Molecular Biology of the Cell
Vol. 19, 3554–3563, August 2008

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

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Submitted October 22, 2007; Revised May 12, 2008; Accepted May 16, 2008
Monitoring Editor: Yixian Zheng

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 relocalizes 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

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–10–1059) on May 28, 2008.

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Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; DNMT1, DNA methyltransferase 1; H3:K9met3, tri-methylated lysine 9 of histone H3; H4:K20met3, tri-methylated lysine 20 of histone H4; pHDB, PH duplication body; RNAi, RNA interference; siRNA, small interfering RNA oligonucleotides.

chromatin compartment (Maison and Almouzni, 2004; Brero *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 epigenetic 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 (Brero *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 relocalizes 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 the cell strongly reduces both DNA duplication of this area and PH reformation. This involves modification of the acetylation status of lysines 8, 12, and 16 of histone H4 and the silencing of major satellite repeats. Very recent studies show that UHRF1 plays a role in maintaining DNA methylation in mammalian cells by recruiting DNMT1 to hemi-methylated DNA (Bostick *et al.*, 2007; Sharif *et al.*, 2007), adding more evidence for a key role of Np95 in PH replication and in the maintenance of the epigenetic modifications required for PH formation.

In this article, we investigated the possibility that Np95 might have a role in the control of large-scale reorganization of chromocenters, thereby possibly contributing to the dynamic changes of these dense chromatin areas that occur during PH replication.

MATERIALS AND METHODS

Cells and Adenovirus

NIH-3T3 cells was grown in DMEM supplemented with 10% fetal bovine serum (FBS). The Np95 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.*, 2001), *dnmt1, 3a,* and *3b* triple knockout (*dnmt* TKO) embryonic stem (ES) cells (Tsumura *et al.*, 2006), 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 manufacture'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 Suv39h1/2dn 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. *dnmt* TKO ES cells were transfected using FuGene HD (Roche) transfection reagent according to the manufacturer's instructions.

For RNA interference, NIH3T3 cells were transfected with 20 nM small interfering RNA (siRNA) duplex using Oligofectamine (Invitrogen). Two rounds of transfection were done for all experiments. Cells were analyzed 24 h after the last transfection. siRNA oligos were from Ambion (Austin, TX), and the targeting sequences were as follows: RNA interference (RNAi) control: AAAACGAGGCAGGAAAGGCGGTT; RNAi Np95: AACGCGGCTTCT-GGTATGATGTT.

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 polyclonals anti-Np95 (Bonapace *et al.*, 2002); anti-HP1α, 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-H:K9met3 and anti-H4:K20 (Abcam, Cambridge, United Kingdom), mouse monoclonal anti-5mC (Calbiochem, Darmstadt, 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 36 h 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. Until hybridization, coverslips with fixed and pretreated cells were stored in 50% formamid/2× SSC at 4°C.

The probe was generated by PCR using 5′-GACGACTTGAAAAATGAC-GAAATC-3′ (MajF1-for) and 5′-CATATTCCAGGTCCTTCAGTGTGC-3′ (MajR1-rev) as primers and pCR4-MajSat9-2 plasmid as template (kind gift from T. Jenuwein, Research Institute of Molecular Pathology and The Vienna Biocenter, Vienna, Austria) and labeled by nick translation using Biotin-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 d 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 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 (DP50; 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).

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% Np40. The Histone:DNA weight ratio was usually 1.5:1. Reconstitution mixes were reduced by dialysis at 26°C from 2 M to 50 mM NaCl/TE/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 $MgCl_2$, 4 mM ATP, pH 8, 2 μg of glutathione *S*-transferase (GST), 1 or 2 μg GST-Np95, or 200 ng of Brg1, or 200 ng of *Drosophila* nuclear extracts and 4 U of SfcI restriction enzyme. Products were separated by agarose gel electrophoresis and visualized by hybridization with radiolabeled pFM218-H5 plasmid DNA.

