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The PHD Domain of Np95 (mUHRF1) Is Involved in Large-Scale Reorganization of Pericentromeric Heterochromatin

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Heterochromatic chromosomal regions undergo large-scale reorganization and progressively aggregate, forming chromocenters. These are dynamic structures that rapidly adapt to various stimuli that influence gene expression patterns, cell cycle progression, and differentiation. Np95-ICBP90 (m- and h-UHRF1) is a histone-binding protein expressed only in proliferating cells. During pericentromeric heterochromatin (PH) replication, Np95 specifically relocates to chromocenters where it highly concentrates in the replication factories that correspond to less compacted DNA. Np95 recruits HDAC and DNMT1 to PH and depletion of Np95 impairs PH replication. Here we show that Np95 causes large-scale modifications of chromocenters independently from the H3:K9 and H4:K20 trimethylation pathways, from the expression levels of HP1, from DNA methylation and from the cell cycle. The PHD domain is essential to induce this effect. The PHD domain is also required in vitro to increase access of a restriction enzyme to DNA packaged into nucleosomal arrays. We propose that the PHD domain of Np95-ICBP90 contributes to the opening and/or stabilization of dense chromocenter structures to support the recruitment of modifying enzymes, like HDAC and DNMT1, required for the replication and formation of PH.

INTRODUCTION

The chromosomal regions that carry constitutive heterochromatin tend to undergo large-scale reorganization and to progressively aggregate to form clusters called chromocenters (Hochstrasser and Sedat, 1987). The biological significance of chromocenters is not yet fully understood. These structures, produced by the higher order conformation of various heterochromatic areas, might represent regions of permanently silenced chromatin (Polo and Almouzni, 2006). They are not static or inaccessible, but dynamic, and display the potential to rapidly adapt to various stimuli that influence gene expression patterns, cell cycle progression, and cell differentiation (Cheutin et al., 2003; Festenstein and Aragon, 2003). Alterations in chromocenter number, dimension, and nuclear distribution have been observed during terminal differentiation and studies in various organisms have convincingly demonstrated a relationship between nuclear topology and transcriptional silencing (Kosak and Groudine, 2004).

These highly compacted chromatin structures are easily detectable in mouse cells; they form the characteristic nodules stained by 4,6-diamidino-2-phenylindole (DAPI) and are mainly constituted of pericentromeric heterochromatin (PH). In mammals, PH is characterized by repeated DNA sequences, by high levels of specifically methylated forms of histone H3 and H4, by deacetylated histone H4, and by methylated DNA. These epigenetic modifications represent binding substrates for chromatin modifiers, like HP1 and MeCP2, that are thought to contribute both to the highly silent environment and to the structural organization of this...
chromatin compartment (Maison and Almouzni, 2004; Brezo et al., 2005). Apparently, the high content of repeated DNA sequences in heterochromatin enhances the condensation and aggregation properties that characterize these heterochromatin regions (Barr and Ellison, 1972).

During middle S phase, these highly compacted structures must be opened to allow the replication machinery to proceed along DNA and to reconstitute the epigenic marks and the silent state of this chromatin area. It has been proposed that these events occur at the pericentromeric duplication body (pHDB; Quivy et al., 2004), in which parental chromatin from the interior is pulled out to the periphery, becomes transiently disrupted during replication, reassembled to form new chromatin, and finally pushed back inside the domain. The molecular mechanisms that produce the dynamic conformational changes of chromocenters are poorly understood although a role for some proteins has been proposed (Brezo et al., 2005).

Np95 is a cell cycle–regulated and histone-binding protein expressed only in proliferating cells and involved in PH replication and formation (Papait et al., 2007). At the time of PH replication, Np95 specifically relocates to chromocenters where it gets highly concentrated in the areas of active replication known as the PH duplication body (pHDB; Quivy et al., 2004). These areas correspond to less compacted DNA where the parental DNA is pulled out from the central core of the chromocenter and replicated. Newly synthesized nucleosomes are then deposited and epigenetically modified to allow the formation of new heterochromatin domains. Np95 is part of the pHDB, and its ablation in mammalian cells by recruiting DNMT1 to hemi-methylated DNA represses silencing of major satellite repeats. Very recent studies show that UHRF1 plays a role in maintaining DNA methylation in the silent state of this chromatin area. It has been proposed that these events occur at the pericentromeric duplication body (pHDB; Quivy et al., 2004), in which parental chromatin from the interior is pulled out to the periphery, becomes transiently disrupted during replication, reassembled to form new chromatin, and finally pushed back inside the domain. The molecular mechanisms that produce the dynamic conformational changes of chromocenters are poorly understood although a role for some proteins has been proposed (Brezo et al., 2005).

