Transcriptional Coactivator PC4, A Chromatin-associated Protein, Induces Chromatin Condensation

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Running Title: PC4, a nonhistone chromatin-associated protein.

Keywords: Chromatin, PC4, AFM, Condensation.

Total word count: Materials and Methods:1537, Rest of the document:9964.
Abstract

Human transcriptional coactivator PC4 is a highly abundant multifunctional protein, which plays diverse important roles in cellular processes including transcription, replication and repair. It is also a unique activator of p53 function. Here we report that PC4 is a bonafide component of chromatin with distinct chromatin organization ability. PC4 is predominantly associated with the chromatin throughout the stages of cell cycle and is broadly distributed on the mitotic chromosome arms in a punctate manner except the centromere. It selectively interacts with core histones H3 and H2B, which is essential for PC4-mediated chromatin condensation as demonstrated by micrococcal nuclease (MNase) accessibility assays, circular dichroism spectroscopy and atomic force microscopy (AFM). The AFM images show that PC4 compacts the 100 kb reconstituted chromatin distinctly as compared to the linker histone H1. Silencing of PC4 expression in HeLa cells results in chromatin decompaction as evidenced by the increase in MNase accessibility. Knocking down of PC4 up-regulates several genes leading to the G2/M check point arrest of cell cycle, which suggests the physiological role of it as a chromatin compacting protein. These results establish PC4 as a new member of chromatin associated protein (CAP) family, which plays an important role in chromatin organization.
Introduction

The Eukaryotic genome is organized into a highly complex nucleoprotein structure, the chromatin. This dynamic chromatin structure is regulated by posttranslational modifications of the core-histones and histone H1, and also by the direct interaction of nonhistone chromatin associated proteins with the different components of chromatin including core octamer and/or linker histones \((60, 2, 8, 56, 20)\). The ATP-dependent chromatin remodeling and histone chaperones (replication dependent and independent) also contribute to the organization of the dynamic chromatin \((1, 37)\). The chromatin fiber bridging proteins (Sir3p, Tup1 and MENT) \((23, 21, 50)\) and nonhistone chromatin associated proteins, which include High Mobility Group proteins (HMGs) \((2, 8, 10, 42)\), Heterochromatin binding Protein 1 (HP1) \((35)\), Methyl CpG binding protein 2 (MeCP2) \((31)\) and Poly ADP-ribose Polymerase 1 (PARP-1) \((28)\) help in chromatin compaction or decompaction through their direct interaction with core-histones and/or DNA. These proteins may also compete or cooperate with histone H1 during this process \((10, 53)\). The interaction of histone H1 with the nucleosomes stabilizes the higher order compact chromatin structure, restricting the ability of the regulatory factors to access their chromatin binding sites \((60, 52, 3)\). Histone H1 is a key factor to aid in the compaction of the chromatin for mitotic chromosomes.

Transcriptional silencing during mitosis occurs in tandem with numerous structural and biochemical changes, which include chromatin condensation and massive increase in protein phosphorylation. These changes trigger the
dissociation of most of the transcription machinery from the condensed chromatin. Nevertheless, few important transcription regulators, for example TBP and some TBP associated factors remain associated with the mitotic chromatin (47, 11 and references therein). Several TAFs associated with the mitotic chromatin get phosphorylated and consequently cannot modulate activator dependent transcription, which is restored upon dephosphorylation (47). Apart from TFIID, some amount of TFIIB also remains associated with the previously active promoters during mitosis, whereas RNA Polymerase II and NC2 (which can function both as an activator and a repressor) are displaced (11, 12). In general association of transcription factors with the chromosome/ chromatin is found to be a highly dynamic process, which depends upon the stages of cell cycle.

The present report focuses on the discovery of a highly abundant, multifunctional transcriptional Coactivator PC4, as a bonafide component of chromatin with distinct functional consequences. PC4 plays an important role in transcription, repair and replication (22, 30, 57, 43). It facilitates activator dependent transcription by RNA polymerase II to ~ 85 fold in vitro, through direct interactions with general transcription factors as well as transcriptional activators (22, 30, 25). This 15-kDa protein interacts with free or DNA bound TFIIA and TBP component of the basal transcription machinery (25) but not with TBP-TFIIB complex or free TFIIB. It cannot interact with highly purified TFIID alone, in the absence of TFIIA (22). Apart from its role in transcription, PC4 can interact with TFIIH (19) as well as to the single stranded DNA, indicating its potential
role in the repair pathway. Recent report shows that PC4 directly interacts with one of the important DNA repair factor, XPG, specifically required for transcription-coupled repair and helps in the repair of oxidative DNA damage (57). However, the DNA binding as well as the interaction with the activators and components of basal transcription machinery are essential for the transcriptional coactivation function of PC4. Interestingly, PC4 inhibits RNA Pol-II phosphorylation and hence Pol-II mediated transcription (46). Furthermore PC4 acts as a potent inhibitor of transcription in regions of unpaired dsDNA, ssDNA and on DNA ends (59). PC4-mediated transcription repression can be relieved by ERCC3 helicase activity of TFIIH (18). Its diverse cellular functions also include its ability to interact with TFIIIC, influencing the process of re-initiation and termination in RNA Polymerase III dependent transcription (58). PC4 can interact with CstF64, thereby has a role in polyadenylation and subsequent transcription termination (9). Recently it has been shown that it also has a role in promoter release and transcription elongation in GAL4- VP16 dependent transcription (19). PC4 can also form complex with HSSB on ssDNA and markedly affect the replication function of HSSB (43).

PC4 can inhibit self-repression of AP2 in a ras transformed cell line and thus can act as a putative tumor suppressor (26). The tumor suppression activity of PC4 could also be through its ability to enhance the p53 function (4). This functional diversity of PC4, its similarity with HMGB1 with respect to its DNA-binding properties, involvement in p53 induction and its cellular abundance, tempted us to investigate whether PC4 is a chromatin- associated protein. We have found that
PC4 is indeed associated with the oligonucleosomes and widely distributed in a punctate manner on the compact metaphase chromosomes. It directly interacts with the core histones H3 and H2B and consequently induces chromatin folding. Significantly, Atomic Force Microscopy of PC4 chromatin complexes showed that PC4-mediated chromatin compaction is distinct from the histone H1 induced higher order fiber formation. Knockdown of PC4 by siRNA in HeLa cells was shown to decondense the chromatin \textit{in vivo} and facilitate the over-expression of several genes. Furthermore, silencing PC4 gene expression using a vector based system (34), led to a G2/M checkpoint arrest, suggesting its role in cell cycle progression. These results establish PC4 as a chromatin-associated protein, which may play an important role in chromatin compaction and chromatin-mediated transcriptional regulation.
Materials and Methods

**Sucrose gradient fractionation of chromatin fragments**

The HeLa cells (~50 X 10^6) were grown in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS). The nuclei were prepared from packed cells suspended in hypotonic buffer (10mM Tris.HCl, 10mM KCl and 15mM MgCl2), followed by 10 min incubation at 4°C. The nuclei were digested with MNase (0.2 U/µl) for 10 and 15 minutes at room temperature in nuclei digestion buffer (10% glycerol, 10 mM Tris-HCl pH8, 3 mM CaCl2, 150 mM NaCl, 0.2 mM PMSF). MNase digestion was stopped by the addition of 10 mM EDTA and the digested chromatin was fractionated on a linear sucrose gradient of 15-40% in NTE buffer (10 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) using Beckman ultracentrifuge (SW60Ti rotor) at 28,500 rpm for 14 hrs. Fractions were analyzed as described in the figure legends.

