Chromatin architecture and the orchestration of gene expression: cell systems to explore epigenetic gene control

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Chapter 4

Pericentromeric heterochromatin domains are maintained without accumulation of HP1

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Abstract

The heterochromatin protein 1 (HP1) family is thought to be an important structural component of heterochromatin. HP1 proteins bind via their chromodomain to nucleosomes methylated at lysine 9 of histone H3 (H3K9me). To investigate the role of HP1 in maintaining heterochromatin structure we used a dominant-negative approach by expressing truncated HP1α or HP1β proteins lacking a functional chromodomain. Expression of these truncated HP1 proteins individually or in combination resulted in a strong reduction of the accumulation of HP1α, HP1β and HP1γ in pericentromeric heterochromatin domains in mouse 3T3 fibroblasts. The expression levels of HP1 did not change. The apparent displacement of HP1α, HP1β and HP1γ from pericentromeric heterochromatin did not result in visible changes in the structure of pericentromeric heterochromatin domains, as visualized by DAPI staining and immunofluorescent labeling of H3K9me. Our results show that the accumulation of HP1α, HP1β and HP1γ at pericentromeric heterochromatin domains is not required to maintain DAPI-stained pericentromeric heterochromatin domains and the methylated state of histone H3 at lysine 9 in such heterochromatin domains.

Introduction

The HP1 (heterochromatin protein 1) gene was first discovered in Drosophila melanogaster as a mutant suppressing position-effect variegation, a phenomenon in which a gene adjacent to a heterochromatin domain shows a mosaic expression pattern (Eissenberg *et al.*, 1990; Festenstein *et al.*, 1999; James and Elgin, 1986). Since then, a variety of HP1 homologues has been found in many eukaryotes (Li *et al.*, 2002). There are at least three HP1 proteins in mammals. HP1α and HP1β are present in heterochromatic regions, whereas HP1γ has been found either exclusively in euchromatin or in both euchromatin and heterochromatin (Cheutin *et al.*, 2003; Horsley *et al.*, 1996; Minc *et al.*, 1999; Minc *et al.*, 2000; Wreggett *et al.*, 1994). HP1 proteins are thought to have a role in gene regulation in a broad range of eukaryotes: from fission yeast to mammals (Hediger and Gasser, 2006; Hiragami and Festenstein, 2005).

The HP1 gene products are proteins of ~180 amino acids, containing two protein-binding domains linked by a flexible hinge region. The chromodomain (CD) located at the N-terminal side of the hinge region specifically binds histone H3 methylated at lysine 9 (H3K9me; Bannister *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Lachner *et al.*, 2001), which is a marker for heterochromatin and is related to gene silencing (Cowell *et al.*, 2002; Sims *et al.*, 2003). HP1 is able to repress gene activity when artificially targeted to a site near a reporter gene (van der Vlag *et al.*, 2000). The

C-terminal part of HP1 contains the chromoshadow domain (CSD), which is partially homologous to the CD (Aasland and Stewart, 1995; Sims *et al.*, 2003). The CSD binds to 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 (Brasher *et al.*, 2000; Li *et al.*, 2002; Nielsen *et al.*, 2001; Smothers and Henikoff, 2000; Thiru *et al.*, 2004). Finally, HP1 can bind RNA, probably through its hinge region. RNA binding is important for the binding of the protein to pericentromeric heterochromatin (Muchardt *et al.*, 2002). HP1 proteins are assumed to have a structural role in defining the condensed heterochromatin state. HP1 dimers may bridge nucleosomes in heterochromatin via H3K9me, resulting in a compact chromatin structure (Jenuwein, 2001; Lachner and Jenuwein, 2002; Nielsen *et al.*, 2001; Singh and Georgatos, 2002). However, there is no direct evidence that HP1 is an essential component of heterochromatin (Singh and Georgatos, 2002). In mouse fibroblasts, pericentromeric heterochromatin constitutes conspicuous nuclear domains that can be visualized by DAPI staining. HP1 proteins are highly accumulated in these domains. A large fraction of the HP1 in heterochromatin domains rapidly exchanges with nucleoplasmic HP1, whereas a small fraction exchanges slowly, as shown by FRAP and FCS studies (Cheutin *et al.*, 2003; Festenstein *et al.*, 2003; Schmiedeberg *et al.*, 2004).

In this study we investigate whether HP1 accumulation is essential for maintaining pericentromeric heterochromatin domains that can be visualized by DAPI staining in mouse fibroblasts. Using a dominant-negative approach in which truncated forms of HP1α and HP1β that lack a functional chromodomain are over-expressed, we show that the apparent displacement of HP1α, HP1β and HP1γ from pericentromeric heterochromatin does not result in visible changes in large-scale chromatin structure of DAPI-stained heterochromatin domains and in H3K9me levels of such domains. Results indicate that the accumulation of HP1α, HP1β or HP1γ at pericentromeric heterochromatin is not required to maintain DAPI-stained pericentromeric heterochromatin domains and the methylated state of histone H3 at lysine 9 in such heterochromatin domains.

