Functional Mechanisms of Human Transcriptional Coactivator PC4, a Bona Fide Nonhistone Component of Chromatin

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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To

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December 2006

Declaration

I hereby declare that this thesis entitled **"Functional Mechanisms of Human Transcriptional Coactivator PC4, a Bona Fide Nonhistone Component of Chromatin"**, is an authentic record of research work carried out by me under the supervision of Prof. Tapas K. Kundu at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and that this work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

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Certificate

This is to certify that the work described in this thesis entitled, **"Functional Mechanisms of Human Transcriptional Coactivator PC4, a Bona Fide Nonhistone Component of Chromatin"**, is the result of the investigations carried out by Ms. Chandrima Das in the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (a Deemed University), Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Date: December 12th 2006

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Acknowledgements

It is my great pleasure to thank my mentor Prof. Tapas K. Kundu for his constant support and encouragement. I have always felt that it is my privilege to get trained under his able guidance. The prolific enthusiasm and excitement he nurtures for Science, leaves us charged and it has propelled my research work forward. I also immensely value and thank him for the kind of academic freedom he has provided me to visualize my research problem. He has inculcated the ability of innovative thinking and a sense of responsibility for my work.

I greatly acknowledge the critical comments and constant encouragement of Prof. M.R.S. Rao towards my work. I would like to acknowledge all the faculty members of MBGU for their valuable suggestions, constant support and help. A special thanks to Dr. Ranga Uday Kumar for encouraging discussions and also providing the FACS Instrumentation facility.

I would like to express my gratitude to our collaborators Prof. P. P. Sadhale and Prof. K. Takeyasu for their encouraging discussions on my work. My sincere thanks to Prof. D. Chatterjee, Prof. M. Vijayan, Prof. A. Surolia and Prof. V. Nagaraja for their interest and valuable suggestions during the progress of my work.

I greatly acknowledge Drs. Koji Hisatake, Utpal Tatu, M.R.S Rao, Shin-ichiro KOJIMA, Lee Kraus and James T. Kadonaga for providing valuable reagents.

I would like to thank my lab seniors Dr. Sourav Banerjee, Dr. Balasubramanyam and specially Dr. Swaminathan and Prashanth for their supportive attitude and extremely helpful suggestions. I am also thankful to the other past lab members- Radhika, Febitha, Altaf, Amresh and Sukanya. I acknowledge all the present lab members Kishore, Kiran, Shrikanth, Arif, Ashok, Nagashankar, Jayasha, Selvi, Sailaja, Deepak, Vedamurthy, Dr. Lingu, Dr. Ravindra, Gowda, Srinivas, all the summer trainees and collaborative research students for a lively lab environment. A special thanks to Kiran and Shrikanth for their constant cooperation and help in generating several reagents which has been extensively used in many experiments that form a part of the thesis.

I would like to thank all my batchmates Rahul, Aparna, Anand, Sonali, Debjani and also to Sutirth and Dr. Malik for helpful discussions. I would like to thank all my friends for

making my stay in JNCASR a memorable experience. I thank Sagarika and Angshuman for their care and concern. A special thanks to Mrs. Kundu for her kind hospitality.

I would like to acknowledge the Central Instrumentation Facility (CIF) of Molecular Biology and Genetics Unit for Confocal Microscopic facility (Carl Zeiss), Circular Dichroism Spectroscopic facility (Jasco) and Microarray facility (Perkin Elmer). A special thanks to Suma for helping me with the confocal imaging.

My stay in JNCASR has been extremely eventful. I got the opportunity to attend the 7th Transcription Assembly Meeting (December 4th –6th, 2003), Indo-Japan Workshop on understanding of chromatin structure function (January 20th –23rd, 2005), Nuclear Architecture: Chromosome-Chromatin Dynamics (December 11th -13th, 2006) organized in JNCASR. I would like to acknowledge the Centre for organizing such wonderful scientific meet. I would also like to acknowledge CSIR, SBC, INSA and JNCASR for the financial support provided to me to attend the 45th Annual Meeting of "The American Society for Cell Biology 2005" (December 10th – 14th, 2005) at San Francisco, California, USA. These conferences and wet-lab workshops has given me a new exposure and encouraged scientific interest in me.

I am thankful to the people of Administrative and Academic section for their support. I would also like to acknowledge the Library, Computer Lab facility and the Animal facility, which have been of great help throughout.

The financial assistance from JNCASR is greatly acknowledged.

I am grateful to my parents, sister, husband and all my family members for their unconditional support, faith and understanding throughout this most cherished endeavor.

CONTENTS

Abbreviations

μg	Micro gram
μl	microlitre
AFM	**Atomic Force Microscopy**
bp	base pair
CAP	**Chromatin Associated Protein**
CD	**Circular Dichroism**
cDNA	Complementary Deoxyribonucleic acid
CHCl3	Chloroform
CPM	counts per minute
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EtOH	Ethanol
FACS	Fluorescence Activated Cell Sorting
hr(s)	hour(s)
kda	kilo Dalton
min(s)	minute(s)
ml	mililitre
mM	mili Molar
MNase	Micrococcal Nuclease
ng	nano gram
nM	nano Molar
PAGE	Polyacrylamide Gel electrophoresis
Ph-OH	Phenol
PMSF	Phenyl Methyl Sulphonyl Fluoride
RNA	Ribonucleic acid
Rpm	revolutions per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic acid
TE	Tris EDTA
TEMED	N,N,N’N’-Tetramethyl-ethylene diamine
UV	ultraviolet

INTRODUCTION

1.1 Chromatin, a dynamic nucleoprotein organization of eukaryotic genome

The Eukaryotic genome is organized into a highly complex nucleoprotein structure, chromatin, which is composed of DNA, wrapped around a core histone octamer and a diverse group of nonhistone proteins involved in maintaining the cellular homeostasis. The dynamic chromatin organization is regulated by three different groups of factors: (i) histone and nonhistone modifying enzymes; (ii) ATP-dependent chromatin remodelling enzymes; (iii) histone chaperones. The posttranslational modifications of the core histones and other nonhisone chromatin proteins (including linker histone H1) lead to differential functional consequences (Wolffe *et al*, 1997; Agresti and Bianchi, 2003; Bustin, 2001; Garcia-Ramirez *et al*, 1992). The ATP-dependent chromatin remodeling and histone chaperones (for core or linker histones) also contribute to the organization

of the dynamic chromatin (Vignali *et al*, 2000; Loyola and Almouzni, 2004; Saha *et al*, 2006). The ATP-dependent chromatin remodeling machinery is responsible for differential positioning of the DNA over the core octamer. Thus the regulatory DNA sequences can be accessed by several factors transiently without altering the overall chromatin structure. The histone chaperones are anionic cellular proteins that help in proper assembly and disassembly of chromatin, preventing nonspecific aggregation. The functional diversity of this class of proteins ranges from histone storage and transport, transcription regulation and repair. The chromatin fiber bridging proteins (eg. Sir3p, Tup1, MENT etc) (Gavin and Simpson, 1997; Georgel *et al*, 2001, Springhetti *et al*, 2003) and other nonhistone chromatin associated proteins (eg. HMGs, HP1, MeCP2, PARP1 etc) (Agresti and Bianchi, 2003; Bustin, 2001; Catez *et al*, 2004; Pallier *et al*, 2003; Li *et al*, 2002; Kriaucionis and Bird, 2003; Kim *et al*, 2004) 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. 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 (Allan *et al*, 1981; Thomas, 1999; Wolffe *et al*, 1997). Taken together the dynamicity of the chromatin is mediated by a diverse repertoire of factors orchestrating the events towards a destined cellular fate.

1.1.1 Nucleosomes to higher order structure:

The unit of chromatin is the nucleosome, which is composed of 146 bp of DNA, wrapped around the core histone octamer. The nucleosomal structure of chromatin follows closely the structural hierarchy as observed in proteins, where the 10 nm filament (beads on a string) may be considered as the primary structure and the secondary structure is the 30 nm filament which folds to create the 100-400 nm compact structure like the tertiary folding of the proteins. The quaternary structure of the chromatin is achieved in the condensed chromosome territory or metaphase chromosome by further folding with the help of several nonhistone

proteins and histone H1 (van Holde and Zlatanova, 1996; Cremer and Cremer, 2001). The varied length of DNA between the two nucleosomes is the linker DNA, a site where different histone and nonhistone proteins bind. The gradual structural organization of the eukaryotic genome is represented in Figure 1.1.

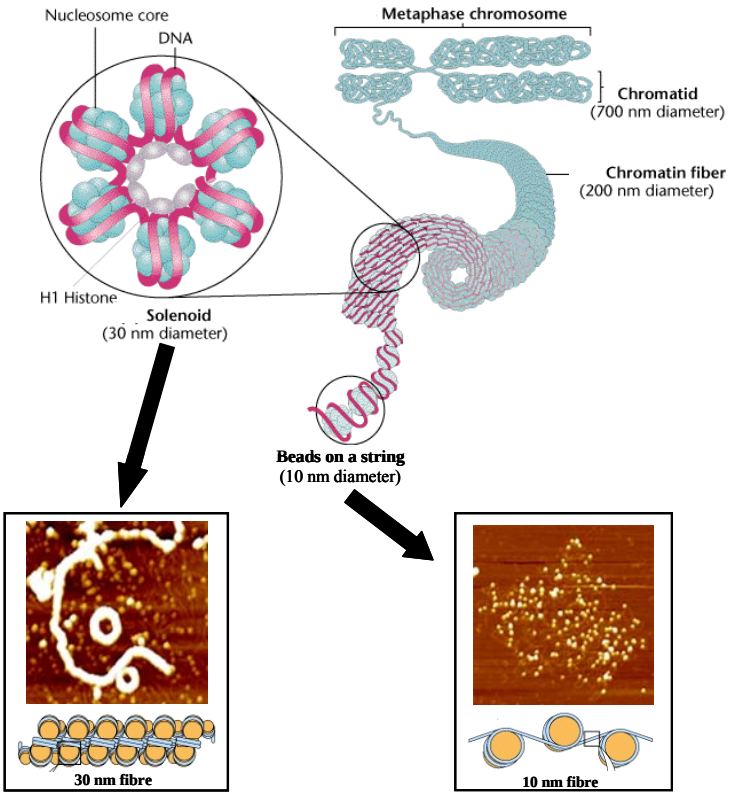


Figure 1.1: Structural organization of the eukaryotic chromosomes: The graded degree of chromatin compaction from the beads on a string till compact metaphase chromosomes.

Structure of a nucleosome core particle:

The X-ray crystal structure of the nucleosome core particle (2.8A°) (Luger *et al*, 1997) shows that 146 bp of DNA wrapped around the histone octamer in 1.65 turns of a flat left-handed superhelix (Figure 1.2). The octamer is divided into four "histone-fold" dimers defined by H3-H4 and H2A-H2B histone pairs. H3-H4 forms a four-helix bundle constituting H3-H4 tetramer. Each H2A-H2B dimer interacts with tetramer and forms a homologous four-helix bundle between H2B

and H4 histone folds. Furthermore, α-helices and coil elements extend from the histone fold regions and are also integral part of the core particle within the confines of the DNA superhelix. The tails of H3 and H2B pass through channels of the DNA superhelix created by two minor grooves. One H4 tail segment makes a strong inter-particle connection, perhaps relevant in maintaining higher order structure of nucleosomes.

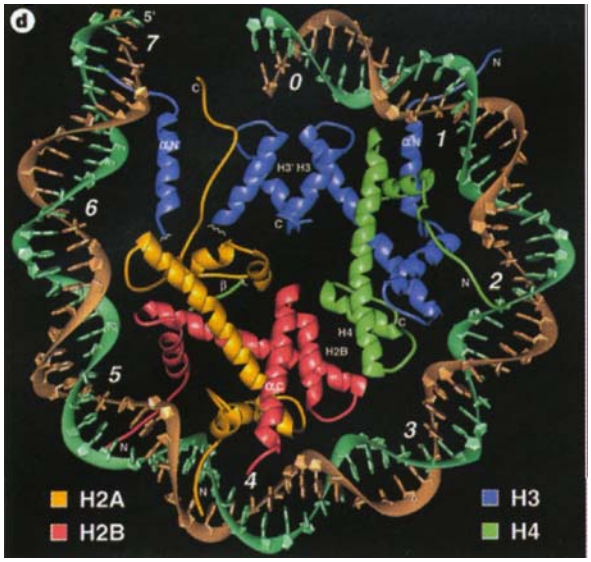


Figure 1.2- Structure of Nucleosome core particle at 2.8 A° resolution (taken from Luger et al, 1997).

Apart from the canonical nucleosomal structure, extensive studies in order to understand the variant nucleosomal structure have been in progress. Since the activation of transcription in a chromatin context is favourable in presence of histone variant H2AZ in nucleosomes, 2.6 A° crystal structure of nucleosome core particle containing H2AZ was reported (Suto *et al*, 2000). Interestingly, H2AZ containing nucleosomes show an alteration in higher order chromatin organization ability as compared to the canonical nucleosomes. The crystal structure of nucleosome containing macro H2A (2.9 A°) has been solved. It was visualized that macro H2A preferentially forms hybrid nucleosomes, which are more floppy in nature (Abbott *et al*, 2004; Chakravarthy *et al*, 2006). H2ABbd containing nucleosomes, on the other hand, are usually present in the active

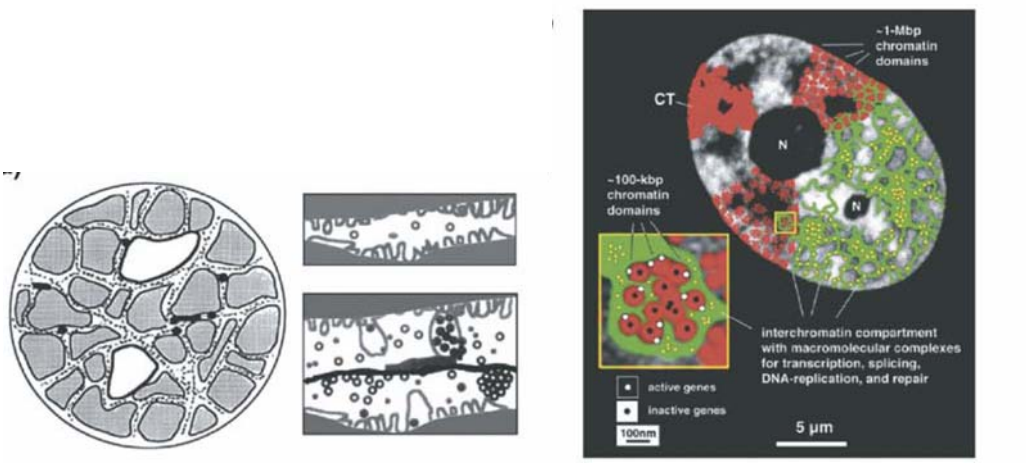
chromatin. The histone fold motif of H2ABbd (and not the N-terminal tail) is responsible for its functional diversity (Bao *et al*, 2004; Doyen *et al*, 2006).
The ordered folding of an array of nucleosomes gives rise to higher order compact chromatin structure. This gives rise to functional chromatin domains as elaborated in the next section.

Higher order structural organization of the chromatin:

A hierarchical organization of chromatin fibres starting from 10 nm beads on a string fibre to 130 nm chromonema fibre establishes the chromatin territories (CT). Although various proposed models argue in favour or against the folding of chromatin forming the CTs, the real *in vivo* picture is not yet been completely established. There are three basic hypotheses (Cremer *et al*, 2006) regarding this issue: (i) higher order chromatin structure (rosettes) are originated from small-scale chromatin loops (SLs) (50-200 kb long) and are the essential building blocks of the CTs (Cremer and Cremer**,** 2001; Zirbel *et al*, 1993) (Figure 1.3 A); (ii) CTs are originated from giant loops (GLs) comprising of 1-2 Mbp DNA (Chubb and Bickmore, 2003; van Driel and Fransz, 2004) (Figure 1.3 B); (iii) involvement of both short loops and giant loops in establishing CTs. The SL model proposes the existence of CTs separated by interchromosomal domain (ICD) (Cremer and Cremer**,** 2001). The active genes are located at the periphery of the CTs, in order to allow a functional interaction with various components of the ICD (Cremer and Cremer**,** 2001). The CT surface can undergo folding, in order to expose the active genes buried in the interior of CTs, resulting in looping out of CTs into ICD (Cremer and Cremer**,** 2001). The GL model proposes an existence of chromatin fibre of several Mb DNA (GL) that is separated from the next GL in the same CT by a 200 kb chromatin linker (Chubb and Bickmore, 2003). Modulation of the size of GLs or linker affects chromatin compactness.
The general belief regarding chromatin architecture is that the actively transcribed genes (housekeeping) or the genes expressed under suitable cellular signals (being

poised for a temporary phase) are located on an open (decondensed) chromatin configuration, while relatively permanently silenced genes (heterochromatin) are

A. Small-scale chromatin loop model



B. Giant loop model

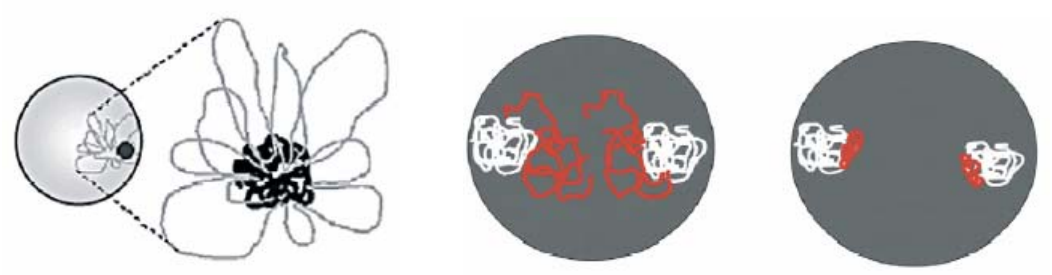


Figure 1.3: The proposed models of chromatin territory architecture (taken from Cremer *et al*, 2006): (A) ICD separating neighboring CTs. Chromatin loops are formed in the ICD. Transcriptionally silent ICD is almost empty. Transcriptionally active ICDs are filled with protein complexes of various nuclear functions. (B) Chromatin consists of GL separated by linker. Each CT contains a single chromocentre surrounded by a rosette of GLs. Chromatin loci of very high transcriptional activity (red) is present outside the CT core (white). These loci collapse back into condensed CTs as soon as transcription ceases.

present on the closed (compact) chromatin loci. However, recent studies indicate that although there is a correlation between open chromatin structure and high gene density, it does not necessarily corroborate with high gene expression. This

is because there are instances of presence of active genes in more compact and silent genes in more open chromatin loci, indicating that open or closed chromatin conformations are not the only determinant of gene expression.

Interestingly, RNA has been known to contribute to the higher order chromatin architectures (Worcel and Burgi, 1972; Zamore and Haley, 2005; Bernstein and Allis, 2005). Such higher order architectures and their hierarchical transition seem to be critical to achieve the genomic activities in cell. To investigate the role of RNA in the formation of 30-80 nm fibre, RNase treatment was carried out to the nucleus isolated from the HeLa cells (Ohniwa *et al*, 2006). This leads to the disruption of the edge of the nucleus and exposes the fibre structure predominantly forming the 30 nm fibre and not beyond this level of compaction. This indicates that RNA is critical in maintaining higher order folded chromatin architecture beyond 30 nm fibre.

The first step in the organization of chromatin is the assembly of histones into the nucleosomes, which has been highlighted in the next section.

1.1.2 Chromatin Assembly to maturation:

The sequential step of events, which leads to the organization of DNA into nucleosomal template, is chromatin assembly. The pathways of chromatin assembly can be classified as - (a) parental nucleosome segregation, (b) *de novo* nucleosome assembly. In the first case, transfer of pre-existing core histones occurs into the nascent chromatids. The *de novo* nucleosome assembly occurs in a stepwise manner. The initial step involves the deposition of H3-H4 tetramer followed by H2A-H2B dimer. The ordered deposition of the histones is carried out by the histone chaperones. The initial nucleosomal template generated has irregular spacing, which is regularized by the involvement of the ATP-dependent chromatin remodeling system (Adams and Kamakaka, 1999; Tyler, 2002; Haushalter and Kadonaga, 2003). Histones are synthesized in bulk during S-phase in cytosol (Osley, 1991) and remain acetylated for a brief period of time till its deposition into the DNA. The acetylation is brought about by Hat1, a B-type

histone acetyl transferase residing in cytosol (Verreault *et al,* 1996; Verreault *et al,* 1998; Imhof and Wolffe, 1999). This HAT acetylates free histone H4 and there after the H3-H4 tetramer is recruited onto the nucleosomes with the help of histone chaperones (Ye *et al*, 2005). After the assembly, the acetylated histones are subsequently deacetylated by HDAC1 and the remodellers carry out the proper nucleosomal spacing leading to a functional chromatin assembly. The final phase is the positioning of the linker histone H1 at the dyad axis. However, linker histone binding to nucleosome is a highly dynamic process (as described in the subsequent section). The steps of chromatin assembly have been represented in Figure 1.4. The functional chromatin domains can be classified into distinct repressed, active or boundary regions as represented in the next section.

Euchromatin, Heterochromatin and Insulator elements:

Chromatin is a highly dynamic functional organization with regions of varying gene expression determined by several factors. Transcriptionally active regions of high gene expression are known as euchromatin. Heterochromatin on the other hand is structurally compact regions of silenced genes. Euchromatin-heterochromatin boundaries are composed of insulator elements. A distinct protein profile and chromatin modification states are found to be in each of these chromatin elements.

Heterochromatin is characteristically silenced chromatin loci. Functional sub classification of heterochromatin can be done with constitutive (always repressed) and facultative (conditionally repressed) states. Centromere and telomeric regions are two condensed heterochromatin foci. Generally the acetylation levels are low and methylation levels are high in heterochromatin regions. Heterochromatin formation initiates with recruitment of sequence-specific DNA binding proteins. In telomeric regions, initial nucleation proceeds with binding of Rap1p, followed by Sir4p, 3p, 2p that deacetylates H4K16 promoting heterochromatin spread (Luo *et al,* 2002).