**Immunofluorescence localization of PC4**

The HeLa and mouse L cells were cultured as monolayers on poly L-Lysine coated glass coverslips in DMEM medium. Cells were processed as described in the supplemental data. Condensed mitotic metaphase chromosomes from mouse L cells were spread using a cytobucket rotor, after swelling the cells with 75 mM KCl and probed with purified polyclonal antibody against PC4, followed by secondary antibody conjugated to rhodamine. To stain the chromosomal DNA Hoechst 33258 (Sigma) was used.
**In vivo and in vitro histone interaction assays**

The *in vivo* PC4-histone interactions were investigated by performing M2-agarose pull down assay from the FLAG-PC4 transfected HeLa whole cell extracts, followed by immunoblotting by anti histone polyclonal antibodies. The histone interaction ability of PC4 was further characterized by incubating 5 µl of Ni-NTA beads with 1 µg of His$_6$-PC4 and 200 ng of recombinant (Xenopus) individual histones H2A, H2B, H3 and H4 in a final volume of 200 µl in BC buffer containing 150 mM KCl supplemented with 30 mM imidazole at 4°C for 2.0 hrs. The beads were washed five times (1 ml each) with the incubation buffers. The Ni-NTA agarose pull down complexes was analyzed by western blotting using anti H2A, H2B, H3 and H4 polyclonal antibodies. Control experiments were performed with 5 µl of Ni-NTA beads incubated with 200 ng of individual recombinant histones H2A, H2B, H3 and H4 in the same buffer. In order to map the domain of histone H3 or H2B involved in the interactions with PC4, GST-pull down assays were performed as described elsewhere (33). GST-tagged deletions of each of histone H3 and H2B - NG (N-terminal + Globular), GC (Globular + C-terminal) and G (Globular) domain were cloned, expressed and purified (Supplementary Fig. 1D, 1E) and interaction studies were done with native PC4 in presence of 150 mM NaCl. For scoring the interaction, GST-pull down assay was done followed by probing with anti-PC4 antibodies. The probability of PC4 interaction with the centromeric histone H3 variant CENP-A was verified by immunopull down assays (by anti-HA-antibody) using the whole
cell extract prepared from the HeLa cells transfected with HA-CENP-A mammalian expression construct.

**Circular Dichroism Spectroscopy**

The Circular Dichroism (CD) spectrum of H1 stripped chromatin (0.6 mg/ml) and complexes with different proteins individually: histone H1, PC4 and HMGB1 were recorded after incubation at 25°C for 90 mins or as indicated in the figures in 10 mM Tris-HCl and 25 mM NaCl, pH7.4. The spectra were recorded at room temperature in a JASCO model J715 spectropolarimeter from 250-300 nm.

**Reconstitution of chromatin template**

The 100 kb chromatin was reconstituted using plasmid DNA and highly purified HeLa core histones as described earlier (24). In brief equal amounts (0.5 µg) of the purified DNA and the histone octamer were mixed in Hi-buffer [10 mM Tris-Cl (pH 7.5), 2 M NaCl, 1 mM EDTA, 0.05 % NP-40, and 5 mM 2-mercaptoethanol], and placed in a dialysis tube (total volume, 50 µl). The dialysis was started with 150 ml of Hi-buffer with stirring at 4 °C. Lo-buffer [10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.05 % NP-40, and 5 mM 2-mercaptoethanol] was added to the dialysis buffer at a rate of 0.46 ml/min, and simultaneously, the dialysis buffer was pumped out at the same speed with a peristaltic pump so that the dialysis buffer contained 50 mM NaCl after 20 h. The sample was collected from the dialysis tube and stored at 4 °C. The chromatin template with the tail-
less core histones was also reconstituted as described above except the ratio of DNA (0.5 µg) to tail-less histones (0.365 µg) was altered to 1.37: 1.

**AFM**

The histone H1 or PC4, were mixed with the reconstituted chromatin and incubated on ice for 5~60 min. The samples were diluted 10-fold by the fixation buffer containing 0.3 % glutaraldehyde, 50 mM NaCl, and 5 mM Hepes-K⁺ (pH 7.5). After fixation with glutaraldehyde for 30 minutes at room temperature, the samples were dropped onto a freshly cleaved mica substrate, which was pretreated with 10 mM spermidine. After 15 min at room temperature, the mica was washed with water and dried under nitrogen. AFM observation was performed with Nanoscope IIIa or IV (Digital Instruments) using the cantilever (OMCL-AC160TS-W2, Olympus) of 129 µm in length with a spring constant of 33-62 N/m in air under the tapping mode. The scanning frequency was 2-3 Hz, and images were captured with the height mode in a 512 x 512 pixel format. The obtained images were processed (plane-fitted and flattened) by the program accompanying the imaging module. For the imaging of the DNA with or without PC4, the sample was diluted by the buffer containing 0.3 % glutaraldehyde, 5 mM Hepes-K⁺ (pH 7.5), and 5 mM MgCl₂, and then put on a freshly cleaved mica substrate immediately. After 15 minutes at room temperature, the mica was washed with water and dried under nitrogen gas. Images of only proteins (H1 and PC4) were recorded upon incubation of the proteins (0.2 µg/µl) in the fixation buffer for 30 min as described above.
RNA interference

The siRNA sequence targeting PC4 gene corresponded to the nucleotides 157-177 of the coding region relative to the first nucleotide of the start codon (sense: “5’r(ACAGAGCAGCAGCAGCAGA)dTT3’”; antisense:
“5’r(UCUGCUGCUGCUGCUGU) dTT 3’”) were synthesized. As a control we used the scrambled RNA with the sequence – sense: “5’r(GAAAGGCAACGACCGACAC)dTT3’”; antisense:
“5’r(GCGAACACUAACGUACCUCAU)dTT3’”). HeLa cells were transfected with siRNA and scrambled RNA using Lipofectamine 2000 Plus (Invitrogen) according to the manufacturer’s protocol. For RT-PCR total mRNA was isolated using Trizol reagent (Invitrogen). The mRNA was subjected to RT-PCR using the enzyme Superscript II to generate the cDNA library. Subsequently PCR was set using gene specific primers for PC4 and β Actin (loading control). The silencing of PC4 expression was also confirmed by performing western blotting analysis and immunofluorescence using purified polyclonal antibodies against PC4. Silencing was also done using a vector-based system where PC4 siRNA (sense: 5’GATCCCCACAGAGCAGCAGCAGCAGATTCAAGAGATCTGCTGCTGC TGCTCTGTTITT3’; antisense: 5’AGCTAAAAACACAGACAGCAGCAGCAGGATCTCTTGAAAATCTTGCTGCT GCTG CTCTGTGG3’) was cloned in tandem with a GFP expression cassette into pGShin2 plasmid (34), a kind gift from Dr. Shin-ichiro KOJIMA. GFP positive cells were sorted for FACS analysis.
Microarray Analysis

The total RNA was isolated from untransfected HeLa cell (control) and PC4 knocked down HeLa cells (siRNA transfected) using RNaeasy kit (Qiagen, CA, catalog no.-74104). The RNA samples were quantified by nanodrop (ND1000 spectrophotometer) and analyzed on formaldehyde –agarose gel. The micromax TSA indirect labeling kit (Perkin Elmer Life Sciences) was used to synthesize the labeled cDNA from 5 µg of total RNA that was further hybridized on the array by the tyramide signal amplification method. All steps were carried out according to manufacturer's recommendations (www.nen.com/pdf/penen264-mmaxaminated_card.pdf).