MATERIALS AND METHODS

Cells and Adenovirus

NIH-3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The NIH-3T3 adenovirus (Ad-Np95), and adenovirus Track (Ad-Track) have been described previously (Bonapace et al., 2002). Mouse Suv39h1/2 double-null (dn; Peters et al., 2007) and wild-type mouse fibroblasts (NIH-3T3) were cultured in DMEM supplemented with 10% fetal bovine serum (Seromed, Berlin, Germany).

DNA Transfection and RNA Interference

NIH-3T3 cells were transfected with vectors expressing myc-tagged Np95 using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturing’s instructions. For experiments in Figure 5, 2 μg of pCDNA3.1-myc-his tag (Invitrogen, Carlsbad, CA) recombinant plasmids containing the wild type (wt), and the deletion mutants indicated in Figure 5 were transfected into NIH3T3 and Suv39h1/2 dn cells using (Lipofectamine, Invitrogen). Forty-eight hours later, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature (RT) for 10 min or pretreated with 0.5% Triton X-100 in PBS for 10 min on ice before fixation. Fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min, followed by incubation in blocking solution (2% bovine serum albumin in PBS) for 30 min.

RESULTS

Functional Ablation of Np95 Induces Clustering of PH

Np95 relocalization to chromocenters at the time of PH replication leads to a high concentration of the protein in PH replication and formation. This involves modification of the acetilation status of lysines 8, 12, and 16 of histone H4 and the decondensation of chromatin regions (Barr and Ellison, 1972). The molecular mechanisms that produce the dynamic conformational changes of chromocenters are poorly understood although a role for some proteins has been proposed (Brezo et al., 2005).

In Vitro Restriction Enzyme Accessibility Assay

Nucleosome arrays were reconstituted on 2.8-kb plasmid pFM218-H5 by high-salt dialysis using a modification of published methods (Baumann et al., 2003). Briefly, plasmid DNA and histones extracted from CV1 cells were mixed in 2 M NaCl/TE buffer plus 0.05% NP-40. The Histone:DNA weight ratio was usually 1:5.1. Restriction mixes were reconstituted and digested with 26°C from 2 to 50× mobility shift/Np40 buffer. For each accessibility assay, arrays contained 25 ng of DNA. Assays were performed for 3 h at 30°C in a buffer containing 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 4 MgCl2, 4 mM ATP, pH 8.6, 2 μg of glutathione S-transferase (GST), 1 or 2 μg GST-Np95, or 200 ng of Brg1, 200 ng of Drosophila nuclear extracts and 4 U of SfiI restriction enzyme. Products were separated by agarose gel electrophoresis and visualized by hybridization with radiolabeled pFM218-H5 plasmid DNA.

Protein Extraction and Immunofluorescence

Proteins were extracted as described previously (Citterio et al., 2004). Triton X-100 extraction was performed by treating with 0.5% Triton X-100 in PBS for 10 min on ice, followed by three washes in PBS, before lysis or immunofluorescence.

FISH and Immunofluorescence

Immunofluorescence procedures were as described previously (Citterio et al., 2004). Antibodies used were as follows: rabbit polyclonal anti-Np95 (Bonapace et al., 2002); anti-H1, α, β, and γ (Euromedex, Strasbourg, France); goat polyclonal anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Mecp2 (Sigma-Aldrich, St. Louis, MO), anti-H3K9me3 and anti-H4-K20 (Abcam, Cambridge, United Kingdom), mouse monoclonal anti-5M (Calbiochem, Bad Soden, Germany). Secondary antibodies were from Jackson Laboratories (Bar Harbor, ME). Nuclear counterstaining was performed with DAPI. Samples for DNA-FISH were mounted in Vectashield antifade (Vector Laboratories, Burlingame, CA), whereas for immunofluorescence, with Mowiol (Calbiochem).