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

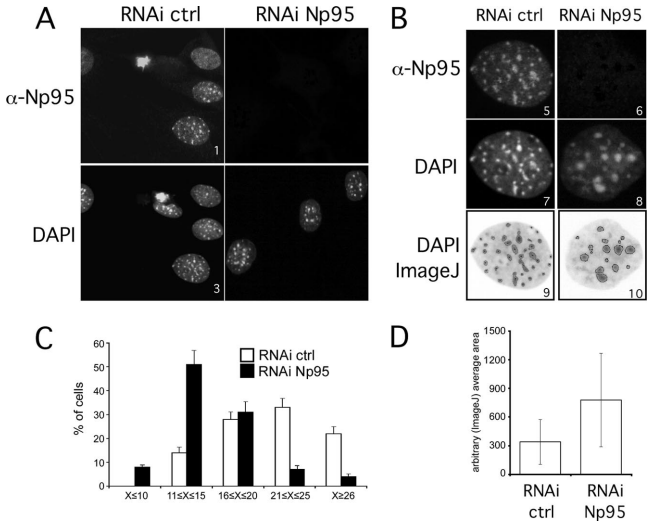

Figure 1. Depletion of NP95 induces clustering of PH. (A) Silencing efficiency of Np95 by siRNA treatment. NIH3T3 cells were grown on coverslips, transfected with an siRNA oligo against Np95 (Np95 RNAi) or with control siRNA oligo (control RNAi), and analyzed 48 h later by immunofluorescence (IF) using antibody against Np95 (pictures 2 and 4). Nuclear counterstaining was visualized with DAPI (pictures 1 and 2). Representative pictures are shown. (B) Quantitative analysis of chromocenter number and size. Single nuclei of NIH-3T3 cells were treated as in A. Pictures 9 and 10 show the same cell nuclei of picture 7 and 8 overlaid with the masks applied to the chromocenters as calculated with the ImageJ software (version 1.32j) for quantitative analysis. (C) Quantitative analysis of chromocenter number in RNAi-Np95–treated cells. Nuclei of 200 cells per condition (RNAi-Np95 and RNAi ctrl treated) were analyzed with the ImageJ software as in pictures 9 and 10 of B. The number of chromocenters were then plotted with the following parameters: number of chromocenters (x) less or equal to 10; $11 \leq x \leq 15$; $16 \leq x \leq 20$; $21 \leq x \leq 25$; and $x \geq 26$. (D) Quantitative analysis of chromocenter size in RNAi-Np95–treated cells. The same cells of panel C were analyzed for the size of the particles calculated on chromocenters by the ImageJ software. The area of each particle is expressed in ImageJ arbitrary units.

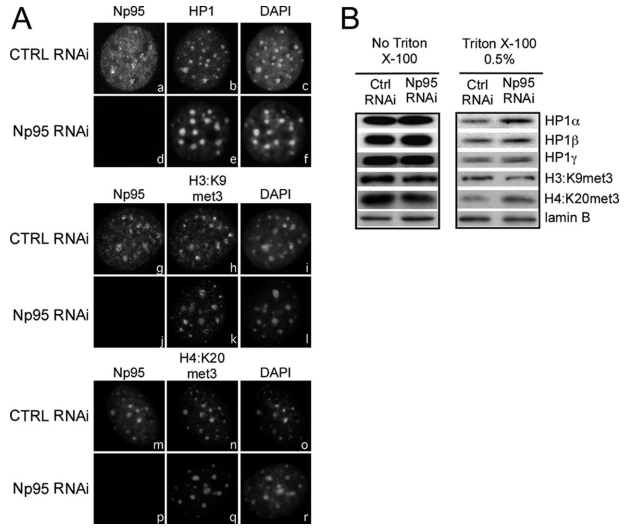

Figure 2. Clustering of PH is independent from the histone H3 methylation pathway. (A) NIH3T3 cells were grown and transfected as in Figure 1. Cells were then stained with anti-Np95 antibody, together with anti-Hp1α, anti-H3:K9 trimethylated (H3:K9met3), and anti-H4-K20 (H4:K20met3) trimethylated, and analyzed by indirect fluorescence. Nuclear counterstaining was visualized with DAPI. Representative pictures are shown. (B) Nuclear lysates from cells treated as in A were pretreated with or without Triton X-100 to extract weakly bound chromatin proteins and were analyzed by Western blot with the indicated antibodies.

these areas. Specific colocalization of Np95 with replication factories, which always corresponds with a less compacted DNA, suggested to us a possible role of the protein in chromocenter dynamics. We reasoned that if Np95 has a role in this process, we would expect that quantitative variations of the protein in the cell would lead to major changes in chromocenter structure.