Materials and Methods

DNA constructs

HP1α and HP1β denote the full-length sequences of the human HP1s. HP1α-Δ(2-39) and HP1β-Δ(2-40) have deletions of amino acids 2 to 39 and 2 to 40 in HP1α and HP1β, respectively. These proteins lack a large part of the chromodomain, including V21 (HP1α) and V23 (HP1β), which are required for MeH3K9 binding (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Platero *et al.*, 1995). HP1α, HP1β, HP1α-Δ(2-39) and HP1β-Δ(2-40) fragments were PCR-amplified and cloned into a tetracycline-inducible vector (pUHD10-3; Gossen and Bujard, 1992) at the N-terminal end of an inserted flag-tag. The truncated HP1β-Δ(2-40) DNA fragment was digested with *EcoRI* and *BamHI* and inserted into pmCherry-C1 (Shaner *et al.*, 2004) resulting in pmCherry-HP1β-Δ(2-40). EGFP-tagged HP1β was kindly provided by drs L. Schmiedeberg and

P. Hemmerich (Schmiedeberg *et al.*, 2004).

Cell culture and transfection
NIH/3T3 mouse fibroblasts were cultured in DMEM supplemented with 10% tetracycline-free fetal bovine serum (BD Biosciences, Clontech, San Jose, CA, USA) in a 5% CO_2 atmosphere. Cells were grown on cover slips coated with Alcian blue 8GS (Fluka, Buchs, Switzerland). Cells were transfected using FuGENE6 (Roche, Basel, Switzerland) by adding 0.5 µg DNA of each plasmid and 6 µl of FuGene6 reagent per milliliter culture medium. Fibroblasts were cotransfected with pUHD15-1, containing the tTA gene, necessary for the Tet-ON system (Gossen and Bujard, 1992). We used pEGFP-C1 (BD Biosciences-Clontech) for expressing enhanced green fluorescent protein (EGFP). NIH/3T3 cells stably expressing EGFP-tagged HP1β were selected using 800 µg/ml G418. A low-expressing cell population was sorted by FACS analysis. Cells expressing EGFP-HP1β were transiently transfected with pmCherry-HP1β-Δ(2-40) and analyzed between 24 and 48 hours after transfection.

Immunolabeling and DAPI staining
Cells growing on cover slips were washed twice with PBS and fixed with 2% formaldehyde for 15 minutes at RT and permeabilized with 0.5% (w/v) Triton X-100 in PBS for 5 minutes. Residual aldehyde groups were blocked for 10 minutes with 0.1 M glycine PBS at RT. Incubation with primary antibodies in PBG (0.5% (w/v) BSA, 0.1% (w/v) gelatin in PBS) was performed for 2 hours at RT and with the secondary antibodies for 1 hour in the same buffer at RT. DAPI staining was carried out by incubation for 5 minutes in 0.4 µg/ml DAPI in PBS.

Antibodies
To detect flag-tagged proteins we used rabbit anti-flag and mouse anti-flag M2 (Sigma-Aldrich, Zwijndrecht, The Netherlands). To detect HP1α mouse anti-HP1α (MaHP1α; Euromedex, Mundolsheim, France; Nielsen *et al.*, 1999) and rabbit anti-HP1α (RaHP1α; Kourmouli *et al.*, 2000) were used. For detection of HP1β we used mouse anti-HP1β (MaHP1β; Euromedex; Nielsen *et al.*, 1999) and rat anti-HP1β (RaaHP1β; Wreggett *et al.*, 1994). We used mouse anti-HP1γ (MaHP1γ; Euromedex; Nielsen *et al.*, 1999) to detect HP1γ. RaHP1α and RaaHP1β were kindly provided by Dr. P. B. Singh. All anti-HP1 antibodies recognize human as well as murine HP1. For *in situ* labeling of H3K9me we used rabbit anti-trimethylated H3K9 (RaH3K9me3; Cowell *et al.*, 2002), also provided by Dr. P. B. Singh, rabbit anti-dimethylated H3K9 (RaH3K9me2; Upstate, Milton Keynes, UK; Nakayama *et al.*, 2001), and rabbit anti-branched (4x) methylated peptide-H3K9 (RaH3K9me-branched; Maison *et al.*, 2002) provided by Dr. T. Jenuwein. As secondary antibodies we used donkey anti-rabbit IgG(H+L)-Cy5, donkey anti-rabbit IgG(H+L)-Cy3, donkey anti-mouse IgG(H+L)-Cy3, donkey anti-mouse IgG(H+L)-FITC, donkey anti-rabbit IgG(H+L)-FITC and donkey anti-rat IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Microscopy and image analysis
Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope equipped with C-Apochromat 63x/1.2 water immersion lenses (Carl Zeiss Jena GmbH, Jena, Germany). All tracks were recorded separately to reduce cross-talk. Three or more midnuclear confocal sections (0.5-1 µm apart) were recorded for every image. We made line scans in the *x-y* plane. Figures were composed in Adobe PhotoShop v. 7.0 (Adobe Systems, San Jose, CA, USA). For quantitative image analysis, maximum intensity projections of the imaged cells (represented in the Supplementary Figure S1) were generated. Average signal intensity was measured for every channel over circular areas of 1.5 μm^2. These circles were placed over five DAPI-dense chromatin regions and five non-DAPI-dense chromatin regions in transfected and non-transfected control cells. The ratio between transfected (dn) and non-transfected cells (wt) for the DAPI, as well as the endogenous HP1 signal, was calculated in individual images as the ratio between the differences between average intensity in DAPI-dense heterochromatic chromatin regions (HC) and non-DAPI-dense euchromatic regions (EC) according to the following: $(HC\text{-}EC)_{dn}/(HC\text{-}EC)_{wt}$.