FISH with a mouse major satellite-specific probe was performed as described in Weierich et al. (2003). In brief, NIH-3T3 cells were fixed with 4% paraformaldehyde (PFA) in 1× PBS at 5°C after DNA transfection. Cells were permeabilized with 0.5% Triton X-100/1% PBS followed by incubation in 20% glycerol and a repeated freezing-thawing step in liquid nitrogen. Finally, incubated in 0.1 N HCl for 5 min. Hybridization, coverslips with fixed and pretreated cells were stored in 50% formamide/2× SSC at 4°C.

The probe was generated by PCR using 5′-GACGAGCTGAAAATGACC-GAATC-3′ (MajF1-for) and 5′-CATATCCAGCTCCTCGTGCAGC-3′ (MajR1-rev) as primers and pCR4-MsaAT9-2 plasmid as template (kind gift from J. Tenuwee, Research Institute of Molecular Pathology and The Vienna Biocenter, Vienna, Austria) and labeled by nick translation using Bioin-16-DUTP (Roche).

Labeled DNA was coprecipitated with salmon sperm DNA and mouse Cot-1. Hybridization mixture in all cases consisted of 50% formamid/10% dextran sulfate/2× SSC. Cells and probe DNA were denatured simultaneously at 75°C for 2 min and hybridization was performed for 2 or 3 days at 37°C on a hot-block in humid conditions, and posthybridization washes were performed with 2× SSC at 37°C and 0.1× SSC at 60°C, respectively. Bio-16-DUTP in major satellite probe was detected by two layers of avidin-Alexa488 (Molecular Probes, Eugene, OR) and FITC-conjugated goat anti-avidin-avidin antibodies (Vector Laboratories).

Cells were analyzed with a fluorescent microscope (BX51; Olympus, Melville, NY) equipped with 100× plan. Pictures were acquired with a color camera (DPS5; Olympus). Blots were digitalized with an Epson scan system (Expression 1600 Pro, Epson, Long Beach, CA).

Picture Management

All pictures were managed with Adobe Photoshop (Adobe Systems, San Jose, CA) and Canvas (Deneba Software, Miami, FL). Quantitative analysis of chromocenters of Figure 1 were done with ImageJ (http://rsb.info.nih.gov/ ij/; National Institutes of Health, Bethesda, MD).

PHD Domain of Np95 and Chromocenters
cells and around 13% in RNAi-ctrl treated cells. The average size of the chromocenters in RNAi-Np95–treated cells is more than double with respect to the RNAi-ctrl cells (Figure 1D).

Clustering of chromocenters (present results) and impairment of PH replication in the absence of Np95 (Papait et al., 2007), together with the specific relocalization of Np95 to PH in middle S phase and association to the areas of less dense chromatin (Papait et al., 2007), argue in favor of a role of the protein in chromocenter dynamics.

Clustering of PH Is Independent from Alterations of HP1, Histone H3:K9, and H4:K20 Methylation

We next checked if this clustering was accompanied by modifications of H3:K9 and H4:K20 trimethylation levels, two of the most relevant epigenetic modifications of this chromatin compartment. We also monitored the distribution and expression level of HP1, a key protein for PH organization.

