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Chapter 3

Truncated HP1 lacking a
functional chromodomain induces
heterochromatinization upon
in vivo targeting

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Abstract

Packaging of the eukaryotic genome into higher-order chromatin structures is tightly related to gene expression. Pericentromeric heterochromatin is typified by accumulations of heterochromatin protein 1 (HP1), methylation of histone H3 at lysine 9 (MeH3K9) and global histone deacetylation. HP1 interacts with chromatin by binding to MeH3K9 through the chromodomain (CD). HP1 dimerizes with itself and binds a variety of proteins through its chromoshadow domain. We have analyzed at the single-cell level whether HP1 lacking its functional CD is able to induce heterochromatinization *in vivo*. We used a lac operator-array-based system in mammalian cells to target EGFP-lac repressor-tagged truncated HP1 α and HP1 β to a lac operator-containing gene-amplified chromosome region in living cells. After targeting truncated HP1 α or HP1 β we observe enhanced tri-MeH3K9 and recruitment of endogenous HP1 α and HP1 β to the chromosome region. We show that CD-less HP1 α can induce chromatin condensation, whereas the effect of truncated HP1 β is less pronounced. Our results demonstrate that after lac repressor-mediated targeting, HP1 α and HP1 β without a functional CD are able to induce heterochromatinization.

Introduction

In the eukaryotic nucleus, various levels of chromatin packaging can be discerned (Horn and Peterson, 2002). The condensation state of chromatin is a key aspect of epigenetic gene control. Different chromatin condensation states are related to the presence or absence of proteins involved in the regulation of gene expression (reviewed in Lachner and Jenuwein, 2002). Pericentromeric heterochromatin, for instance, is typified by the accumulations of heterochromatin protein 1 (HP1), methylation of histone H3 at lysine 9 (MeH3K9) and global histone deacetylation (reviewed in Dillon, 2004; Maison and Almouzni, 2004). The molecular mechanisms underlying the structural and functional aspects of heterochromatin are still unresolved.

In mammals, three isoforms of HP1 have been identified, HP1 α , HP1 β and HP1 γ , showing different subnuclear distributions. HP1 α is primarily found in pericentromeric heterochromatin. HP1 β and to a much lesser extent HP1 γ are also present in pericentromeric heterochromatin (Cowell *et al.*, 2002; Horsley *et al.*, 1996; Li *et al.*, 2003; Minc *et al.*, 1999; Wreggett *et al.*, 1994). HP1 proteins have emerged as key structural and regulatory components for the assembly of compact chromatin (Maison and Almouzni, 2004). HP1 consists of a chromodomain (CD) at the amino terminus and a chromoshadow domain (CSD) at the carboxy terminus, separated by a hinge domain (HD). The CD recognizes MeH3K9, mediating binding to chromatin (Bannister *et al.*, 2001; Cowell *et al.*, 2002; Jacobs and Khorasanizadeh, 2002; Jacobs

et al., 2001; Lachner *et al.*, 2001; Nielsen *et al.*, 2002). The CSD interacts with a consensus peptide that is present in a number of proteins, including the CSD of HP1 itself, thereby targeting proteins to heterochromatin and forming HP1 dimers (Thiru *et al.*, 2004). The conserved CD and CSD are essential for the function of HP1. The HD of HP1 is less well characterized; a few studies indicate that it plays a direct role in HP1 function (Meehan *et al.*, 2003).

Recently, we showed that targeting of intact HP1 α and HP1 β is sufficient to induce large-scale chromatin compaction, enhanced tri-MeH3K9 and recruitment of proteins involved in heterochromatinization (Verschure *et al.*, 2005). In the present study, we investigate whether HP1 that lacks a functional CD is able to induce heterochromatinization. Our results demonstrate that when HP1 α or HP1 β without a functional CD is targeted to chromatin, it is able to induce heterochromatinization.