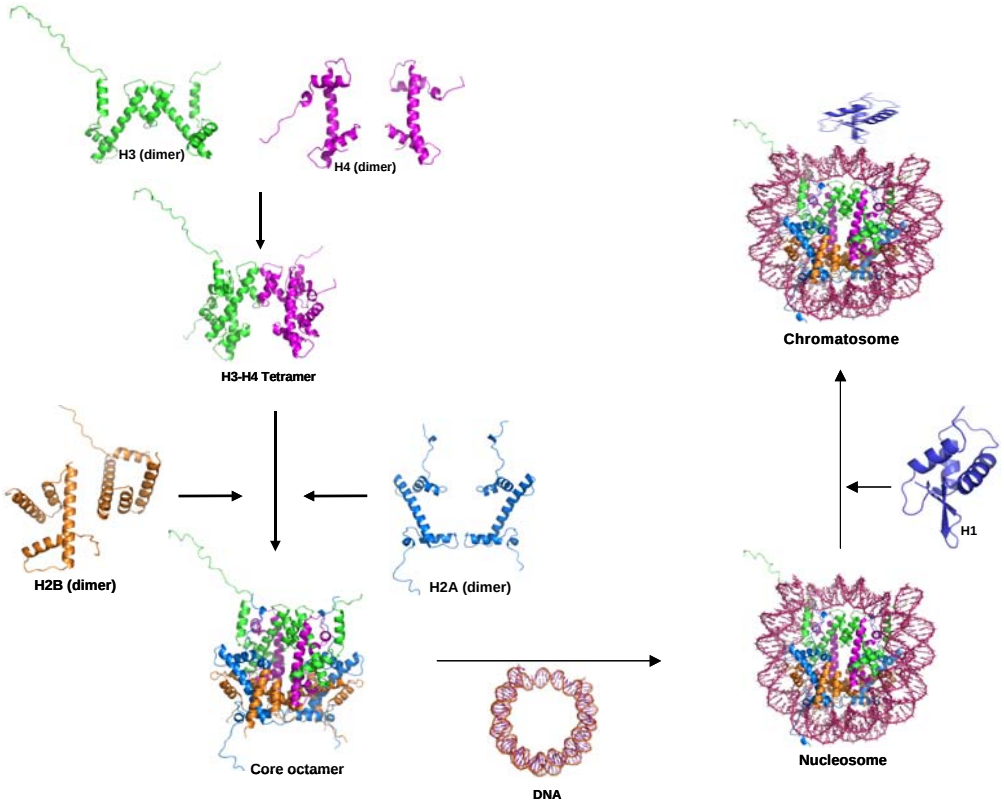


Figure 1.4: Structural organization of a chromatosome: The steps of chromatin assembly involve formation of a H3-H4 tetramer followed by H2A-H2B dimer. Finally there is formation of the core octamer with a double stranded DNA wrapped around it. Histone H1 is positioned at the dyad axis of the nucleosome forming the chromatosome.

In case of centromeric region, short stretches of repetitive DNA is activated by RNAi pathway leading to the recruitment of Suv39H1 (histone methyl transferase) (yeast homologue is Clr4). H3K9 gets methylated and leads to the docking of HP1 (yeast homologue is Swi6) that results in silencing of the locus. Suv39H1 is recruited on the adjacent histones by HP1 and thereby lead to the heterochromatin spread (Maison and Almouzni, 2004; Nielsen *et al*, 2001). H3K9 methylation is further enhanced by removal of H3K9 and K14 acetates by the HDAC complex (yeast homologue Clr3). Methylation of H3K4 (by Set9) prevents the association of NuRD chromatin remodeling and deacetylase complex and Suv39H1 histone methyltransferase. H3K9 methylation by Suv39H is also prevented by H3K9 (and possibly H3K14) acetylation or H3S10 phosphorylation.
The euchromatin - heterochromatin boundary is efficiently maintained by the insulator elements. H4K16 acetylation is an important modification (Shorgren-Knaak *et al*, 2006), which demarcates the heterochromatin boundary. Sas2p is responsible for maintaining the global genome acetylation of H4K16 (Suka *et al,* 2002; Kimura *et al,* 2002; Kimura and Horikoshi, 2004) that act as landmark preventing the spread of heterochromatin to euchromatin boundaries. Incorporation of this modification into nucleosomal arrays inhibits formation of 30 nm fibre and also prevents the chromatin remodeling machinery ACF to mobilize the nucleosomes. Thus a single histone modification regulates both higher order chromatin structure and functional interactions between a nonhistone protein and chromatin fibre. Recently, the class III general transcription factor, TFIIIC, has been found to be involved in global chromatin structural organization (Noma *et al*, 2006) being a component of the boundary element throughout the genome. It was found that the mat barriers of yeast, which contained hypoacetylated histones is devoid of H3K4 methylation. The barrier function is provided by a sequence stretch of consecutive five copies of B-box elements, the binding site of TFIIIC. These B box sequence elements function as chromosome organizing clamps where TFIIIC facilitate heterochromatinization as well as maintenance of heterochromatin barrier. A recent study has shown Poly-ADP-ribosylation regulates the activity of CTCF at the insulators (Yu *et al*, 2004). The

insulator within the imprinting control region of Igf2-H19 loci, where CTCF binds is dependent on PARP activity. This was specific to the maternal allele and was found to be functional CTCF binding sites. Detailed proteomic and genomic analysis of the boundary elements of yeast has been carried out (Tackett *et al*, 2005). A large repertoire of proteins including DNA Pol ε (replication factor), Isw2, Top 2 (chromatin remodeling proteins), Sas3-Spt16 (chromatin modifying complex) and distinct histone modification status (hypoacetylation of K5, K8, K12 and hyperacetylation of K16) was important for maintaining a faithful boundary region (Tackett *et al*, 2005).

The functional dynamics of chromatin is generated by a wide repertoire of nonhistone chromatin-associated proteins as discussed in the following section.

1.1.3 Nonhistone chromatin-associated proteins:

The nonhistone chromosomal proteins are defined as those proteins (excluding the histones) that isolate together with DNA in purified chromatin. This class of proteins overlaps in general with the classes of acidic nuclear proteins and is under extensive investigation. These proteins possess distinct chemical, physical and biological properties that contrast sharply with those of histones. A large repertoire of nonhistone chromatin associated proteins is involved in dynamic chromosomal organization. These proteins exhibit limited tissue specificity, as most of these proteins are present in all tissues of the organisms. However, quantitative variations are observed across tissue samples. Nonhistone chromosomal proteins are key players in maintaining dynamic chromatin organization and are thus important regulators of gene expression from the chromatin template. A class of these proteins can compact the chromatin into higher ordered structural organization, while others may cause decompaction thereby activating transcription from an open chromatin template (Elgin and Weintraub, 1975; McBryant *et al*, 2006).

The first identified nonhistone chromatin protein involved in maintaining dynamic chromatin structure is linker histone H1. Linker histone H1 has large

number of tissue specific variants regulating chromatin architecture. 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 (Wolffe *et al*, 1997; Thomas, 1999; Zlatanova *et al*, 2000; Hizume *et al*, 2005). Loss of histone H1, however, up-regulates and down-regulates expression of specific genes, suggesting that histone H1 is able to affect the transcription both positively and negatively (Alami *et al*, 2003; Hellauer *et al*, 2001; Shen and Gorovsky, 1996). Histone H1 is the key factor to aid in the compaction of the chromatin for mitotic chromosomes and the molecular determinants of condensed chromatin structure, including structure-function of kinetochores are formed in interphase (Thomas, 1999). Histone H1 interacts with the chromatin in a dynamic fashion (two sub-populations of H1 with differential mobility exists in the cell) and a rapid exchange of H1 occurs in euchromatic and heterochromatic regions (Misteli *et al*, 2000; Catez *et al*, 2006). Core histone acetylation reduces the H1 dwell time on chromatin (Misteli *et al*, 2000).

The modulation of chromatin structure as induced by histone H1 can be summarized in the following Figure 1.5 (taken from Catez *et al*, 2006). Briefly the steps involve an initial nonspecific binding to the linker region. This is followed by the globular domain induced proper positioning of H1 in the nucleosomal dyad axis. Subsequently structural changes are induced by H1 into the chromatinised template.

The dynamic interaction of H1 with chromatin has been represented in the Figure 1.6 (taken from Catez *et al*, 2006). (i) Histone H1 induces structural alteration of the chromatin (as illustrated before). The modulation of chromatin structure can be also through (ii) tighter binding of cofactors (eg. Msx1) as mediated by H1, (iii) posttranslational modification of histone tails (eg. phosphorylation of histone H1 or acetylation of core histone H3), (iv) site-specific or (v) global competitors of H1 in the chromatin.

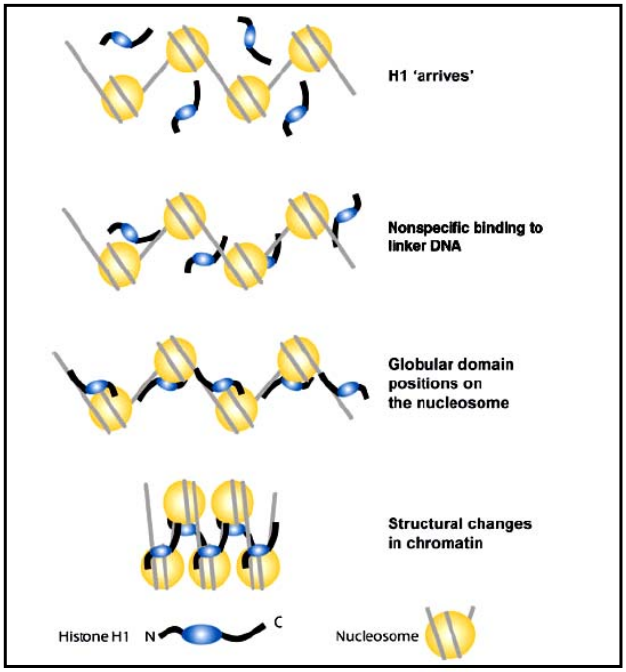


Figure 1.5- Stepwise modulation of chromatin structure as induced by histone H1 (taken from Catez *et al*, 2006).

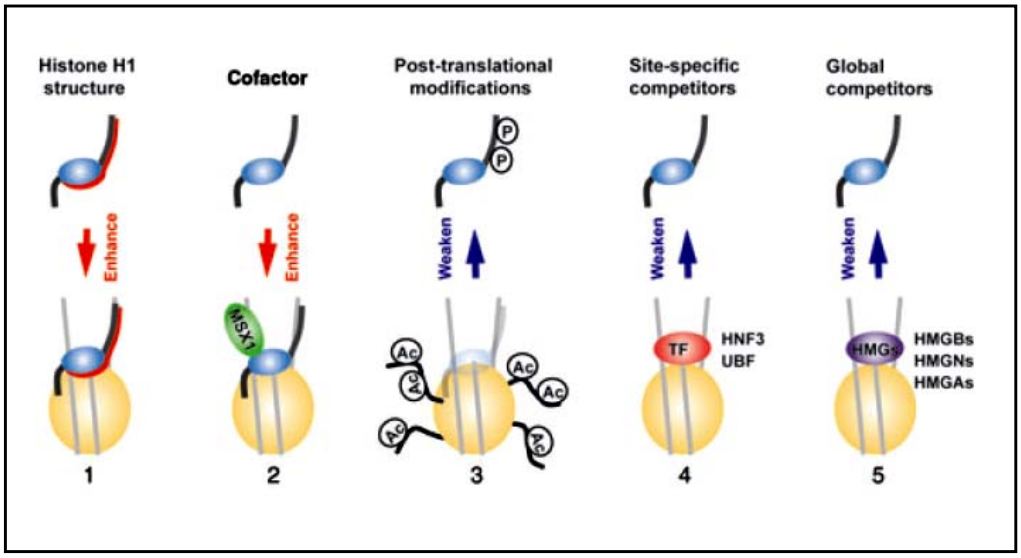


Figure 1.6: Dynamic interactions of histone H1 with chromatin (taken from Catez *et al*, 2006).

Apart from linker histone H1, there are several other nonhistone structural components of chromatin. Exploring the mechanism of chromatin folding

mediated by these nonhistones revealed distinct *in vivo* pathways to be operative (Figure 1.7). Oligomeric status of the nonhistone proteins play an important role in the formation of higher order structure mediated by these proteins. Furthermore, posttranslational modifications of the chromatin or the chromatin-associated proteins also act as the functional switch in chromatin folding. However, some of the mechanisms are unique and need to be yet explored. The following scheme summarizes the role of nonhistone chromatin associated proteins in chromatin folding.

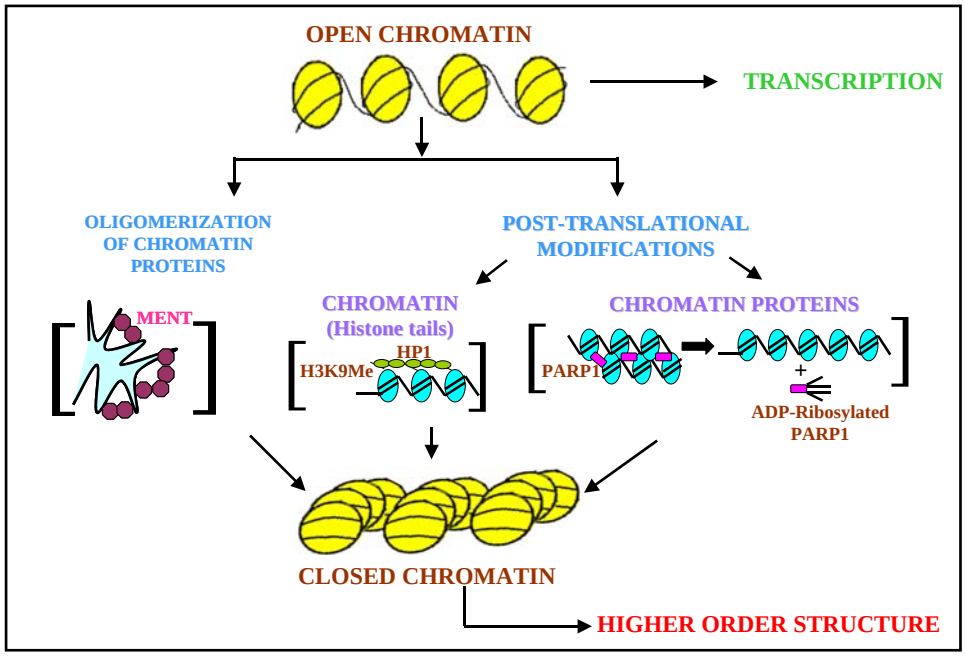


Figure 1.7: Functional mechanisms of chromatin compaction by nonhistone chromatin associated proteins: The different mechanisms of chromatin folding include (a) oligomerization of chromatin proteins, (b) post translational modifications of histones or the other nonhistone chromatin proteins.

The diverse repertoire of nonhistone chromatin proteins has been further sub-grouped depending upon their physiological functions.

i. Nonhistone chromatin proteins in maintenance of chromosomal structure during mitosis:

A large repertoire of nonhistone chromatin protein is involved in faithful maintenance of compact chromatin structure during mitosis. These proteins (either in native or posttranslationallly modified form) are involved in maintenance of chromatin architecture. The functional attributes of some of these proteins, which include Topoisomerase II, SMC family members (Cohesins and Condensins), chromokinesin KIF4, HCF-1 and the oncoprotein DEK, has been discussed here.

DNA topology is altered by Topoisomerase II, which participates in a variety of cellular processes including chromosome compaction and segregation, replication and transcription. Topoisomerase II localizes in the scaffold/ matrix fraction of the interphase nuclear and the mitotic chromosomes (Berrios *et al*., 1985). Depletion of Topoisomerase II from mitotic extracts prevents chromatin compaction as shown in the *in vitro* studies (Adachi *et al*., 1991). Furthermore, mutants defective in Topoisomerase II are unable to condense their chromosomes in prophase. Inactivation of Topoisomerase II leads to a partially compacted metaphase chromosomes remaining as an entangled mass. Detailed mechanistic study suggest that the Topoisomerase II removes catenation between the sister chromatids in the last step of chromatin compaction (Wang, 2002).

SMC family of chromatin proteins, which can form multi-component complexes, are also involved in the structural maintenance of chromosomes (Hirano, 2006). Important members of this family are Cohesins and Condensins. This family of proteins is largely involved in chromosome condensation, including segregation, organization of loops, positive supercoiling of DNA and linking sister chromatid cohesion with chromosome condensation (Lam *et al*, 2006; Gruber *et al*, 2006). Cohesin complex remain chromatin associated from Telophase to the entire Interphase and is required for sister chromatid cohesion. Condensins on the other

hand are specifically enriched on the axial structure of the sister chromatids during mitosis. There are two types of Condensin complex, Condensin I and II, which are composed of same SMC subunit but with different non-SMC subunits having distinct functions (Ono *et al*., 2003, Hirota *et al*, 2004).

Accurate chromosome alignment at metaphase and a proper segregation of condensed chromosome is a complex process. Human chromokinesin HKIF4A is a chromosome-associated molecular motor involved in chromosome condensation and segregation during mitosis (Mazumdar *et al*, 2004). Depletion of HKIF4A results in defective prometaphase organization, chromosome mis-alignment at metaphase, spindle defects and chromosome mis-seggregation. HKIF4A interacts with Condensin I and II complexes and HKIF4 depletion leads to chromosome hyper condensation, suggesting its requirement for normal chromatin architecture (Mazumdar *et al*, 2004).

The abundant human chromatin-associated factor HCF-1 is a heterodimeric complex of HCF1N and HCF1C and plays a critical role in cell cycle progression. Mechanistic investigation shows HCF1N promotes G1 phase progression, whereas HCF1C regulates M-phase by maintaining H4K20 monomethylation (by regulating the levels of the enzyme PR-SET7) (Julien and Herr, 2004). HCF1C depletion leads to extensive mitotic defects, including defective chromosome alignment and segregation (Julien and Herr, 2004).

DEK was first isolated as part of a fusion protein expressed in a subtype of acute myeloid leukemia (AML) with chromosomal translocations (von *et al,* 1992). It was reported to be a site-specific DNA binding factor. It was also found to associate with the splicing complexes and also to the mRNA in a splicing dependent manner (McGarvey *et al*, 2000). It is a proto-oncogene, which alters the DNA structure by introducing supercoils (Alexiadis *et al*, 2000). The change in DNA topology, as introduced by DEK, was only observed with chromatin (but not with naked DNA) and depends on the presence of H2A-H2B dimer (Alexiadis

et al, 2000). The ability to change the DNA topology gave the initial indication that DEK protein might reside onto the cellular chromatin. Detailed experimental analysis indicated that it is a nuclear phospho-protein that remains chromatin associated throughout all the stages of cell cycle (Kappes *et al*, 2001). It was found to be present in both active and repressed chromatin foci. It can oligomerize and thereby alter chromatin architecture in a phosphorylation-dependent manner (as discussed).

ii. Nonhistone chromatin proteins in chromatin silencing:

A large repertoire of proteins is responsible for maintenance of the repressed chromatin states. These proteins include methyl CpG binding proteins, Heterochromatin proteins, Polycomb group of proteins, SIR proteins, MENT complex and Thanatos Associated Proteins, which has been elaborated in this section.

There are six Methyl CpG binding proteins (MBP) characterized so far (Klose and Bird, 2006). Kaiso is an atypical member having Zinc- finger domain to recognize methylated DNA (Prokhortchouk, 2001) and POZ/BTB domain to repress transcription. MBD1 uses its methyl-binding domain (MBD) to bind methylated DNA sequences, where as three Zinc-binding domain (CxxC) binds specifically to non-methylated CpG sequences. MBD1 also has a C-terminal transcription repression domain (TRD). MBD2 possesses an overlapping MBD and TRD domain and a GR repeat at the N-terminus. MBD3 contains a conserved MBD domain that cannot recognize methylated DNA sequence (because of some crucial amino acid change). MBD4 has a canonical MBD domain and a C-terminal glycosylase domain at the C-terminal involved in DNA repair function. MeCP2 is the founding member of this family, having a conserved MBD and an adjacent TRD.

MBD1 associates with the histone methyl transferase SetDB1, coupling DNA methylation and histone methylation (Wang *et al*, 2003). DNMT1 associates with

PCNA, during S- phase, and tracks DNA polymerase complex (Sarraf and Stancheva, 2004). MBD1 also associates with replication factor CAFp150, facilitating the histone methylation marks to newly replicated methylated DNA. MBD1 is involved in bridging cell cycle events to re-establish histone methylation marks at loci containing DNA methylation.

MBD2 binds to the methylated promoter in IL4 gene, in naïve Th cells and maintain silenced state of gene (Hutchins *et al*, 2002). On differentiation into Th2 cells, transcriptional activator GATA3 can bind. GATA3 competes out MBD2 at IL4 promoter, leading to transcription activation. Thus the level of IL4 transcription is proportional to occupancy of the promoter by GATA3, but inversely proportional to MBD2 occupancy.

MeCP2 is a dynamic repressor of the neuronal genes and is localized chiefly in the centromeric heterochromatin region (binds chiefly by its MBD domain). A direct correlation between the neurodegenerative disease Rett syndrome and MeCp2 levels are noted. Screening of candidate genes in patients showed that ~80% cases of Rett syndrome patients, there is a discernable mutation in the MeCP2 gene. MeCP2 binds to methylated DNA at the promoter of several neuronal and some imprinted genes, maintaining silencing through the recruitment of Sin3a-HDAC corepressor complex (Jones *et al*, 1998). Though MeCP2 has a preferential binding to methylated CpG islands, a consecutive location of adjacent (A/T)4 stretches are present (Klose *et al*, 2005). In the case of bdnf gene activation, in neuronal cells cascade of events results in MeCP2 phosphorylation. This phosphorylation leads to dissociation of MeCP2 corepressor complex from the promoter leading to gene activation (Chen *et al*, 2003). Interestingly, studies with nucleosomal array templates showed that MeCP2 forms oligomeric suprastuctures, in unmethylated array template as well (Georgel *et al*, 2003). The ability to condense chromatin was found to be resident in regions other than MBD domain. The ability to assemble novel secondary structure might also lead to chromatin silencing.