To quantify the levels of EGFP-tagged HP1β in living cells before and after pmCherry-HP1β-Δ(2-40) expression, we imaged the cells on a Zeiss Axiovert 200M widefield fluorescence microscope, equipped with a 100x Plan-Apochromat (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany) and a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, UK). The objective was temperature-controlled with an objective heater and cells were examined in microscopy medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM D-glucose and 20 mM HEPES) at 37°C. Images were recorded with a cooled

CCD camera (Coolsnap HQ, Roper Scientific, Tucson, AZ, USA). A 375-490 excitation filter, 490 long-pass dichroic mirror and 525-40 band-pass emission filter were used for EGFP imaging (monochromator: 470 nm ± 20 nm). A 375-580 excitation filter, 585 long-pass dichroic mirror and 620-60 band-pass emission filter was used for mCherry imaging (monochromator: 550 nm ± 20 nm). The average pixel intensity in the nucleus of the imaged EGFP-HP1β-expressing cells was quantified using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA). The distribution of pixel intensities (ranging from 0-1600) of 50 cells of each population (transfected and non-transfected) was plotted in a histogram (32 bins with a width of 50).

Results

Approach

We used a dominant-negative approach to analyze the role of HP1 in maintaining pericentromeric heterochromatin in the interphase mouse nucleus. Human HP1α and HP1β were cloned as full-length FLAG-tagged proteins (HP1α-flag and HP1β-flag) and as FLAG-tagged truncated proteins (HP1α-Δ(2-39)-flag and HP1β-Δ(2-40)-flag) that lack most of the CD, which is essential for binding to H3K9me (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Platero *et al.*, 1995). To identify transfected cells, a FLAG-tag was positioned at the C-terminal end of all constructs. We used 3T3 mouse fibroblasts, which have large pericentromeric heterochromatin domains that are easily visible after DAPI staining. Cells were co-transfected with the tetracycline-inducible vector pUHD15-1 and the wild-type or mutant HP1 construct in the absence of tetracycline or doxycycline. Expression of all transfected proteins (full-length and truncated HP1) was detectable 12 hours after transfection using an anti-flag antibody (data not shown). After 24 hours, cells with high expression levels were analyzed for HP1 localization and DAPI-stained heterochromatin domains.

Immunofluorescent labeling, using mouse anti-flag (figure 1) and rabbit anti-flag antibodies (data not shown), showed that the transfected wild-type HP1α-flag and HP1β-flag proteins are targeted to the DAPI-stained heterochromatin domains in the nucleus, as shown by others (Cheutin *et al.*, 2003; Horsley *et al.*, 1996; Minc *et al.*, 1999; Wreggett *et al.*, 1994). The same anti-flag antibodies showed that HP1α-Δ(2-39)-flag and HP1β-Δ(2-40)-flag proteins are localized in the nucleus. As expected, HP1α-Δ(2-39)-flag and HP1β-Δ(2-40)-flag did not accumulate in the DAPI-dense areas, since they lack the CD (figure 2, red and cyan channels). In most of these cells the anti-flag signal is homogeneously distributed in the nucleus, whereas in some cells the anti-flag signal is more concentrated at the periphery of the cell nucleus. Our results agree with the notion that the CD is required for localization of HP1α and HP1β in DAPI-stained pericentromeric heterochromatin through H3K9me binding (Bannister *et al.*, 2001; Cowell *et al.*, 2002; Jacobs and Khorasanizadeh, 2002; Lachner *et al.*, 2001).

Truncated HP1α and HP1β displace endogenous HP1α, HP1β and HP1γ from pericentromeric heterochromatin

Endogenous HP1α, HP1β and HP1γ in non-transfected cells are located in the nucleus and accumulate in DAPI-dense pericentromeric heterochromatin domains (figure 2, S1; Cheutin *et al.*, 2003; Horsley *et al.*, 1996; Minc *et al.*, 1999; Minc *et al.*, 2000; Wreggett *et al.*, 1994). To establish whether truncated HP1 displaces the endogenous protein from heterochromatin domains, cells were transfected with HP1α-Δ(2-39)-flag (figure 2A, B, figure S1), or HP1β-Δ(2-40)-flag (figure 2C-E, figure S1). After 24 hours, cells were fixed and fluorescently labeled with antibodies against flag-tagged transfected proteins (figure 2, red channel and figure S1) and with antibodies against HP1α, HP1β or HP1γ (figure 2, green channel and figure S1) and stained with DAPI (figure 2, cyan channel and figure S1). For detection of HP1α we used two different antibodies, *i.e.* a mouse anti-HP1α monoclonal antibody (MaHP1α; figure S1B and F) and a rabbit anti-HP1α polyclonal antibody (RaHP1α; figure 2A, 2C and S1) that gave identical results. Cells expressing HP1α-Δ(2-39)-flag showed a strong increase in labeling intensity by MaHP1α but not by RaHP1α labeling. This indicates that MaHP1α recognizes the transfected HP1α-Δ(2-39)-flag protein, whereas RaHP1α does not. For detection of endogenous HP1β we used a mouse anti-HP1β monoclonal antibody (MaHP1β; figure S1) and a rat anti-HP1β monoclonal antibody (RaaHP1β; figure 2B, 2D, S1). Both anti-HP1β antibodies did not recognize the mutant HP1β-Δ(2-40)-flag protein. To detect HP1γ we used a monoclonal mouse anti-HP1γ (MaHP1γ; figure 2E, S1). The specificity of all antibodies has been tested elsewhere (see references in the materials and methods section). As a control we analyzed non-transfected cells in the same preparation to relate HP1 accumulation at pericentromeric heterochromatin in control cells to the amount of HP1 in these domains in transfected cells. To allow semi-quantitative assessment of the co-localization and intensity of the different labels, line scans were made in the *x*-*y* plane through intense DAPI domains in transfected (figure 2 A2, B2, C2, D2 and E2) and in non-transfected control cells (figure 2 A1, B1, C1, D1 and E1).