Materials and methods

Construction of plasmids

A plasmid expressing the enhanced green fluorescent protein (EGFP)-dimer lac repressor-simian virus 40 nuclear localization signal (NLS) fusion protein under control of the F9-1 promoter, called p3'SS-EGFP-dimer lac repressor was used as the basis for these studies (Tumbar *et al.*, 1999). This plasmid was modified as described previously to generate EGFP-lacR-*Ascl*-NLS (NYE4; Nye *et al.*, 2002). HP1 α and HP1 β correspond to full sequences of the human HP1. Both human HP1 α and HP1 β have only a minor amino acid difference from the Chinese hamster HP1 α and HP1 β (for HP1 β one amino acid difference in the hinge domain and for HP1 α one amino acid difference in the CD, two in the HD and two outside of the domains). HP1 α - Δ (2-39) and HP1 β - Δ (2-40) have deletions of amino acids 2 to 39 and 2 to 40 in HP1 α and HP1 β , respectively. These proteins lack a large part of the CD, including V21 (HP1 α) and V23 (HP1 β), which are required for MeH3K9 binding (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Platero *et al.*, 1995). Full-length HP1 α and HP1 β as well as HP1 α - Δ (2-39) and HP1 β - Δ (2-40) fragments were PCR amplified using primers that contain *Ascl* sites in frame. The PCR products were digested with *Ascl* and ligated into the *Ascl*-digested EGFP-lacR-*Ascl*-NLS (NYE4) vector to create full-length HP1 α and HP1 β as well as HP1 α - Δ (2-39) and HP1 β - Δ (2-40) C-terminal in-frame fusions with the EGFP-lacR-*Ascl*-NLS (NYE4) vector. All regions of constructs that had undergone PCR were sequenced to ensure fidelity.

Cell culture and transfection assay

We used the CHO-derived cell line RRE_B1, which contains large amplified genomic domains (several tens of mega basepairs) consisting of arrays of lac operator binding sites, in an extended, often fibrillar conformation, suggesting a euchromatin-like structure (Robinett *et al.*, 1996a). The *in situ* appearance of the RRE_B1 cell line as visualized with the light microscope is described in detail in Verschure *et al.* (2005). Cells were cultured in F12 Ham's media without hypoxanthine or thymidine supplemented by 10% dialyzed fetal bovine serum (HyClone Labs, Logan, Utah, USA) and 10 μ M methotrexate at 37°C in a 5% CO₂ atmosphere. Transient transfections were performed with FuGENE6 reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions, using 500 ng of DNA and 6 μ l FuGENE6 reagent per milliliter of culture medium using cells growing on coverslips. Fresh medium was added 24 hours after transfection. After 48 hours, cells were rinsed with phosphate buffered saline (PBS) and used for immunofluorescent labeling.

Immunofluorescent labeling

Immunofluorescent labeling was performed as described previously (Verschure *et al.*, 2002). Briefly, cells were fixed for 10 minutes at 4°C in 2% (w/v) formaldehyde in PBS. After fixation, cells were permeabilized

with 0.5% (w/v) Triton-X 100 in PBS for 5 minutes and incubated in PBS containing 100 mM glycine for 10 minutes. Subsequently, cells were incubated for 1 hour at 37°C with the primary antibodies diluted in PBG: PBS containing 0.5% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) gelatin (Sigma, St Louis USA). The following primary antibodies were used: rabbit anti-tri-MeH3K9 (Nakayama *et al.*, 2001), mouse anti-HP1 α and anti-HP1 β (Euromedex; Mundolsheim France; Nielsen *et al.*, 2001). After several washes with PBS, cells were incubated with the appropriate secondary antibodies, using Cy3-conjugated donkey anti-rabbit antibody or Cy3-conjugated donkey anti-mouse antibody (Jackson, West Grove, PA, USA). Secondary antibodies were diluted in PBG. Incubations were performed for 1 hour at room temperature. Cells were then rinsed with PBS at room temperature and DNA staining was performed with 0.4 μ g/ml DAPI 33258 (Sigma) in PBS. Slides were mounted in Vectashield (Brunschwig, Burlingame, CA, USA), stored at 4°C and analyzed within 24 hours. As a control, the primary antibody was omitted.

Confocal scanning laser microscopy

All experiments were performed at least twice in duplicate. Images were recorded using a Zeiss LSM 510 (Zeiss, Jena, Germany) confocal laser scanning microscope, equipped with a 100x/1.23 NA oil immersion objective. We used an argon laser at 488 nm in combination with a helium neon laser at 543 nm to excite green and red fluorochromes simultaneously. Emitted fluorescence was detected with a 505-530 nm bandpass filter for the green signal and a 560 nm longpass filter for the red signal. Three-dimensional (3D) images were scanned as 512x512x32 voxel images (sampling rate: 49 nm lateral and 208 nm axial).