The key player involved in maintaining the heterochromatin structure is Heterochromatin Protein1 (Maison and Almouzni, 2004). HP1 was originally identified as a DNA binding protein involved in specific type of gene repression termed position effect variegation. This effect was because of the proximity of the euchromatic genes towards heterochromatic regions. There are three definitive HP1 isoforms in human HP1-α, HP1-β and HP1-γ. Although the amino acid sequences and structural organization of these proteins are highly similar, there are differences in their localization. They are primarily associated with the centromeric heterochromatin regions. However, HP1-α and HP1-γ are also localized in the euchromatic sites. HP1 proteins contain the following conserved domains: conserved N-terminal Chromodomain (chromatin-organization modifier), a variable hinge region and a conserved C-terminal Chromoshadow domain.

Functional diversity of HP1 is achieved by being an important platform for interactions among different factors. This list includes chromatin modifiers, transcription regulators, replication and cell cycle implicated factors and nuclear structural proteins. H3K9-trimethylation by Suv39H1 acts as the docking site for HP1 recruitment and consequent establishing the heterochromatin silencing. The binding of HP1 is also influenced by phosphorylation status of H3S10 amino acid by Aurora B Kinase as the cells enter mitosis. HP1 does not bind to the doubly modified H3K9MeS10P (Hirota *et al*, 2005, Fischle *et al*, 2005). Thus phosphorylation being a key event in regulating cell cycle controls the heterochromatin formation induced by HP1. Other histone modifications might also regulate HP1 binding. Modification of H1.4 variant at K26 recruits HP1. Furthermore, modification of HP1 (phosphorylation at the hinge region) also regulates the interaction of HP1 to different proteins. Interestingly, interaction of HP1 with variant nucleosomes is distinct from that of canonical nucleosomes. HP1α binds to condensed higher order chromatin structure and alters the individual chromatin fibre without cross-linking them. These features are enhanced by an altered nucleosomal surface created by H2AZ. Thus the

nucleosomal surface can regulate the formation of distinct higher order chromatin structures mediated by chromatin protein HP1α (Fan *et al*, 2004).

HP1 can promote transcriptional repression or activation depending on the chromatin context and its interacting partners. Genes embedded in the heterochromatin region require compact chromatin structure as induced by HP1, for their normal expression level. The association of HP1 at the promoters with Transcription Initiation Factors (TIFs) might promote activation through the recruitment of coactivators. Another potential role of HP1 is reported in its ability to stabilize the mRNA transcripts, thereby having a positive effect on transcription. The transcriptional repression function of HP1 is primarily expressed in docking at the H3K9Me sites, leading to the formation of a compact chromatin structure. In the case of the genes under the regulation of Cyclin E promoter, HP1 can be recruited to through the association with Rb and promotes histone methylation and subsequent gene repression. HP1 also associates with the factors of basal machinery. TBP associated factor TAFII130 can recruit HP1 to promoters and hinder activation, possibly by blocking the association by the activators. HP1 also promote repression by interacting with the corepressors like TIF β. HP1 being a docked onto the H3K9 (by the chromodomain), serve as a platform for bridging the interaction among different other proteins. HP1 can also associate with the chromatin through interaction of DNA binding proteins (eg Zinc Finger Protein ZNF) with its chromoshadow domain.

At the early onset of development, PcG protein complexes establish a transcriptionally silenced state at those genes that are initially repressed (Francis *et al*, 2004). PcG proteins are necessary to initiate epigenetic modifications, and continual PcG expression is required to maintain these modifications later in development. PcG complexes interact with regulatory element termed polycomb response elements (PRE), which consist of several hundred base pairs, located at a wide separation from the enhancer and promoter elements. There are various mechanisms of PcG mediated chromatin compaction (Lund and van Lohuizen, 2004). PcG complex nucleates at the PRE and spreads via cooperative interactions

along the chromatin fibre. According to another school of thoughts, PcG complex assembles at the PRE, leading to the looping out of chromatin in order to establish contact with the regulatory regions. Polycomb proteins are localized onto the surface rather than core of the condensed chromatin domains. Polycomb mediated silencing is established through a selective steric hindrance for the transcription and remodeling factors to access the chromatin. Polycomb complex can bridge two separated chromatin arrays and has multiple nucleosome binding sites. It preferentially binds to the nucleosomes rather than the linker DNA. Electron microscopic studies have established the role of Polycomb Repressive Complex 1 in condensing nucleosomal arrays (Francis *et al*, 2004). The chromatin compaction is independent of the N-terminal tail of histones as revealed by the electron microscopic studies, although the tails are shown to influence PRC1 binding *in vivo*. PRC1- nucleosome interaction facilitates looping out of chromatin (Shao *et al*, 1999). PcG complex are found to be freely dissociable from the chromatin and allow for epigenetic modifications by other proteins that would compete for the binding sites. These modifications would facilitate the binding of PcG complexes with an increased affinity, thus reestablishing silenced chromatin states.

Silent information regulator (SIR) proteins function in trans to repress transcription from HML and HMR loci in budding yeast. Although the complete picture of SIR protein recruitment, SIR complex assembly and the molecular mechanism of silencing have not yet been established, some generalization can be made regarding the roles of SIR proteins. SIR1 directs the silencing factor recruitment to the chromatin through a well-characterized interaction with Orc1 (component of origin recognition complex). SIR1 further recruits SIR2/SIR4 complex, through a direct interaction with SIR4. SIR2 is a NAD-dependent deacetylase which hypoacetylates the histones with the next level of recruitment of SIR3P by SIR1/2/4 complex. SIR3P binding leads to the heterochromatin spread *in vivo*. SIR3P can interact with DNA, mononucleosomes and chromatin array. SIR3P can also bind to both extended and folded nucleosomal arrays

indicating that the histone tails are not an absolute requirement for the chromatin folding. Consistently SIR3P can bind to tailless nucleosomal template, though with slightly lesser efficiency. SIR3P has multiple chromatin binding sites existing as an oligomer and allows for bridging of individual nucleosomes. These bridged structures results in large supramolecular assemblies whose physical properties transcend those of typical 10 nm- 30 nm fibres. SIR3P binds progressively to the nucleosomal array leading to the formation of a high molecular weight complex without changing the overall compaction of the array. Self-association of SIR3P and also its interaction with the chromatin fibre leads to a unique chromatin structure, which may be a global representation of silenced chromatin. Thus SIR3P functions by mediating the reorganization of canonical chromatin fibre into functionally specialized higher order chromosomal domains.

Myeloid and erythroid nuclear termination stage specific protein (MENT) is a developmentally regulated and a highly abundant nuclear protein found in terminally differentiated avian blood cells belonging to serine protease inhibitor (serpin) family. This protein was localized in the condensed chromatin fraction chiefly in the heterochromatin (Grigoryev *et al*, 1999). MENT can bind to naked DNA and form tramline structure wherein two or more DNA molecules come to the proximity. It can bind also to the nucleosomes but in a histone-tail independent manner. The protein oligomerization plays a major role in MENT-dependent chromatin condensation. MENT binds to the chromatin through an AT-hook domain within the M-loop region (Springhetti *et al*, *et al*, 2003). MENT also possesses a Reactive Centre Loop (RCL), which was important for its oligomerization property (Springhetti *et al*, *et al*, 2003). MENT induces formation of higher order chromatin structure of 30-50 nm diameter with the entry-exit sites of the DNA always oriented towards the center of the fibre ultimately leading to the formation of stacks or coils of chromatin. MENT induced chromatin compaction occurs through the formation of a zipper-like structure bridging the DNA molecules similar to histone H1. Interestingly, both MENT and H1 are present in the purified chromatin, indicating both can impose

additive constraints to the chromatin structure. Two independent events leads to MENT induced heterochromatinization: i) the nucleosomal linkers are brought to proximity by the proteins which can then ii) act as a bridging molecule because of its oligomeric status Interestingly, ordered binding of MENT to linker DNA promoting chromatin folding was via its unique M-loop domain, whereas bridging of chromatin fibres is facilitated by MENT oligomerization mediated by the RCL.

Thanatos Associated Protein THAP7 is a member of a large class of proteins containing novel THAP domain (a C2H2 signature zinc finger domain). There are at least 12 human proteins that containing the highly conserved THAP domain, located at the N-terminus. Interestingly, THAP7 was found to bind to hypoacetylated histone tail and thus to the chromatin (Macfarlan *et al*, 2005). It interacts with the corepressors (HDAC3 and NCoR) bringing about transcriptional repression (Macfarlan *et al*, 2005). Thus it is an important transcriptional regulator as well as signal transducer protein (transmitting the repressive signal through histone H4 deacetylation).

iii. Nonhistone chromatin proteins as regulator of gene expression:

A subgroup of nonhistone chromatin proteins can act as global activators or coactivators of transcription and thus have regulatory roles in gene expression. Leading in this list is the High mobility group of proteins which is an established coactivator, has been discussed in this section. Recently discovered chromatin-associated protein Myc, which is an established transcription factor and Poly ADP Ribose Polymerase1 enzyme which has important roles is switching a repressed chromatin structure to a transcriptionally amenable state are also discussed in this section.

High mobility group of proteins (HMGs) are the most abundant nonhistone chromatin proteins that exert global genomic functions in establishing active or inactive chromatin domains. HMGs not only regulate the expression of a specific

subset of genes, they also contribute to fine tune transcription in response to rapid environmental changes. They show dynamic interaction ability with nucleosomes, transcription factors, remodeling machineries and also with linker histone H1.

HMG proteins were discovered as acid extractable components of chromatin that showed high electrophoretic mobility. They were considered to function as architectural components that can modify the structure of DNA and chromatin that promotes various DNA-dependent functions (Bustin, 1999). HMG proteins can be classified into three families (Bustin, 2001):

a) HMGA proteins containing AT hooks. These are nine amino acid stretches that are although unstructured in solution, can bind to AT rich sequences in the DNA minor groove.

b) HMGB proteins contain conserved Box sequence stretches. These are 80 amino acid stretches that bind minor groove of DNA with no sequence specificity as such.

c) HMGN proteins bind the nucleosomes, between the DNA spirals and the histone octamer.

HMGAs are involved in transcription control of specific genes, being key factors of enhanceosomes. HMGA1a distorts the DNA thereby increasing the affinity of the other proteins for their binding sites. It also facilitates the protein-protein interactions with other transcription factors (Reeves, 2001; Bianchi and Agresti, 2005). HMGAs participate in higher order chromatin structure, by binding to Scaffold/Matrix Associated Regions (S/MAR), which are segments of AT rich DNA that are part of the nuclear matrix. HMGA1a preferentially localizes in the heterochromatin regions and binds to condensed chromosomes during mitosis (Harrer *et al*, 2004). It is a highly dynamic protein, with a preferable longer residence time in condensed chromosomes. HMGA1a interact with many chromatin proteins like hnRNA, SF3A (splicing factor), helicases and chromatin assembly factors (Sgarra *et al*, 2005). A recent report has focused on the role of HMGA proteins as structural components of senescence-associated heterochromatin foci, showing its role as a probable tumor suppressor (Narita *et al*, 2006).

HMGB proteins are capable of bending (allosteric transitions) DNA, thereby promoting the binding of other proteins. HMGBs are also involved in transcription regulation and can act both as a transcriptional coactivator and repressor (Agresti and Bianchi, 2003). Although the initial report suggested that HMGB1 is not stably associated with the chromatin in somatic cells (Falciola *et al*, 1997), later investigations suggested that there are two subpopulations of HMGB- free or associated with condensed chromatin, which gets rapidly exchanged, similar to the dynamic localization of histone H1. It can directly interact with the nucleosomes targeting the linker DNA, the binding site of linker histone H1 (Catez *et al*, 2004). Although the concentration of histone H1 is ~10 times higher than HMGB1, quite surprisingly, former cannot compete out the latter for binding to the linker DNA. However, HMGB1 does not bind to the nucleosomal core particle although it shows ability to interact with the core histones inducing chromatin decompaction. Its core histone interacting domains have been shown to be important for the probable chromatin perturbation and enhancement of p53 mediated transcription (Banerjee and Kundu, 2003). The dynamic interactions of HMGB and histone H1 lead to completely different cellular fates. While H1 can compact chromatin and lead to higher order structure formation, HMGBs can fluidize chromatin facilitating nucleosome remodeling. The DNA bending ability (Thomas and Travers, 2001) also helps in recruitment of TBP to the promoter region leading to the preinitiation complex formation (Sutrias-Grau, 1999). The DNA bending also helps in stabilizing other transcription factor binding thereby activating gene expression. HMGB1 is also a prominent marker in determining cellular status, and has been shown to have cytokine like function (Muller *et al*, 2004; Bonaldi *et al*, 2003). They are found to be tightly bound to the apoptotic chromatin and are released as an inflammatory response during necrosis (Scaffidi *et al*, 2002).

HMGN are nucleosome-binding proteins, which can change the chromatin architecture enhancing transcription and replication. It has been shown to be a chromatin-specific transcription coactivator (Paranjape *et al*, 1995). They have nucleosome binding domain and an acidic chromatin-unfolding domain (CHUD).

They are highly dynamic in function, with a short residence time onto the chromatin, competing with histone H1 (Catez *et al*, 2004).

There are 18 distinct proteins belonging to the Poly ADP Ribose Polymerase (PARP) family. PARP-1 and PARP-2 catalyzes the polymerization of ADP-ribose units from donor NAD^+ to target proteins. PARP family members share conserved catalytic domain (of ~50 amino acids), called "PARP Signature". The other functional domains of PARP family members are- BRCT ("BRCA1 C-terminus like" DNA biding domain), automodification domain, ankyrin repeats, macro domain and WWE domain that dictate their unique activities (D'Amours *et al*, 1999). PARP-1 plays role in regulation of chromatin structure and gene expression in response to specific cellular signals. The targets of enzymatic activity of PARP-1 are PARP-1 itself (primary *in vivo* target), core histones, linker histone H1 and a variety of transcription related factors (Ogata *et al*, 1981; Huletsky *et al*, 1989; Kraus and Lis, 2003). Auto ADP ribosylation of PARP-1 leads to a dramatic reduction in its DNA binding ability and hence leads to a loss of compact chromatin structure.

PARP-1 is a nucleosome binding protein, similar to histone H1, preferentially binding at the dyad axis contacting the linker DNA where it exits the nucleosomes. Through this binding PARP-1 acts as a structural component of the chromatin promoting the formation of higher order structure. However, addition of NAD+ can reverse this effect by auto ADP ribosylation of PARP-1 and consequently its dissociation from the chromatin. Although the enzymatic activity of PARP-1 enzyme is not essential for its nucleosome binding, however it is important for NAD+ dependent release. PARP-1 can provide an interesting mechanism of transcriptional control. While chromatin bound, PARP-1 promotes a transcriptionally repressed state, poised for activation (since nucleosomes themselves are potent stimulators of PARP-1 activity), in the presence of NAD+ PARP-1 is released by auto-ADP-ribosylation shifting the chromatin to a more transcriptionally active conformation (Kim *et al*, 2004). The process of activation or repression of PARP-1 is made reversible, by the enzyme PARG (Poly-ADP-

ribose-glycohydrolase) that can remove the poly-ADP-ribose units from PARP-1 enabling it to rebind to the nucleosomes. Interestingly, PARP-1 has been found to be associated with mitotic spindle (Earle *et al*, 2000). Recent studies suggest that increased production of poly-ADP-ribose during mitosis plays an essential role in the assembly and structure of bipolar spindle (Smith and de Lange, 1999; Kanai *et al*, 2003).

Myc family of transcription factors (C, N and L-Myc) is proto-oncogene that is involved in regulation of cell growth and proliferation. Recent reports suggest, Myc is involved in maintenance of active chromatin. N-Myc disruption leads to chromatin compaction and a vast change in histone modifications (Knoepfler *et al*, 2006). Myc widely influences chromatin structure by upregulating the levels of GCN5 HAT and hence the acetylation status of histones (Knoepfler *et al*, 2006). Myc knockouts also influence the methylation status of histones leading to an elevation of H3K9Me (Knoepfler *et al*, 2006). Genome-wide large-scale CHIP analysis has lead to the identification of the epigenetic marks important for Myc binding to the chromatin. A stretch of chromatin with H3K4/K79 methylation and H3 acetylation is a pre-requirement for *in vivo* Myc binding (Guccione *et al*, 2006). These euchromatic islands, with preassembled basal transcription machinery recruited onto it, are the preferred sites for Myc binding (without any sequence preference as such).

Functional interaction amongst chromatin proteins:

The *in vivo* chromatin organization is a multifaceted event, where a functional cooperation between several chromatin proteins plays essential role. The interaction amongst chromatin proteins and its functional consequence is being explored. Cooperative binding of chromatin-repressing proteins such as HP1 and polycomb group with nucleosome arrays has long been thought to promote heterochromatin spread. Two other key components of mammalian heterochromatin that plays an important role in higher order chromatin

organization are HP1-α and histone H1. Phosphorylation of H1 disrupts this interaction providing a signal for disassembly of higher order chromatin structure. A recent report shows the regulation of HP1-α binding and heterochromatin formation is functionally regulated by histone H1 (Hale *et al*, 2006) and is independent of H3K9Me, which is considered as an important heterochromatin mark.

HMG proteins and histone H1 modulates the chromatin compactness affecting the factors to access nucleosomal targets. Histone H1 leads to a stable higher ordered chromatin structure thereby decreasing the access of several factors to nucleosomes thereby causing chromatin compaction. HMG proteins on the other hand decompacts the chromatin, leading to easier accessibility of the different regulatory factors to the chromatin. HMG proteins compete with histone H1 for chromatin binding sites (Catez *et al*, 2004). The effects are observed in both euchromatin as well as heterochromatin region regardless of chromatin compaction and histone modifications, indicating it to be a global genome wide phenomenon.

An extensive study, however, indicates PARP-1 and H1 occupy distinct nucleosomal fractions. Thus although both PARP-1 and H1 both colocalize in the chromatin, they are directed to distinct chromatin domains by distinct signals (Kim *et al*, 2005). Posttranslational histone modification might be such a regulatory signal governing the functional diversities of these two molecules (Jenuwein and Allis, 2001).

Recent studies have shown a functional interaction between nonhistone chromatin proteins for common cofactors. There is found to be an intriguing connection between PARP-1 and SIRT1 at the molecular level (Zhang, 2003). Increased PARP-1 activity leads to a depletion of NAD+ levels and a consequent reduction in HDAC activity of SIRT1, indicating a functional interaction between the two (Pillai *et al*, 2005). In a similar manner activation of SIRT1 leads to a loss in PARP-1 activity (Kolthur-Seetharam *et al*, 2006). The modulation of chromatin structure may also follow a correlation when deacetylation of nucleosomal

histones by SIRT1 leads to the formation of condensed chromatin structure where PARP-1 gets recruited.

Functional consequence of knock down of nonhistone chromatin proteins:

Knock down of chromatin-associated proteins leads to different cellular fates. Several secondary effects have been observed upon knocking down these proteins, apart from alteration in chromatin architecture. Depletion of linker histone H1 leads to an alteration of global chromatin architecture, including reduced nucleosome repeat length and local chromatin compaction ability, but affects a specific subset of genes, specifically those involved in imprinting (Shen and Gorovsky, 1996; Fan *et al*, 2005). Detailed functional analysis has been performed after knocking down each of the specific classes of HMG proteins. Experiments were done with genetically engineered mice completely lacking HMGB1. These genetically engineered mice developed normally, until just after birth they succumbed to lethal hypoglycemia (Calogero *et al*, 1999). Cell lines lacking HMGB1 grew normally, but GR mediated activation of gene expression was found to be impaired, leading to abnormal control of a particular group of genes essential for regulating sugar metabolism. HMGN1 modulates the repair rate of UV-induced DNA lesions in chromatin (Birger *et al*, 2003). HMGN1-/- embryonic fibroblasts are hypersensitive to UV. By reducing the compactness of the chromatin, HMGN1 facilitates the access of UV-damaged DNA sites and hence enhance the rate of DNA repair in the chromatin (Birger *et al*, 2003). Knocking out HMGN2 (heterozygotes or homozygotes) however generated no specific cellular phenotypes as examined in lymphoid lineage of cells. This points out to the fact that there may be a functional redundancy to a certain extent, between HMGN group. The AT hook binding HMGA1 also have important function in Nucleotide Excision repair (NER). Knockdown of HMGA1 leads to abrogation of UV sensitivity, indicating an intricate association of HMGA class of proteins to chromatin repair pathway (Adair *et al*, 2005).

The proto-oncogene DEK knockdown protects the cells from apoptotic cell death, since the mode of action of DEK involves direct involvement of p53 (Wise-Draper *et al*, 2006). PARP1 is activated by cellular oxidative stress leading to the activation of Angiotensin II leading to cardiac hypertrophy. Molecular mechanism of Angiotensin II induced hypertrophy was assessed using wild type (PARP+/+) and PARP deficient (PARP-/-) mice (Pillai *et al*, 2006). Furthermore, knockdown of PARP1, a novel coactivator of β-Catenin/TCF4 activator complex, inhibits transcription and proliferation of colorectal cancer cells (Idogawa *et al*, 2005). Transcription factor c-Myc has an important role in development of breast cancer. siRNA-mediated silencing of this protein reduced tumor growth in nude mice, indicating it to be a potential therapeutic target (Wang *et al*, 2005). Knockdown of Methyl CpG binding domain protein (MBD2) stimulated DNA hypomethylation and also affected the transcript level of NBR2 (Near BRCA1 2) gene, but not that of BRCA1 (Auriol *et al*, 2005). Hence the reported literature suggests that knockdown of these chromatin-associated proteins affects several other physiological pathways, apart from its role in chromatin organization.