As Figure 2 shows, no significant alterations of these three percentromeric markers are observed in RNAi-Np95 experiments. The increased size of HP1, H3:K9met3, and H4:K20met3 dots observed in the absence of Np95, in fact, parallels the variations observed for the DAPI staining (cf. in Figure 2A, pictures b, e, h, k, n, and q, respectively, with c, f, i, l, o, and r). Western blots performed on protein extracts obtained from Np95-depleted or control cells indicate no major alterations of these markers, even when cells were pretreated with Triton X-100 to extract weakly bound chromatin proteins and were analyzed by Western blot with the indicated antibodies.
distribution of Np95 in Suv39h1/2dn cells (a kind gift of Dr. T. Jenuwein; Figure 3). We found that 1) Suv39h1/2dn cells grow much slower than NIH-3T3 cells and in the majority of the asynchronous growing cells Np95 appears as diffused as H3:K9met3 (Figure 3A; arrows indicate the cells in which Np95 colocalizes with chromocenters), a typical well-known pattern of Np95 in G1 cells (Uemura et al., 2000; Miura et al., 2001; Papait et al., 2007); 2) in synchronized Suv39h1/2dn cells, as expected, Np95 appears diffused at the G1/S boundary, but shows a higher concentration at the chromocenters at the onset of S phase and clearly relocalizes to the dense DAPI dots at the time of heterochromatin replication with a pattern indistinguishable from NIH-3T3 cells (Uemura et al., 2000; Papait et al., 2007; Figure 3B, lanes Middle S, II and IIIA) and it is part of the pHDB (Quivy et al., 2004; Figure 3C). We conclude that Np95s distribution is independent from the trimethylation status of lysine 9 of histone H3.

Altogether, these experiments indicate that in the absence of Np95 the clustering of PH we detect occurs independently from the nuclear distribution pattern and methylation status of H3:K9 and H4:K20 and from the expression levels of HP1. These results suggest that chromocenter aggregation observed in the absence of Np95 is due to mechanisms that are independent from the known epigenetic markers that are critical for gene silencing and PH organization. They also show that lower amounts of Np95 induce the clustering of PH, suggesting that this protein might have a role in the regulation of chromocenter number and size.

Overexpression of Np95 and of ICBP90 Mislocalizes HP1α, -β, and -γ from PH

To verify this hypothesis, we overexpressed Np95-ICBP90 in NIH-3T3 cells. In Figure 4, we show that infection with a recombinant adenovirus expressing Np95 (AdNp95; Bonapace et al., 2002), but not the control adenovirus (Ad-TRACK), causes an alteration of the immunofluorescence distribution of HP1 in a dose-dependent manner (see Figure 4C for the overexpression levels of Np95 after infection). HP1 is displaced from the heterochromatic DAPI spots and becomes diffused in the nucleoplasm (cf. pictures b with e, h, and m in Figure 4, A and B). The effect on HP1 was confirmed by transfection experiments, which showed that overexpression of recombinant myc-tagged Np95-wt protein displaced all three forms of endogenous HP1 (HP1α, -β, and -γ) from PH (Figure 4D; cf. pictures b, h, and p, respectively with e, m, and s). Confocal microscopy corroborated the results (Figure 4E; cf. pictures b with e). The same outcome was obtained with transfection of myc-tagged ICBP90-wt
protein, indicating that higher cellular levels of either the human and the murine proteins displace HP1 from PH (Figure 4F; cf. pictures b with e).

**Overexpression of Np95 Produces Large-Scale Changes in Chromocenters Structure Independently from the H3:K9 Trimethylation Pathway, from DNA Methylation and from the Cell Cycle**

Surprisingly, we also observed major changes in size, form, and distribution of the chromocenters that seemed to gradually dissolve after overexpression of both Np95 and the human orthologue ICBP90 (Figure 4, A and B; cf. respectively, pictures c, i, and n with f, n, and t, respectively). At moderate levels of Np95 overexpression (MOI 60), DAPI nodules begun to change their characteristic “spot” pattern by assuming a more diffuse distribution in the nucleus. At higher levels of expression (multiplicity of infection [MOI 120]), DAPI spots became completely dispersed, and the chromocenters were no longer distinguishable in the nucleus.

Fluorescent in situ hybridization with a major satellite probe showed that dispersion of DAPI-bright chromocenters is accompanied by a similar decondensation of pericentromeric satellite DNA (Lehnertz et al., 2003; Figure 4G, cf. picture b with e).