The microarrays used in this study (human19kv7) were procured from the Microarray center, University Health Network, Toronto, Ontario. Each array carries 19,200 spots from the human genome, arranged in 48 individual arrays of 400 spots each. Measurement of the fluorescence corresponding to hybridization intensities was performed with the ScanArray Express Microarray Acquisition System (Perkin Elmer) Data were acquired and analyzed by using QUANTARRAY software (Packard Biosciences Version-III). The Genorm.pl software (Genotypic Technology, Bangalore) was used for normalization of the array. Six arrays that included four biological repeats were performed. Each array was done with control versus PC4 knockdown, including a reverse dye hybridization to control for potential dye bias. After doing various statistical analyses and ranking, the four best-quality arrays, corresponding to three forward reactions and one dye swap were selected to calculate the mean fold change.
Clustering of gene expression data was carried out using CLUSTER (Eisensoftware – tree and cluster). One pair of control array (Forward and dye swap) was done using RNAs from untransfected HeLa cells vs. scrambled RNA transfected HeLa cells to test whether the global gene expression change was the result of the transfection or not.

**Cell Cycle Analysis**

HeLa cells were transfected with pGShin2 (vector) or PG7 (PC4 siRNA cloned into pGShin2 plasmid). Propidium Iodide (PI) staining was done as described elsewhere (14). Double positive cells (for GFP and PI) were sorted and analyzed by flow cytometry for the cell cycle distribution. A three-way statistical analysis of variance (ANOVA) was performed using Statistica 5.2B (STATSOFT INC.) software.

**For the following methods please see the Supplementary section:**

1. Expression and purification of recombinant proteins.

2. Synchronization and differential permeabilization of cells.

3. Preparation of histone H1 stripped chromatin.

4. Processing the cells for immunofluorescence.
Results

PC4 is associated with all the chromatin fractions

The human transcriptional coactivator PC4 is a highly conserved nuclear protein, which plays diverse roles in cellular function. Based on the following facts, (i) its ability to bind DNA (59), (ii) undergo post-translational modifications (acetylation and phosphorylation) (32), (iii) act as a transcriptional coactivator (22, 30) and (iv) its cellular abundance, we speculate that PC4 may perform its nuclear functions by tethering to the chromatin. To examine the association of PC4 to the chromatin, we used sucrose density gradient fractionated nucleosomal fragments obtained from HeLa nuclei, partially digested by MNase. The fractionated nucleosomal fragments were analyzed on a 1% agarose gel (Fig. 1A), to detect the presence of nucleosomal DNA in a particular fraction. The same fractions were also subjected to immunoblotting (Fig. 1B), to confirm PC4 association with the nucleosomes. The results show that indeed PC4 is only present in the fractions where nucleosomes are detected (Fig. 1B, panel I), as validated by the presence of histone H3 (Fig. 1B, panel III). To confirm the proper fractioning of the nucleosomal fragments and associated proteins, we also subjected the fractions to immunoblotting analysis using HMGB1 monoclonal antibody (Fig. 1B, panel II). As reported previously (15), HMGB1 was distributed over all the fractions, unlike PC4. Significantly the general transcription factor TFIIA was present in the non-chromatin fraction (Fig. 1B, panel IV, lane 16) but not in the chromatin fractions (Fig. 1B, panel IV, lane 2-15), indicating that
association of PC4 with the chromatin is not non-specific. Taken together these results suggest that PC4 is predominantly associated with the chromatin.

**PC4 is broadly distributed on metaphase chromosomes**

The direct association of PC4 with the mitotic chromatin was further confirmed by analyzing the PC4 distribution in mitotic chromatin and cytosolic fractions of nocodazole- treated HeLa cells, by immunoblotting. Histone H3 and HSC70 antibodies were used as nuclear and cytosolic markers respectively. As expected histone H3 was detected only in the mitotic chromatin fraction and interphase nuclear fraction, whereas the cytosolic protein HSC70 was found in cytosolic fractions of mitotic and interphase cells. Interestingly, PC4 was detected only in the nuclear fraction of interphase and mitotic cells, but not in the cytosolic fraction (Supplementary Fig. 2C, compare lanes 1 and 3 vs lanes 2 and 4). The presence of PC4 in the nuclear fractions prompted us to investigate the strength of association of PC4 to the chromatin. We have addressed the affinity of PC4 to the chromatin by treating HeLa cells with two different types of detergents with diminishing strengths, NP40 (Fig. 1C, lanes 3 and 4) and digitonin (Fig. 1C, lanes 5 and 6). NP40 is a stronger detergent as compared to digitonin (13). Treatment of the cells with only buffer was taken as the control (Fig. 1C, lanes 1 and 2). Though the digitonin treatment could not dissociate PC4 from the chromatin (Fig. 1C, panel I, lanes 5 and 6), the stronger detergent NP40 could release some amount of PC4 in the supernatant (Fig. 1C, panel I, lanes 3 and 4). This indicates that the binding of PC4 to chromatin is not as strong as core histones and also the
linker histone H1, which remained associated with the chromatin upon NP40 treatment (as shown by the western blotting using histone H3 and H1 antibodies) (Fig. 1C, panel II and III, lanes 3 and 4). Presence of HSC70 (a cytoplasmic marker) only in the supernatant fraction irrespective of the types of detergent treatment confirms the experimental integrity of the system (Fig. 1C, panel IV, lanes 3 and 5). These data suggest that PC4 is tightly bound to the chromatin although the binding affinity is not as strong as core histones or linker histone H1. In order to visualize the chromatin association of PC4, immunofluorescence localization of PC4 was performed in HeLa and mouse L cells using affinity purified highly specific polyclonal PC4 antibody. The results show a predominant localization of PC4 in the nucleus of both the cell lines, as expected (data not shown). The nuclear association of PC4 was further investigated during the mitotic division of HeLa cells. As it has been depicted in Fig. 2A, PC4 was found to be associated with the chromosomes throughout the different stages of mitosis, indicating its association with individual metaphase chromosomes. To find out the chromosomal distribution of PC4, chromosome spreads were made from metaphase arrested mouse L cells and HeLa cells and probed with the PC4 antibody. Significantly, it was found that PC4 is distributed throughout the entire chromosome arms in both mouse L-cells (Fig. 2B) and HeLa cells (data not shown) in a punctate manner without any apparent chromosome specificity. Interestingly, PC4 is not associated with the chromatin in the centromeric region (Fig. 2B, merge and also indicated by arrows).
The relative amount of PC4 in the different stages of cell cycle was also assessed, biochemically (45). HeLa cells were arrested in G0/G1 stage of cell cycle by serum starvation for a period of 3 days, followed by serum replenishment for 3 hrs and a consistent increase was observed in the amount of PC4 upon serum stimulation (Fig. 2C, compare lanes 3 vs 4). Furthermore the amount of PC4 was also substantially high when the cells were arrested in G1/S phase of cell cycle by a double Thymidine and Hydroxyurea block (Fig. 2C, lane 5). On the other hand, Nocodazole treatment, leading to a pre-metaphase arrest showed a large amount of PC4 present in the Mitotic stage as compared to Interphase (Fig. 2C, compare lane 1 vs 2). These results therefore suggest that though PC4 is present throughout all the stages of cell cycle, as observed by immunofluorescence studies, there is substantial difference in the amount of the protein in the different stages of cell cycle. The higher amount of PC4 present in Mitotic stage as compared to Interphase, led us to investigate the strength of interaction of PC4 with the chromatin in these stages of cell cycle. The treatment of chromatin with 0.2% NP40 also did not lead to a complete removal of PC4 from the chromatin fraction (Fig. 2D, panel III). In fact it was observed that the amount of PC4 released in the supernatant fraction in mitotic stage was lesser than the interphase stage indicating a tighter association of PC4 to the mitotic chromatin (Fig. 2D, panel III, compare lanes 1 vs 3). In contrast both histone H3 and H1 was found to be tightly bound to the chromatin fraction in mitotic and interphase stage of cell cycle (Fig. 2D, panel I and II), as treatment with 0.2% NP40 could not mobilize these proteins.
Taken together, these data suggest that PC4 is a bonafide nonhistone chromatin associated protein.