Posttranslational modifications of histones or nonhistone chromatin associated proteins regulate chromatin structure. In the following section the role of these epigenetic marks in regulating chromatin structure has been highlighted.

1.1.4 Epigenetic markers as regulator of chromatin dynamics:

The heritable changes in gene expression evidenced by DNA and histone modifications without any alteration of the coding sequence is collectively termed as epigenetics. Epigenetic codes are set up by modifications on the DNA (methylation) or on the histones (acetylation, methylation, phosphorylation etc) (Quina *et al*, 2006).

a. DNA Methylation:

DNA methylation at the CpG islands has been found associated with long term gene silencing and has a direct correlation with the developmental stages of cell cycle. A class of DNA methyl transferase (DNMT3) causes *de novo* methylation

of the DNA during early embryonic stages. These enzymes methylate pericentric heterochromatin on the inactive X chromosomes. Another class of such enzymes (DNMT1) is involved in maintenance of methylation marks in the genome. They preferentially target the hemi-methylated DNA created during the semi-conservative mode of replication thereby maintaining the genetic imprinting patterns. However, these subclasses of DNA methyltransferases are functionally interdependent as DNMT3 can substitute DNMT1 in cellular functions.

There are different mechanisms for the DNA methylation-dependent gene silencing. DNA methylation in the cognate DNA-binding sequence of some transcription factors can result in inhibition of DNA binding. By blocking the activators from binding to their target sites, DNA methylation directly inhibits transcriptional activation. The other mechanism is through recognition of methylated DNA sequences by the methyl CpG binding proteins (MBPs) and recruit corepressor molecules to silence chromatin and also modify the surrounding regions. In addition to the DNA methyl transferase activity, the DNMTs are also physically linked to Histone Deacetylases and Methyltransferases. In this case addition of methyl group to DNA is linked to transcriptional repression and chromatin modification. DNA methylation within the coding sequence of the gene can also have a dampening effect on transcription elongation. MBPs are involved in this aspect either directly or by their effects on surrounding chromatin structure. To understand the methylation dependent alteration of chromatin structure, AFM studies have been performed. Chromatin fibres were reconstituted on unmethylated and methylated CpG sequence in presence or absence of linker histones. The results indicated that DNA methylation induced fibre compaction occurs only in the presence of linker histones (Karymov *et al*, 2001). However, other reports suggest poly ADP-ribosylated H1e maintains the undermethylated state of CpG islands in the promoter of housekeeping genes and MeCP2 can cause displacement of linker histone H1 from chromatin. Hence a complicated network of molecular interactions modulates chromatin structure.

b. Reversible acetylation of histones and nonhistone proteins:

The enzymatic covalent modifications of histones and nonhistones, which lead to differential functional consequences, include acetylation, methylation, phosphorylation, sumoylation and ubiquitylation. The most well explored posttranslational modification is reversible acetylation, which is catalyzed by Histone Acetyltransferases (HATs) and Deacetylases (HDACs). The broad classification of HATs and HDACs are represented in Figure 1.8.

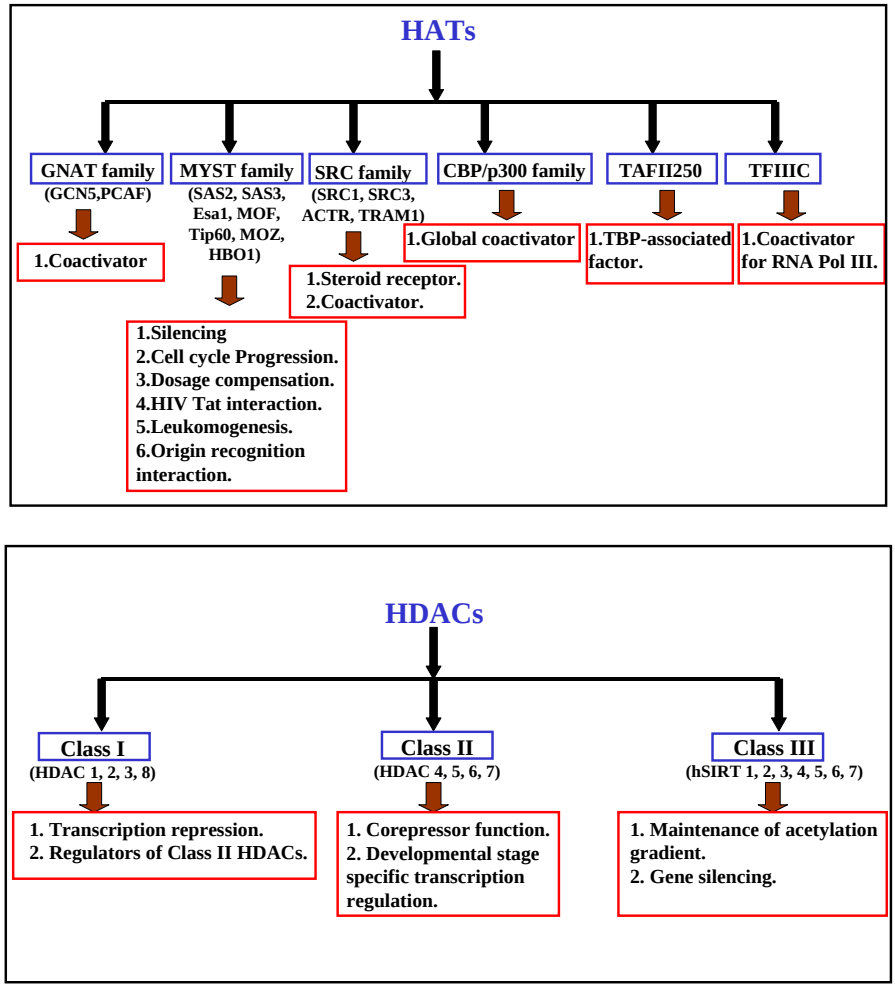


Figure 1.8: Classification of HATs and HDACs: The different classes of HATs and HDACs are grouped and the functions of each group are summarized.

The functional consequence of histone acetylation has been summarized in Table 1.1.

Table1.1: Functional consequence of histone acetylation

Sites	Functional Consequences	Enzymes
H3 K9	Transcription ⇧	SAGA (Sc)
H3 K14	Transcription ⇧	p300, PCAF
H3 K18	Transcription ⇧	p300,CBP,SAGA
H3 K23	Transcription ⇧	CBP, SAGA
H4 K5	Transcription ⇧	p300
H4 K8	Transcription ⇧	P300, PCAF
H4 K12	Histone deposition	Hat1 (Sc)
H4 K16	Sequence specific transcription factor binding	ATF2

Apart from the histones, the acetyltransferases have a broad range of substrate specificity which include transcription factors, coactivators, hormone receptors, structural proteins, histone chaperones and hence are known as Factor Acetyltransferases (FAT). Reversible acetylation of these factors can lead to an altered DNA binding ability influencing the chromatin structure. Furthermore, there occurs a transition between an open and closed chromatin structure as a consequence of the reversible acetylation. Interestingly, acetylated histones and nonhistone proteins can act as specific signaling platform promoting critical interactions with several factors leading to an open chromatin structure facilitating transcription. However, the interplay of factor acetylation, chromatin remodeling and histone chaperones coordinately regulates the transcription. Represented here in the Table 1.2 and 1.3 is the functional consequences of acetylation and deacetylation of nonhistone substrates by specific acetyltansferases and deacetylases.

Table 1.2: Functional consequence of Acetylation of the various groups of nonhistone proteins

Class of proteins	Nonhistone substrates	HATs	Functional consequence of acetylation
DNA binding transcription factors			
1.	p53	p300/CBP, PCAF	Enhanced DNA binding and hence transcription.
2.	C-Myb	p300/CBP, GCN5	Enhanced DNA binding and hence transcription.
3.	MyoD	p300/CBP, PCAF	Enhanced DNA binding and hence transcription.
4.	E2F	p300/CBP, PCAF	Enhanced DNA binding and hence transcription.
5.	GATA-1	p300/CBP	Enhanced DNA binding and hence transcription.
6.	EKLF	p300/CBP	Enhanced globin gene expression.
Non-histone chromatin associated proteins			
1.	HMGB1/B2	CBP	Enhanced ability to bend DNA
2.	HMGN1/HMGN2	p300, PCAF	Weakens the ability to bind to nucleosome core particle
3.	HMGA	p300/CBP PCAF	Enhanceosome disruption. Enhanceosome assembly.
General transcription factors			
1.	TFIIB	Autoacetylated	Stabilizes interaction between TFIIB and TFIIF and stimulates transcription.
2.	TFIIEβ	PCAF, p300/CBP, TAFII250	Unknown
3.	TFIIF	p300/PCAF	Unknown
Viral Proteins			
1.	Tat	PCAF p300/CBP	Enhanced transcription

Table 1.3: Functional consequence of Deacetylation of the various groups of nonhistone proteins

Class of proteins	Nonhistone substrates	HDACs	Functional consequence of deacetylation
DNA binding transcription factors			
1.	p53	HDAC1, Sir2	Reduced DNA binding ability and hence transcription.
2.	MyoD	HDAC1	Impedes cell differentiation.
3.	E2F	HDAC1	Transcription repression.
4.	GATA1	HDAC5	Transcription repression.
Viral Proteins			
1.	Tat	HDAC1	Transcription repression.

c. Methylation:

Methylation of gene expression correlates with both active as well as repressed chromatin states (Shilatifard, 2006). The site of modifications on the histone tails chiefly determines this functional specificity as represented in Table 1.4.

Table 1.4: Functional consequences of histone methylation:

Sites	Functional Consequences	Enzymes
H3 R2	**In vitro site**	**CARM1**
H3 K4	**Transcription⇧**	**SET7/ SET9**
H3 K9	**Transcription⇩**	**G9a**
H3 R17	**Transcription⇧**	**CARM1**
H3 R26	**Transcription⇧**	**CARM1**
H3 K27	**Transcription⇩**	**G9a (Mm)**
H3 K36	**Gene repression**	**Set2 (Sc)**
H3 K79	**Telomeric silencing**	**Dot1**
H4 R3	**Transcription⇧**	**PRMT1**
H4 K20	**Transcriptional silencing, Mitotic chromosome condensation**	**Pr-SET7/ SET8**

The site-specific K methylation is involved in transcriptional activation or repression, while the R methylation is involved in transcriptional activation. The K-methyltransferases are found frequently in a protein complex (eg. EZH2 is a functional component of polycomb group of proteins or Suv39H1-mediated trimethylation of H3K9 leading to the docking of HP1) establishing heterochromatinization. Summarized here are the classification of methyltransferases and the functional outcome of methylation in Figure 1.9. Although methylation has been considered as a comparatively stable mark, active turnover of methyl groups on histones may proceed by the method of replacement or covalent modifications by another class of enzymes, which function as demethylases (eg PADI4 or LSD1).

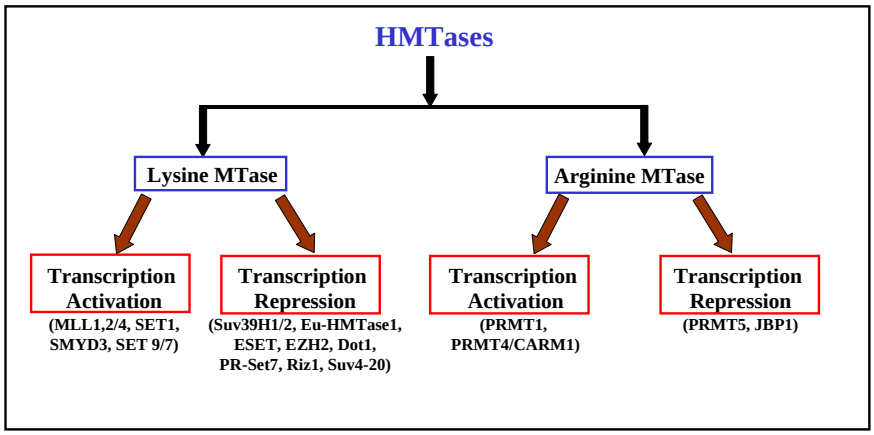


Figure 1.9: Classification of Histone Methyltransferases and their functional role.

Methylation is considered to be a modification of larger half-life and therefore contribute to the epigenetic memory. H3K9Me is associated with the heterochromatin assembly and to the stable silencing of genes where as H3K4Me and methylation of some H4 residues are related to the transcriptional activation. The differential pattern of histone methylation on the open reading frame of a transcribed gene like H3K36Me can play a role as a noninheritable marker for the "transcriptional memory" of recently transcribed genes (discussed later). H3K79Me is present as a broad modification throughout the open reading frame

of transcriptionally active genes. Although methylation is considered to be a stable modification recently a class of enzymes has been reported that are capable of removing methylation of Lysine via an oxidative reaction (LSD1) (Shi *et al*, 2004) or by antagonizing R methylation by conversion into Citrulline (PADI4) (Cuthbert *et al*, 2004). Recently a class of proteins (~30) containing the JmjC domain with histone lysine H3K9Me demethylase activity has been reported (Tsukada *et al*, 2006).

Unlike the acetyltransferase there are few nonhistone substrate of methyltransferase. Histone H1 gets methylated although the physiological significance of this event is not yet known (Wisniewski *et al*, 2006). The tumor suppressor p53 gets methylated by Set 9 (Chuikov *et al*, 2004). Methylation leads to a functional activation of p53, similar to acetylation. HMGA1 (a) protein gets methylated, although biological consequences are not yet established. The first DNA-binding AT-hook domain (R25) gets both mono and dimethylated. There are several other proteins belonging to the RNA processing pathway, which are found to get methylated. The transcriptional coactivator p/CIP (SRC-3/AIB1/ACTR/RAC3) binds to hormone-receptor complex and facilitates transcription by directly recruiting CBP/p300 and CARM1. The coactivator p/CIP gets methylated specifically by CARM1 (Naeem *et al*, 2006). This causes its rapid turnover as a result of enhanced degradation. Methylation of p/CIP also leads to an impaired ability to interact with CBP. This is an important example of coactivator methylation in hormone signaling (Naeem *et al*, 2006). Transcription activation of HNF4 (a nuclear receptor) follows two distinct functional modes in a methylation-dependent manner (Barrero and Malik, 2006). Firstly PRMT1 methylates DNA binding domain of HNF4 and enhances the affinity of HNF4 binding to DNA. PRMT1 is then recruited to the ligand-binding domain of HNF4. This is followed by p300 recruitment and consequently an alteration in the nucleosomal architecture leading to the PIC assembly.

d. Phosphorylation:

Phosphorylation of histones is intricately linked to cell cycle as described later. The differential functional consequence of phosphorylation of histone tails has been summarized in the following Table 1.5:

Table 1.5: Functional consequences of histone phosphorylation

Sites	Functional Consequences	Enzymes
H3 S10	Transcription ⇧	Snf1 (Sc), Rsk2
H3 S28	Mitotic chromosome condensation	Aurora-B
H4 S1	Unknown	?

A wide repertoire of nonhistone proteins gets phosphorylated with distinct functional consequence. Condensin protein gets phosphorylated (Takemoto *et al*, 2004) during different stages of cell cycle. Interestingly, the phosphorylation sites in interphase (CK2-mediated) and mitotic stage (Cdc2-mediated) are found to be distinctly different. Cdc2-mediated phosphorylation leads to: (i) the chromosomal targeting of Condensins, (ii) stimulation of its biochemical activity (like supercoiling activity). CK2-mediated phosphorylation, on the other hand, plays a negative role by reducing the biochemical activity of Condensins (Takemoto *et al*, 2006). The phosphorylation of neuronal MeCP2 regulates its nuclear translocation induced during cellular differentiation and/or maturation (Chen *et al*, 2003). Phosphorylation of PARP-1 by ERK1/2 (Kauppinen *et al*, 2006) is essential for its activity after DNA damage. Phosphorylation of Sir3p by Slt2p MAPK is directly linked to a shortened lifespan of yeast (Ray *et al*, 2003). Decrease in phosphorylation levels of certain proteins is also important cellular signals. Hypophosphorylation of architectural chromatin protein DEK occurs during induction of apoptosis and can be considered as a specific signal (Tabbert *et al*, 2006).

e. ADP Ribosylation:

Poly ADP ribose polymerase catalyzes the ADP ribosylation reaction and has both histones as well as nonhistone substrates (Hassa *et al*, 2006). Poly ADP Ribosylation of histone H2A (E residue) occurs in response to double strand DNA breaks and appear to play important role in DNA repair. There is also an increase in the level of auto ADP ribosylation of histone H1 in case of DNA damage (Kun *et al*, 2002). There are a number of chromatin proteins, which get ADP ribosylated in mitotic and interphase stages of cell cycle. Interestingly, the poly ADP ribose polymerase enzyme (PARP1), itself undergoes auto ADP ribosylation and causes dynamic chromatin alteration (as discussed). HMG1, 2, 14 and 17 also gets ADP-ribosylated (Tanuma *et al*, 1983). The tumor suppressor p53 (Wesierska-Gadek *et al*, 1996), and chromatin repair factor XRCC1 are also found to get this modification. The process of ADP ribosylation is found to be reversible by virtue of the enzyme poly-ADP-ribose-glycohydrolase (Realini and Althaus, 1992).

f. Ubiquitination:

Ubiquitination of histones directly regulates gene expression (Shilatifard, 2006). H2A ubiquitination has a direct functional consequence in spermatogenesis (Baarends *et al*, 1999). Histone H3K27Me and H2AK119Ub are absolute requirement for PcG gene silencing (Ringrose *et al*, 2004) and X-chromosome inactivation. H2B ubiquitination on the other hand has functional implication in meiosis and transcriptional activation. H2B can be monoubiquitinated by the enzyme Rad/Bre1 (Wood *et al*, 2005). This acts as a regulatory mark in signaling for histone methylation by COMPASS. Factors involved in telomere-associated gene silencing also showed that monoubiquitination is required for methylation. Interestingly, Paf1 complex, which is associated with elongating RNA Polymerase II, is required for histone monoubiquitination and thus methylation by playing a role in recruitment of factors (like COMPASS) to the transcribing polymerase (Wood *et al*, 2005). Ubiquitination is a reversible process.

Monoubiquitinated H2B can be deubiquitinated by the enzyme Ubp8, a component of the SAGA histone acetyltransferase complex that is required for transcription initiation (Henry *et al*, 2003; Daniel *et al*, 2004). Another deubiqitinating enzyme is Ubp10/DOT4, which is specifically involved in disrupting telomeric silencing and is also involved in deubiquitination of H2B (Emre *et al*, 2005).

g. Sumoylation:

Sumoylation (small ubiquitin related modifications) are involved in chromatin structure and gene regulation. This modification happens in case of histones as well as nonhistone proteins having distinct fate. All the four core histones get sumoylated, which has a negative role in transcription (Shiio and Eisenman, 2003). Sumoylation of MeCP2 acts as a nuclear transport signal. SUMO modification is involved in MBD1 (Methyl CpG binding protein) and MCAF1 interaction inducing chromatin silencing (Uchimura *et al*, 2006). Upon stress induction, sumo-conjugation inactivates transcription factor c-Myb.

h. Biotinylation:

Biotinylation of histone H4 (K12) is a new posttranslational modification. There is a drop in the biotinylation status of histone H4 in response to the DNA double strand breaks (Kothapalli *et al*, 2005). This was found to be an early signaling event arising before phosphorylation of H2B and poly-ADP ribosylation of H2A.

i. Formylation:

Formylation is yet another novel posttranslational modification of linker histone H1, which is reported recently (Wisniewski *et al*, 2006). The functional consequence of this modification is not elucidated yet.

Epigenetic interplay regulating gene expression:

Although the reversible acetylation or methylation leads to specific functional state, *in vivo* these modifications occur in combination leading to a destined outcome. Multiple histone modifications act synergistically or antagonistically towards a particular function.

Promoter DNA methylation is accompanied by the concomitant deacetylation resulting in gene silencing. This occurs through the recruitment of Methyl CpG binding proteins (MeCP2), which in turn recruits the HDACs leading to a repressed chromatin state.

H3S10P facilitates H3K14Ac along with H3K4Me. This also leads to H3K9 acetylation thereby preventing methylation and consequent transcriptional repression (Cheung *et al*, 2000).

H2BK123 monoubiquitination is a necessary prerequisite for H3K24 and H3K79 methylation resulting in an open chromatin structure (Sun and Allis, 2002).

Upon estrogen stimulation CBP mediated acetylation of H3K18 and K23 is followed by H3R17 methylation by CARM1 (Daujat *et al*, 2002).

LSD1 acts on trimethylated H3K4 making it unmethylated, which is negatively regulated by H3K9 acetylation and H3S10 phosphorylation. Thus deacetylation and dephosphorylation are necessary for the demethylation (Forneris *et al*, 2005).

Functional cooperativity between p300, PRMT1 and PRMT4 in activating p53 function (An *et al*, 2004), is yet another example of cross talk among the different enzymes in establishing a particular cellular fate.

The cis-trans prolyl isomerase regulates the confirmation of H3Pro38 (towards trans form), promoting the methylation of H3K38 by Set 2, promoting the transcriptional elongation phase (Nelson *et al*, 2006).

The functional diversity of chromatin is further brought about by specific histone variants, which are elaborated in the next section.