In cells transfected with HP1α-Δ(2-39)-flag, HP1α labeling with RaHP1α showed a dispersed distribution of the HP1α antigen throughout the nucleus, lacking accumulation in the DAPI-stained pericentromeric regions (figure 2A, S1). Labeling with MaHP1α also showed no accumulation of HP1α in DAPI-dense domains in HP1α-Δ(2-39)-flag transfected cells, compared to non-transfected control cells (figure S1). This indicates that endogenous HP1α is displaced from the DAPI-stained pericentromeric heterochromatin domains by the truncated protein. Strikingly, in HP1α-Δ(2-39)-flag transfected cells, HP1β labeling with RaaHP1β (figure 2B, S1) or MaHP1β (figure S1) also showed no accumulation of endogenous HP1β in DAPI-stained chromocenters. These results show that truncated HP1α displaces endogenous HP1α and HP1β from DAPI-stained pericentromeric heterochromatin.

In HP1β-Δ(2-40)-flag transfected cells, HP1β did not accumulate in the DAPI-

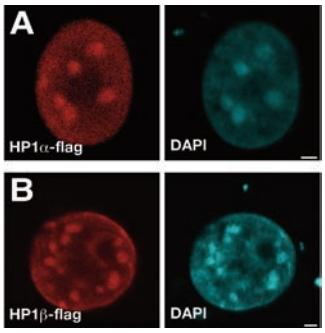

Figure 1. Distribution of transfected full-length HP1α-flag and HP1β-flag in mouse fibroblasts. Cells transfected with HP1α-flag (A) and HP1β-flag (B) were fluorescently immunolabeled 24 hours after transfection. The red signal (mouse anti-flag) shows the distribution of transfected HP1α-flag (A) and HP1β-flag (B). The cyan channel shows the DAPI staining. Bars represent 2 µm. Individual midnuclear optical sections are shown.

Figure 2. See next page for legend.