**Preferential interactions of PC4 with different core histones**

The stable association of PC4 to the chromatin could occur through its non-specific DNA binding ability (32), interaction with bookmarked general transcription factors (47, 11, 12), other nonhistone chromatin-associated proteins (35, 31) or direct interactions with the core histones. Direct interactions of several nonhistone chromatin-associated proteins with the core histones have been shown to contribute to their association with the chromatin (51, 40). In order to examine the histone interacting ability of PC4 in vivo the FLAG tagged PC4 mammalian expression vector was transfected into the HeLa cells. The expression of this construct was confirmed by western blotting analysis using both anti-FLAG and anti-PC4 antibodies (data not shown). FLAG-tagged PC4 was pulled down by M2-agarose beads, from whole cell lysates prepared from transfected cells and the pull down complex was analyzed by immunoblotting with highly specific anti-histone antibodies. It was found that PC4 could efficiently pull down all the core histones (Fig. 3A, lane 2). Furthermore, the PC4-GST construct could also pull down the core histones (Fig. 3A, lane 4) from the whole cell extract, but not the GST alone (Fig. 3A, lane 3). In order to find out the specific site of interaction(s) of PC4 on the nucleosome, histone interaction experiments were carried out using recombinant individual core histones and His$_6$-PC4. The results show that PC4 bound to the Ni-NTA beads could predominantly pull down histone H3 and H2B.
The amount of histones H2A and H4 pull down by PC4 was found to be almost negligible, as compared to H3 and H2B (Fig. 3B, compare lane 3 of panels I and IV vs II and III). These data argue that PC4 directly interacts with histones, with a distinct preference for histone H3 and H2B. Interestingly, PC4 did not show any interaction with histone H1 (Fig. 3B, panel V, lane 3). We further analyzed the relative strength of PC4 interaction with the core histones. For this purpose the PC4-core histone complex was washed with increasing concentration of salt in the washing buffer. PC4-histone interaction was found to be quite stable up to 200 mM salt concentration beyond which the complex could barely be detected (Fig. 3C, compare lane 4 vs 5).

Site of interaction on the core histone occasionally determines the structural and functional role of chromatin interacting nonhistone proteins. Therefore we investigated the domains of histone H3 and H2B involved in the PC4 and histone contact. Three GST-fused deletion mutants, consisting of NG (N-terminal + Globular), GC (Globular + C-terminal) and G (Globular) domains of each of histone H3 and H2B (Supplementary Fig. 1D, 1E) were constructed. The western blotting analysis shows that PC4 interacts quite efficiently with GC and G domains of both histones H3 and H2B as compared to that of full length (FL) (Fig. 3D, panel I and II, compare lanes 2 vs 4 and 2 vs 5). This indicates that the Globular domain of histone H3 and H2B is the preferential interaction site for PC4. Interestingly, presence of N-terminal tail along with the globular domain (i.e. NG) only significantly inhibits the interaction of PC4 with the core histones,
(Fig. 3D, compare lanes 2 vs 4) indicating that the N-terminal tail rather plays a negative role in this phenomenon.

PC4 is broadly distributed over all the chromosome arms except the centromeric region, as evidenced by chromosomal localization of PC4 (by immunofluorescence). If the chromosomal localization of PC4 were a result of its interaction with histone H3, the absence of PC4 over the centromere could be attributed to its inability to interact with the centromeric variant of histone H3, CENP-A. Therefore, we were interested to investigate whether PC4 interacts with CENP-A. The mammalian expression construct of HA-tagged CENP-A clone was transfected into the HeLa cells and the expressed protein was pulled down by anti-HA-sepharose beads. Immuno-blotting of the pulled down complex using PC4 and histone H4 antibodies revealed that CENP-A could efficiently interact with histone H4 (Fig. 3E, panel II, lane 2) (62), while PC4 did not show any detectable interaction with CENP-A (Fig. 3E, panel I, lane 2). Taken together these results suggest that PC4 binds to the chromatin through the direct interaction with globular domain of core histones H2B and H3 but not the centromeric variant of histone H3, CENP-A.

**PC4 induces chromatin condensation**

The stable chromatin association, direct interaction with the core histones and uniform (punctate) distribution over the metaphase chromosome arms, suggests that PC4 may have a specific role to play in chromatin organization. The effect of PC4 in the chromatin organization was addressed by employing circular
dichroism spectroscopy using the H1 stripped chromatin fiber. Incubation of PC4 with H1-stripped chromatin decreased the molar ellipticity (peak) value of the chromatin spectra, indicating that PC4 is inducing condensation of the chromatin (Fig. 4A). This observation was further confirmed by the addition of equi-molar amount of histone H1 in a separate reaction using an equivalent amount of H1 stripped chromatin. The results show that histone H1 decreases the ellipticity value to the same extent as compared to PC4. Addition of HMGB1, which dynamically interacts with chromatin, could not alter the chromatin spectra as expected (10) (Fig. 4A). Interestingly, an equimolar amount of PC4 could not alter the ellipticity peak value of total DNA isolated from the HeLa cells, indicating the necessity of a chromatin template in general (specifically histones) for PC4 to induce the chromatin compaction (Fig. 4B). In order to visualize the PC4-mediated chromatin condensation, we subjected the 100 kb reconstituted chromatin (Fig. 4C) with either PC4 or H1 complexes to Atomic Force Microscopy (AFM). Significantly, though histone H1 induced the formation of expected higher ordered fiber structure (Fig. 4D), incubation of the reconstituted chromatin with PC4 led to the formation of distinct compact globular structure (Fig. 4E). In agreement with the circular dichroism spectroscopic data, addition of recombinant PC4 to the purified DNA (Fig. 4G) had no visual effect on the folding of the DNA molecules (Fig. 4G vs 4F).