To this end, we treated NIH-3T3 cells with siRNA against Np95, which resulted in a reduction of Np95 protein. This caused clustering of PH, visualized by a reduction in the number (Figure 1A; cf. picture 3 with 4; and 1B: cf. picture 7 with 8) and an increase in the size (Figure 1B; cf. picture 7 with 8) of the intense DAPI spots corresponding to chromocenters. We performed a quantitative analysis with ImageJ software (NIH; version 1.32j) to calculate the number and the size of chromocenters in RNAi-control (ctrl) and RNAi-Np95–treated cells (Figure 1B, pictures 9 and 10). The analysis of ~200 cells per experiment per treatment is shown in Figure 1, C and D. In RNAi-Np95 experiments, 88% of the cells display 20 or less chromocenters, whereas in RNAi-ctrl experiments <42% of cells display this number of chromocenters (Figure 1C), as shown previously for NIH-3T3 cells (Cerda *et al.*, 1999). The differences become more obvious when counting the number of cells that display 15 or less chromocenters: more than 57% in the RNAi-Np95–treated 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 pericentromeric 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 proteins weakly bound to chromatin (Figure 2B). A slight increase in HP1α and H4:K20met3 was observed.

A recent study by Wong and coworkers (Karagianni *et al.*, 2008) has shown that ICBP90 and Np95 specifically bind to H3:K9met3 and that in Suv39h1/2dn cells Np95 is delocalized. We performed a detailed analysis of the cell cycle