1.1.5 Role of histone variants in chromatin structure:

There are multiple copies of histone genes, which express mostly in the S-phase of cell cycle, encoding for the bulk of cellular histone proteins. These variants impart distinct nucleosomal structures and are involved in wide ranges of function (Sarma and Reinberg, 2005; Kamakaka and Biggins, 2005, Henikoff *et al*, 2004). Histone H1 has a large number of subtypes- H1.1, H1.2, H1.3, H1.4, H1.5, $H1^{oo}$, H1t, $H1^{o}$ (Khochbin, 2001, Khochbin and Wolffe, 1994, Kamakaka and Biggins, 2005).

Among the core histones H2A have the largest number of variants- H2AX, H2AZ, macro H2A and H2Abbd (Rogakou *et al*, 1998; Chadwick *et al*, 2001; Angelov *et al*, 2004; Costanzi and Pehrson, 1998; Suto *et al*, 2000). The variants of H3 include H3.2, H3.3 and CENP-A (H3.1 being the canonical histone) (Palmer *et al*, 1991; Tagami *et al*, 2004; Hake and Allis, 2006). Recently H2BFWT, a variant of histone H2B, has been identified (Boulard *et al*, 2006).

The functional role of histone variants in organizing higher order chromatin structure has been explored. Presence of H2A.Z assisted chromatin folding into a 30 nm fiber, but reduced fiber-fiber interactions and aggregation, similar to fiber containing acetylated histones. CENP-A containing nucleosomes also form higher order structure by orienting themselves to form the base of the kinetochore excluding the canonical nucleosomes containing histone H3.

The histone H1 variants can be sub-grouped into several classes: (i) Somatic (H1.1, H1.2, H1.3, H1.4, H1.5), (ii) Replacement ($H1^{o}$), (iii) Germ cell specific (H1t) (iv) Oocyte specific ($H1^{oo}$) variants. The histone H1 variants are mostly involved in chromatin condensation and formation of higher order chromatin structure.

The functional role of these subtypes has been summarized in Table 1.6.

Posttranslational modifications of the histone variants:

The epigenetic modifications of histone variants regulate the genome function. As per the “H3 barcode hypothesis” (Hake and Allis, 2006), mammalian H3 variants, although remarkably similar in sequence, undergoes differential degree of

posttranslational modifications that act as specific signatures thereby creating chromosomal territories influencing the epigenetic state during cellular differentiation and development. The epigenetic marks of H3.2 permit a more repressed chromatin structure, where as that of H3.3 entail a more active chromatin structure. Most of these modifications are absent prior to the incorporation of these variants into the chromatin (except H3K9Me). Non-nucleosomal H3.1 contains more H3K9Me1 compared to H3.3. Interestingly, H3.3 also shows K9/K14 diacetylation indicating a transcriptionally amenable chromatin structure. Pericentric heterochromatin region are rich in H3.1 having H3K9Me3. Studies on the posttranslational modifications of other histone variants are ongoing.

Table 1.6: Cellular functions of the histone variants:

	Cellular Functions	Involved Histone Variants
Histone H2A variants		
1.	DNA repair and recombination	H2AX
2.	Chromosome segregation and prevention of heterochromatin spread (barrier or insulator element)	H2AZ
3.	Transcription repression, X chromosome inactivation	MacroH2A
4.	Transcription activation	H2ABbd
Histone H2B variant		
1.	Specific epigenetic marker for assembly of mitotic chromosomes	H2BFWT
Histone H3 variants		
1.	Associated with facultative heterochromatin and is involved in transcription repression	H3.2
2.	Involved in transcription activation	H3.3
3.	Kinetochore assembly	CENP-A
Histone H1 variants		
1.	Chromatin compaction and formation of higher order structure	All H1 variants

1.2 Chromatin compaction- a necessary prerequisite for diverse cellular phenomenon: Factors affecting chromatin architecture across cell cycle

Eukaryotic chromosomes undergo massive structural alteration and spatial rearrangements as they prepare to condense to metaphase chromosomes. The chromosomal DNA undergoes very high degree of contraction, as a result of which there is modulation of histone-DNA interactions.

1.2.1 ***Stages of cell cycle:***

The cell division cycle is a tightly coordinated sequence of events that results in the faithful replication of the genetic material from a parent cell to two identical daughter cells. It has two distinct phases- Interphase (I) and Mitosis (M). Interphase can be further divided into G1, S and G2 sub-stages. DNA replication takes place in S-phase. G1 and G2 are preparatory stages leading to S or M phases respectively. Mitotic stage can be subdivided into four distinct stages- Prophase, Metaphase, Anaphase and Telophase having distinct chromosomal architecture. Prophase is marked by distinct chromosomal condensation, disappearance of nuclear membrane and dramatic changes in the microtubule organization beginning with bipolar spindle formation. During metaphase the compact chromosomes are aligned onto the center of the spindle pole. Anaphase is marked by the separation of each sister chromatids and their migration towards the edge of the spindle pole. Telophase marks the end of mitosis, when each chromatid reaches the edge of spindle pole. Subsequently nuclear envelops for the two individual daughter nuclei are reformed and the chromosomes decondense. Nuclear division is followed by cytokinesis and at the end two daughter cells each containing a complete set of chromosomes is formed.

Several molecular events happen during the stages of cell cycle. The alteration of chromatin structure as a consequence of posttranslational modifications of various chromatin components have been elaborated in the following section.

1.2.2 Posttranslational modification of canonical and variant histones and the consequent effect on cell cycle:

Eukaryotic cell cycle is marked by reversible histone modifications, which occurs to coordinate the structural transitions that take place during major cellular processes including transcription, replication and repair. Histone H1, H3 and H2A shows patterns of phosphorylation throughout the cell cycle stages. Histone H1 phosphorylation leads to the formation of condensed metaphase chromosomes. The graded increase in H1 phosphorylation levels occurs across S and G2 phase, reaching a hyper-phosphorylated state at metaphase. However, all phosphate groups are lost by Telophase. Interestingly, the hyper-phosphorylation of H1 in metaphase stage occurs in unique sites and thus phosphorylation mark is considered as a mitotic trigger leading to a faithful separation of chromosomes during interphase. However, contrasting reports suggests that H1 hyper-phosphorylation is not an absolute requirement for chromatin condensation. The recent reports elucidate the site-specific phosphorylation of H1 in mitotic and interphase stages having different functional consequences. The phosphorylation sites are Ser residues (of SPKK motif) during interphase and Thr residues (of TPKK motif) during mitotic stage (Sarg *et al*, 2006). Thus the phosphorylation of Thr residues are directly connected to the chromatin condensation visualized during mitosis (Sarg *et al*, 2006). Histone H1 phosphorylation also regulates ATP-dependent chromatin remodeling machinery (Dou *et al*. 2002**)** and also transcription of some specific genes (Dou *et al*. 1999; Herrera *et al*. 1996; Chadee *et al*. 2002)**.** This indicates H1 phosphorylation has a direct connection to an open chromatin architecture regulating cell cycle and transcription.

A strong correlation is observed between core histone H3 phosphorylation and chromatin compaction (Gurley *et al*. 1978; Paulson and Taylor 1982; de la Barre *et al*. 2000; De Souza *et al*. 2000). Interphase stage does not show H3 phosphorylation. Mitotic stage, however, shows H3 phosphorylation, which is lost at terminal stage of Telophase. Histone H3 phosphorylation at S10 during mitosis is severely modifies H3-H4 interactions leading to an alteration in nucleosomal

structure in metaphase stage. Thus general protein kinase inhibitors treated to the mitotically arrested cells, leads to a dephosphorylation of H3 and a consequent decompaction of chromatin. Recent studies, however, show a cross-talk between phosphorylation of histone H3 Ser 10 and acetylation or methylation of histone H3 (Cheung *et al*. 2000; Lo *et al*. 2000; Edmondson *et al*. 2002; Rea *et al*. 2000). H3S10P mediated by Aurora Kinase disrupts the interaction of HP1 to the H3K9Me residue (Hirota *et al*. 2005; Fischle *et al*. 2005). Apart from H3S10P, other H3T3P and H3T11P also occur during mitosis (Polioudaki *et al*. 2004; Preuss *et al*. 2003). H3S28P by Aurora Kinase B directly connects to mitotic chromosome condensation (Goto *et al*. 2002). Centromeric histone H3 variant CENP-A gets phosphorylated at S7 residue and in absence of this modification causes mislocalization of Aurora B, phosphatase PP1γ1 and inner centromere protein (INCENP) (Zeitlin *et al*. 2001). Phosphorylation of H3.3 takes place at S31 residue only in late prometaphase and metaphase and such modifications occur adjacent to the centromeric region (Hake *et al*. 2005). Current evidences shows that along with from H3S10P, a combination of H3K9Me or H3K9,14Ac play important roles in regulating cell cycle related chromosome dynamics and transcriptional activation. A recent report focuses the role of histone methylation in direct connection to cell cycle. E2F dependent regulation of Cyclin E1 (involved in regulating G1/S cell cycle checkpoint) gene was shown to correlate with H3K9 acetylation / methylation. Furthermore, the methylation of R17 and R26 is important for activating the Cyclin E1 gene by CARM1 establishing a direct connection of methylation to gene expression (chromatin decompaction) in a cell cycle dependent manner (El Messaoudi, *et al*, 2006). Apart from the modification of the canonical histones the modification of histone variants regulates the final epigenetic state (Loyola *et al,* 2006). As per the proposed model the early acting enzymes can modify free H3.1 and H3.3 establishing a pre-nucleosomal modification status. However, the late acting enzymes act in specific chromatin sites (like H3.3K9Me2 or H3.3K9,14Ac) leading to specific destined fate. These results suggest that a combination of different histone modifications execute specific physiological outcome.

The core histone H2B N-terminal tail plays important role in chromatin compaction (de la Barre *et al*. 2001). The phosphorylation of H2BS14 by Sterile 20 kinase is directly connected to chromosome condensation as visualized during apoptosis (Ajiro 2000; Cheung *et al*. 2003). H2BS10P modulates chromatin structure during meiosis (Ahn *et al*. 2005). H2BS33P is essential for transcriptional activation events that promote cell cycle progression (Maile *et al*. 2004). The core histone H2A remains phosphorylated throughout the cell cycle. Enhancement of H2A phosphorylation induces chromosome compaction, concomitant with H3 phosphorylation. H2AT119P by NHK-1 is an important event that regulates mitotic and meiotic progression (Cullen *et al*. 2005). Histone H2A variant γ-H2AX (H2AXS139P) leads to the recruitment of DNA damage response proteins in response to DNA double strand breaks (Fernandez-Capetillo *et al*. 2002; Nakamura *et al*. 2004; Morrison *et al*. 2004). Recent studies establish a direct interaction between mammalian MDC1 and γ-H2AX is an important requirement for DNA damage response (Stucki *et al*. 2005). Phosphorylation of H4 increases during the cell cycle similar to H2A (Barber *et al*. 2004). There is a drop in H2A or H4 phosphorylation in early S phase (Barber *et al*. 2004). Recent reports suggest that histone H4 phosphorylation has an important role in DNA double-strand break repair, cell-cycle progression and gene expression. Phosphorylation of H2A and H4 are found to be evolutionarily conserved modification that might be having separate roles in mitotic and interphase stage of cell cycle (Barber *et al*, 2004).

A specific class of proteins is found as integral chromatin component throughout the stages of cell cycle. The functional roles of such proteins have been elaborated in the following section.

1.2.3 The role of general transcription factors in "bookmarking" the genes:

Gene "bookmarking" is a mechanism of epigenetic memory to transmit the pattern of active genes through mitosis (Sarge and Park-Sarge, 2005). Genes that exist in a transcriptionally active state are marked by certain proteins or a

definitive modification state before mitosis, which helps in the assembly of transcription machinery in early G1 ensuring the similar pattern of gene expression in daughter cells as that of the parental cell. 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 (Chen *et al*, 2002; Segil *et al*, 1996). Several TAFs associated with the mitotic chromatin get phosphorylated and consequently cannot modulate activator dependent transcription, which is restored upon dephosphorylation (Segil *et al*, 1996). 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 (Chen *et al*, 2002; Christova and Oelgeschlager, 2002). Another classic example of bookmarking is seen in case of the stress inducible gene hsp70. Transcription factor heat shock factor 2 (HSF2) binds to the promoter element (heat shock element-HSE) in mitosis, recruits protein phosphatase 2A (PP2A) and interacts with condensisns to promote dephosphorylation and consequent inactivation of the condensing complex preventing chromatin compaction (Xing *et al*, 2005). Knocking down HSF2 decreases hsp70 induction and survival of stressed cells in G1 (Xing *et al*, 2005), demonstrating the *in vivo* significance of the gene bookmarking phenomenon. Recent report provides another evidence of the molecular mechanism of bookmarking in the hepatocyte nuclear factor 4 (HNF4) gene, contributing towards prevention of a permanent silenced loci during the repressed state by maintaining a distal enhancer – proximal promoter communication (Hatzis *et al*, 2006). Mitosis associated protein kinase (MAPK) activation down regulates HNF4 expression, by dissociation of several factors thereby disrupting the enhancer-promoter complex. However, the promoter region remains marked by components of TFIID as well as HNF-6 and HNF1α with retention of

nucleosomal acetylation status (Hatzis *et al*, 2006) that could lead to the HNF4 gene expression under suitable cellular signal establishing the enhancer-promoter complex formation by recruitment of the necessary factors leading to the assembly of transcription machinery.

1.2.4 Chromatin remodeling in connection to cell cycle:

Chromatin remodeling in context of cell cycle is a largely unexplored area. The remodeling complexes have roles in cell proliferation and are regulators of genes involved in cellular transformation. These remodelers are shown to act as putative tumor suppressor. Cyclin E directly interacts with the SWI/SNF remodeling complex, and also gets phosphorylated by the Cyclin E-Cdk2 complex. This phosphorylation is thought to regulate the activity of the SWI/SNF remodeling complex thereby directly connecting chromatin remodeling with the cell cycle.

1.3 Gene expression in the chromatin context

Transcription is an ordered recruitment of a set of factors, which helps in delineating the coding sequences of the genome. In eukaryotic system the transcription regulation is a complex set of events involving three distinct RNA Polymerases (Polymerase I, II and III for catalyzing rRNA, mRNA and tRNA and 5s rRNA transcripts) for transcribing distinct set of genes, corresponding general transcription initiation factors, gene specific transcription regulatory factors (eg. activators or repressors) and a variety of coregulatory factors (eg. coactivators or corepressors) that act through chromatin modifications facilitating the formation of preinitiation complex. Thus multiple regulatory steps coordinately regulate eukaryotic gene expression (Lieb and Clarke, 2005; Mellor, 2006).

The order of events from a chromatin template initiates with the binding of transcription activator, which is a highly cooperative event. The activator can recruit the coactivator complexes, which acts as a bridging molecule between the activator and the basal transcription machinery, the latter being recruited in a stepwise manner, leading to the formation of a functional preinitiation complex.

The transcription initiation is followed by elongation and termination, which are again multiple factor dependent steps leading to the first level of gene expression.

1.3.1 Basal Transcription Machinery:

The core promoter region has the TATA box and the INR elements, which are the consensus sites for the assembly of the Preinitiation Complex (PIC). The sequence of events initiates with the recruitment of TBP component of TFIID at the minor groove leading to a bending of the DNA thereby bringing about distant upstream and downstream sequences in close proximity. TFIIA stabilizes this interaction, followed by recruitment of TFIIB, which serves to selectively assemble TFIIF-RNA Pol II complex. Finally TFIIE and TFIIH are recruited to form the functional PIC. Transcription initiation leads to the transition from of closed complex to open complex following the RNA Polymerase II CTD phosphorylation. Recent studies demonstrate that specific methyltransferases are also recruited by transcription elongation complex (PAF) following the phosphorylation of RNA Pol II CTD (Hampsey and Reinberg, 2003). Another school of thoughts suggests that instead of sequential or ordered recruitment of PIC, RNA Pol II holocomplex gets recruited to the promoter region as a whole.
The functional role of the general transcription factors (Roeder, 1996; Woychik and Hampsey, 2002; Thomas and Chiang, 2006) has been summarized in the following Table 1.7

1.3.2 General Transcription Cofactors:

Earlier studies to elucidate the mechanism of activator-dependent transcription activation showed that apart from the initiation factors activated transcription did require additional factors like coactivator or mediator (Featherstone, 2002). In human cells the Upstream Stimulatory Activity (USA) fraction was resolved into a number of positive and negative cofactors. The positive factors included PC1, PC2, PC3/Dr2, PC4 and ACF (Meisterernst *et al*, 1991; Kretzschmar *et al*, 1994; Ge and Roeder, 1994; Kretzschmar *et al*, 1993).

Table 1.7: General Transcription Factors

	General Transcription Factors	Subunits	M. Wt. (kDa)	Functions
1.	TFIID (TBP, TAFs)	1 (TBP) 12 (TAFs)	38 (TBP) 15-250 (TAFs)	Initiation of PIC formation by TBP (TATA elements). TBP interacts with Activators, Coactivators and TFIIB. TAFs act as coactivators and are required for promoter recognition (for non-TATA elements). TAFII250 has both kinase (TFIIF phosphorylation) and acetyl transferase (core histone acetylation) activity.
2.	TFIIA	3	12, 19, 35	Stabilizes TBP binding and TAF-DNA interaction. TFIIA shows anti-repression function. It interacts with specific transcription activators, TAFII110 and certain coactivators.
3.	TFIIB	1	35	RNA Pol II-TFIIF complex gets recruited. Interacts with TFIID and Pol II-TFIIF complex that is essential for start site selection.
4.	TFIIF	2	30, 74	Facilitates promoter targeting of Pol II. Destabilize non-specific Pol II-DNA interactions. Phosphorylation facilitates transcription initiation and elongation.
5.	RNA Pol II	12	10-220	Essential enzyme catalyzing mRNA synthesis. Although its initial recuitment is in a dephosphorylated state, its largest subunit CTD has a heptapeptide (YSPTSPS) repeat which gets phosphorylated to enter into elongation phase. Interacts with several transcription factors, coactivators, mediator and DNA response element through protein-protein and protein-DNA interaction.
6.	TFIIE	2	34, 57	Facilitates TFIIH recruitment. Stimulates ATP hydrolysis to provide energy for helicase and kinase activity of TFIIH.
7.	TFIIH	9	35-89	Promoter melting using helicase activity is mediated by TFIIH. Phosphorylates Pol II CTD, which is important for elongation process. TFIIH can phosphorylate other coactivators as well as established roles in Nucleotide Excision Repair (NER).

Other chromatographic techniques also led to the identification of several cofactors like CofA, PC5, PC6, and HMG2 (Shykind *et al*, 1995; Stelzer *et al*,

1994). A functional component of PC1 is Poly ADP Ribose Polymerase (Kaiser and Meisterenst, 1996). PC2 is a multiprotein complex having specific functional aspects as elaborated later. PC3 is DNA Topoisomerase I that facilitates structural change in DNA. PC4 is a 15 kDa protein which could activate activator-dependent transcription to ~85 fold (Ge and Roeder, 1994; Kretzschmar *et al*, 1994). Two components closely related to PC4- p52 (shows broad specificity and functional activity similar to PC4) and p75 (shows activator selectivity but is less active as compared to PC4) were obtained from same fraction as PC4. PC5 and PC6 functions along with the regulatory factors for efficient PIC formation. HMG2 is homologous to HMG1, which can induce DNA bending for efficient binding of activators and basal machinery components for activating transcription. PC1, PC3, PC4 and HMG2 show nonspecific DNA binding ability. They have been hypothesized to act as chromatin architectural proteins that stabilize the PIC in conjunction with activator binding. Furthermore, the ability of these factors to alter the topological or structural constraints of DNA indicated probable gene specific functions *in vivo*. However, PCs show the ability to act independently *in vivo*.

One of the positive cofactor was identified as the Mediator (PC2), a 500 kDa multi-protein complex. The Mediator complex (highly conserved from yeast to humans) was found to interact with RNA Polymerase II (Malik and Roeder, 2000). Human Mediator complex was first discovered in association with thyroid hormone receptor (TR). This TRAP/SMCC complex (TR Associated Protein) was found to be essential for TR-dependent transcription. TRAP/Mediator complex function dynamically at two levels – (a) in formation of PIC assembly in association with the activator (like VP16 and p53) and coactivators (like PC4); (b) in modulation of RNA Polymerase II activity following a structural alteration (by a direct interaction with RNA Pol II). The TRAP and SMCC complex was initially discovered separately and later on found to be identical. This 1.5 MDa complex contains ~25 proteins and has been implicated in tissue specific transcriptional activation. Several other mammalian mediator complexes have been discovered including- DRIP, ARC, CRSP, and NAT (Gu *et al*, 1999; Ito *et*

al, 1999; Fondell *et al*, 1999; Malik *et al*, 2000; Rachez *et al*, 1999; Naar *et al*, 1999; Ryu *et al*, 1999; Jiang *et al*, 1998; Sun *et al*, 1998).

A class of cofactors termed negative cofactors (NCs) that can repress transcription include Ada/Mot1, NC1 and NC2 (Auble *et al*, 1994; Meisterernst *et al*, 1991; Meisterernst and Roeder, 1991; Inostroza *et al*, 1992). TBP is released by Ada, where as NC1 binds tightly to TBP competing out TFIIA and thereby both can prevent PIC formation. However, contradictory studies suggest HMGB1 to be a functional component of NC1 (Ge and Roeder, 1994). NC2, a two subunit (NC2α and NC2β) containing protein shows remarkable homology to core histones H2A and H2B (Goppelt *et al*, 1996). It can also bind to TBP thereby competing out TFIIA and TFIIB and inhibiting PIC formation (Meisterernst and Roeder, 1991).