Image analysis

For a semi-quantitative analysis of the spatial relationship between the relative spatial distributions of components in dually labeled cells, line scans were made. The signal intensities of the two labels are plotted along a line through the nucleus. The line scan method provides a rapid and straightforward way to determine to what extent two components colocalize. To quantitatively analyze changes in large-scale chromatin structure, we applied 3D-image-analysis tools as described in a previous study (Verschure *et al.*, 2005). Briefly, 3D images of the amplified chromosome region were acquired and attributes of the 3D structure were determined. A region of interest, *i.e.* the EGFP-labeled chromosome region, is defined. The images were Gaussian filtered to reduce noise with a size of 20 pixels ($\sigma = 250$ nm). For each set we record the intensity gradient over the chromatin region. The distribution of the intensity gradient is plotted in a box-plot. The second and third quartiles of the observed values are within the box, the median value is shown by the thick horizontal line, the vertical small lines show the first and fourth quartile of the observed values. We used the Wilcoxon non-parametric test to calculate the *P*-value giving the probability that two populations are different from each other.

Results

Experimental approach

To investigate the ability of HP1 lacking a functional CD to induce heterochromatinization, we used the CHO cell line RRE_B1. This cell line contains a large amplified chromatin region consisting of arrays of lac operator binding sites (Robinett *et al.*, 1996). When visualized *in vivo* by binding of EGFP-tagged lac repressor (lacR), the amplified region occupies a large nuclear area and displays an open chromatin structure, suggesting a euchromatin-like state (Robinett *et al.*, 1996). These cells constitute a useful system to investigate euchromatin/heterochromatin transitions. In a previous study, we have shown that targeting of full-length HP1 α or HP1 β to the lac operator arrays induced chromatin compaction, enhanced tri-MeH3K9 and the recruitment of endogenous HP1 and the histone methyltransferase SETDB1

(Verschure *et al.*, 2005). These results demonstrated that HP1 binding is sufficient to trigger heterochromatinization.

DNA constructs were made encoding truncated human HP1 α and HP1 β , lacking the C-terminal part of their CD, tagged with EGFP-lacR: EGFP-lacR-HP1 α - Δ (2-39) and EGFP-lacR-HP1 β - Δ (2-40). Two controls were used: (1) similar constructs that code for full-length wild-type HP1 α and HP1 β instead of the truncated protein and (2) a construct coding for EGFP-lacR. Proteins coded by all constructs bind to the amplified chromatin region via their lacR domain. As expected, after transient transfection in cells without lacR-binding sites, CD-less HP1 does not accumulate in pericentromeric heterochromatin, since it lacks the binding site for MeH3K9 (data not shown). In contrast, EGFP-lacR full-length HP1 does accumulate in pericentromeric domains (Verschure *et al.*, 2005). These findings agree with the notion that the CD is responsible for localization of HP1 α and HP1 β in pericentromeric heterochromatin through MeH3K9 binding (Bannister *et al.*, 2001; Cheutin *et al.*, 2003; Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Nakayama *et al.*, 2001; Nielsen *et al.*, 2002).

To analyze whether targeting of truncated HP1 results in changes in the chromatin structure we measured three different parameters: (1) tri-MeH3K9 using immunofluorescent labeling with an anti-tri-MeH3K9-specific antibody, (2) accumulation of endogenous HP1 α or HP1 β and (3) changes in *in vivo* 3D chromatin structure of the EGFP-marked amplified chromatin domain, imaged by confocal microscopy followed by quantitative image analysis.

Histone H3 lysine 9 methylation

MeH3K9 creates a binding site for the CD of HP1, whereas the CSD interacts with a variety of proteins, including HP1 itself (Aasland and Stewart, 1995; Bannister *et al.*, 2001; Cowell *et al.*, 2002; Jacobs and Khorasanizadeh, 2002; Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Lechner *et al.*, 2005; Nielsen *et al.*, 2002; Smothers and Henikoff, 2001; Thiru *et al.*, 2004). Recently, we showed that lacR-mediated targeting of full-length HP1 α or HP1 β results in strong heterochromatinization, including an increase of tri-MeH3K9 and recruitment of HP1 and a histone methyltransferase (Verschure *et al.*, 2005). Figure 1 shows that in cells transfected with EGFP-LacR-HP1 α - Δ (2-39) or EGFP-LacR-HP1 β - Δ (2-40) the level of tri-MeH3K9 is considerably increased in the amplified chromatin domain. In addition, tri-MeH3K9 is present diffusely throughout the nucleus. Line scans confirm that enhanced methylation levels colocalize with the binding of CD-less HP1 to the chromatin array. This phenomenon is observed in all the cells transfected with either full-length (Verschure *et al.*, 2005) or truncated HP1 α or HP1 β (figure 1). In control cells transfected with EGFP-lacR the methylation level is not changed (figure 1A-D). These observations show that CD-less HP1 is able to recruit a histone methyltransferase directly or indirectly, similar to wild-type HP1.

