatography buffer (7 M deionized urea, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 2 mM DTT, 0.2 mM PMSF) and loaded onto a Q Sepharose column in front of a SP Sepharose column (both GE Healthcare, Freiburg Germany). After washing with five column volumes of urea chromatography buffer, the Q Sepharose column was bound with DNA and contaminating proteins was removed. Histone proteins were eluted from the SP Sepharose column using a linear gradient from 0.1 to 0.6 M NaCl. Purified histones were dialyzed extensively against ddH\textsubscript{2}O, lyophilized and stored at −80°C.
Native chemical ligation. 0.2 mM of H3A1-20, A21C and 1 mM of N-terminal histone peptide (1–20) with a C-terminal thioester group were ligated for 24 h in 100 mM potassium phosphate, 3 M guanidine-HCl, 0.2% (v/v) mercaptoan, 0.5% (v/v) thiphenol, pH 7.9 at 25°C with vigorous mixing. The crude reaction mixture was dissolved into 25:75:0.1 acetonitrile/water/trifluoroacetic acid, diluted fivefold into SAU-200 buffer (7 M deionized urea, 20 mM sodium acetate (pH 5.2), 1 mM EDTA, 1 mM DTT, 200 mM NaCl), applied to a Hi-Trap SP-Sephacore high performance cation exchange column (GE Healthcare, Freiburg, Germany), and eluted with a linear NaCl gradient from 200 to 600 mM. Protein samples were dialyzed extensively against 2 mM DTT at 4°C, lyophilized and stored at −80°C.

Site-specific installation of H3K9me3. H3K9C, C110A was expressed and purified as WT histones. 5 mM of mutant H3 was reduced for 1 h at 37°C in the dark with occasional mixing. After 2.5 h of incubation, 10 mM of fresh DTT was added and the reaction was allowed to proceed for another 2.5 h at 50°C. The alkilation reaction was quenched with 700 mM 2-mercaptoethanol, the crude reaction mixture was dialyzed 50-fold into SAU-200 buffer. Alkylated histones were purified by anion exchange chromatography as described above.

Reconstitution of histone octamers. Lyophilized purified WT core histones H2A, H2B, H4 and WT or modified H3 were dissolved in unfolding buffer and mixed to equimolar ratios. The histone mixture was extensively dialyzed at 4°C against RB high buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, 1 mM DTT, pH 7.5) with at least three changes of dialysis buffer. Histone octamers were concentrated to 10–20 mg ml−1 using Amicon Ultra centrifugal filter units (Millipore, Billerica, USA) and purified on a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare, Freiburg, Germany). Peak fractions were pooled and concentrated to at least 2 mg ml−1. Histone octamers were stored in 30% (v/v) glycerol at −20°C.

Reconstitution of chromatin templates. Mononucleosomes were reconstituted on a 187 bp DNA fragment containing the ‘601’ sequence flanked on each site by a linker of 20 bp55. Oligonucleosomes were reconstituted on a 12 × 200 bp × 601 bp template56. Plasmids carrying the respective inserts were purified using a Giga kit (Qiagen, Hilden, Germany). Templates for reconstitution were released from plasmids by restriction digestion with BstBI and dialyzed at 4°C for 30 min against RB high buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5, 20 mM NaCl, 0.1 mM DTT, pH 7.5) over a 36 h period using a RB high buffer that was continuously replaced by RB low buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.5) in the dark with occasional mixing. After 2.5 h of incubation, 10 mM of fresh DTT was added and the reaction was allowed to proceed for another 2.5 h at 50°C. The alkilation reaction was quenched with 700 mM 2-mercaptoethanol, the crude reaction mixture was dialyzed 50-fold into SAU-200 buffer. Alkylated histones were purified by anion exchange chromatography as described above.

Fluorescence correlation spectroscopy. All fluorescence measurements were performed with a pulsed custom-built mode-locked Ti:Sa-Laser and a computer-controlled two-photon fluorescence microscope setup. Detailed description of the experimental setup and the measurement procedure are available on request. In all cases 10 nM of the acceptor labelled species, ATTO 610-labelled nucleosomal array, was incubated with 20–200 μM of recombinant hHP1Δ in 10 μl of 10 mM triethanolamine HCl (pH 7.3), 0.1 mM EDTA, 150 mM NaCl at 4°C. For each sample the fluorescence correlation curve, fluorescence intensity and anisotropy values were determined at 22°C. Every data point was averaged over a minimum of 15 single measurements of 10 s each. Excitation was by two-photon mode. The fluorescence data were analysed using the correlation function

\[ G(t) = \frac{I(t)}{I(0)} - 1, \]

and the equation for free diffusion of particles

\[ G(t) = N^{-1} - \left(1 + \frac{r^2}{D t^2}ight)^{-1} \left(1 + r^2 + \frac{r^2}{D t^2}ight)^{-0.5}, \]

where \(I(t)\) is the fluorescence intensity measured at time \(t\), \(N\) the particle number, \(t_d\) the diffusion time, and \(r_d = 0.25\) the axis ratio of the focal region, considering the dimensions of a Gaussian beam waist.