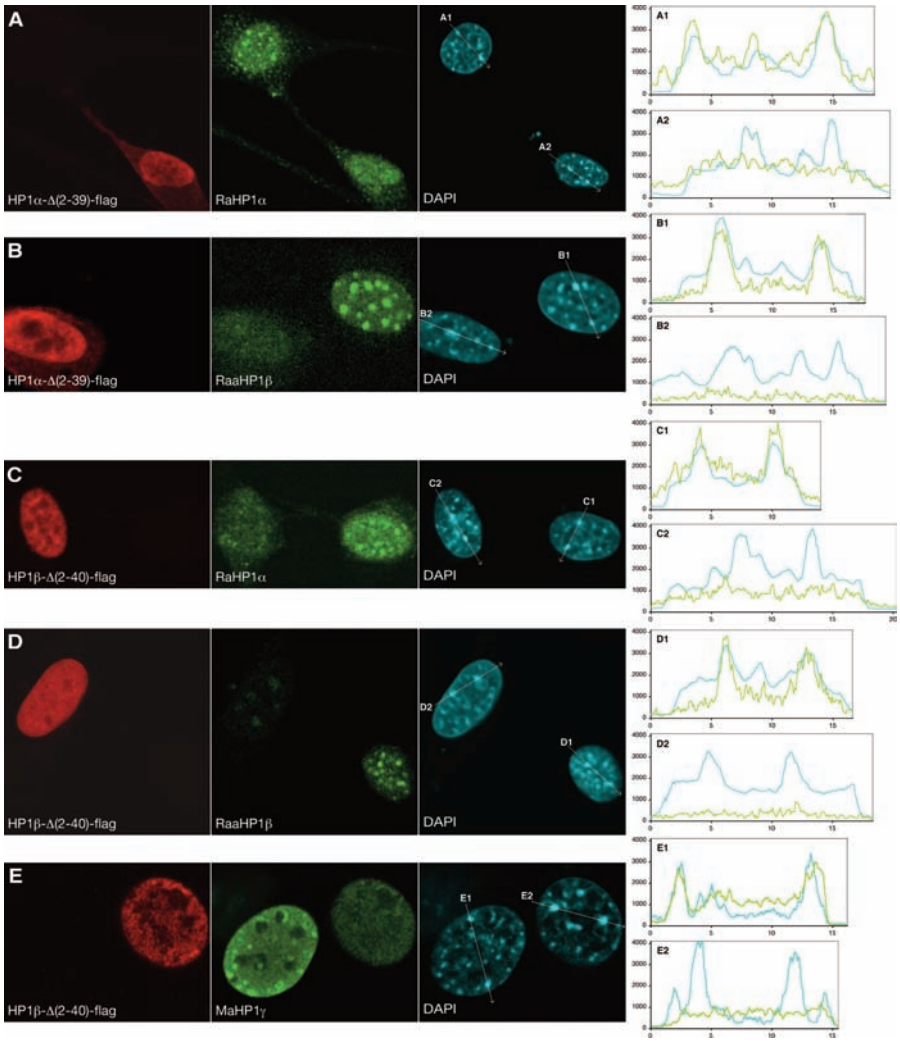

Figure 2. Spatial distribution of transfected truncated HP1, endogenous HP1 and DAPI-stained heterochromatin domains. Cells transfected with HP1α-Δ(2-39)-flag (A and B) and HP1β-Δ(2-40)-flag (C-E) were fluorescently labeled 24 hours after transfection. The red signal (Maflag in A-D and Raflag in E) shows the transfected HP1α-Δ(2-39)-flag (A and B) and HP1β-Δ(2-40)-flag (C-E). The green signal shows the distribution of endogenous HP1α, HP1β and HP1γ after labeling with RaHP1α (A and C), RaaHP1β (B and D) and MaHP1γ (E). The cyan signal shows the DAPI staining. On the right, intensity profiles along the lines shown in the DAPI images show endogenous HP1α, HP1β and HP1γ (green line) and DAPI staining (cyan line). A1, B1, C1, D1 and E1 are line scans in control cells and A2, B2, C2, D2 and E2 in transfected cells. The *x*-axis shows the distance in μm; the *y*-axis represents signal intensity in arbitrary units. Individual midnuclear optical sections are shown.

stained pericentromeric domains and was dispersed throughout the nucleoplasm, as shown by labeling with two different antibodies, *i.e.* RaaHP1β (figure 2D, S1) and MaHP1β (figure S1). Moreover, in HP1β-Δ(2-40)-flag transfected cells RaHP1α (figure 2C, S1), MaHP1α (figure S1) and MaHP1γ (figure 2E, S1) labeling did not show any accumulation of HP1α and HP1γ in DAPI-stained pericentromeric domains. To verify that the displacement of endogenous HP1 is not a consequence of the transfection procedure, fibroblasts were transfected with a plasmid containing only the enhanced green fluorescent protein (eGFP) gene and were subsequently immunofluorescently labeled with MaHP1α and MaHP1β. No changes in the distribution or intensity of endogenous HP1 were observed, compared to non-transfected cells (data not shown). Therefore, it is unlikely that the observed changes in HP1 distribution after expression of the mutant HP1 proteins are an artifact of the transfection procedure.

In addition to the analysis of endogenous HP1 levels in fixed cells using anti-HP1 antibodies, we analyzed the dominant-negative effect of transfecting cells with truncated HP1 in living cells. To this end we created a mouse fibroblast cell line stably expressing EGFP-tagged HP1β. In these EGFP-HP1β-expressing cells, HP1β was found to accumulate in DAPI-stained pericentromeric heterochromatin domains and to occur diffusely throughout the nucleoplasm (figure 3A). Transfection of these EGFP-HP1β-expressing cells with mCherry-tagged HP1β-Δ(2-40) resulted in a homogeneous nuclear distribution of both the truncated and the wild-type HP1β proteins (figure 3B, C). These results confirm the antibody labeling data in figures 2, 5 and S1, showing that truncated HP1 protein that lacks a functional CD displaces full-length HP1 from the DAPI-dense nuclear domains.

To examine whether expression of truncated HP1β-Δ(2-40) results in a decrease of the HP1β concentration, we determined the expression level of cells stably expressing EGFP-HP1β with and without transfection with mCherry-HP1β-Δ(2-40) (figure 3). The fluorescence intensity of EGFP-HP1β integrated over the nucleus was determined 24 hours after transfection in cells expressing high levels of mCherry-HP1β-Δ(2-40) (based on the red signal) and in untransfected cells. The distribution of EGFP-HP1β fluorescence measured in transfected and non-transfected cells was fitted with a Gaussian distribution yielding average EGFP-HP1β fluorescence intensities of 161 ± 26 and 162 ± 40 for non-transfected and transfected cells, respectively. These results

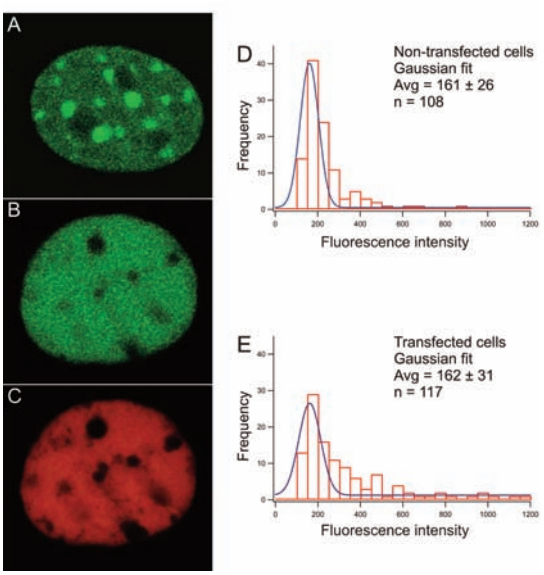

Figure 3. Spatial distribution of EGFP-tagged HP1β in cells transfected with or without mCherry-HP1β-Δ(2-40). Cells stably expressing EGFP-tagged HP1β show that HP1 is accumulated in pericentromeric heterochromatin domains (A). EGFP-tagged HP1β-expressing cells were transfected with mCherry-tagged HP1β-Δ(2-40) (B and C). The green signal shows the EGFP-tagged HP1β (B). The red signal shows the transfected mCherry-tagged HP1β-Δ(2-40) (C). The fluorescence intensity of EGFP-HP1β in cells transfected with mCherry-tagged HP1β-Δ(2-40) was compared to non-transfected cells. A histogram of the Gaussian distribution of the EGFP-HP1β fluorescence intensity in transfected (E) and non-transfected cells (D) is shown. The *x*-axis represents the average fluorescence intensity in arbitrary units; the *y*-axis shows the number of cells.

show that expression of truncated HP1β does not change the cellular concentration of full-length EGFP-HP1β (figure 3D, E). These results show that truncated HP1α and truncated HP1β protein, which lack a functional CD, not only expel their full-length HP1 counterpart from the DAPI-stained pericentromeric heterochromatin domains, but also the two other HP1 homologues. This suggests that the three HP1 homologues interact in heterochromatin.