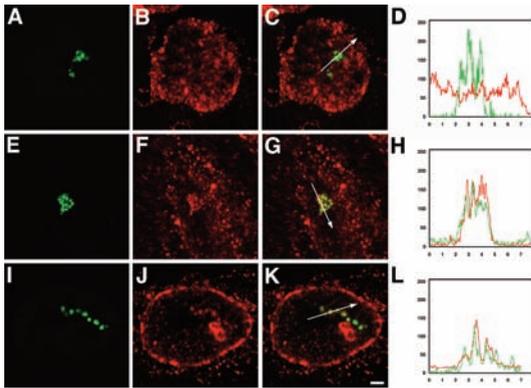


Figure 1. Enhanced tri-MeH3K9 at the amplified chromosome region. RRE_B1 cells transfected with EGFP-lacR (as a control) (A-D), EGFP-lacR-HP1 α - Δ (2-39) (E-H) or EGFP-lacR-HP1 β - Δ (2-40) (I-L), were immunofluorescently labeled with an antibody against tri-MeH3K9 after 48 hours. The green signal shows the EGFP-tagged chromosomal array and the red signal marks the distribution of the immunolabeled tri-MeH3K9. Three-dimensional images were recorded. Images shown are individual mid-nuclear optical sections. Figure D, H and L show line scans through the

nuclei shown in A-C, E-G and I-K, respectively. The position of the line scans is shown by the white arrow. Nuclei in A-C, E-G and I-K are the same scale; bar represents 2 μ m.

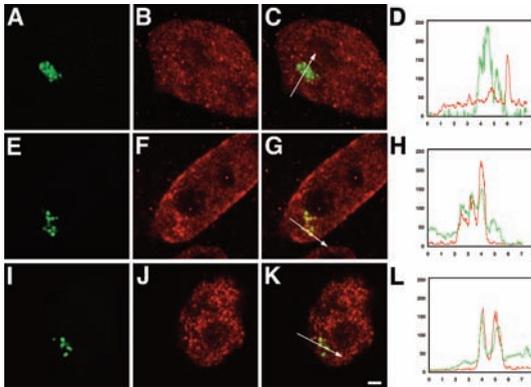


Figure 2. Recruitment of endogenous HP1 at the amplified chromosome region. RRE_B1 cells transfected with EGFP-lacR (A-D), EGFP-lacR-HP1 α - Δ (2-39) (E-H) or EGFP-lacR-HP1 β - Δ (2-40) (I-L) were immunofluorescently labeled after 48 hours with an antibody against HP1 β (A-D) and (E-H) or against HP1 α (I-L). Three-dimensional images were recorded. Images shown are individual mid-nuclear optical sections. The green signal shows the EGFP-lacR-tagged chromosomal array and the red signal marks the distribution of the immunolabeled endogenous HP1 proteins. Figure D, H and L show line

scans through the nuclei shown in A-C, E-G and I-K, respectively. The position of the line scans is shown by the white arrow. Nuclei in A-C, E-G and I-K are the same scale; bar represents 2 μ m.

Recruitment of endogenous HP1

LacR-mediated targeting of wild-type HP1 α or HP1 β results in local accumulation of endogenous HP1 α and HP1 β at the chromatin array. Here we analyze whether targeting of HP1 without a functional CD has the same effect. Cells transfected with EGFP-LacR-HP1 α - Δ (2-39) were fluorescently labeled with an antibody against HP1 β , and cells transfected with EGFP-LacR-HP1 β - Δ (2-40) with an antibody specific for HP1 α . Figure 2 shows that both the truncated proteins are able to recruit a considerable amount of endogenous HP1 to the amplified chromatin domains. Line scans confirm that endogenous HP1 accumulates at the sites where the CD-less HP1 protein is bound

(figure 2). In control cells transfected with EGFP-lacR the distribution of endogenous HP1 is not changed (figure 2A-D). These results indicate that targeting of HP1 without the CD domain enables the recruitment of endogenous HP1.