Immunodetection of overexpressed and fixed cells with antibodies against trimethylated H3:K9 and H4:K20 shows that the distribution of these PH markers mirrors the altered DAPI staining (Figure 5A; cf. respectively, pictures 4 and 6 with 13 and 15; and 7 and 9 with 16 and 18). Strikingly, the decondensation effect of chromocenters is observed also in Suv39h1/2dn double null cells, which lack H3:K9met3, and in *dnmt* TKO ES cells, which lack DNA methylation. (Tsumura et al., 2006; Figure 5A; cf. respectively, pictures 19 and 21 with 22 and 24 for the Suv39h1/2dn cells; in this case the specific Ab utilized is anti-5-methyl-cytosine; and 26 with 28 for TKO cells). MeCP2 has been shown to play a relevant role in chromocenter dynamics and to induce chromocenter clustering during terminal differentiation and in overexpression independently from the H3:K9 trimethylation pathway (Brero et al., 2005). Figure 5A (pictures 10, 11, and 12) shows that overexpression of Np95 delocalizes MeCP2, which also mirrors the altered DAPI staining. This
Figure 5. Large-scale alterations of chromocenters induced by the overexpression of Np95 are independent from DNA methylation, the methylation of histone H3:K9, H4:K20, of MeCP2 and of cell cycle. (A) NIH-3T3, Suv39h1/2dn and TKO cells were grown and transfected with the pcDNA3.1-Np95-myc-His6-tagged plasmid. At very high levels of overexpression and after pretreatment with Triton X-100, however, a slight decrease of the tightly chromatin-bound levels of MeCP2 and HP1 are seen (Figure 5B, right panel). Nevertheless, at a multiplicity of infection 60 (MOI 60), which is sufficient to induce large-scale chromocenter modifications (see Figure 4A), the levels of H3:K9met3, H4:K20met3, MeCP2, and HP1 are comparable to controls (cf. in Figure 5B, lanes Ad-Np95 60, all antibodies, with Track 120).

The delocalization of HP1 and the dissolving effect on chromocenters was also independent of the cell cycle. Overexpression of Ad-Np95 in serum-starved NIH-3T3 cells, a cell cycle phase in which the protein is completely absent, had the same effect as that in asynchronously growing cells (Figure 5C).

The pattern of bromodeoxyuridine (BrdU) incorporation in growing cells after transfection of recombinant myc-tagged Np95-wt reflected the altered DNA organization. No specific replication foci were observable, and the BrdU was distributed homogenously throughout the nucleus (Figure 5D), although it resulted less intense, once more indicating that higher amounts of Np95 profoundly modified chromatin organization.

Altogether, these results indicate that large-scale chromocenter modifications induced by the overexpression of Np95 are independent of the H3:K9met3-H4:K20met3-HP1 pathways, DNA methylation, and the clustering activity of MeCP2, and are independent from the cell cycle.

The PHD Domain Is Essential for the Large-Scale Changes in Chromocenter Structure

To investigate which domain of Np95-ICBP90 is involved in large-scale chromocenter modifications, we performed overexpression experiments in NIH3T3, Suv39h1/2dn, and TKO cells with various recombinant Np95 deletion mutants.

Np95 contains several protein domains. Progressing from the N to the C terminus, these are: a Ub-like domain, a putative nuclear localization signal, a PHD domain, an SRA-YDG domain and a RING finger domain (Figure 6B). In a previous study, we showed that Np95 is a RING-type E3 ubiquitin ligase (Citterio et al., 2004). The SRA-YDG domain has been implicated in the control of DNA methylation (Unoki et al., 2004; Bostick et al., 2007; Johnson et al., 2007; Sharif et al., 2007), the recruitment of HDAC (Unoki et al., 2004), and transcriptional silencing of major satellites (Papait et al., 2007). We constructed deletion mutants of each of these domains (Figure 6B) and evaluated the effects of their expression on the stability of chromocenters by immunofluorescence with antibodies against HP1α and by staining the PH areas with DAPI. Only those constructs retaining the PHD domain (Figure 6B, Np95-wt, -1,719, -1,590, -1,419, -82,782, and -Δ-SRA) were able to disaggregate chromocenters and to delocalized HP1α from PH regions (Figure 6A; for HP1, cf. images 12, 13, 14, 15, 17, and 18 with 16, 19, and 20; for DAPI, cf. images 22, 23, 24, 25, and 27 with 26, 29, and 30). Deletion of the PHD domain only (Figure 6A, Δ-PHD mutant) is sufficient to impair the large-scale modifications observed with the wild-type protein in either NIH-3T3, Suv39h1/2dn or dnmt TKO cells. It has been shown that a RING point domain, but not ICBP90wt causes large-scale chromocenter changes (Karagianni et al., 2008). Our experi-