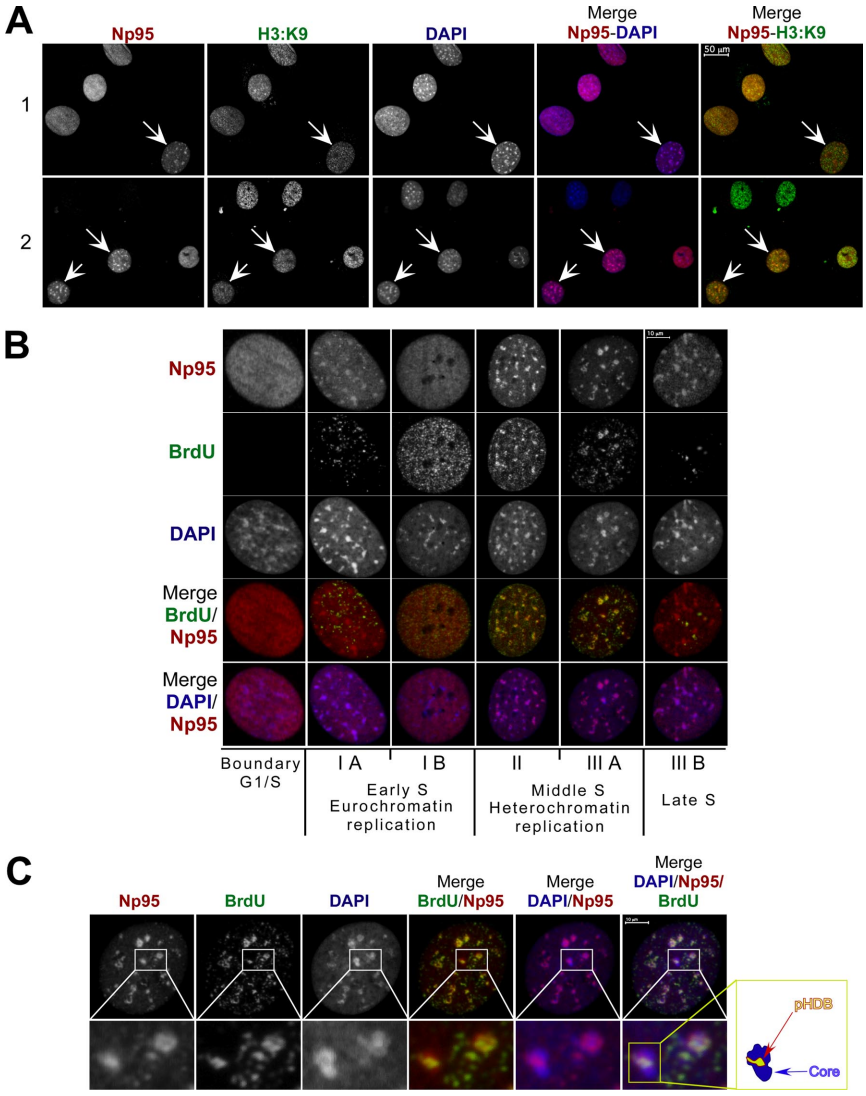

Figure 3. Np95s distribution is unaffected in Suv39h1/2dn cells. (A) Colocalization between Np95 and chromocenters in Suv 1/2 dn cells. Asynchronously growing Suv39h1/2dn cells were grown on coverslips and analyzed 48 h later by immunofluorescence (IF) using antibody against Np95 (red) and lysine 9 of histone H3 (green). Nuclear counterstaining was visualized with DAPI. Arrows indicate the cells that display colocalization. (B) Distribution of Np95 during S Phase. Suvh1/2 dn cells were synchronized in G0. At different times after release, cells were pulse-labeled with BrdU and then stained with anti-Np95 together with anti-BrdU. Nuclear counterstaining was visualized with DAPI. The distribution of nuclear DNA replication sites was classified into the five major types of patterns during S-phase that have been shown to be identical in primary, immortalized, and transformed mammalian cells: IA and IB, Early S and euchromatin replication; II and IIIA, Middle S, pericentromeric heterochromatin replication; IIIB, Late S, replication of centromeric heterochromatin and other structures (Ma *et al.*, 1998; Dimitrova and Berezney, 2002). (C) Np95 is part of pHDB. Suv39h1/2dn cells in mid-S phase were pulse-labeled with BrdU and then stained with anti-Np95 together with anti-BrdU. Nuclear counterstaining was visualized with DAPI. Representative pictures are shown. The insets correspond to magnifications of the areas indicated. On the right is a schematic representation of the pHDB of the inset.