1.3.3 Activator recruits coactivators:

Several specific examples of activator-mediated recruitment of coactivators are reported. The DNA octamer element (ATTTGCAT) is a regulatory sequence for several genes (eg. Immunoglobulin or H2B promoter). Ig promoters are activated in B lymphoid cells while H2B promoters are activated in S phase of the cell cycle. Oct family of proteins is sequence specific (octamer DNA element) transcription and DNA replication factor, which has POU domain as the DNA binding domain. The B cell specific coactivator OCA-B acts in conjunction with the activator Oct protein (Oct1) for transcribing Ig promoters (Luo and Roeder, 1995; Siegel *et al*, 2006). This is the first example of tissue specific promoter regulatory mechanism, where coactivator creates the specificity. OCA-S (containing p38 or GAPDH as a part of this multi-component complex), on the other hand, is the coactivator for H2B transcription in S-phase (Zheng *et al*, 2003).

1.3.4 Coordinated interplay of factors for transcription from chromatin template:

Chromatin transcription is a functional coordination amongst several factors orchestrating the first step of gene expression (Gregory and Horz, 1998). Activators recruit numerous proteins including ATP-dependent chromatin remodeling complexes along with histone chaperones to effectively regulate transcription in a cell cycle dependent manner. Several biochemical, genetic and structural studies have contributed to the novel insights into transcription, as well as functional significance of posttranslational histone modifications having specific consequences. Studies regarding transcription elongation show how chromatin structure may be maintained after RNA Polymerase II traverses a nucleosome. Further novel role of RNA Polymerase II in mRNA maturation, surveillance and export to the cytoplasm are also explored.

A functional interaction between HAT and remodeling complexes has been monitored prior to transcription initiation (Fry and Peterson, 2002; Workman *et al*, 2006; Clayton *et al*, 2006). In case of yeast HO promoter, activator Swi5p recruits SWI/SNF, which is further responsible for the binding of Gcn5p to the promoter element. Gcn5p mediated histone acetylation is a prerequisite for the recruitment of another activator, SBF, finally forming the PIC assembly (Cosma *et al*, 1999). In case of human IFN-β promoter, viral infection generates a signal that induces binding of a group of activators (like NFκB) and leads to the formation of enhanceosome complex. This complex promotes the recruitment of Gcn5p, which acetylates nucleosomes in the promoter region. Upon acetylation there is recruitment of SWI/SNF, which remodels the chromatin facilitating the PIC assembly (Agalioti *et al*, 2000). In the case of α1-AT gene, HNF-1 and two general transcription factors TBP and TFIIB, remain bound to the promoter region, even before recruitment of the remodeling complex. Suitable cellular signals lead to a recruitment of RNA Polymerase II and other general transcription factors leading to the formation of PIC. However, another activator

HNF-4 leads to the recruitment of remodelers (SWI/SNF) and HATs (CBP, PCAF) to the promoter after the PIC formation (Soutoglou and Talianidis, 2002).

The FACT complex is a major determinant of RNA Polymerase II transcription elongation through nucleosomes (Orphanides *et al*, 1998; Belotserkovskaya *et al*, 2003; Mason and Struhl, 2003; Saunders *et al*, 2003; Sims *et al*, 2004; Reinberg and Sims, 2006). It can destabilize the nucleosomes by removing H2A-H2B, to enable RNA Polymerase II passage. The histone chaperone activity of this complex, leads to the reestablishment of nucleosome structure after the passage of RNA Polymerase II. Like FACT, there are other factors (SPT5, SPT6 which can displace the hyperacetylated histone octamer), which remain associated to the transcriptionally active genes and have predicted role in transcription elongation. Analogous to the FACT, remodeling complex (SWI/SNF, RSC) facilitates exchange of H2A-H2B. ATP dependent SWR1 complex can selectively exchange H2A variant (including H2AZ) (Mizuguchi *et al*, 2004). *In vivo* FACT and SWR1 may be coordinated to facilitate the exchange of H2A by H2AZ.

The role of histone acetyltransferases has been elucidated in details, as a determinant in regulating the transcription (H3 K9, 14 acetylation are marks of active transcription). Recently it has been reported that autoacetylation induced conformational change and subsequent dissociation of p300 acts as a catalytic switch leading to preinitiation complex assembly (Black *et al*, 2006). Present studies, however, highlight the role of histone methyltransferases recruited by RNA Polymerase II in actively transcribing genes and the functional consequence of this (Eissenberg and Shilatifard, 2006). Elongating RNA Polymerase II can recruit HMTases to methylate H3K4 (by Set1 containing complex) and K36 (by Set2 containing complex), in the body of actively transcribing genes. Although H3K9Me (Suv39H1-mediated) is a repressive mark observed in the heterochromatin regions, recent reports suggest the presence of H3K9Me (mediated by G9a) are also present in the euchromatic regions (Tachibana *et al*, 2002; Rice *et al*, 2003). It has been reported that H3K9 methylation status has been increased in the actively transcribing genes (including the housekeeping genes), and there is a drop of his marker level in repressed genes. Along with an

increase in H3K9Me in active genes, there is a deposition of HP1γ (Vakoc *et al*, 2005). Blocking transcription elongation seems to reduce H3K9Me marks as well as HP1γ levels. Coimmunoprecipitation of phosphorylated (Ser2, Ser5) RNA Polymerase II (indicative of transcription elongation stage) brings HP1γ, suggesting a dual interaction of HP1γ with RNA Polymerase II and H3K9Me (Vakoc *et al*, 2005). These finding suggests a mechanism of deposition of HP1γ on active genes by elongating RNA Polymerase II. According to transcription coupled chromatin renewal model, the removal of methyl marks occurs by the displacement of methylated nucleosmes by advancing RNA Polymerase II (Vakoc *et al*, 2005). The loss of methyl marks occurs along with the drop in RNA Polymerase II activity. Another possibility is the involvement of a demethylase (LSD1), which removes the H3K9Me marks as repression occurs.

Recent reports also establish that histone are evicted and deposited during transcription by RNA Polymerase II. Histone chaperone Asf1 (a H3-H4 chaperone) associates with the promoters and coding regions of transcriptioally active genes and is capable of activator mediated eviction of histones at the PHO5 promoter (Schermer *et al*, 2005; Korber *et al*, 2006). Interestingly, Asf1 can lead to histone H3 removal and deposition during Polymerase II elongation a process that also involves FACT and Spt6, suggesting a stepwise manner of nucleosome assembly and disassembly (Schwabish and Struhl, 2006). Recent co crystal structure of Asf1 with H3-H4 heterodimer provides insight on the mechanism of histone removal by Asf1 (English *et al*, 2006). The C-terminus of H4 undergoes dramatic conformation change upon Asf1 binding thereby preventing H3-H4 tetramer formation. Recently acetylation dependent (p300-mediated) activation of transcription by another histone chaperone NPM1 (H3-H4, H2B and H1 chaperone) has also been reported (Swaminathan *et al*, 2005) and nucleosome disassembly is the hypothesized mechanism. Another report focuses on the role of histone chaperone Nucleolin (a H2A-H2B chaperone), possessing activity like FAT complex and helping in the remodeling of SWI/SNF of the variant nucleosomes (containing macro H2A, but not H2ABbd) (Angelov *et al*, 2006). Histone chaperone JDP2 (H2A-H2B, H3-H4), can inhibit p300-mediated histone

acetylation (Pan *et al*, 2003; Jin *et al*, 2006) and may regulate transcription affecting the step of chromatin assembly. Rtt109, another histone chaperone, is involved in maintenance of H3K56 acetylation status and is associated with elongating form of RNA Polymerase II (Schneider *et al*, 2006).

1.4 Human Positive Coactivator PC4- a multi-functional protein

Human Positive Coactivator PC4 (p15) was purified from the upstream stimulatory activity (USA) fraction and could enhance the activator-dependent RNA Polymerase II mediated transcription to ~85 fold (quantitatively the largest among all the coactivators from USA). This protein was discovered by two independent groups and was found to be a multifunctional, highly conserved protein across species.

1.4.1 Domain organization and the Structural aspects of PC4:

127 amino acid containing protein PC4 has a structured C-terminal domain (62-127 amino acids), while the N-terminal 62 residues are highly dynamic in function. There are two **Se**rine rich **Ac**idic domains (SEAC) from residues 9-22 and 50-58, separated by **Lys**ine rich domain (LYS) from residues 22 to 50 (Werten *et al*, 1998) (Figure 1.10). The residues 22-87 have the double stranded DNA binding ability and coactivation function. The residues 63-127 are single stranded DNA (ssDNA) binding and dimerization domain and are highly conserved amongst species (from mammals to yeast). Yeast PC4 also known, as Sub1/Tsp1 is a 293 amino acid protein (Henry *et al*, 1996; Knaus *et al*, 1996) containing a Carboxyl terminal extension of unknown significance and also contains fewer Ser residues as compared to the human counterpart.

The crystal structure of PC4 CTD showed that the biological unit is a dimer with two ssDNA binding channels running in opposite direction to each other. Each of the monomers consist of a curved four stranded anti – parallel β-sheet followed by a 45° kink α helix (Brandsen *et al*, 1997). Recently PC4 in complex with a 20-mer oligonucleotide structure reveals β surface of the homodimer interact with

juxtaposed 5 nucleotide DNA regions running in opposite direction (Werten and Moras, 2006). R86, F77, W89 are three highly conserved residues of PC4 having specific functions to play. R86 is involved in H-bonding interaction with DNA via water, while F77 and F89 are involved in stacking interactions with DNA bases and are thus essential for the DNA binding ability of PC4 (Werten and Moras, 2006).

1.4.2 Role of PC4 in various DNA templated phenomena:

PC4 is a highly abundant, multifunctional protein, which plays an important role in transcription, repair and replication (Ge and Roeder, 1994; Kretzschmar *et al*, 1994; Pan *et al*, 1996; Wang *et al*, 2004).

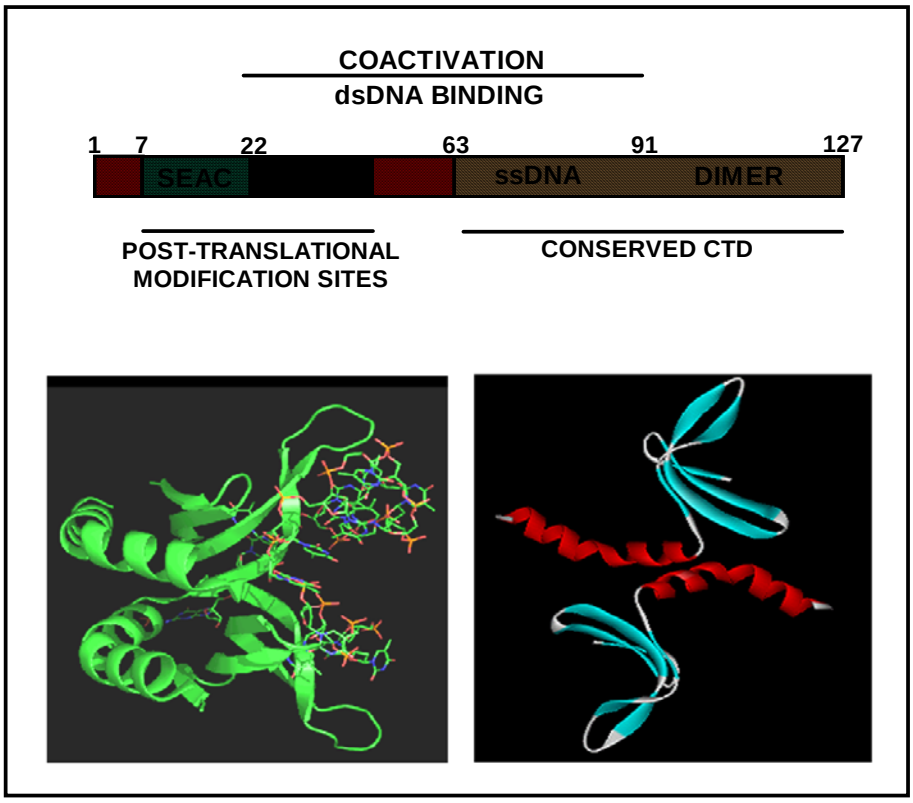


Figure 1.10: Human transcriptional coactivator PC4: The upper panel shows the domain organization of PC4. The lower panel represents the structure of PC4 CTD either in combination with the DNA or alone (in a dimeric state of interaction).

a. Transcription-

PC4 facilitates activator dependent transcription by RNA polymerase II, through direct interactions with general transcription factors as well as transcriptional activators (eg. p53, VP16, Tat, CTF1, NFκB, Sp1, BRCA1, AP2) (Banerjee *et al*, 2004; Ge and Roeder, 1994; Haile and Parvin, 1999; Kannan and Tainsky, 1999; Holloway *et al*, 2000). This 15-kDa protein interacts with free or DNA bound TFIIA and TBP component of the basal transcription machinery (Kaiser *et al*, 1995) but not with TBP-TFIIB complex or free TFIIB. It cannot interact with highly purified TFIID alone, in the absence of TFIIA (Ge and Roeder, 1994).

In contrast to human PC4, yeast Sub1 fails to bind TFIIA and interacts with TFIIB (Sub1 was first discovered as a suppressor cold sensitive TFIIB mutation) leading to transcription activation *in vivo*. Apart from its role in transcription, PC4 can interact with TFIIH (Fukuda *et al*, 2003) 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 (Wang *et al*, 2004). 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, recent reports indicate PC4 activates p53 function *in vitro* and *in vivo* (Banerjee *et al*, 2004). This can be attributed to the DNA bending ability of PC4, a novel functional aspect of PC4 (unpublished data). However, Contrasting reports suggest that PC4 inhibits RNA Pol-II phosphorylation and hence Pol-II mediated transcription (Schang *et al*, 2000). Furthermore, PC4 acts as a potent inhibitor of transcription in regions of unpaired dsDNA, ssDNA and on DNA ends (Werten *et al*, 1998). PC4-mediated transcription repression can be relieved by ERCC3 Helicase activity of TFIIH (Fukuda *et al*, 2003). Its diverse cellular function also includes its ability to interact with TFIIIC, influencing the process of re-initiation and termination in RNA Polymerase III dependent transcription (Wang and Roeder, 1998). Recently it has been shown that it also has a role in promoter release and transcription elongation in GAL4- VP16 dependent

transcription (Fukuda *et al*, 2004). Interestingly, Sub1, the yeast homologue of PC4, has also established function of preventing premature transcription termination by interacting with the CTD modifiers, thereby having a role in transcription elongation. PC4 can also interact with CstF64, thereby has a role in polyadenylation and subsequent transcription termination (Calvo and Manley, 2001). Hence the role of PC4 in transcription regulation is throughout all the stages starting from initiation, elongation till termination. Recently bioinformatic analysis of PC4 promoter reveals several transcription factor-binding site (eg. p53, AP2, NFκB) indicative of an auto-regulatory loop involved in maintaining dynamic functional regulation (unpublished data). Downstream promoter elements are regulatory elements that add diversity to the promoter architecture of RNA Polymerase II transcribed genes. Recent report shows apart from the GTFs, transcription from DPE (downstream promoter element) and not DCE (downstream control element) requires both PC4 and CK2. Thus PC4 has selective role to coordinate transcription from specific downstream promoter element and CK2 can act as a switch converting the functioning of transcription machinery from one downstream element to another (Lewis *et al*, 2005).

b. Replication-

PC4 can also form complex with Human single stranded DNA binding (HSSB) protein on ssDNA and markedly affect the replication function of SV40 virus (Pan *et al*, 1996). Furthermore, it also plays important role in replication of adeno-associated virus by interacting with the Rep proteins (Muramatsu *et al*, 1998).

c. Repair-

Apart from the aspects of transcription-coupled repair, PC4 can also inhibit AP2 self-repression in a *ras* transformed cell line and thus can act as a putative tumor suppressor (Kannan and Tainsky, 1999). The tumor suppression activity of PC4 could also be through its ability to enhance the p53 function *in vivo* (Banerjee *et al*, 2004).

1.4.3 Interacting partners of PC4:

The multifunctional protein PC4 can interact with a wide repertoire of proteins with a distinct functional consequence. The functional implications of various PC4 interacting partners are summarized in the following Table 1.8:

Table 1.8: PC4 interacting partners

	Interacting partner	Function
Basal Machinery Components		
1.	TFIID (TBP)-TFIIA Complex	Activates RNA Polymerase II transcription.
2.	TFIIH	PC4 mediated repression in certain promoters is relieved by TFIIH helicase activity.
3.	TFIIIC	Activates RNA Polymerase III transcription.
Transcriptional Activators		
1.	p53	Activates p53 transactivation, possibly through DNA bending.
2.	AP2 (α)	AP2 self repression is released by interaction with PC4
3.	VP16	Activates transcription initiation, promoter release and elongation phase.
4.	Tat	Enhanced activation of HIV LTR.
5.	OCA B	Activation of Oct1-OCAB transcription in B cells.
6.	HNF-4	Activation of tissue-specific (liver) gene expression.
Replication Factors		
1.	HSSB (RPA)	Activates SV40 virus replication.
2.	Rep68, Rep78	Activates Adeno associated virus replication.
Repair factors		
1.	XpG	Transcription coupled repair
Others		
1.	CstF64	Transcription termination

1.4.4 ***Posttranslational modifications of PC4:***

PC4 undergoes posttranslational modifications having distinct functional consequences. Acetylation and Phosphorylation are two such well-studied posttranslational modifications, which can act as regulatory switch for PC4. Acetylation is mediated by the functional activity of p300 enzyme both *in vitro* and *in vivo*. At least two Lysine resides (in the canonical p300 motifs) are found to get acetylated both *in vitro* (Kumar *et al*, 2001) and *in vivo* (unpublished data). Recent results also indicate PC4 can be deacetylated by the HDAC1 (unpublished data). Although *in vitro* studies show that phosphorylation of PC4 can take place by the enzyme Casein Kinase II (CKII) and Protein Kinase C (PKC), mass spectrometric studies show that *in vivo* hyperphosphorylation of PC4 is mainly mediated by CK2 (Ge *et al*, 1994). The sequence of PC4 with the canonical phosphorylation sites mediated by CK2 and PKC has been represented in the Figure 1.11. There are 7 phosphorylation sites by CKII at the SEAC domain of PC4 (Ge *et al*, 1994). However, TFIIH and TAFII250 subunit of TFIID can also phosphorylate PC4 in the PIC complex. The functional consequences of acetylation and phosphorylation are antagonistic in nature. Acetylation enhances dsDNA-binding ability of PC4, where as its phosphorylation leads to the loss of the same (Kumar *et al*, 2001). Interestingly, phosphorylation negatively regulates the acetylation of PC4, while the reverse can happen (Kumar *et al*, 2001). Furthermore, phospho-PC4 completely looses its transcription coactivation function (Ge *et al*, 1994). Interestingly, recent reports suggest that acetylated-PC4 can activate p53 function (unpublished data). Thus phosphorylation - acetylation dependent functional switch is critical in explaining the functional diversity of PC4.

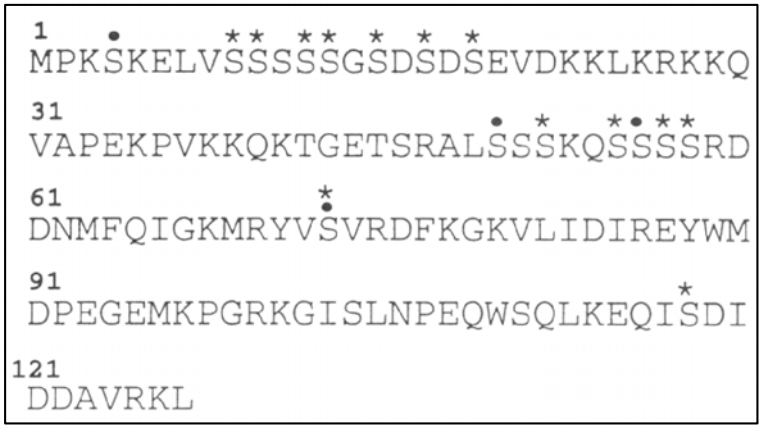


Figure 1.11: Amino acid sequence of PC4 with the canonical phosphorylation sites mediated by CK2 (represented by asterisks) and PKC (represented by dots).

1.5 Aim and scope of the present study

Chromatin is a highly dynamic and functional base of eukaryotic genome. The maintenance of higher ordered compact chromatin structure is interplay of several factors. Nonhistone chromatin-associated proteins play an important role in this process. Interestingly, we have found that human transcriptional coactivator PC4is a nonhistone chromatin component with distinct functional consequence. It is a highly abundant, multifunctional nuclear protein, which plays diverse important roles in the vital cellular processes including transcription, replication and DNA repair. Furthermore, PC4 acts as a putative tumor suppressor probably through its ability to enhance the p53 function and inhibition of AP2- mediated self-repression. This functional diversity of PC4 and its close functional similarity to HMGB1 (a highly dynamic nonhistone chromatin protein) with respect to its DNA binding ability, involvement in the p53 activation of function, cellular abundance, dynamic interaction with histones prompted us to investigate whether PC4 is a chromatin-associated protein. In agreement with our speculation we find that PC4 is not only associated with the chromatin, but is also involved in the higher ordered organization of chromatin architecture. 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. The transcriptional coactivation and chromatin organization functions are quite contrasting and we find that posttranslational modifications of PC4 play the key regulatory role in molecular switching of the transcriptional coactivator to a chromatin condensing protein. Presumably, PC4 is primarily involved in the maintenance of facultative heterochromatin. PC4 is thus a bona fide nonhistone component of chromatin, having posttranslational modification dependent distinct functional switch from a transcriptional coactivator to a chromatin organizing protein.

MATERIALS AND METHODS

2.1 General Methods:

2.1.1 Preparation of competent cells

BL21pLysS, DH5α and JM109, *E. coli* strains were grown overnight in 5 ml Luria Broth (LB- 10 gm/litre Tryptone, 5 gm/litre Yeast extract, 10 gm/litre NaCl), from a frozen glycerol stock. The overnight culture is then streaked in a LB-Agar (1.5% agar in LB medium) plate and grown in 37°C. Subsequently a single colony was inoculated into 500 ml of medium A (10 mM MgSO4 and 11 mM Glucose supplemented into 500 ml LB) and grown till mid log phase (OD600 = 0.3). The culture was cooled and pelleted at 2000 rpm for 10 mins at 4°C, subsequently resuspended in 5 ml of medium A at 4°C. The cells were pelleted again at 2000 rpm/10 mins/4°C and finally resuspended in 25 ml storage buffer B (36% Glycerol, 12 mM MgCl2, 12% PEG8000). 100 µl aliquots of resuspended cells were made and stored at -80°C.