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ments in at least three type of mouse cells (NIH-3T3, Suv39h1/2dn, and TKO) indicate that overexpression of Np95wt or ICBP90wt or of the deletion mutants Δ-SRA, Δ-RING, Δ-ubiquitin-like domain (82-782), but not Δ-PHD always causes large-scale chromocenter modifications. These data suggest that the known functions of Np95 (ubiquitin ligase activity determined by the RING domain, and HDAC recruitment and control of DNA methylation attributed to the SRA-YDG domain) are not involved in the ability of Np95 to disaggregate the chromocenter structures. They indicate, instead, a specific role for the PHD domain of Np95 in this process.

Np95 Increases the Access of Restriction Enzymes to DNA Packaged into Nucleosomal Arrays

During PH replication in middle S phase, the bulk of Np95 specifically relocates to and gets highly concentrated in the chromocenters where it occupies the areas of less compacted DNA that correspond to “replication factories.” The disaggregating effects we observe on chromocenters in overexpression experiments suggest that, in living cells, Np95 might contribute to the opening and/or stabilization of the dense chromatic structure to support the access and recruitment of modifying enzymes required for replication and formation of PH.

We therefore used in vitro experiments to investigate if Np95 is able to facilitate the access to modifying enzymes (restriction enzyme SfcI) to DNA (pFM218-H5 plasmid) packaged into nucleosome arrays (Figure 7A). This type of assay has been used by various authors to assess the chromatin accessibility activity of various protein complexes in vitro (Almer et al., 1986; Varga-Weisz et al., 1997; Boyer et al., 2000; Shen et al., 2000; Alexiadis and Kadonaga, 2002). Recombinant GST-Np95 was incubated with reconstituted chromatin in the presence or absence of the restriction enzyme SfcI, separated by agarose gel electrophoresis, and visualized by hybridization with radiolabeled pFM218-H5 plasmid DNA. Figure 7B shows that 1 or 2 μg of GST-Np95 (Figure 7B, lanes 6 and 7), 2 μg of ICBP90 (Figure 7B, lane 14), and 200 ng of the positive control (Drosophila nuclear extracts; Figure 7B, lane 5), but not 2 μg of GST alone (Figure 7B, lane 4), increase access of a restriction enzyme to packaged DNA (Figure 7B, cf. lanes 4 and 5 with lane 6). Figure 7 further shows that deletion of the PHD domain, but not of the SRA domain strongly reduces this activity, although it does not abolish it (Figure 7B, cf. lanes 6–7 with 8–9, 10–11, and 12–13).

Altogether, these experiments indicate that Np95, by means of its PHD domain, is able to disaggregate chromo-

Figure 6. The PHD domain is essential for large-scale rearrangement of chromocenters. (A) Overexpression of deletion mutants of Np95. NIH-3T3, Suv39h1/2dn, and TKO cells were grown as in Figure 3B and transfected with the indicated pcDNA3.1-Np95-myc-His6–tagged recombinant deletion mutants of Np95. Cells were then stained with antibodies against myc to visualize the transfected cells and with HP1α (not for TKO cells). Cell nuclei were counterstained with DAPI. Representative pictures are shown. (B) Schematic representation of wild type and mutants of Np95 proteins used in this experiment. All constructs were myc-His6 tagged.