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

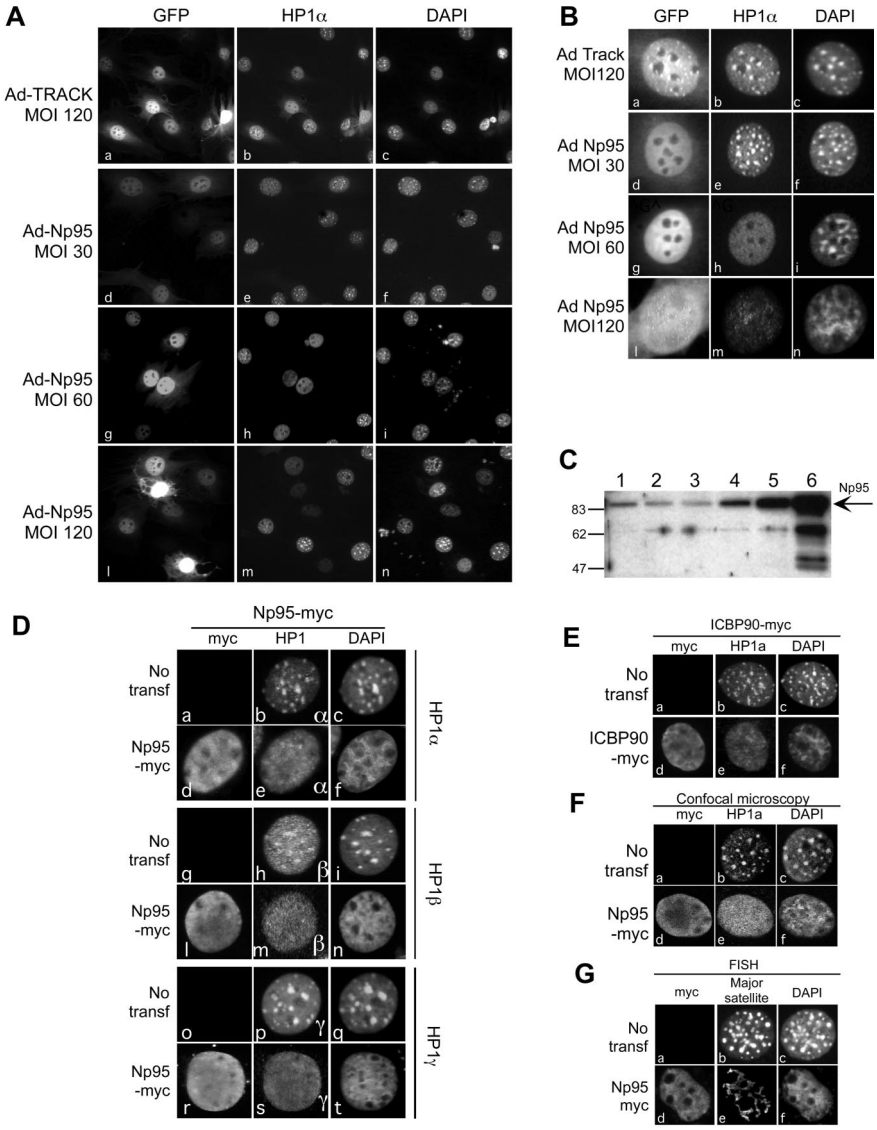

Figure 4. Large-scale alterations of chromocenters induced by the overexpression of Np95. (A and B) Overexpression of GFP-adenovirus expressing recombinant Np95 (Ad-Np95; Bonapace *et al.,* 2002). NIH-3T3 cells were grown on coverslips and infected with either a Np95 recombinant adenovirus (Ad-Np95; MOI 30, 60, and 120) or nonrecombinant E1A defective adenovirus (Ad-Track; MOI 120) as a control. Forty-eight hours later the cells were fixed and analyzed by immunofluorescence (IF) using antibody against HP1α, whereas nuclear counterstaining was visualized with DAPI. (A) Low-magnification images; (B) high-magnification images of representative cells. (C) Western blot of the overexpressed cells. Forty-eight hours after infection with Ad-Np95 or Ad-TRACK as a control the NIH-3T3 cells were harvested and lysed, and Western blotting was performed with an antibody against Np95. Lanes are as follows: 1) No infection; 2) Ad-TRACK MOI 60; 3) Ad-TRACK MOI 120; 4) Ad-Npo95 MOI 30; 5) Ad-Npo95 MOI 60; 6) Ad-Npo95 MOI 120. (D and E) Overexpression of a recombinant pcDNA3.1-Np95- and pcDNA3.1-ICBP90-myc-His_6-tagged plasmid. NIH-3T3 cells were transfected with the pcDNA3.1-Np95-myc-His_6-tagged plasmid (D) or pcDNA3.1-ICBP90-myc-His_6-tagged plasmid (E). Forty-eight hours later the cells were then stained with anti-myc antibody to visualize the transfected cells, together with either anti-HP1α, anti-HP1β, and anti- HP1γ. Nuclear counterstaining was visualized with DAPI. Representative pictures are shown. (F) Confocal microscopy analysis of overexpression of Np95. NIH-3T3 cells were transfected as in D). Forty-eight hours later the cells were then stained with anti-myc antibody, together with either anti-HP1α, and analyzed by confocal microscopy. (G) Effects of Np95 overexpression on Major satellite DNA. NIH-3T3 cells were transfected as in D). Forty-eight hours later the cells were then hybridized with a Biotin-16-dUTP labeled probe against the mouse Major satellite and detected with FITC-conjugated goat-anti-avidin antibodies.

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. picture c with f, i, and n; and 4D; cf. pictures c, i, and q 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).