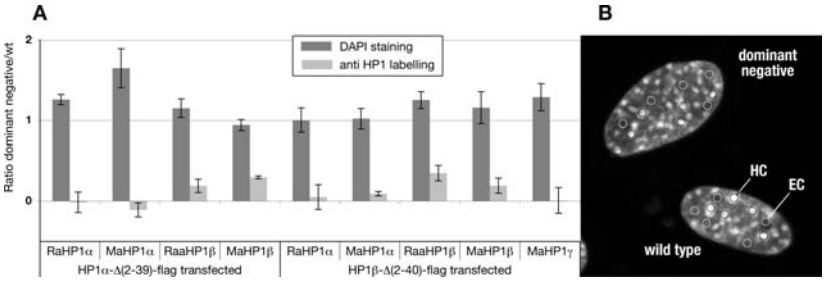

Figure 4. Quantitative analysis of the spatial distribution of transfected truncated HP1, endogenous HP1 and DAPI-stained heterochromatin domains. Maximum intensity projections of the imaged cells (see figure S1) were generated. The average signal intensity was measured for every channel over circular areas of 1.5 μm^2. These circles were placed over five DAPI-dense chromatin regions and five non-DAPI-dense chromatin regions in transfected and non-transfected cells (B). The ratio between cells transfected with HP1α-Δ(2-39)-flag or HP1β-Δ(2-40)-flag versus non-transfected control cells, for the DAPI signal (dark grey bars) as well as for the endogenous HP1 signal (light grey bars) was calculated. This was done in individual images as the ratio of the differences between average intensity in DAPI-dense chromatin regions (HC) and non-DAPI-dense euchromatic chromatin regions (EC) in transfected (dn) and non-transfected cells (wt): $(HC\text{-}EC)_{dn}/(HC\text{-}EC)_{wt}$ (A). The error bars represent the standard error between the different images.

Loss of HP1α and HP1β from heterochromatin does not result in changes in large-scale heterochromatin structure

We performed a quantitative analysis of the relative amount of DAPI staining and endogenous HP1 levels in cells transfected with HP1α-Δ(2-39)-flag or HP1β-Δ(2-40)-flag and in untransfected control cells. Results demonstrate that there is no change in DAPI staining after transfection with either HP1α-Δ(2-39)-flag or HP1β-Δ(2-40)-flag. The ratio of DAPI stain in transfected versus non-transfected cells is close to unity (figure 4). In contrast, the ratio for the HP1 signal, using different antibodies (*i.e.* RaHP1α, MaHP1α, RaaHP1β, MaHP1β and MaHP1γ) is close to zero (figure 4). These measurements show in a quantitative manner that, after expression of HP1 that lacks the chromodomain, most of the endogenous HP1 is relocated from DAPI-dense chromatin regions to the nucleoplasm, whereas the DAPI staining remains unchanged.

To analyze whether the loss of HP1α and HP1β from DAPI-stained pericentromeric heterochromatin domains is correlated with a decrease in H3K9me level, we analyzed the distribution of H3K9me 24 hours after transfection with HP1α-Δ(2-39)-flag, HP1β-Δ(2-40)-flag, or with both simultaneously. We labeled H3K9me with three different antibodies: RaH3K9me2, RaH3K9me3 and RaH3K9me-branched. The RaH3K9me-branched antibody is described to have a high affinity for H3K9me3, to significantly cross-react with di- and trimethylation of several other H3 lysine positions, and to decorate pericentromeric heterochromatin and the inactive X-chromosome (Maison *et al.*, 2002; Perez-Burgos *et al.*, 2004; figure 5B, D). No detectable change in H3K9me labeling was observed with the RaH3K9me3 and RaH3K9me-branched antibodies 24 hours after transfection with either HP1α-Δ(2-39)-flag (figure 5A, B), HP1β-Δ(2-40)-flag (figure 5C, D), or with HP1α-Δ(2-39)-flag and HP1β-Δ(2-40)-flag simultaneously (data not shown). Similarly, no change in the dimethylation level of H3K9 was observed (figure 5E). The same results were obtained 48 hours after transfection, using RaH3K9me2, RaH3K9me3 and RaH3K9me-branched antibodies (data not shown). These results show that, although all three endogenous HP1 proteins are, to a large degree, displaced from DAPI-stained pericentromeric heterochromatin domains by HP1α-Δ(2-39)-flag or HP1β-Δ(2-40)-flag, this does not result in a visible reduction of H3K9me levels in these domains.

We showed that the overall structure, size and nuclear distribution of DAPI-stained pericentromeric heterochromatin domains remained unaffected as the three HP1 proteins are displaced (figures 2, 5, S1). These observations demonstrate that the visible accumulation of HP1α, HP1β and HP1γ is not essential for maintaining pericentromeric heterochromatin structure as visualized by DAPI staining and by immunofluorescent labeling of H3K9me2, H3K9me3 or H3K9me-branched.