Figure 7. Np95 induces chromatin template accessibility to a restriction enzyme in an in vitro assay. (A) Schematic representation of the chromatin accessibility assay. Naked DNA was cut by the restriction endonuclease SfcI; if nucleosomes were reconstituted on the DNA, the enzyme was unable to cut; the presence of a remodeler could mobilize histones and allow DNA cutting by SfcI. (B) Np95 increased template accessibility of the SfcI restriction enzyme to chromatinized DNA. Reconstituted nucleosomal arrays that were incubated in the presence either 2 μg of GST (lane 4), or 1 μg (lanes 6, 8, 10, 12) or 2 μg (lane 7, 9, 11, 13) of wt-Np95, PHD, SRA, PHD-SRA deletion mutants (lanes 8 and 9), or 2 μg of wt-ICBP90 (lane 14) were separated by agarose gel electrophoresis and visualized by hybridization with radiolabeled pFM218-H5 plasmid DNA. Nicked (N), linear (L), and supercoiled (S/C) forms of uncut pFM218-H5 plasmid DNA species (lane 1) are indicated to the left. The appearance of complete (C1, C2; left of pictures) or partial (p; right of pictures) SfcI digestion products are indicated.
centers in vivo and to facilitate the access to DNA modifying enzymes in vitro. We suggest that Np95-ICBP90, when it specifically relocates to PH in middle S phase and becomes highly concentrated in the pHDB, could actively participate to render the chromatin of chromocenters more dynamic and potentially more accessible to modifying enzymes, such as HDAC and DNMT1.

DISCUSSION

In this article, we provide evidence that Np95 determines large-scale modifications of chromocenters and that the PHD domain is required for this process. Depletion of Np95 leads to a reduction in the number and a size increase of chromocenters, whereas overexpression of Np95-ICBP90 induces decondensation of chromocenters.

Remarkably, the dissolving effect on chromocenters is independent from the H3:K9/K4:K20met3/H1P1 pathway, from DNA methylation, and from the cell cycle. In Suv39h1/2dn and in dmtTKO cells, indeed, overexpression of Np95 leads to the disappearance of the DAPI nodules. In NIH-3T3 cells, neither the histone trimethylation pattern in PH nor the expression levels of H1P1 are significantly affected after Np95 depletion or overexpression. This, however, is not surprising because several studies show that the H3:K9 methylation pathway is not involved in chromocenter dynamics. Accumulation of H1P1 in PH regions does not cause large-scale chromocenter modifications (Mateos-Langerak et al., 2007). The number and size of chromocenters in Suv39h1/2dn cells are comparable to control cells (Peters et al., 2001). Large-scale rearrangements of PH induced during terminal differentiation of muscle cells and by overexpression of MeCP2 are independent from H3:K9met3 and from H1P1 levels (Brero et al., 2005). In plants, the methyltransferase suvl4 mutant has normal chromocenters although H3:K9met2 is strongly reduced (Jasencakova et al., 2003; Naumann et al., 2005; Zemach et al., 2005).

It has also been suggested that extensive DNA methylation is not necessary for PH clustering. The DNA methyl-binding protein MeCP2 was shown to assemble secondary chromatin structures independently from the methylated DNA-binding domain and from DNA methylation (George et al., 2003). However, during muscle terminal differentiation the MBD domain of MeCP2 and of other MBD-containing proteins seems to be necessary and sufficient to increase pericentromeric clustering (Brero et al., 2005). In plants, disruption of chromocenter structures during dedifferentiation of specialized mesophyll cells into undifferentiated protoplasts is not accompanied by changes in DNA or H3:K9 methylation and in transcriptional reactivation of silent genomic elements (Tessadori et al., 2007). The severe DNA hypomethylation mutants dnm1-5, dnm1-2, met1-1, and hgot only show a limited decondensation of chromocenter heterochromatin (Mittelsten Scheid et al., 2002; Probst et al., 2003). Deletion of Np95 results in a drastic reduction of DNA methylation levels in mouse ES cells and embryos (Bostick et al., 2007; Sharif et al., 2007). Our results indicate that loss of Np95 increases chromocenters size and that overexpression of Np95 in TKO cells disaggregates chromocenters, once more suggesting that the chromocenter dynamics we observe is independent from DNA methylation.