The distinct difference in the AFM images of histone H1- mediated chromatin folding and PC4 induced compaction, tempted us to investigate further the mechanistic details of the chromatin organization by these two proteins. In order
to quantitate the chromatin condensation, dose dependent condensation of the histone H1-stripped chromatin from HeLa cells was compared between H1 and PC4 by circular dichroism spectroscopy (Fig. 5A-B). Though PC4 seems to be less efficient as compared to histone H1, gradual increase of the protein concentration decreased the ellipticity value in a regular fashion (Fig. 5A vs 5B). The AFM images of similar experiments using the 100 kb reconstituted chromatin and varying concentrations of histone H1 and PC4 (expressed in the ratios of core histone: H1 or PC4) (Fig. 5C-F or 5G-J) showed that PC4-mediated chromatin globule formation is achieved optimally at the equimolar ratio of core histone and PC4 (Fig. 5I). Further increase in the concentration of PC4 did not increase the size of the globule (Fig. 5I vs 5J). However, when core histone: histone H1 molar ratio was increased to 1:1.25, a highly folded fiber structure could be observed (Fig. 5E vs 5F).

Furthermore, we have also analyzed the time-dependent chromatin organization by PC4 and the linker histone H1. Interestingly, histone H1 could fold the chromatin very rapidly (within 5 mins) as revealed by both the CD spectroscopic analysis (Fig. 6A) and AFM images (Fig. 6C-F). On the other hand chromatin compaction (formation of globular structure) by PC4 was found to be a gradual process, which required at least 15 mins to initiate the compaction process (Fig. 6B and 6G-J). These results suggest that though both histone H1 and PC4 induce chromatin condensation, the type and mode of actions are distinctly different.
Interaction with core histones is essential to induce chromatin compaction by PC4

PC4 interacts with core histones H3 and H2B in vitro and induces chromatin condensation. However, the functional requirement of histone interaction in this phenomenon needs to be established. In order to address the connection between histone interaction and chromatin condensation by PC4, we made different deletion constructs of PC4 (1-62, 1-87, 22-127, 62-127) as shown in supplementary Fig. 1B. It was found that except PC4 (1-62), all the other PC4 deletion mutants could interact with both the core histone H3 and H2B (Supplementary Fig. 3B). Based on these results an internal deletion construct of PC4, PC4 Δ62-87 was made (Fig. 7A). As expected PC4 Δ62-87 could not interact with core histones H3 and H2B (Fig. 7B, panels I and II, lane 3). These deletion mutants of PC4 were then used in the CD spectroscopic analysis. Interestingly, it was observed that except PC4 (1-62) (Supplementary Fig. 3C) and PC4 Δ62-87 (Fig. 7C), all the other mutants could induce chromatin condensation with a varying ability to condense chromatin as compared to the full-length protein. The AFM images using reconstituted chromatin and PC4 Δ62-87 further confirms these results. Though the equi-molar amount of PC4 could efficiently induce the chromatin globule formation (Fig. 7E), the addition of PC4 Δ62-87 showed negligible effect on the reconstituted chromatin images (Fig. 7F), suggesting that PC4 induce the chromatin compaction through the direct interactions with the core histones.
PC4 interacts with the tail-less globular domains of histones (H3 and H2B) quite efficiently (Fig. 3D) and the role of N-terminal tail is rather negative. To investigate the functional validity of these interactions, 100 kb chromatin template was reconstituted using tail-less octameric histones. As reported previously tail-less histone could be organized into a chromatin template similar to the wild type histones (17) (compare Fig. 8A vs 8B). In agreement with the histone interaction data we observed that PC4 could efficiently condense the chromatin, reconstituted with the tail-less histones (Fig. 8C). Thus, for the PC4-mediated chromatin compaction the flexible N-terminal tails of histones may not be essential.

**Knocking down of PC4 expression alters chromatin organization (in vivo), gene expression and cell cycle progression**

In order to validate the chromatin condensation by PC4 in vivo, PC4 expression was knocked down by RNA interference, using a double stranded (21 bp) RNA duplex, homologous to PC4 mRNA. A scrambled RNA of same base composition and similar length was used as a control for these experiments. The knockdown of PC4 was confirmed by immunoblotting (Fig. 9A), immunofluorescence (data not shown) and RT-PCR (Supplementary Fig. 5A). We next investigated the effect of PC4 repression on the global chromatin folding in human cells by the MNase accessibility assay. The equal amount of chromatin used in the experiment was confirmed by western blotting using antibodies against different core histones and histone H1 (Supplementary Fig. 5B). The results showed that while the MNase pattern of the chromatin isolated from scRNA transfected HeLa cells resembled
that of the untransfected control, the chromatin of the siRNA transfected HeLa cells was more susceptible to the MNase digestion (Fig. 9B lanes, 2 and 3 vs lane, 4). Taken together, these data suggest that the silencing of PC4 decompacts the higher ordered chromatin structure in vivo. These results were further confirmed by subjecting the chromatin isolated from siRNA and scRNA transfected cells, in a multiple time point MNase digestion assay. In agreement with the single time point of digestion the chromatin isolated from siRNA transfected cells was found to be more accessible to MNase (Fig. 9C, lanes 2-4 vs 5-7).

Since siRNA knockdown of PC4 opens up the chromatin as evidenced in the MNase accessibility assays, the absence of PC4 would presumably upregulate a substantial number of genes in the cells. To investigate the effect of PC4 knockdown on the global gene expression, we carried out genome wide differential expression analysis in siRNA transfected HeLa cells using microarray. The expression profile analysis identified 128 up-regulated genes and 49 down-regulated genes in response to PC4 knockdown. In all experiments, a substantial number of the affected genes were of unknown function. We have clustered the genes according to the level of their expression (Fig. 9D). The extensive table with all of the differentially expressed genes grouped into functional groups is available as supplemental data, Table I. The control experiment was carried out with the untransfected HeLa cells and scrambled RNA transfected HeLa cells did not show any differential regulation (data not shown). In order to validate the microarray data, two candidate genes were chosen and after knocking down of PC4 expression, their expression levels were compared by Real Time PCR.
analysis. It was found that, as compared to the scRNA transfected HeLa cells there was an enhancement in the expression of RPL10 gene upon PC4 siRNA transfection (Fig. 9E). On the other hand S100A11 gene expression was reduced upon PC4 siRNA transfection, as compared to the scRNA transfected cells (Fig. 9F). These results were in agreement with the microarray data. The down-regulation of several genes in the absence of PC4 is not surprising since it is a positive coactivator. The up-regulation of a large number of genes suggests that at least partially, knocking down of PC4 results in a global opening of the chromatin.