Large-scale chromatin structure

In a recent study, we showed that lacR-mediated binding of wild-type HP1 induces considerable chromatin compaction (Verschure *et al.*, 2005). Figure 3 shows that HP1 without a functional CD is also able to induce a similar condensation of chromatin, as can be visualized by confocal microscopy of the EGFP-tagged amplified chromatin domain. In many cells targeting of truncated HP1 induces condensation of the amplified chromatin regions in a cluster or chain of highly condensed spherical chromatin domains. There is significant variability in the appearance of the chromatin domains in a population of otherwise identical cells for full-length HP1 (Verschure *et al.*, 2005), for CD-less HP1 and for control cells (figure 3). Therefore, we carried out a quantitative analysis of the change in compaction of the chromatin structure of the array. As a measure for the degree of compaction we used a parameter that quantifies the discreteness of the individual subdomains in the amplified chromatin domain (Verschure *et al.*, 2005). In control cells transfected with EGFP-lacR, such subdomains are poorly discernable (figure 3A). In contrast, after HP1-induced chromatin condensation (either wild-type or truncated HP1), the individual domains can be clearly discerned (figure 3B-E). As a quantitative parameter we measured the gradient of the EGFP signal over such chromatin subdomains. The analysis was carried out on 3D images using novel image-analysis software as described in greater detail by Verschure *et al.* (2005). For each experiment several tens of cells were analyzed (*n*-values are shown in table 1).

Results are summarized in figure 4 and table 1. Figure 4 shows the median value (thick horizontal line) of the observed values, the second and third quartiles (open box) and the first and fourth quartiles of the detected values (thin vertical lines). The results confirm the large cell-to-cell variation observed earlier (Verschure *et al.*, 2005). Statistical analysis (Wilcoxon test; table 1) shows that CD-less HP1 α induces significant chromatin condensation, similar to wild-type HP1 α and HP1 β . Although condensed chromatin is seen in a number of cells transfected with EGFP-LacR-HP1 β - Δ (2-40), the difference with the control cell population (transfected with EGFP-lacR) is statistically not significant. This suggests that CD-less HP1 α is more effective in condensing chromatin than CD-less HP1 β . In contrast, both truncated proteins are similarly effective in inducing enhanced tri-MeH3K9 and in recruiting endogenous HP1 according to light-microscopy evaluation (figures 1 and 2). Unexpectedly, our results also show that the effect of chromatin condensation after targeting full-length HP1 α is more pronounced than the effect of full-length HP1 β . Apparently, both wild-type HP1 α and CD-less HP1 α are more effective than their HP1 β counterparts.

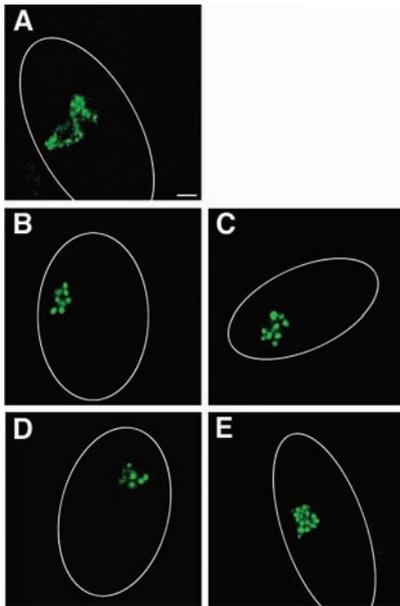


Figure 3. Local chromatin compaction of the amplified chromosome region. RRE_B1 cells were transfected with EGFP-lacR-HP1 α , EGFP-lacR-HP1 β , EGFP-lacR-HP1 α - Δ (2-39) or EGFP-lacR-HP1 β - Δ (2-40) fusion constructs or with EGFP-lacR as a control. Three-dimensional images were collected 48 hours after transfection. Images shown represent individual mid-nuclear optical sections illustrating the EGFP signal of the amplified chromosome region. The chromosomal array in a control nucleus transfected with EGFP-lacR (A) has the appearance of an extended fibrillar structure. After HP1 α (B) or HP1 β (C) targeting, the amplified chromosome region is compacted to a cluster of distinct spherical subdomains. After targeting of HP1 α - Δ (2-39) (D) or HP1 β - Δ (2-40) (E) similar compaction of the large-scale chromatin structure was observed. The approximate position of the nuclear envelope is shown by the white line. Nuclei in A-E are the same scale; bar represents 0.9 μ m.