Immunodecoration 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

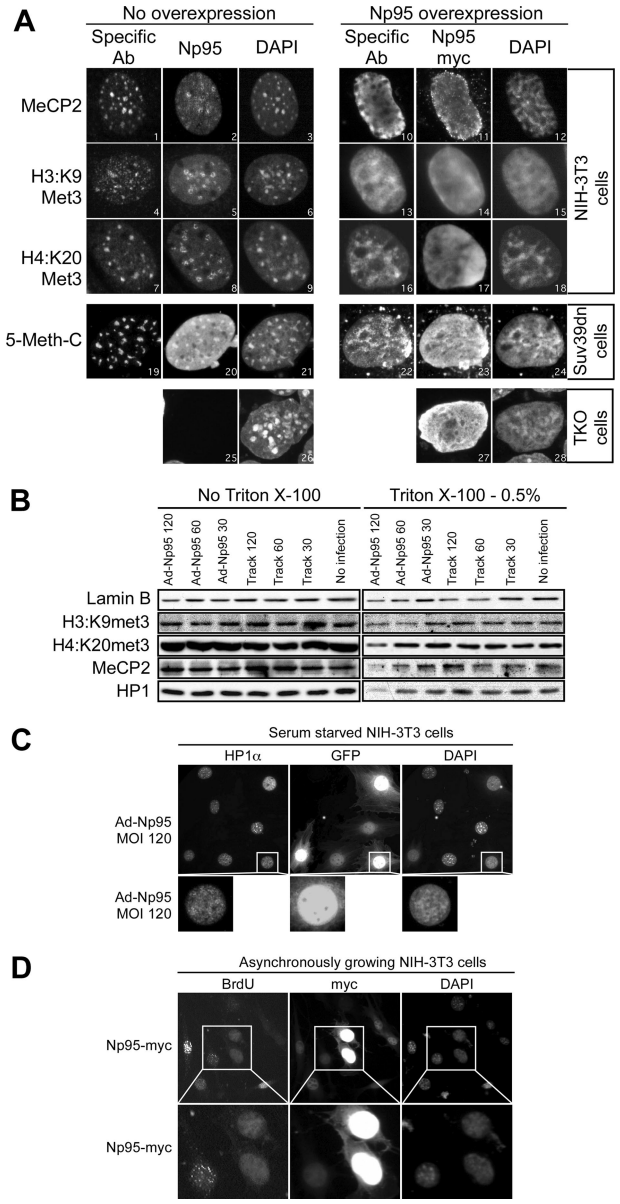

Figure 5. Large-scale alterations of chromocenters induced by the overexpression of Np95 are independent from DNA methylation, the methylation of histone H3:K9, H4:K20, of MeCP2 and of cell cycle. (A) NIH-3T3, Suv39h1/2dn and TKO cells were grown and transfected as in Figure 3B with the pcDNA3.1-Np95-myc-His_6-tagged plasmid. Forty-eight hours after transfection, the cells were immunostained with antibodies against MeCP2, H3:K9met3 and H4:K20met3 (NIH-3T3) or against 5-methyl-cytosine (5-Meth-C; Suv39h1/2dn). Nuclei were counterstained with DAPI. Representative pictures are shown. (B) Overexpression of Np95 in NIH-3T3 cells does not modify the methylation pathway that leads to the formation of H3:K9met3 and H4:K20met3. NIH-3T3 cells were grown and infected as in Figure 3A. Forty-eight hours after infection, nuclear lysates were obtained from samples with or without Triton X-100 pretreatment. Western blotting was then performed using the indicated antibodies. (C) NIH-3T3 cells were grown on coverslips, serum-starved, and infected with Ad-Np95 MOI 120. Forty-eight hours later the cells were fixed and stained with antibody against HP1α. Infected cells express GFP. Nuclei were counterstained with DAPI. The single-cell field is the enlargement of the boxed cell in the top panel. (D) NIH-3T3 cells, asynchronously growing on coverslips, were transfected with Np95-myc. Forty-eight hours later the cells were pulse-labeled for 10 min with BrdU, fixed, and stained with anti-myc and anti-BrdU antibodies.

suggests that overexpression of Np95 is able to counteract the chromocenter clustering effect of MeCP2.

Western blot experiments conducted on protein extracts from cells overexpressing Np95 showed no significant variations in the overall methylation state of H3:K9 or H4:K20 or in the level of expression of HP1 or MeCP2 (Figure 5B, left panel). At very high levels of overexpression and after pretreatment with Triton X-100, however, a slight decrease of the tightly chromatin-bound levels of HP1 and MeCP2 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-