2.1.2 Transformation

The competent cells are thawed on ice and 1 µg DNA was added onto it, followed by incubation for 30 mins. Brief heat shock was given at 42°C for 90 sec. After 5 mins incubation on ice, 1ml LB was added and the cells were grown at 37°C for 45 mins. Cells are plated on LB-Agar containing suitable antibiotic for selection and grown at 37°C.

2.1.3 DNA purification

Isolation of plasmid DNA using Qiagen mini or maxi prep kits was done according to the manufacturer's protocol. For purifying plasmids used for transfection in the mammalian cells Qiagen endo-free maxi prep kit was used.

Qiagen PCR purification kit was used to purify defined length PCR products as per the protocol supplied by the manufacturer. Products after restriction enzyme digestion were eluted from agarose gel using Qiagen gel elution kit following manufacturer's protocol.

2.1.4 Isolation of total mRNA

2 x 10^6 HeLa cells were washed in ice-cold phosphate buffer saline (PBS- 172 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4). After pelleting the cells in PBS, 100 µl of Trizol reagent (Invitrogen) was added. The cells were resuspended by mild vortexing and incubated at room temperature for 5 mins followed by centrifugation at 12000 rpm for 10 mins at 4°C. To this 27 µl CHCl3 was added with vortexing, incubated at room temperature for 15 mins and centrifuged at 12000 rpm for 15 mins at 4°C. The upper phase was taken and re-extracted with 200 µl CHCl3. An equal volume of isopropanol was added and centrifuged at 12000 rpm for 20 mins at 4°C to precipitate the RNA. The RNA pellet was resuspended in 70 µl of Diethyl Pyrocarbonate (DEPC) treated water (1ml DEPC is added to 1 litre miliQ water followed by overnight incubation at RT, autoclaved). Re-precipitation of RNA was done by 7 µl of 3M NaOAc (pH-5.2) and 77 µl isopropanol, followed by centrifugation at 12000 rpm for 20 mins at 4°C. The RNA pellet was washed in 80% EtOH, air dried and dissolved in appropriate volume of milliQ water.

2.1.5 cDNA synthesis by Superscript II RT

1 µg isolated RNA was taken in a 12 µl reaction volume containing 40 pmoles of oligodT and incubated at 70°C for 10 mins, followed by quick chilling on ice for 10 mins. The reaction was subsequently continued by the addition of 10 mM DTT, 0.5 mM dNTP mix and 5X First Strand Synthesis, in a PCR based thermal cycle. The initial step was 42°C/2 mins, followed by the addition of Superscript RT II (Invitrogen) and continuation of

thermal cycles as 42°C for 50 mins and 70°C for 15 mins. The cDNA was subsequently used for PCR amplification using gene-specific primers.

2.1.6 Gel Electrophoresis

(a) Agarose gel electrophoresis: In order to analyze and purify DNA samples, agarose gel electrophoresis was employed. As per the gel percentage required amount of agarose (Sigma) was added to 1X TBE (0.09 M Tris borate and 0.002 M EDTA) and dissolved by heating in a microwave oven. The DNA samples were made in 6X loading buffer (for 1X- 0.25% Bromophenol Blue, 0.25% Xylene cyanol in 40% Sucrose), loaded on the gel and electrophoresed at 100V in 1X TBE. Post-run the gels were stained in ethidium bromide (10 μg/100 ml water) with gentle rocking, followed by destaining in water followed by visualization on the UV lamp, in a Gel Documentation System (Biorad).

(b) SDS-polyacrylamide gel electrophoresis: Different proteins were resolved according to their molecular weights in an SDS- PAGE. The separating (or resolving) gels were made with different percentages of acrylamide (30% Stock solution of acrylamide: bis-acrylamide :: 29:1), 0.375 M Tris-HCl (pH-8.8), 0.1% SDS, 0.1% APS and 8% TEMED. The components of stacking gel included 5% acrylamide 0.375 M Tris-HCl (pH-6.8), 0.1% SDS, 0.1% APS and 8% TEMED. Protein samples were made in 5X SDS sample buffer (for 1X- 50 mM Tris-HCl pH 6.8, 100 mM DTT, 0.1% Bromophenol blue, 10% Glycerol), heated at 90°C for –10 mins, before loading into gel. The gel was electrophoresed in Tris- glycine- electrophoresis buffer (25 mM Tris, 250 mM Glycine pH8.3, 0.1% SDS). The gel was stained in Coomassie Brilliant Blue (CBB) (45% MeOH, 10% glacial CH3COOH, 0.25% CBB), followed by destaining in Destaining solution (30% MeOH, 10% glacial CH3COOH).

(c) Native-polyacrylamide gel electrophoresis: In order to visualize DNA-protein or nucleosome-protein complexes Native PAGE was run. 4.5% of Native PAGE was casted using 4.5% Acrylamide, 0.5X TBE (0.045 M Tris borate and 0.001 M EDTA) 0.1% APS and 8% TEMED.

(d) Urea-polyacrylamide gel electrophoresis: In order to visualize the transcripts generated by *in vitro* transcription reaction, Urea PAGE was run. 5% Urea PAGE was casted using 5% Acrylamide, 0.5X TBE (0.045 M Tris borate and 0.001 M EDTA), 21

gm Urea, 0.1% APS and 8% TEMED. The samples were loaded in the Transcription loading buffer (8M Urea, 0.005% Bromophenol Blue and Xylene cyanol).

2.1.7 Western Blot Analysis

The proteins were resolved in a 12% SDS-PAGE. The gel was incubated for a period of 15 mins in Transfer Buffer (25 mM Tris, 192 mM Glycine, 0.038% SDS and 20% MeOH). As per the dimensions of the gel, PVDF membrane was cut, activated in MeOH, and kept in transfer buffer for a period of 10 mins. Proteins were transferred from the gel to the membrane using a semidry western transfer apparatus (Biorad) at 25 V, for a period of time depending upon the Molecular Weight of the protein. The blot was blocked in 5% skimmed milk solution in PBS for a minimum period of 5 hrs. Primary and HRP- conjugated Secondary antibody was made in 2.5% skimmed milk solution (in PBS containing 0.05% Tween20). After blocking, the blot was incubated with Primary antibody for a period of 3 hrs at 4°C, followed by washes in PBS containing 0.05% Tween20 for a period of 15 mins, 4 times at room temperature. This was followed by incubation with Secondary antibody for a period of 3 hrs at 4°C, followed by similar set of 4 washes at room temperature. The blot was developed using the Pierce Super Signal West Pico Chemiluminiscence Kit, as per the manufacturer's protocol. The blots were exposed in X-Ray films (TMX-Kodak), for different time points and developed using GBX-Developer-Fixer Kit (Sigma).

2.1.8 Estimation of Nucleic Acids and Proteins

(a) Nucleic acids: Concentration (C) of the nucleic acids was estimated by measuring the absorbance using UV spectrophotometer at 260 nm (A_{260}). The concentration was estimated as follows,

(i) For dsDNA, C= A_{260} x 50 ng/µl

(ii) For mRNA, C= A_{260} x 40 ng/µl

(iii) For oligonucleotides, C= A_{260} x 33 ng/µl

The purity of the samples was further ascertained by A_{260}/ A_{280} ratios.

(b) Proteins: The total protein concentration of whole cell lysates was estimated with the Biorad Protein Estimation Reagent as per manufacturer's protocol, by measuring absorbance at 595 nm (A_{595}).

Furthermore for estimation of the concentration of purified proteins, visual comparisons were also done with BSA standards of varying concentration range run in a SDS PAGE gel.

(c) Quantifying the autoradiogram band: This was done using the Image Gauge Software of the Phosphorimager (Fuji Film).

2.1.9 Culturing Mammalian cells

The human cervical carcinoma cell line, HeLa, embryonic kidney cell line HEK293 and 293T and mouse L-cell line were grown in the commercial Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with L-Glutamine (Sigma), Penicillin-Streptomycin-Amphotericin B (Sigma) and 10% Foetal Bovine Serum (FBS) (Hyclone) according to manufacturer's protocol. The cells were grown at 37°C in a 5% CO2 supply and an 80% relative humidity of in a CO2 incubator. After 70% confluency was attained, the cells were dislodged from the substratum by 0.025% Trypsin-EDTA (Sigma) for 5-10 mins followed by inactivation using FBS, and subcultured in 1:2 ratio.

2.1.10 Culturing Insect cells

To express and purify human proteins using respective baculo viruses the *Spodoptera frugiperda* ovarian cell line Sf21 was cultured in TC100 medium (Sigma), supplemented with 0.1% Pluronic F-68 solution (Sigma), 10µg/ml Gentamycin (Sigma) and 10% FBS (Hyclone) at 27°C in a BOD incubator. After attaining confluency, the cells were dislodged from the substratum using a cell scraper and subcultured in a 1:3 ratio.

2.2 Cloning, purification of proteins and antibody raising

2.2.1 Cloning:

2.2.1.1 Cloning of Flag-PC4 mammalian expression construct

PC4 gene was cloned in mammalian expression vector P-Flag-CMV TM-2 (Sigma), at Hind-III and Xba-1 enzyme sites. The sequences of the primers are:

Forward Primer: 5′ CCC AAG CTT ATG CCT AAA TCA AAG GAA C 3′

Reverse Primer: 5′ GCT CTA GAT TAC AGT TTT CTT ACT GCA TC 3′

The clone was confirmed by restriction digestion with Hind-III and Xba-1 *enzymes*.

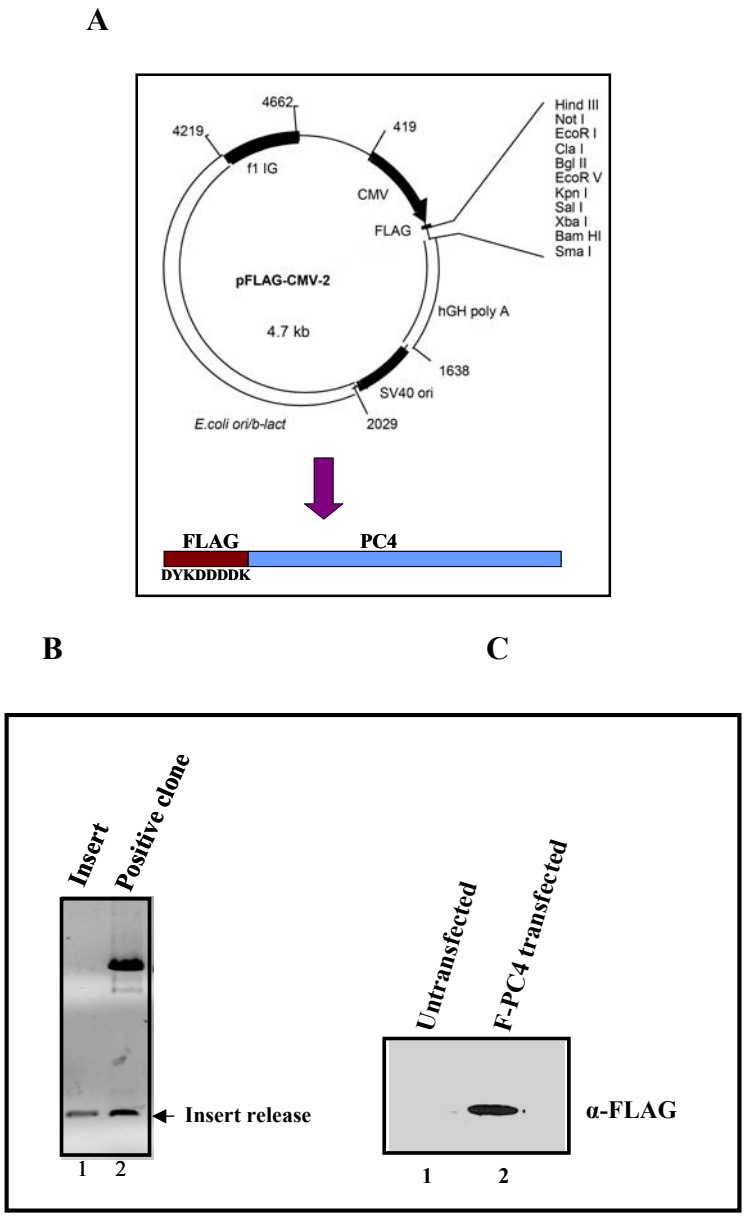


Figure 2.1- Cloning of FLAG tagged PC4 mammalian expression construct: (A) Mammalian expression construct FLAG-PC4 was cloned into pFLAG-CMV2 plasmid with Hind III and XbaI sites. (B) The clone was confirmed by checking the insert release after restriction digestion with the same enzymes. (C) The expression of FLAG-PC4 was confirmed by transfecting the construct into HeLa cells followed by western blotting analysis, probing with α-FLAG antibody.

2.2.1.2 Cloning of Histone H3 and H2B deletion mutants, bacterial expression construct

Deletions of each of histone H3 and H2B - NG (N-terminal + Globular), GC (Globular + C-terminal) and G (Globular) domain were cloned in pGEXCD vector at NcoI and Xho I enzyme sites. The clones are confirmed by PCR.

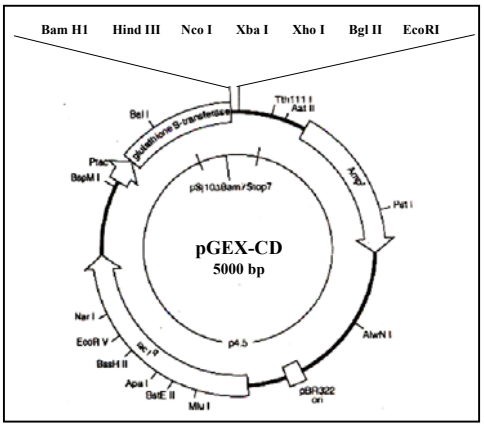


Figure 2.2- GST-tagged histone deletion mutants were cloned into pGEX-CD bacterial expression vector.

Table: Primers used to clone GST-H3 FL and the deletion mutants:

	Clones (GST-tagged)	**Primer Sequences** (FP: Forward primer; RP: Reverse primer)
1.	H3FL	FP: 5′ CAT GCC ATG GCC CGT ACC AAG CAG ACC GCC 3′ RP: 5′ CCG CTC GAG TTA AGC CCT CTC GCC TCG GAT TC 3′
2.	H3NG	FP: 5′ CAT GCC ATG GCC CGT ACC AAG CAG ACC GCC 3′ RP: 5′ CCG CTC GAG TTA GCC TCG GAT TCT GCG GGC 3′
3.	H3GC	FP: 5′ CAT GCC ATG GGC CTG CTC ATC CGC AAA CTG 3′ RP: 5′ CCG CTC GAG TTA AGC CCT CTC GCC TCG GAT TC 3′
4.	H3G	FP: 5′ CAT GCC ATG GGC CTG CTC ATC CGC AAA CTG 3′ RP: 5′ CCG CTC GAG TTA GCC TCG GAT TCT GCG GGC 3′

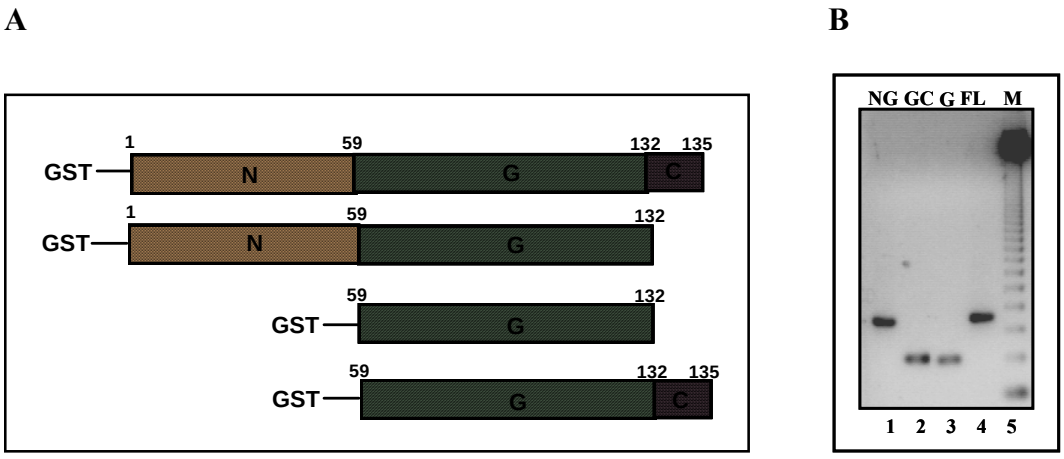


Figure 2.3. Clones of FL and deletions of H3: (A) Schematic representation of GST-tagged FL and deletions of H3. (B) Confirmation of the positive clones by PCR amplification.

Table: Primers used to clone GST-H2B FL and the deletion mutants:

	Clones (GST-tagged)	**Primer Sequences** (FP: Forward primer; RP: Reverse primer)
1.	H2BFL	FP: 5′ CAT GCC ATG GCC AAG TCC GCT CCA GCC CCG 3′ RP: 5′ CCG CTC GAG TTA CTT GGC GCT GGT GTA CTT GG 3′
2.	H2BNG	FP: 5′ CAT GCC ATG GCC AAG TCC GCT CCA GCC CCG 3′ RP: 5′ CCG CTC GAG TTA CAG CAG CAG TCG GAC CGC 3′
3.	H2BGC	FP: 5′ CAT GCC ATGGGC AGT TAT GCC ATT TAC GTG 3′ RP: 5′ CCG CTC GAG TTA CTT GGC GCT GGT GTA CTT GG 3′
4.	H2BG	FP: 5′ CAT GCC ATG GGC AGT TAT GCC ATT TAC GTG 3′ RP: 5′ CCG CTC GAG TTA CAG CAG CAG TCG GAC CGC 3′

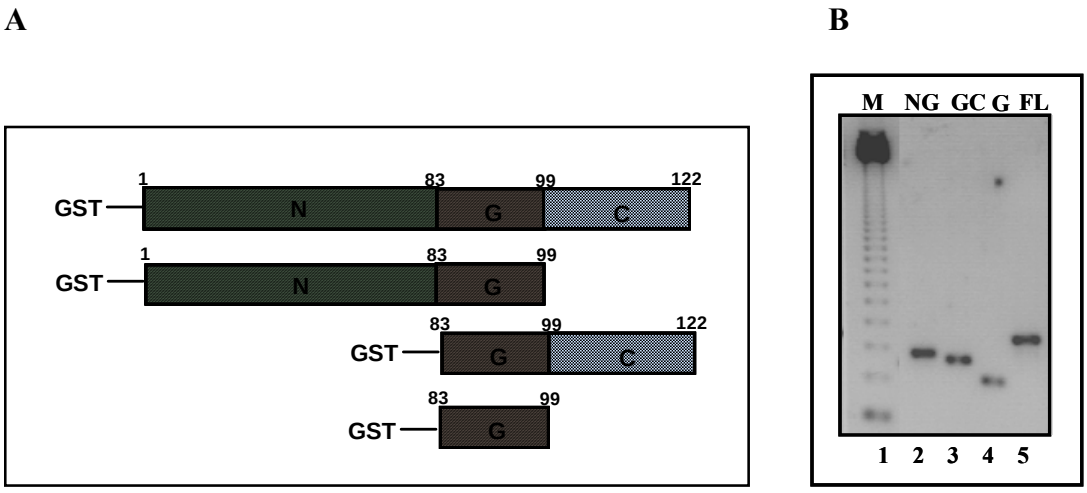


Figure 2.4. Clones of FL and deletions of H2B: (A) Schematic representation of GST-tagged FL and deletions of H2B. (B) Confirmation of the positive clones by PCR amplification.

2.2.2 Purification of proteins:

2.2.2.1 Purification of Native PC4

The untagged recombinant PC4 was purified (Gu and Roeder, 1994) using a two-column purification protocol using heparin sepharose followed by phosphocellulose P11 columns. *E. coli* BL21pLys cells were transformed with the PC4 expression vector in presence of 100 μg/ml Ampicillin resistance marker. Single colony was inoculated from the transformed plate into 100 ml LB medium containing 100 μg/ml Ampicillin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 100 μg/ml Ampicillin and grown till the OD600 reached 0.6 at 37°C. The culture was induced with 0.5 mM IPTG and grown further for 3 hrs at 37°C. The cells were harvested at 6000 rpm for 10 mins at 4°C. The cells were resuspended in homogenization buffer BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 300 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) and sonicated with 4 burst of 30 sec at a setting of 7. The lysate was cleared by centrifugation at 16000 rpm for 30 mins at 4°C. The lysate was passed through a BC300 pre-equilibrated Heparin Sepharose column. The column was washed with BC300 and eluted with BC500 (20 mM Tris-HCl pH7.4, 20% Glycerol,

0.2mM EDTA, 500 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). All the fractions were analyzed by 15% SDS PAGE. PC4 containing fractions were pooled and the protein was loaded onto a pre-equilibrated (with BC500) phosphocellulose P11 (Whatman) column. The column was washed with BC500 and eluted with BC850 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 850 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). The peak fractions were pooled, dialyzed in BC100 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C.