The altered gene expression pattern, upon knocking down of PC4 in HeLa cells suggests that it may play a significant role in the cell cycle regulation. We designed a vector based knocking down system to probe into the role of PC4 in cell cycle. In agreement with the shRNA vector mediated silencing of the PC4 gene (Fig. 10A), Hoechst staining, followed by confocal microscopic imaging (Fig. 10B) of the control (vector transfected) and knock down of PC4 (PG7 transfected) showed differential density of compaction of chromatin DNA (Fig. 10B compare panel I vs II). The PC4 knock down cells lost most of the densely packed chromatin (Fig 10B, panel II). Further we also observed a significant reduction in the number of metaphase plates upon silencing PC4 gene expression in comparison to the control (data not shown). However, after the control and PG7 transfection, GFP positive cells were sorted and demarcated as a sub-population R1, represented in the Dot plot analysis (Fig. 10C, panel I and II). Cell cycle analysis of R1 population show that the percentage of cells in G1 + S phase
of cell cycle is 52.4% in control and 27.95% upon PC4 knock down. On the other hand, there was an increase in G2/M cell population from 13.73% in control to 46.12% in PG7 transfection. A drop in pre-G1 cell population was also observed – 33.87% and 25.94% in control and PG7 transfection respectively. These results have been represented in the Histogram analysis (Fig. 10C, panel III and IV). Three consecutive repeats of the experiment indicate that upon PC4 knock down there is a ~ 2 fold drop in G1 + S and a consecutive (~3 fold) increase in G2/M cell population (Fig. 10D), suggesting a G2/M cell cycle arrest. Statistical analysis of the FACS results by ANOVA indicated that the observation made is significant as reflected by the standard parameters $F_{2, 4} = 11.12; p < 0.02$. These results reflect that the nonhistone chromatin component PC4 is involved in chromatin compaction and has significant role to play in maintenance of cell cycle.
Discussion

Multifunctional, highly abundant, nuclear proteins are often associated with chromatin having distinct functional consequences (7, 38, 29). Though PC4 was originally discovered as a positive coactivator for RNA polymerase II (Pol-II) driven, activator dependent transcription from the DNA template, further analysis showed that PC4 is also needed for replication (43), repair (57) and the proper termination of multiple rounds of Pol-III transcription (58). This functional diversity prompted us to investigate PC4 from a broader perspective, and in agreement with our speculation, we have found that indeed PC4 is stably associated with the chromatin through all the stages of the cell cycle. The distinct and punctate appearance of PC4 on the metaphase chromosomes, suggests its role in chromatin organization. We have shown that PC4 induces chromatin compaction and the formation of a very distinct type of globular structure as revealed by CD spectroscopy and AFM analysis. Knocking down of PC4 by siRNA, rendered the *in vivo* chromatin much more accessible to MNase and led to upregulation of several genes, suggesting the cellular role of PC4 as a nonhistone chromatin organizing protein. Silencing of PC4 gene expression (by a vector based system, PG7) also caused a G2/M checkpoint arrest indicating its function in cell cycle progression.

The stable association of PC4 to the chromatin was confirmed by the fact that on treatment with a weak detergent digitonin (13) PC4 remained associated with the chromatin (Fig. 1C, lane 6, Panel I). It was also observed that on treatment with
NP40 substantial amount of PC4 was still bound to the chromatin. In case of HMGB1, though digitonin treatment could release the protein from chromatin to a lesser extent, exposure to NP40 led to the dissociation of more than 70% HMGB1 (15). Taken together these data suggest that PC4 is more stably associated with the chromatin as compared to HMGB1. However the affinity of PC4 to the chromatin is not as high as histone H3 or H1. The treatment with NP40 could not mobilize core histone H3 and the linker histone H1 from the chromatin (Figure 1C, Panel II, III). The mechanism of high affinity association of PC4 to the chromatin is yet to be elucidated. We have shown that PC4 preferentially interacts with core-histones H3 and H2B (Fig. 3B). Most of the other chromatin-associated proteins also interact with the core histones directly (51, 40, 39, 55, 44) except HMGN2, which does not interact with the free histones (8). However it is not known whether interaction with the core histones is important for the chromatin association and consequent function of these proteins. Detailed domain analysis, showed that globular domain of histone H3 or H2B is the preferential docking site of PC4 (Fig. 3D). The N-terminal tails of the histones were found to have an inhibitory effect on the interaction with PC4. In case of the polycomb group of protein PRC1, the bridging of nucleosomes is also found to be independent of histone N-terminal domain (48). Functional role of the flexible N-terminal tail of histones has been further underscored when it was observed that PC4 could condense chromatin reconstituted with the tail-less histones as visualized by AFM (tail-less core octamer: PC4:: 4:1) (Fig. 8C). In fact when the stoichiometry of tail-less octamer: PC4 :: 1:1, individual nucleosomes could not be observed (data
not shown), rather the entire chromatin fiber condensed into a large globule, unlike distinct condensed zones observed with intact core octamer used in the chromatin reconstitution maintaining the same stoichiometry (Fig. 5I). Presence of PC4 throughout the chromosome arms with the exception of centromeric region (Fig. 2B, arrows), strongly argues that PC4 is associated with the metaphase chromatin through its interaction with the histones. The fact that the centromeric region contains an altered form of nucleosomes with H3 being replaced by its variant CENP-A (49) and our finding that PC4 predominantly interacts with histone H3 but not to the centromeric variant CENP-A strongly supports this hypothesis. Furthermore it also suggests that in vivo PC4 prefers to interact with the canonical nucleosomes rather the centromeric nucleosomes (6) containing the histone variants CENP-A.

The data regarding the stable and regular association of PC4 with the chromatin, definitely suggest a significant role of PC4 in chromatin organization. By employing circular dichroism spectroscopy (which measures the conformational change of the chromatin/DNA) (36, 27), visualization of chromatin compaction (upon ectopic addition of PC4) by AFM and RNA- interference mediated knock down of PC4, we have shown that indeed PC4 stimulates the chromatin condensation both in vitro and in vivo. The CD spectral data showed that PC4 folds the histone H1 stripped chromatin to the same extent as that of histone H1. Although the role of histone H1 in the chromatin condensation is not clearly understood, as per the general consensus the linker histone induced contraction of the inter-nucleosomal angle (not the bending of the linker DNA) is responsible for
the organization of the solenoid structure and its further folding (54). However, PC4 folds the chromatin into a very distinct type of higher ordered globular structure unlike the linker histone H1 induced folded fiber (compare Fig. 4D vs 4E). There are few chromatin interacting proteins that are known to form this kind of structure which include the Polycomb group of proteins (17) and MENT protein (50). Both of these proteins cause chromatin condensation in vivo and in vitro. The functional cooperation of these types of proteins including PC4 with the linker histone H1 presumably establishes the cell cycle specific physiological organization of chromatin domains. We have found that the PC4 mutants that are not capable of interacting with the core histones H3 and H2B could not fold the H1-stripped chromatin. These data clearly indicate that interaction with nucleosomal histones is essential to induce the chromatin condensation by PC4.

The possible mechanism of PC4-mediated chromatin condensation could be through the linking of different widely separated nucleosomes by PC4 through the direct interaction with the histones, resulting in looping out of chromatin. These loops may be further condensed by PC4, in a similar manner, giving rise to the large globular structures observed in our AFM studies. Further investigation is necessary to elucidate the molecular details of the condensation process.