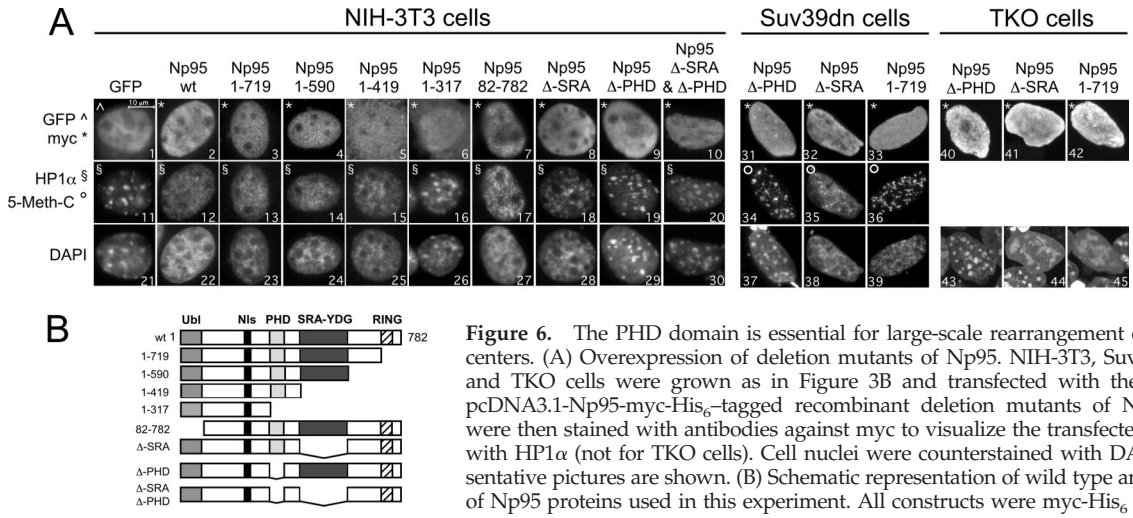

Figure 6. The PHD domain is essential for large-scale rearrangement of chromocenters. (A) Overexpression of deletion mutants of Np95. NIH-3T3, Suv39h1/2dn, and TKO cells were grown as in Figure 3B and transfected with the indicated pcDNA3.1-Np95-myc-His_6–tagged recombinant deletion mutants of Np95. 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-His_6 tagged.

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 relocalizes 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-

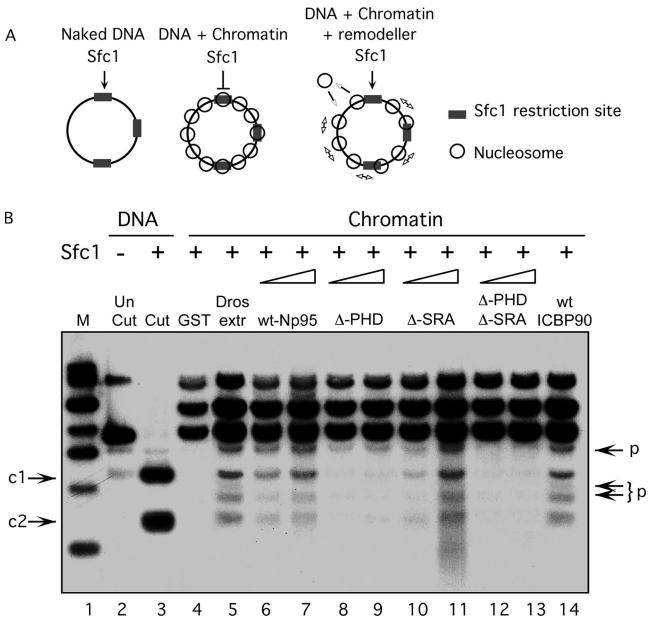

Figure 7. Np95 induces chromatin template accessibility to a restriction enzyme in an in vitro assay. (A) Schematic representation of the chromatin accessibility assay. Naked DNA was cut by the restriction endonuclease SfcI; if nucleosomes were reconstituted on the DNA, the enzyme was unable to cut; the presence of a remodeller 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 and9), 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/HP1 pathway, from DNA methylation, and from the cell cycle. In Suv39h1/2dn and in *dnmt* TKO 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 HP1 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 HP1 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 HP1 levels (Brero *et al.,* 2005). In plants, the methyltransferase *suvh4* 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 (Georgel *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 percientromeric 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 *ddm1-5*, *ddm1-2*, *met1-1*, and *hog1* 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-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 Dnmt3L (Jia *et al.,* 2007), BHC80 (Lan *et al.,* 2007), ACF-1 (Eberharter *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, relocalizes 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 relocalizes 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-HP1 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.

ACKNOWLEDGMENTS

We thank Dr. Roberto Valli and Prof. Manuela Maserati for their help in FISH experiments; Prof. Peter Becker (Ludwig-Maximilians-Universität, Munich, Germany) for the gift of *Drosophila* nuclear extracts; Prof. Thomas Jenuwein for providing us with the Suv39h1/2dn cells; and Prof. Masaki Okano (Center for Developmental Biology, RIKEN, Kobe, Japan) for the TKO cells. We thank the core facilities of the IGBMC. This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC), the Rett Syndrome Research Foundation, the Deutsche Forschungsgemeinschaft, and from Fondazione Cariplo. The transfected Cell Array platform thanks the Cancéropôle du Grand Est, the Région Alsace, the Conseil Général du Bas Rhin, and the Communité Urbaine de Strasbourg. R.P. was supported by a fellowship from AIRC.

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