Fluorescence polarization measurements. Experiments were performed in 10 mM triethanolamine HCl (pH 7.4), 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT. Titration series of 10 μl volume in 384-well plates were read multiple times on a Plate CHAMELEON II plate reader (HIDEX Oy, Turku, Finland). Multiple readings and independent titration series were averaged after data normalization57.

Isothermal calorimetry. ITC measurements were performed on a ITC200 calorimeter (Microcal, Malvern, UK) at 25°C in binding buffer (10 mM triethanolamine HCl (pH 7.5), 20 or 150 mM NaCl, 0.1 mM EDTA). Reaction heats were recorded by sequences of 37 injections of 1.8 mM hHP1Δ, spaced at 120 s intervals, into 250 μl of 60 mM H3 histone peptides, nucleosomes or oligonucleosomal arrays under constant stirring at 100 rpm. Injection #1–10: 0.5 μM each; injection #11–32: 1 μl each; injection #33–37: 2 μl each). Heats of dilution, obtained by the titration of hHP1Δ into the reaction, were subtracted from raw data before analysis. Raw data were integrated, normalized and the apparent heat change (ΔQ) of the reaction was plotted against the molecular ratio using the Origin software. For the determination of apparent enthalpy changes (ΔH), the molar association constant (Kₐ), and the stoichiometry (n) of the interaction, non-linear least-square fitting of the ΔQ values was performed by the Origin software using a binding model of one set of identical binding sites.

Surface plasma resonance. SPR measurements were performed on a Biacore 2000 instrument (GE Healthcare, Buckinghamshire, UK) at 298 K in 10 mM triethanolamine HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.005% (v/v) Tween-20. Biotinylated ligands were immobilized on streptavidin-coated sensor chips (GE Healthcare, Buckinghamshire, UK). Before immobilization, sensor chips were conditioned as described by the manufacturer. To obtain low surface densities (5–24 RU), 5 mM biotinylated H3 peptide was injected at a low rate of 10 μl-min⁻¹ using varying contact times. High surface densities (>700 RU) were prepared by injecting 3.5 mM peptide (10 μl-min⁻¹) until the SA surface was saturated. Peptide-containing binding curves were regenerated by applying three short pulses of 0.05% (v/v) SDS followed by an injection of 1.5 mM NaCl for baseline stabilization. For binding assays, hHP1Δ proteins were injected at 24–50 μM (serial twofold dilutions) for 2 min (30 μl/min). Dissociation was recorded for up to 5 min. A streptavidin surface without ligands served as reference. In addition, blank runs were performed for each measurement. Data evaluation was performed by steady-state analysis assuming a Langmuir 1:1 binding model using BLAevaluation 4.1 and Prism 5.04 (GraphPad, CA, USA) software. Binding to the unmodified proteins were injected at 24–50 μM (serial twofold dilutions) for 2 min (30 μl/min). Dissociation was recorded for up to 5 min. A streptavidin surface without ligands served as reference. In addition, blank runs were performed for each measurement. Data evaluation was performed by steady-state analysis assuming a Langmuir 1:1 binding model using BLAevaluation 4.1 and Prism 5.04 (GraphPad, CA, USA) software. Binding to the unmodified
peptides was analysed with maximum binding (RUmax) set to the corresponding

Scanning force microscopy. Nucleosomal arrays and recombinant HP1β were
dissolved in 0.1% Triton-X100 and incubated for 30 min at RT. Freshly prepared EDC (Sigma-Aldrich, Germany) was added to 15 mM and incubated for 30 min at RT. In the case of a higher

Chemical cross-linking and mass spectrometry. 13.4 nM H3K9me3 oligonucleosomes were mixed with 2 µM HP1β in 1 ml cross-linking buffer (20 mM sodium phosphate (pH 6.4), 100 mM NaCl, 0.1% (v/v) Triton-X100) and incubated for 30 min at RT. The sample was adjusted to ≤ 0.1% (v/v) and 3 µg trypsin (Promega, Mannheim, Germany) was added for 16 h at 37°C. Tryptic peptides were purified using Sep-Pak SPE C18 cartages (Waters, Dublin, Ireland) and separated using size-exclusion chromatography4,25. Each peptide-containing fraction was analysed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Database searching was performed using data26. All cross-linking-containing peptide fragment spectra were manually evaluated26. Crosslinks were visualized with x![http://www.crosslinkviewer.org](http://www.crosslinkviewer.org).

Ultraviolet-mediated protein-protein cross-linking. 34 nM oligonucleosomal arrays were incubated with 8 µM standard or photo crosslinkable HP1β in 100 µl binding buffer (10 mM triethanolamine (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.1% (v/v) Triton-X100) and incubated for 30 min at RT. The mixture was placed into wells of a custom-made metal sample holder on ice and irradiated with ultraviolet light (365 nm, 8 W lamps (Vilber Lourmat, Eberhardzell, Germany)): the distance between the light source and sample was set ~5 cm for 3 × 10 min with intermittent mixing. After irradiation, the samples were collected into 1.5 ml low-binding tubes (Eppendorf, Hamburg, Germany). MgCl₂ was added to 5 mM and chromatin and bound proteins were recovered by centrifugation at 16,100g for 30 min at 4°C. Pellets were washed once with 0.5 ml binding buffer and resuspended in 10 µl SDS loading buffer and boiled for 5 min.