The expression of PC4 was knocked out efficiently by duplex siRNA or vector based system (PG7) (34) in HeLa cells. As expected, knocking down of PC4 significantly increased the accessibility of MNase to the HeLa chromatin indicating that PC4 is involved in the global compaction of the chromatin. The Hoechst stained images of the nuclei after knocking down PC4 by PG7 also
shows chromatin decompaction unlike the distinct condensed regions observed in the control (vector transfected). These data demonstrate that indeed the multifunctional coactivator is involved in the organization of higher order chromatin structure.

The established functions of PC4 suggested that it could be an essential gene for the cells. Therefore knocking down of PC4 was expected to cause the down regulation of a vast majority of genes. However, the data presented in the Figure 10 and Table 1 (supplemental data), clearly indicates that by siRNA-mediated knockdown of PC4 the number of genes that are upregulated is 3-fold more than the number of down regulated genes. To best explain these observations we propose that the absence of PC4 causes at least partial opening of different chromatin territories and facilitates transcription. Though negative role of PC4 in transcription has been scarcely reported (18, 61), the number and the fold expression of upregulated genes prompts us to suggest that PC4 strongly interacts with the core histones and thereby induces chromatin condensation to repress the gene expression. Surprisingly we noticed that, although PC4 is a multifunctional general transcription coactivator and chromatin organizing protein, knocking down of it affects relatively fewer numbers of genes. Presumably, the functional redundancy of other transcriptional coactivator and chromatin proteins with PC4 could help to restore the regulation of several genes under this condition. Significantly knocking down of three H1 genes (H1c, H1d, H1e) (50% of the total H1) in mouse ES cells, caused a dramatic change in chromatin organization, but in agreement with our present observation, affected a fewer number of genes (29
genes) (16) as compared to PC4 (177 genes). It would be interesting to find out the alteration of global gene expression upon knocking down of both PC4 and these H1 genes. Detailed analysis of the candidate genes picked up in microarray upon knocking down PC4, revealed that there are a number of cell cycle regulatory genes (like CDC10), and those belonging to the signal transduction cascades (like MAPK4, MAP3K7IP1, WNT5B), that are differentially expressed. Interestingly, CDC10 is downregulated, which is an important component of the transcription complex in the S-phase of cell cycle (5). Furthermore there are two candidates, belonging to the chromatin-associated protein (CAP) family- STK4 and SAFB, which are also upregulated upon PC4 knockdown. SAFB induces chromatin condensation and has inhibitory role in cell proliferation (41). FACS analysis after PC4 knockdown shows a drop in G1+ S and an increase in G2/M population of cell cycle, establishing its role in cell cycle progression.

The present finding that the global transcriptional coactivator, PC4 is a chromatin-associated protein inducing chromatin folding in vitro as well as in vivo reveals a new facet of this highly conserved nuclear protein. Its ability to interact with histones suggests that this versatile nuclear factor could play a significant role in chromatin dynamics, regulating replication, repair and transcription. In order to understand the mechanism of PC4 function in the chromatin context, the functional correlation of PC4 with histone H1 and other nonhistone chromatin proteins (for example HP1, HMGs and PARP1) should be addressed. In this context it could be speculated that the posttranslational modifications of PC4 may
regulate its multifunctional activity, ranging from chromatin organization to transcription.
Acknowledgements

We thank Drs. Koji Hisatake, Utpal Tatu, M.R.S Rao and Shin-ichiro KOJIMA for providing valuable reagents and V. Swaminathan, Sutirth Dey and M. Shakarad for helpful discussions. This work is supported by JNCASR, Department of Science and Technology and the Department of Biotechnology program support to IISc for genomics initiative (PPS), Government of India. KT would like to thank Special Co-ordination Fund (104041500002) and COE Research Grant (13CE2006) from Ministry of Education, Culture, Sports, Science and Technology of Japan. TK is a recipient of UICC fellowship and National Bioscience Career Development Award, DBT, Govt. of India. SL was supported by EMBO postdoctoral fellowship. KB and SG are Research Fellows of CSIR, India.
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Figure legends:

Figure 1: PC4 cofractionates with HeLa Nucleosomes in sucrose gradient:
(A) HeLa nuclei were partially digested with micrococcal nuclease (MNase) and fractionated on a 15-40% sucrose gradient. Individual fractions were deproteinised, and the alternative fractions were resolved on a 1 % agarose gel and visualized by ethidium bromide staining. (B) Corresponding fractions were analyzed by western blotting for the presence of PC4 (panel I), HMGB1 (panel II) histone H3 (panel III) and TFIIA (panel IV), using respective antibodies. In panel IV, lane 16 corresponds to the non-chromatin fraction. The lane rP stands for the recombinant proteins (PC4 / HMGB1 / H3 / TFIIA). (C) Subcellular localization of PC4 and its relative affinity to chromatin. The cells were incubated in a buffer with 0.1% NP40 or 40 µg / ml digitonin. After the incubation the supernatants (S) and the remnants of permeabilised cell pellets (P) were analyzed by western blotting using antibodies against PC4 (I), histone H3 (II), histone H1 (III) and HSC70 (IV).

Figure 2: Association of PC4 to the chromosome in different stages of mitosis: (A) HeLa cells were fixed and stained for PC4 with the purified polyclonal antibody against PC4 followed by FITC conjugated secondary antibody and for DNA with Hoechst. Representative cells at different stages during mitosis: prophase (I), prometaphase (II), metaphase (III), anaphase (IV), telophase (V) and interphase (VI) are shown. Green indicates chromosome
stained with PC4 antibodies and the blue, staining of the DNA with Hoechst. (B) Distribution of PC4 on mitotic chromosomes. The condensed mitotic metaphase chromosomes from mouse L cells were spread on a slide and stained with Hoechst for DNA (I). The chromosomes probed with purified polyclonal antibody against PC4, followed by secondary antibody conjugated to rhodamine (II). The third panel (III) shows a merge of the antibody and DNA stained images. One of the individual chromosomes has been highlighted to indicate the centromere (by arrow) in the bottom panel. (C) Distribution of PC4 throughout the different stages of cell cycle. Relative amounts of PC4 present during Mitosis (lane 1), Interphase (lane 2), G0/G1 arrest (by serum starvation) (lane 3), release of G0/G1 arrest (upon serum stimulation) (lane 4), G1/S arrest (lane 5) in comparison to asynchronous cell population (lane 6) were assessed by probing with anti-PC4 antibodies in western blotting analysis. As loading control western blotting analysis was done with anti-Actin antibodies (panel II). (D) Comparative affinity of PC4 to the chromatin during Interphase and Mitotic stages of cell cycle. The Mitotic and Interphase stage cells were incubated in a buffer with 0.2% NP40. After the incubation the supernatants (S) (lanes 1 and 3) and the remnants of permeabilised cell pellets (P) (lanes 2 and 4) were analyzed by western blotting using antibodies against PC4 (I), histone H1 (II) and histone H3 (III).