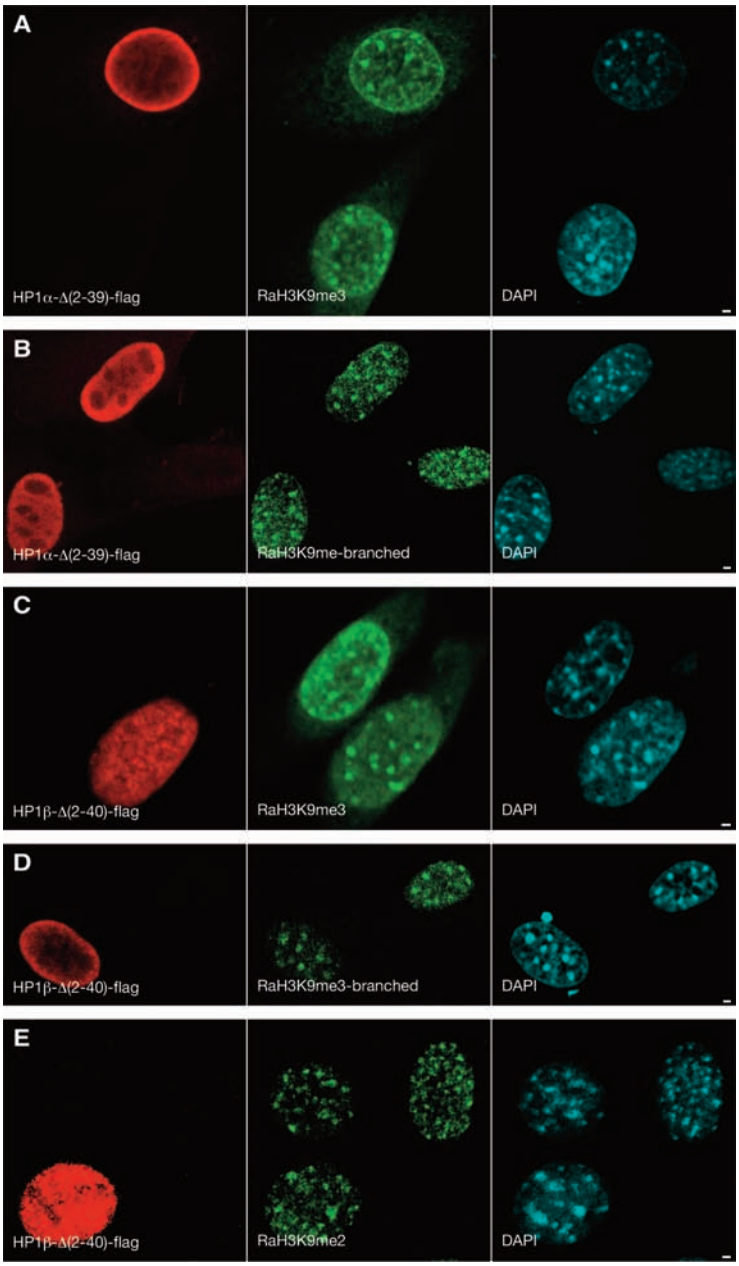

Figure 5. Spatial distribution of H3K9me in cells transfected with truncated HP1α and HP1β. Cells transfected with HP1α-Δ(2-39)-flag (A and B) and HP1β-Δ(2-40)-flag (C-E) were fluorescently labeled 24 hours after transfection. The red signal (mouse anti-flag) shows the transfected HP1α-Δ(2-39)-flag (A and B) and HP1β-Δ(2-40)-flag (C-E). The green signal shows distribution of H3K9me detected by either RaH3K9me3 (A and C), RaH3K9mebranched (B and D), or RaH3K9me2 (E). The cyan signal represents DAPI staining. Bars represent 2 μm. Individual midnuclear optical sections are shown.

Discussion

HP1 is thought to play an important role in heterochromatin organization and the control of gene expression (Hediger and Gasser, 2006; Hiragami and Festenstein, 2005). This involves the specific binding of the CD to H3K9me, dimerization via its CSD and recruitment of a variety of proteins that specifically interact via HP1 with H3K9me-rich chromatin domains (Brasher *et al.*, 2000; Cowell *et al.*, 2002; Jenuwein, 2001; Lachner and Jenuwein, 2002; Nielsen *et al.*, 2001; Singh and Georgatos, 2002; Thiru *et al.*, 2004). HP1 dimerizes through its CSD and may bridge two H3K9me-containing nucleosomes, inducing a higher-order packing of heterochromatin (Jenuwein, 2001; Lachner and Jenuwein, 2002; Nielsen *et al.*, 2001; Singh and Georgatos, 2002; Thiru *et al.*, 2004). It has also been proposed that HP1 dimers bind to methylated K9 and K27 on a single histone H3 tail, instead of binding to methylated K9 on nearby nucleosomes (Jacobs and Khorasanizadeh, 2002). HP1 may also play a role in the maintenance of the heterochromatin state through cell division, via its interactions with chromatin assembly factor 1 (CAF1), H3K9 methyltransferase Suv39 and the DNA methyltransferases Dnmt1 and Dnmt3a (reviewed in Maison and Almouzni, 2004). In this study we used a dominant-negative approach to interfere with HP1 function to obtain insight into the role of HP1α, HP1β and HP1γ in maintaining DAPI-stained pericentromeric heterochromatin domains in mouse fibroblasts.