Alterations in chromocenter structure by Np95 must, therefore, depend on different processes. We show that PHD is the domain required in vivo for the profound alterations of chromocenters structure after overexpression of Np95-ICBP90. It has been shown that a RING point domain, but not ICBP90wt, causes large-scale chromocenter changes (Karagianni et al., 2008). Our experiments in at least three type of mouse cells (NIH-3T3, Suv39h1/2dn, and TKO) indicate that overexpression of Np95-ICBP90wt or of the deletion mutants Δ-RING, Δ-SRA, Δ-RING, Δ-ubiquitin-like domain (82-782), but not Δ-PHD, always causes large-scale chromocenter modifications.

Significant sequence differences have been found in PHD domains, and accordingly, various activities have been assigned to this domain (Bienz, 2006). The PHD domain is an important chromatin-binding module and is crucial for the function of proteins within chromatin-associated complexes that display chromatin-modifying activities, like Dmnt3L (Jia et al., 2007), BHC80 (Lan et al., 2007), ACF-1 (Eberhardt et al., 2004), and BPTF (Wysocka et al., 2006). The deletion or functional impairment of the C-terminal PHD finger of ACF1 profoundly affected the nucleosome mobilization capability of associated SNF2H in trans. Some of these complexes have been implicated in DNA replication (Corona and Tamkun, 2004) and the ACF1–SNF2H complex specifically in PH replication (Collins et al., 2002).

Our in vitro experiments show that the PHD of Np95 is required to facilitate the access of a restriction enzyme (SfcI) to DNA packaged into nucleosome arrays, suggesting that in vivo this domain might enhance Np95s chromatin-binding ability and favor the recruitment of chromatin modifiers. Indeed, the PHD domain of Np95 has a role in chromatin binding, although the SRA is critical (Citterio et al., 2004). Although a recent publication (Karagianni et al., 2008) shows that the PHD and SRA domains of Np95 are required for preferential binding to H3:K9met3 and that Np95 in Suv39h1/2dn cells does not bind heterochromatin, our experiments performed on those cells clearly show that the protein distribution is not affected by that genetic background (Figure 3). In synchronized Suv39h1/2dn cells, Np95 exhibits foci of PH staining over a more diffuse pattern at the onset of S phase, relocates to chromocenters at the time of PH replication and is part of the pHDB, as it does in NIH-3T3 cells. A possible interpretation of this discrepancy is that Suv39h1/2dn cells grow slower and most cells are in G1, a cell cycle phase in which Np95 is well known to appear diffused in the nucleoplasm (Uemura et al., 2000; Miura et al., 2001; Papait et al., 2007).

The PHD-mediated large-scale PH reorganization might reflect changes that occur at a yet unknown level of chromatin organization and disrupts the structure of chromocenters, producing an open chromatin configuration. This view is reinforced by our FISH experiments, which show that major satellite DNA is disaggregated along with chromocenters. At the time of PH replication, the bulk of Np95 relocates to the chromocenters and always associates with the pHDB, which corresponds to the less dense chromatin areas. In these partially disaggregated chromocenter areas, parental DNA is pulled out and duplicated by the replication machinery (Quivy et al., 2004). We propose the hypothesis that the PHD domain of Np95 is involved in PH replication and formation by contributing to the “opening” of this chromatin compartment during replication. The SRA domain would recruit HDAC1 (Unoki et al., 2004; Papait et al., 2007), contributing to the establishment of the repressive environment that produces major satellite transcriptional repression (Papait et al., 2007). Our results showing chromocenter clustering and impairment of PH replication after Np95 depletion, are an indirect confirmation of this hypothesis. The results described here, together with our previous studies, tend to suggest that higher amounts of this protein would contribute to a “proliferating” competence of the
cells, which is consistent with the observation of Np95-ICBP90 overexpression in many tumors (Mousli et al., 2003; Crnogorac-Jurcevic et al., 2005; Jenkins et al., 2005). Indeed, large-scale positional or structural modifications of heterochromatin have key roles in cellular transformation (Zink et al., 2004) and may involve epigenetic regulation of gene expression (van Driel et al., 2003).

In conclusion, we propose that Np95-ICBP90 has an important role for chromocenter dynamics that is accomplished independently from the H3-K9-H4-K20-H1 pathway and from DNA methylation and that the PHD domain has an important role for chromocenter dynamics and might actively participate to the replication and reformation of PH domains in middle S phase.

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