**Figure 3: PC4 interacts with histones:** (A) To find out the histone interaction ability of PC4 in vivo, HeLa cells were transfected with FLAG-PC4 (F-PC4) mammalian expression construct. The expressed F-PC4 was pulled down by M2-


Agarose beads, and the complex was subjected to western blotting analysis using different antibodies as indicated (lane 2). Lane 1, untransfected control, lanes 3 and 4, pull down complexes obtained from HeLa whole cell extract incubated with GST and PC4-GST. (B) The in vitro interactions were assessed by incubating 1 µg of His$_6$-PC4 bound to Ni-NTA beads with 200 ng of individual recombinant core histones (H2A, H2B, H3 and H4) and the linker histone H1. The complexes were pull down and analyzed by western blotting. Lane 1, individual histones (input); lane 2, the histones incubated with only Ni-NTA agarose; and lane 3, individual histone incubated with Ni-NTA agarose bound to His$_6$-PC4. (C) The strength of interaction of PC4 with the histones was checked by stringency washes with the buffer containing increasing concentration of salts, 100 (lane 3), 200 (lane 4), 300 (lane 5), 400 (lane 6) and 500 (lane 7) mM. (D) Mapping the domain(s) of core histones H3 (panel I) and H2B (panel II) involved in the interaction with PC4: Different deletion mutants were subjected to GST-pull down followed by western blotting analysis using anti-PC4 polyclonal antibodies. PC4 incubated with GST (lane 1), FL (Lane 2), NG (lane 3), GC (lane 4), G (lane 5) domains of the deletion mutants of H3 (panel I) and H2B (panel II). (E) CENP-A does not interact with PC4: HA tagged CENP-A construct was transfected into HeLa cells, the expressed protein was pull down by anti-HA antibody and presence of interacting proteins for example, PC4 (panel I, lane 2) and histone H4 (panel II, lane 2) were analyzed by western blotting. rP, IP and PIS indicate recombinant protein, immunopulldown and pre-immuneserum control respectively. All the interactions were done in presence of 150 mM NaCl.
Figure 4: PC4 induces chromatin condensation: Circular dichroic (CD) spectra of histone H1 stripped chromatin incubated with PC4, H1 and HMGB1 (A). CD spectra of DNA incubated with increasing concentrations of PC4 (B). (C-E) PC4 condense the chromatin fiber into a distinct globular structure: AFM images of the 106 kbp reconstituted chromatin fibers (C) incubated with histone H1 (D) and PC4 (E). The molar ratio of histone H1 (or PC4) to the histone octamer was 1:1. Upon 60 mins incubation on ice the complexes were fixed by 0.3 % glutaraldehyde, mounted on mica and observed under AFM. The 106 kb plasmid DNA similarly incubated with (F) or without (G) PC4 at the same ratio and processed for AFM imaging (see methods).

Figure 5: Comparative dose dependent condensation of chromatin fibers by histone H1 and PC4: Effect of increasing concentration of histone H1 (A) and PC4 (B) on the circular dichroic spectra of histone H1 stripped HeLa chromatin. (C-J) AFM images of the chromatin incubated with various amount of histone H1 (C-F) or PC4 (G-J). PC4 or H1 were mixed with the reconstituted chromatin in 50 mM NaCl; at the molar ratios of the histone octamer to PC4 or H1 of 4:1, 2:1, 1:1, and 1:1.25, respectively.

Figure 6: PC4 induced chromatin condensation is a slow process: Histone H1 (A) and PC4 (B) were incubated with H1 stripped HeLa chromatin at different time points (5, 15, 30 and 60 mins) and subjected to circular dichroism
spectroscopy. (C-J) In order to visualize the time dependent condensation brought about by PC4 the salt dialyzed reconstituted chromatin and H1 (C-F) or PC4 (G-J) were mixed at the 1:1 molar ratio of the histone octamer to PC4 or H1. After keeping on ice for 5 min, 15 min, 30 min, and 60 min, they were fixed by 0.3 % glutaraldehyde and observed under AFM.

**Figure 7: Histone interaction ability is essential for chromatin condensation by PC4:**

Full length and mutant PC4 (A) were incubated with core histones and analyzed by western blotting with antibodies against histone H3 (B, panel I) and H2B (B, panel II). (C) Comparative analysis of chromatin condensing ability of PC4 and PC4 \( \Delta 62-87 \) visualized through CD spectroscopy. (D-F) AFM images of the reconstituted chromatin (D) with PC4 (E) and histone interaction deficient PC4 mutant (F). PC4 or PC4 mutant were incubated for 90 mins with the reconstituted chromatin at the molar ratio of the histone octamer to PC4 was 1:1 and the samples were processed for AFM as described above.

**Figure 8: Histone tails are not essential for PC4-mediated chromatin compaction:**

AFM images of the reconstituted chromatin with wild type (A), tail-less histones (B) and the effect of adding PC4 to the chromatin reconstituted with tail-less histones (C). The molar ratio of the histone octamer to PC4 was 4:1.
Figure 9: siRNA mediated knocking down of PC4 expression induces chromatin decompaction (in vivo) and global gene expression: (A) The expression of PC4 after transfection of siRNA and scRNA was verified by Western blotting analysis. (B) After knocking down of PC4, chromatin was isolated from untransfected (lane, 2), scRNA transfected (lane, 3) and siRNA transfected (lane, 4) HeLa cells and were subjected to partial MNase digestion and analyzed on a 1% agarose gel. (C) Similar MNase digestions were also carried out at three different time points with the chromatin isolated from siRNA and scRNA transfected HeLa cells. Lane 1, 123 bp ladder; lanes 2-4, chromatin isolated from scRNA transfected HeLa cells subjected to 5, 10, 15 mins of MNase digestion and lanes 5-7, same time points of MNase digestions were carried out with chromatin isolated from siRNA transfected HeLa cells. (D) Microarray analysis of gene expression upon PC4 knock- down by siRNA. Hierarchical clustering of the gene expression profiling data obtained by cDNA microarray analysis of siRNA- mediated PC4 knock down HeLa cells. Lanes 1-3, forward reaction and lane 4, dye swap. (E, F) Real Time PCR analysis of the up regulated and down regulated candidate genes, RPL10 and S100A11 upon knocking down of PC4 expression validate the microarray data.

Figure 10: Effect of Knocking down of PC4 on the cell cycle: PC4 expression was silenced by using a vector based system having GFP in the expression cassette. (A) Silencing of PC4 expression was checked by western blotting analysis using anti PC4 antibodies. Lane 1, untransfected, lane 2, vector (pGShin2) transfected and lane 3, PG7 (vector having PC4 siRNA) transfected.
(B) Hoechst staining images of pGShin2 (panel I) and PG7 (panel II) transfected HeLa cells. (C) FACS analysis of HeLa cells upon knocking down PC4 gene expression. GFP positive cells were sorted, and then PI stained cells were analyzed from this sub-population, to look into the effect of silencing PC4 gene expression. Dot plot (panels II and I) and Histogram analysis (panels III and IV) of pGShin2 and PG7 transfected cells are represented in C. (D) The difference in G1 + S, G2/M and PreG1 cell population upon transfecting pGShin2 and PG7 have been shown in a bar graph.
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