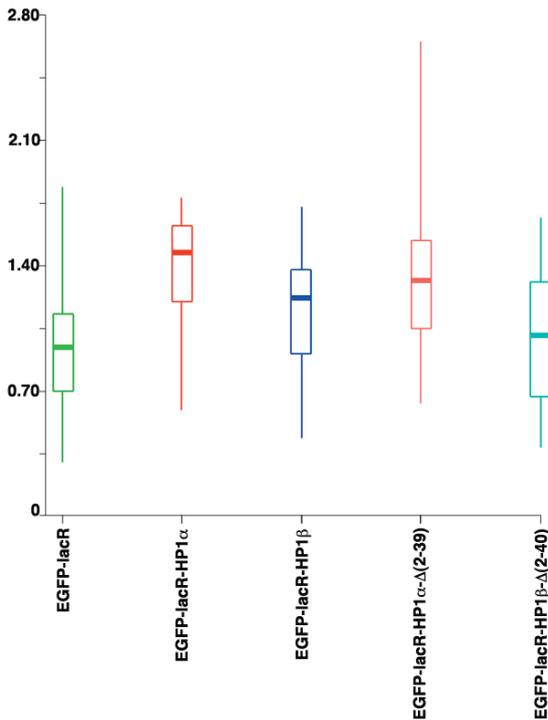


Figure 4. Quantitative analysis of the change in chromatin structure. Three-dimensional images of RRE_B1 cells transfected with EGFP-lacR-HP1 α , EGFP-lacR-HP1 β , EGFP-lacR-HP1 α - Δ (2-39), EGFP-lacR-HP1 β - Δ (2-40) or EGFP-lacR as a control were acquired 48 hours after transfection. We measured the gradient of the EGFP-signal intensity over the amplified chromosome region. The results are shown as a box-plot of the measured values for cells transfected with EGFP-lacR (green), EGFP-lacR-HP1 α (dark red), EGFP-lacR-HP1 β (dark blue), EGFP-lacR-HP1 α - Δ (2-39) (orange) and EGFP-lacR-HP1 β - Δ (2-40) (light blue). The second and third quartiles of the observed values are within the box (open box), the median value is shown by the thick horizontal line, the vertical small lines show the first and fourth quartiles of the observed values.

Discussion

Heterochromatin is a specialized chromatin state that is typically condensed, late replicating, and displays a diminished gene activity. The HP1 protein family is involved in the formation of heterochromatin. The observation that HP1 specifically binds to MeH3K9 via its chromodomain (CD), directly links these proteins to epigenetic gene-control mechanisms (Lachner and Jenuwein, 2002). In the present study we analyze *in vivo* and at the single-cell level whether HP1 that lacks a functional CD can induce heterochromatinization. We use three parameters to assess euchromatin/heterochromatin transitions: (1) enhanced tri-MeH3K9, (2) recruitment of endogenous HP1 α and HP1 β , and (3) condensation of chromatin. Results show that CD-less HP1 α and HP1 β are able to cause tri-MeH3K9 and recruitment of endogenous HP1 α and HP1 β . Targeting of truncated HP1 α induces chromatin compaction, similar to what is observed after targeting full-length HP1 α and HP1 β (Verschure *et al.*, 2005). The effect of truncated HP1 β is less pronounced. Our data demonstrate that both CD-less HP1 α and CD-less HP1 β are able to induce heterochromatinization, CD-less HP1 α being more effective than CD-less HP1 β .