Expressing truncated HP1α and HP1β, lacking a functional CD, resulted in displacement of the endogenous HP1α, HP1β and HP1γ from the visible DAPI-stained pericentromeric heterochromatin domains (figure 2). It did not result in a significant decrease in full-length HP1 expression (figure 3). Remarkably, loss of HP1 did not result in a visible change in the structure of pericentromeric heterochromatin domains, as visualized by DAPI staining, and did not result in a visible change in the methylation state of H3K9 (figures 2, 4, 5, S1). It is known that the CD is responsible for specific binding of HP1 to H3K9me in heterochromatin (Bannister *et al.*, 2001; Cheutin *et al.*, 2003; Cowell *et al.*, 2002; Jacobs and Khorasanizadeh, 2002; Lachner *et al.*, 2001). Therefore, as expected, HP1 lacking a CD did not accumulate in DAPI-stained heterochromatin domains (figure 2, S1), in contrast to wild-type HP1α-flag and HP1β-flag, which did accumulate in these domains (figure 1). Evidently, interaction of the CD of the endogenous HP1 with H3K9me is not sufficient to keep the proteins associated with DAPI-stained heterochromatin domains. Most likely, the truncated polypeptides sequester endogenous proteins that are necessary to stabilize the interaction of HP1 with H3K9me. Because expression of each of the truncated proteins individually resulted in the apparent displacement of HP1α, HP1β and HP1γ from DAPI-stained pericentromeric heterochromatin, it is likely that these three HP1 proteins interact directly or indirectly *in vivo*.

Our results are consistent with studies showing that the CD is necessary but not sufficient for HP1 to bind H3K9me-containing nucleosomes (Smothers and Henikoff,

2001; Thiru *et al.*, 2004) and that HP1β as a monomeric protein is not stably associated with pericentromeric heterochromatin (Thiru *et al.*, 2004). Our data show that when most endogenous HP1 is displaced from DAPI-dense heterochromatin, the level and distribution of tri- and dimethylated H3K9me remains unchanged. This demonstrates that accumulation of HP1α, HP1β and HP1γ is not required for keeping histone H3K9 in its methylated state in DAPI-stained heterochromatin domains. In this context, labeling with the H3K9me-branched antibody as well as HP1α accumulation at pericentromeric heterochromatin were lost after incubation with RNAse and after culturing in the presence of a histone deacetylase inhibitor (Maison *et al.*, 2002; Muchardt *et al.*, 2002). Besides, Gilbert *et al.* (2003) reported that during differentiation of chicken erythrocytes HP1 is completely lost, whereas there is only a modest reduction in H3K9me. Obviously, maintaining H3K9me levels does not require the continuous presence of HP1 proteins.

Interestingly, Peters *et al.* (2001) presented evidence that DAPI-dense domains, where HP1 accumulates, remain present in cells that fully lack the histone methyltransferases Suv39h1 and 2. In these cells heterochromatin domains do not contain H3K9me. From these and our results it can be concluded that HP1 binding and H3K9me are independent processes and are not required simultaneously for heterochromatin stability. Recently, we showed that *in vivo* targeting of HP1α or HP1β to an amplified chromosomal region, causes local chromatin condensation, enhanced trimethylated H3K9me and recruitment of histone methyltransferases (Verschure *et al.*, 2005). Targeting of the HP1 lacking a functional CD also caused heterochromatinization of the amplified chromosome region (Brink *et al.*, 2006; chapter 3 of this thesis). These results show that normal binding of HP1 through its CD domain, as well as artificial binding of HP1 without a CD through the lac operator/lac repressor interaction, is sufficient to trigger heterochromatin formation. The present studies indicate that HP1 is not required for maintaining the DAPI-stained heterochromatin domains.

HP1 in heterochromatin is remarkably dynamic and exchanges rapidly with its soluble pool (Cheutin *et al.*, 2003; Festenstein *et al.*, 2003; Schmiedeberg *et al.*, 2004). HP1 is bound in heterochromatin domains in at least two modes, one that exchanges rapidly and one that is released slowly. The ratio between these pools ranges from approximately 8:1 in NIH/3T3 fibroblasts (Schmiedeberg *et al.*, 2004) to close to 3:1 in mouse T-cells (Festenstein *et al.*, 2003), probably depending on cell type and chromatin state. The function of the fast and the slow-exchanging HP1 subfractions is unknown. Our results show in fixed as well as in living cells that in mouse fibroblasts most HP1 proteins can be removed from DAPI-stained pericentromeric heterochromatin without a visible change in large-scale chromatin structure or a significant decrease in H3K9me. It is possible that a small fraction of the strongly bound fraction of HP1 molecules, *e.g.* the slowly exchanging fraction, remains present

in the dominant-negative experiments. Light-microscopic analysis is not sufficiently sensitive to decide this unambiguously. However, our data clearly show that most HP1 in visible pericentromeric domains is dispensable for maintaining typical heterochromatin features, including strong DAPI staining, H3K9 methylation and the heterochromatin size and shape. Our findings indicate that local accumulation of HP1α, HP1β and HP1γ is not essential for the maintenance of DAPI-stained pericentromeric heterochromatin.

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Supplementary Figure S1. Spatial distribution of transfected truncated HP1, endogenous HP1 and DAPI-stained heterochromatin domains. Cells transfected with HP1α-Δ(2-39)-flag and HP1β-Δ(2-40)-flag were fluorescently labeled 24 hours after transfection. The anti-flag signal (first column) shows the transfected HP1α-Δ(2-39)-flag cells and the transfected HP1β-Δ(2-40)-flag cells. The distribution of endogenous HP1 is shown in the second column; HP1α after labeling with RaHP1α or with MaHP1α, HP1β after labeling with RaaHP1β or with MaHP1β and HP1γ after labeling with MaHP1γ. The DAPI staining is shown in the right column.