Generation of HP1β–EGFP mESCs. The mouse HP1β coding sequence was cloned into the pCAGGS–EGFP–IRES–Puro plasmid containing a chicken beta-actin promoter. Stable mHP1β–EGFP mESC lines were generated by transfecting 6 µg PGLP-linearized vector into mouse WT ES cells.

HP1β ChIP-seq. For crosslink MNase ChIP of HP1β, 10° TET2 WT mouse ES cells were collected. Cells were crosslinked with 1% (v/v) formaldehyde in 10 ml of PBS for 10 min at RT, then quenched with 0.125 M glycine for 5 min. Cells were washed 1 × with PBS, and resuspended in 1 ml of EZ Nuclei Isolation Buffer (Sigma-Aldrich, Steinheim, Germany). The cytoplasmic supernatant was discarded and the pellet was flash frozen with liquid N₂. Nuclei were washed 1 × with MNase wash buffer (50 mM Tri-HCl (pH 8.0), 1.5 mM DTT, 1 mM PMSF, 1 × Protease Inhibitor Cocktail (Roche, Indianapolis, USA)) and resuspended in MNase Digestion Buffer (10 mM Tri-HCl (pH 7.5), 4 mM MgCl₂, 1 mM CaCl₂, 1 mM PMSF, 1 × Protease Inhibitor Cocktail). Chromatin was digested to mono-, di- and tri-nucleosomes with the addition of micrococcal nuclease (NEB, Ipswich, USA) and incubation at 37°C for 30 min. Digestion was quenched with the addition of the ChIP-seq Sample Prep Master Mix (New England Biolabs, Ipswich, USA) at 4°C for 10 min with inter-mixing. After centrifugation, the samples were fixed with 0.05% (v/v) formaldehyde/PBS for 10 min at RT. Cells were permeabilized with 0.5% (v/v) Triton-X100 (PBS) for 5 min at RT and incubated with 200 ng ml⁻¹ DAPI (PBS) for 5–10 min. Cells were mounted in Vectashield (Vectorlabs, CA, USA). Images were acquired using a Leica SP5 confocal microscope with a 63 × oil immersion lens (Leica Mikrosysteme, Wetzlar, Germany). P values (according to Student’s t-test) were calculated using GraphPad QuickCals (GraphPad, CA, USA).

Transfection of cells and fluorescence microscopy. U2OS 2×GFP were kindly provided by Dr Supriya Prasanth (University of Illinois, IL, USA) and maintained in DMEM (high glucose + Glutamax) (GIBCO/Invitrogen, Darmstadt, Germany) supplemented with 10% (v/v) FCS (Fetal bovine serum (FBS), Sigma-Aldrich–Gene-en-Layaye, France), 50 µg ml⁻¹ lysisyn (Carl Roth, Karlsruhe, Germany) and 200 µg ml⁻¹ G418 (Clontech, Saint-Germain-en-Laye, France). For fluorescence microscopy, cells were grown on coverslips in a six-well plate without antibiotics. 500 ng plasmid DNA was transfected using Lipofectamine LTX and Plus reagent (Invitrogen, Darmstadt, Germany). 20 h post-transfection, cells were treated with 1 µg ml⁻¹ doxycycline for 24 h for induction of rTA expression. Cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde/PBS for 10 min at RT. Cells were permeabilized with 0.5% (v/v) Triton-X100 (PBS) for 5 min at RT and incubated with 200 ng ml⁻¹ DAPI (PBS) for 5–10 min. Cells were mounted in Vectashield (Vectorlabs, CA, USA). Images were acquired using a Leica SPS confocal microscope with a 63 × oil immersion lens (Leica Mikrosysteme, Wetzlar, Germany). P values (according to Student’s t-test) were calculated using GraphPad QuickCals (GraphPad, CA, USA).

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Author contributions
K.H.-H., S.S. and W.F. designed the study and analysed data. K.H.-H. and S.S. did biochemical and biophysical analysis of hHP1β/chromatin interaction and effects. S.S. performed SFM studies, while K.H.-H. and S.K. investigated the cellular role of hHP1β/chromatin interaction. M.N., A.C. and H.U. performed and analysed MS data of crosslinks. C.C. and M.L. performed native ChIP-seq experiments. I.A.D. and T.J. performed ChIP-seq studies using tagged HP1β. T.B. performed all bioinformatics analyses. H.M.Z. and B.Z. set up and analysed SPR experiments. W.P. and P.J.W. established and analysed FCS measurements. B.W. and H.N. helped with photo cross-linking analysis. N.K. did chromatin-clustering analysis of hHP1β with tailless histones. D.S. made peptides for NCL. K.H.-H and W.F. wrote the manuscript. All authors commented on the final draft.

Additional information
Accession codes: HP1β ChIP-seq datasets were deposited at the Gene Expression Omnibus (GEO) of National Center for Biotechnology Information under the accession number GSE71114.

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