Table 1

Populations analyzed	number of cells	<i>P</i> -value	significant
Control – wild-type HP1 α	57 - 27	< 0.0001	yes
Control – wild-type HP1 β	57 - 53	0.001	yes
Control – CD-less HP1 α	57 - 27	< 0.0001	yes
Control – CD-less HP1 β	57 - 29	0.29	no
Wild-type HP1 α – wild-type HP1 β	27 - 53	0.0008	yes

Table 1. Statistical evaluation of the structural analysis. Statistical evaluation of the differences in chromatin structure after targeting EGFP-lacR-tagged constructs; differences between control cells transfected with EGFP-lacR and cells transfected with EGFP-lacR-HP1 α , EGFP-lacR-HP1 β , EGFP-LacR-HP1 α - Δ (2-39) or EGFP-LacR-HP1 β - Δ (2-40) (rows 1 through 4) and between EGFP-lacR-HP1 α and EGFP-lacR-HP1 β (row 5). Shown are the *P*-values determined in a Wilcoxon statistical test, indicating the probabilities that two populations (column 1) are different, the number of cells that were analyzed (column 2) and the interpretation in terms of significance of the difference, choosing a cut-off value of *P*=0.05 (column 3 and 4).

Comparison of HP1 α and HP1 β

In the present study, we compared the effect of targeting HP1 α with that of HP1 β (both wild-type and CD-less HP1 proteins) on heterochromatinization of an amplified chromatin region. Results show that the effect of chromatin condensation after targeting full-length HP1 α is more pronounced than that of full-length HP1 β . Similarly, CD-less HP1 α is more effective in inducing chromatin compaction than truncated HP1 β . In contrast, both proteins are similarly effective in inducing enhanced tri-MeH3K9 and in recruiting endogenous HP1. The cause of this difference between HP1 α and HP1 β in their ability to induce chromatin compaction is unclear. The amino

acid sequence of both proteins differs only marginally (Minc *et al.*, 1999). In the present study, we used human HP1 in Chinese hamster cells, but there is only a minor amino acid difference between human HP1 α and HP1 β and Chinese hamster HP1 α and HP1 β (see materials and methods). Therefore, it is unlikely that the difference in effect between HP1 α and HP1 β is related to less efficient binding of the human HP1 in Chinese hamster cells. The difference in effect between HP1 α and HP1 β is probably related to the fact that HP1 α and HP1 β have distinct nuclear and mitotic distributions and differ in cell cycle-related phosphorylation (Minc *et al.*, 1999). HP1 α is phosphorylated throughout the cell cycle, whereas HP1 β remains unphosphorylated. HP1 α and HP1 β are predominantly located in centromeric heterochromatin. A third member of the HP1 family, HP1 γ is found in euchromatin and to a much lesser extent in heterochromatin. In a recent study, using a dominant-negative approach by over-expressing truncated HP1 lacking a functional CD, we show that endogenous HP1 α or HP1 β are lost from mouse chromocenters without changing their condensed chromatin structure (Mateos-Langerak *et al.*, 2007; chapter 4 of this thesis). Together, these observations indicate that full-length HP1 α and HP1 β as well as CD-less HP1 α and HP1 β are sufficient to induce heterochromatinization, but are not necessarily required to maintain the condensed state.

Three HP1 domains

The CD of HP1 recognizes MeH3K9 and is essential for the association of HP1 with heterochromatin. HP1 can form homodimers and bind a variety of other proteins through its CSD (Aasland and Stewart, 1995; Bannister *et al.*, 2001; Cowell *et al.*, 2002; Jacobs and Khorasanizadeh, 2002; Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Lechner *et al.*, 2005; Nielsen *et al.*, 2002; Smothers and Henikoff, 2001; Thiru *et al.*, 2004). It is known from *in vitro* studies that HP1 also binds to DNA and linker histones through its HD (Meehan *et al.*, 2003). Furthermore, the HD is involved in targeting HP1 to heterochromatin through an RNA binding activity (Muchardt *et al.*, 2002).

It is generally assumed that the interaction of HP1 with MeH3K9 facilitates the formation of heterochromatin and represses gene activity (Lachner *et al.*, 2001). Our results show that when HP1 without a functional CD is targeted to chromatin, here via the lac repressor/lac operator interaction, its ability to induce heterochromatin is maintained. This indicates that the CSD and HD are sufficient to recruit and bind the protein factors required for the heterochromatinization process. Interestingly in this context, Stewart *et al.* (2005) recently showed that H3K9 methylation and HP1 can also act independently. Probably, incorporation of HP1 into heterochromatin is a multi-step process, involving interactions with histone methyltransferases, binding to MeH3K9 and possibly stabilization by RNA components (Maison and Almouzni, 2004; Muchardt *et al.*, 2002). Our data suggest that the complex process of heterochromatin formation does not require the CD domain if HP1 is bound to chromatin in a CD-independent manner.

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