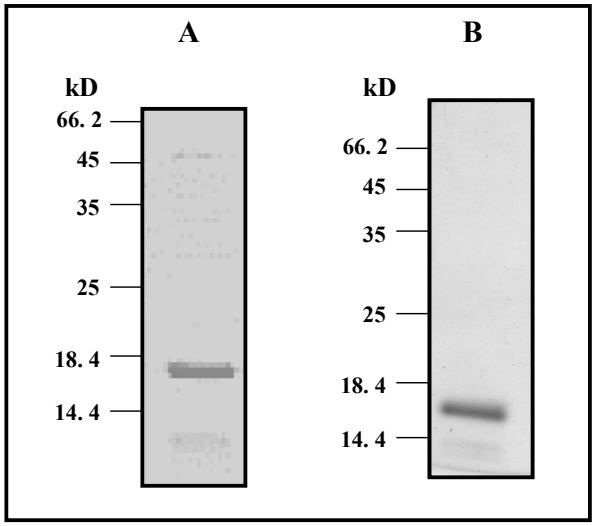


Figure 2.5- Native recombinant PC4 purified by two-columns- Heparin Sepharose (A) followed by Phosphocellulose p11 (B).

2.2.2.2 Purification of PC4-GST

The PC4-GST (Fukuda et al, 2004) was expressed in *E. coli* and PC4-GST sepharose beads were prepared. *E. coli* BL21pLys cells were transformed with PC4-GST expression vectors and grown in 100 ml LB medium containing 100 μg/ml Ampicillin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 100 μg/ml Ampicillin and grown at 37°C followed by induction at 0.4 OD600 with 0.4 mM IPTG for 3 hrs. The cells were harvested, homogenized in BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 300 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM

β Mercaptoethanol), sonicated and centrifuged to clear the prepared cell lysates. Pre-equilibrated GST beads (Pharmacia) were incubated with the cleared lysate for 2 hrs at 4°C in an end-to-end shaker. After incubation the beads were pelleted at 2000 rpm/10 mins/4°C, washed with BC500 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 500 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) (10 times), analyzed by a 12% SDS PAGE and stored in -80°C.

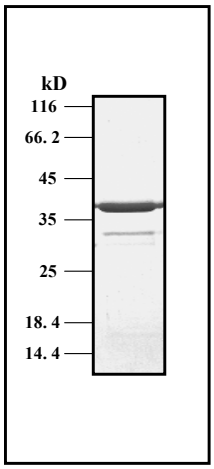


Figure 2.6- Purified protein profile of bacterially expressed PC4-GST.

2.2.2.3 Purification of His_6-PC4 and its deletion constructs

The His_6 tagged recombinant PC4 was purified using Ni-NTA agarose (Novagen). His_6 – tagged PC4 deletions 1-62, 1-87, 22-127, 62-127 and 62-87 were expressed and purified till homogeneity in a similar way. *E. coli* BL21pLys cells were transformed with the individual expression vectors and grown in 100 ml LB medium containing 50 μg/ml Kanamycin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 50 μg/ml Kanamycin and grown at 37°C followed by induction at 0.4 OD600 with 0.4 mM IPTG for 3 hrs. The induction conditions for other clones were: 0.6 OD600 and 0.6 mM IPTG for 1-62 PC4, 0.4 OD600 and 0.6 mM IPTG for 1-87 PC4, 0.4 OD600 0.4 mM IPTG for 22-127 PC4 and 0.5 OD600 and 1.0 mM IPTG for 62-127 PC4. The culture was harvested, homogenized in BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mM KCl, 0.1% NP40, 15 mM Imidazole, 2 mM PMSF and 2mM β

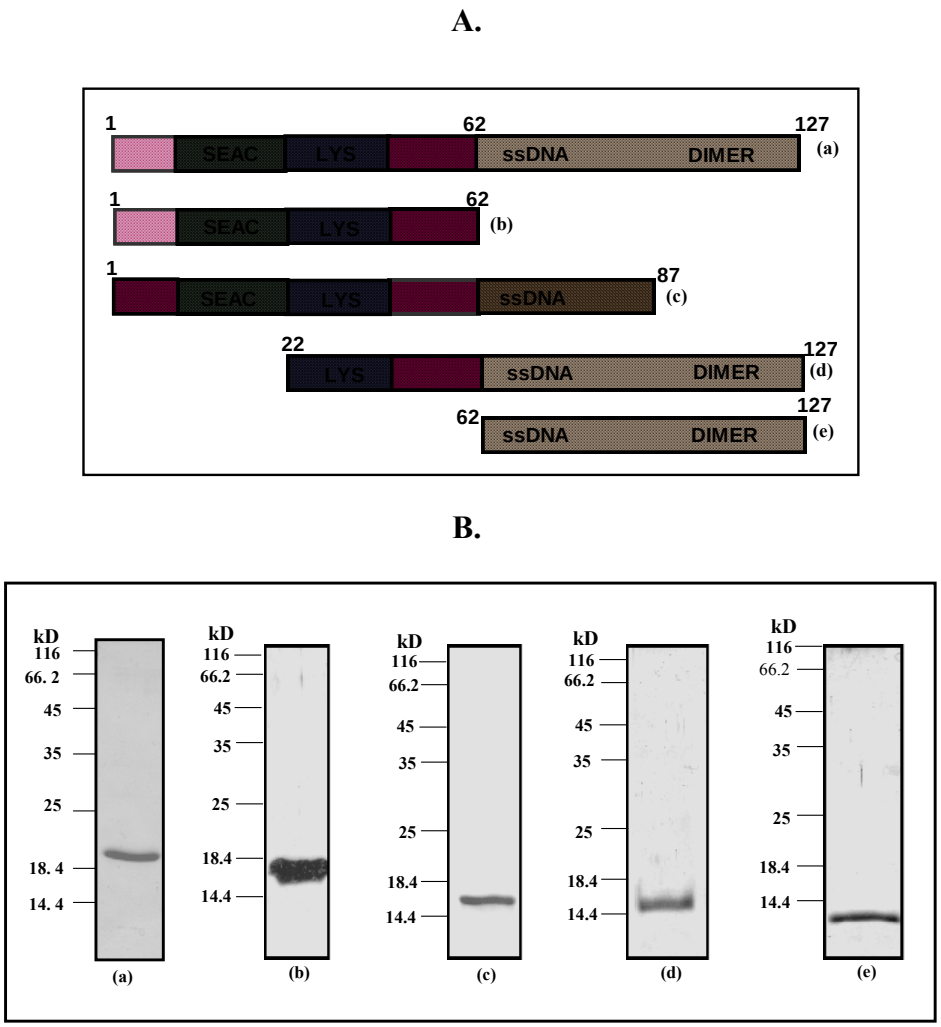
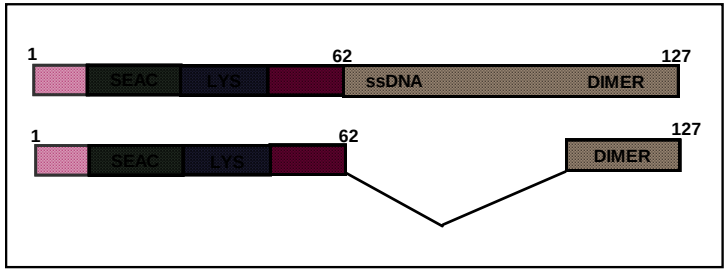
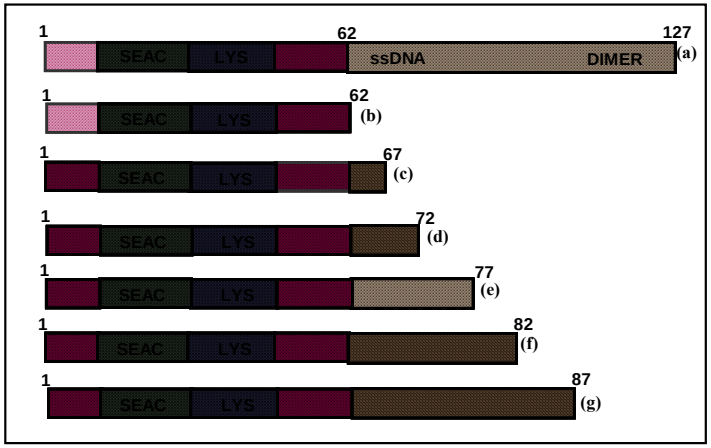


Figure 2.7- (A) Diagrammatic representation of His6-PC4 and its deletion mutants. (B)- Purified protein profile of His6-PC4 (a) and its deletion mutants- 1-62 (b), 1-87 (c), 22-127 (d), 62-127 (e).

A.


1
62
127
SEAC
LYS
ssDNA
DIMER
1
62
127
SEAC
LYS
DIMER

B.


1
62
127
SEAC
LYS
ssDNA
DIMER
(a)
1
62
SEAC
LYS
(b)
1
67
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LYS
(c)
1
72
SEAC
LYS
(d)
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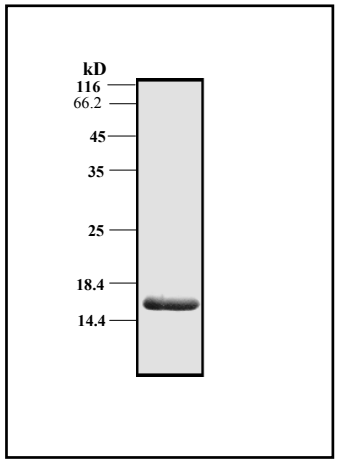
LYS
(f)
1
87
SEAC
LYS
(g)

C.


kD
116
66.2
45
35
25
18.4
14.4

D.

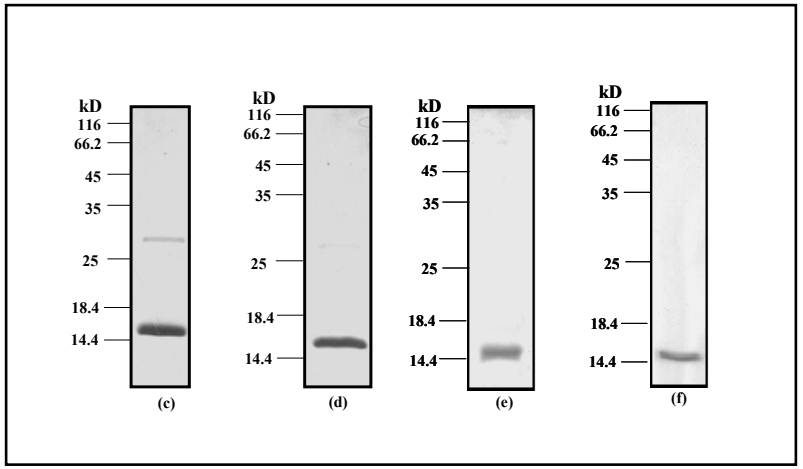

kD
116
66.2
45
35
25
18.4
14.4
(c)
kD
116
66.2
45
35
25
18.4
14.4
(d)
kD
116
66.2
45
35
25
18.4
14.4
(e)
kD
116
66.2
45
35
25
18.4
14.4
(f)

Figure 2.8- A, B. Diagrammatic representation of the internal deletion mutant PC4 (del 62-87) (A) and the finer deletion mutants spanning 62-87 amino acid residues (B). C, D. Purified protein profile of PC4 (del 62-87) (C) and the finer deletion mutants (1-67 PC4 (c), 1-72 PC4 (d), 1-77 PC4 (e) and 1-82 (f) PC4) (D) respectively.

Mercaptoethanol) sonicated and centrifuged to clear the prepared cell lysates. The cleared lysate was incubated with pre-equilibrated Ni-NTA agarose beads for a period of 3 hrs in an end-to-end shaker at 4°C. The resin was washed five times with the wash buffer (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 600 mM KCl, 0.1% NP40, 30 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol) and packed into an EconoFast column (Biorad). The protein was then eluted using elution buffer (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP40, 250 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol), and analyzed in a 15% SDS PAGE. The semi-purified protein was passed through Heparin Sepharose column. The column was equilibrated by BC100 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 100 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) and washed in the same buffer, while elutions were carried out by BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) for all the proteins except 22-127 PC4 which was eluted with BC500 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 500 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol).

The induction condition followed for purifying the internal deletion mutant and the finer deletion mutants (1-67 PC4, 1-72 PC4, 1-77 PC4, 1-82 PC4) was 0.6 mM IPTG at 0.6 OD600 and similar purification protocol was followed as described previously.

2.2.2.4 ***Purification of human core histones***

Human core histones were purified from HeLa nuclear pellet (Kundu et al, 2000). The nuclear pellet was resuspended in Buffer A (100 mM Potassium phosphate buffer pH6.7, 0.1 mM EDTA, 10% Glycerol, 0.1 mM PMSF, 0.1 mM DTT, 630 mM NaCl) and homogenized in a Dounce's homogenizer (Wheaton) with pestle B for 30 mins on ice. The suspension was cleared by centrifugation at 14000 rpm for 20mins at 4°C. Supernatant was incubated with Hydroxyapatite BioGel HTP (Biorad) presoaked in 10

mM Potassium phosphate buffer pH 6.7 (0.5 gm beads per ml of nuclear pellet), and incubated for 3 hrs at 4°C in an end-to-end shaker. The resin was washed with buffer A with 630 mM NaCl in centrifuge. The beads were packed into an Econoglass column and washed overnight with the same wash buffer. The core histones were eluted in buffer A containing 2 M NaCl and analyzed in a 15% SDS PAGE, dialysed in BC100, aliquoted and stored in -80°C.

For isolating hyper acetylated core histones, HeLa cells were previously treated with Histone Deactylase inhibitors (100nM TSA and 1 mm NaBu) for 24 hrs and siilar purification protocol was followed. Hyperacetylation was confirmed by western blotting analysis with anti-AcH3 antibodies.

2.2.2.5 ***Purification of recombinant Xenopus individual histones***

Recombinant core histones (Xenopus) H2A, H2B, H3 and H4, which comes in inclusion bodies, were purified by denaturation in 8M urea followed by renaturation (Luger et al, 1997). *E. coli* BL21 cells harboring the expression vector for the individual histones were grown in 100 ml LB medium containing 100 μg/ml Ampicillin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 100 μg/ml Ampicillin and grown at 37°C followed by induction at 0.8 OD600 with 0.4 mM IPTG for 3 hrs. The cells were harvested and resuspended in wash buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 1 mM Benzamidine, 1 mM Beta-mercaptoethanol) and stored in -20°C. The cell suspension was thawed, sonicated and the lysate was clarified by centrifugation. The pellet was washed with wash buffer containing Triton X100. The detergent was removed by giving successive washes in wash buffer (without detergent). The pellet containing inclusion bodies was soaked in DMSO for 30 mins at 22°C. 6 M Guanidium Hydrochloride containing 20 mM NaOAc pH 5.2, 1 mM DTT was added slowly, and the unfolding was allowed to proceed for 1 hr at 22°C. The solubilized inclusion bodies were centrifuged to remove the particulate materials and the supernatant was subjected to extensive dialysis for refolding in the refolding buffer (10 mM Tris HCl pH 7.5, 2 M NaCl, 1 mM EDTA and 1 mM Beta-mercaptoethanol). The soluble fractions were separated by centrifugation. The proteins in soluble fractions were analyzed in a 15% SDS PAGE.

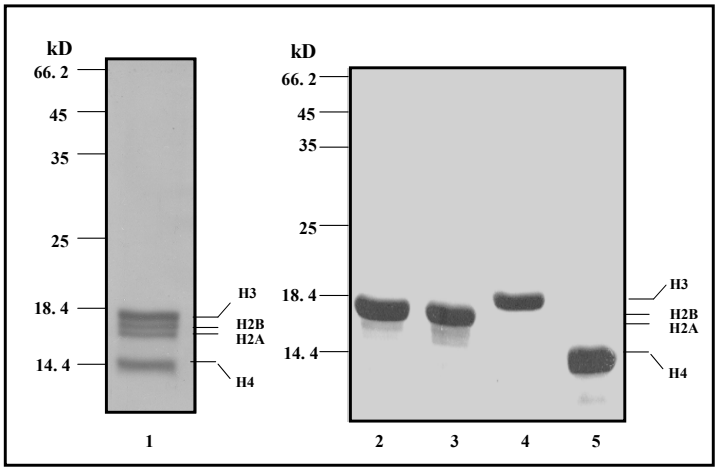


Figure 2.10- Purified protein profile of core histones (lane 1) purified from HeLa nuclear pellet and xenopus recombinant histones (lanes 2-5) expressed in bacteria.

2.2.2.6 ***Purification of His_6-HMGB1***

His_6-HMGB1 was expressed in *E. coli* and purified by Ni-NTA (Novagen) column chromatography. *E. coli* BL21pLys cells were transformed with His6-HMGB1 expression vectors and grown in 100 ml LB medium containing 50 μg/ml Kanamycin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 50 μg/ml Kanamycin and grown at 37°C followed by induction at 0.4 OD600 with 0.4 mM IPTG for 3 hrs. The culture was harvested, homogenized in BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mM KCl, 0.1% NP40, 15 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol) sonicated and centrifuged to clear the prepared cell lysates. The cleared lysate was incubated with pre-equilibrated Ni-NTA agarose beads for a period of 3 hrs in an end-to-end shaker at 4°C. The resin was washed five times with the wash buffer (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 600 mM KCl, 0.1% NP40, 30 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol) and packed into an EconoFast column (Biorad). The protein was then eluted using elution buffer (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP40, 250 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol), and analyzed in a 15% SDS PAGE. The semi-purified protein peak fractions were pooled and passed

through a pre-equilibrated (in BC100 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol)) phosphocellulose P11 column after adjusting the salt concentration to 100 mM. The column was washed with BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 300 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) and eluted with BC500 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 500 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). The peak fractions were pooled, dialysed in BC100 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C.

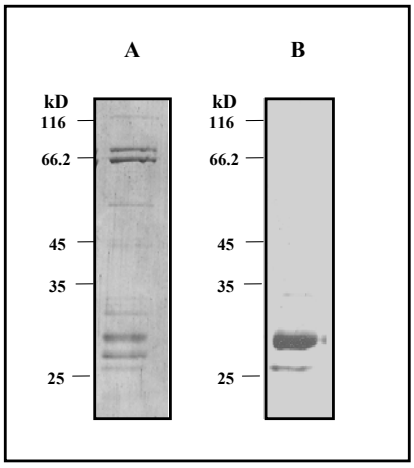


Figure 2.11- Purified protein profile of HMGB1. A two-column purification protocol was followed- Ni-NTA Agarose (A) followed by Phosphocellulose p11 (B).

2.2.2.7 ***Purification of Histone H3 and H2B deletion mutants***

GST tagged constructs of histones H3 and H2B deletions NG (N-terminal + Globular), GC (Globular + C-terminal) and G (Globular) domain were expressed in *E. coli* and purified as individual proteins conjugated to GST sepharose beads. *E. coli* BL21pLys cells were transformed with the expression vectors for each of these proteins and grown in 100 ml LB medium containing 100 μg/ml Ampicillin and grown for 12 hrs at 37°C. The culture was inoculated into 900 ml LB containing 100 μg/ml Ampicillin and grown

at 37°C followed by induction at 0.4 OD600 with 0.4 mM IPTG for 3 hrs. The cells were harvested, homogenized in BC300 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 300 mM KCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), sonicated and centrifuged to clear the prepared cell lysates. Pre-equilibrated GST beads (Pharmacia) were incubated with the cleared lysate for 2 hrs at 4°C in an end-to-end shaker. After incubation the beads were pelleted at 2000 rpm/10 mins/4°C, washed with BC500 (20 mM Tris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 500 mM KCl, 0.1% NP40, 2 mM PMSF and 2 mM β Mercaptoethanol) (10 times), analyzed by a 15% SDS PAGE and stored at -80°C.

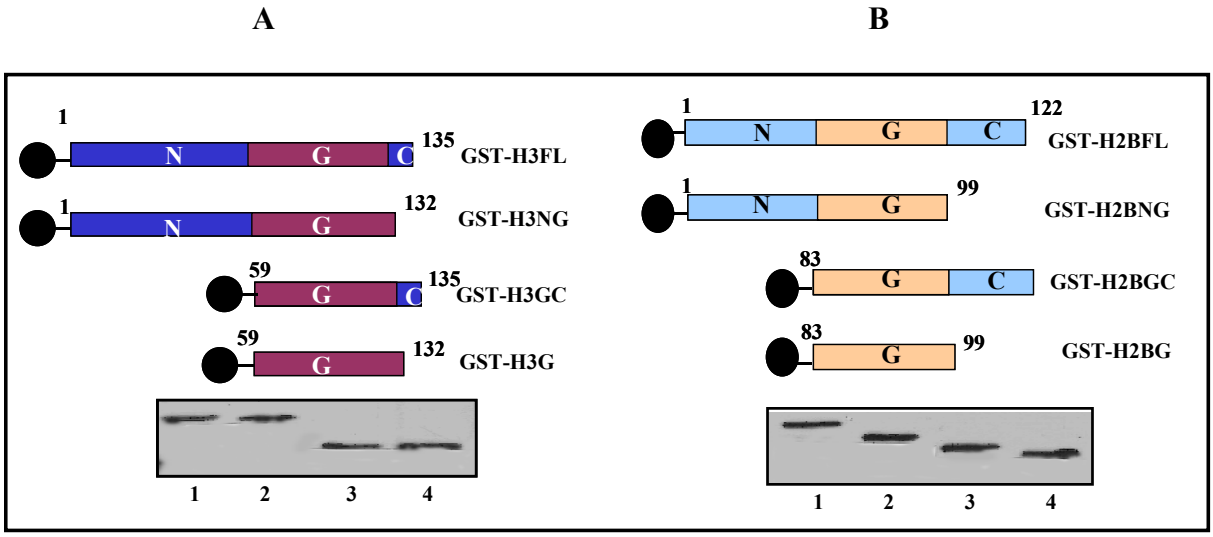


Figure 2.12- Purified protein profile of GST-tagged H2B and H3 deletion mutants. (A, B) Lane1-GST, Lanes 2-5 –FL, NG, GC and G domains of H2B (A) and H3 (B).

2.2.2.8 Purification of human histone acetyltransferase p300

Full length His_6 tagged p300 was purified from baculovirus (p300) infected *Spodoptera frugiperda* (Sf21) insect ovary cell line. The infection time was around 72 hrs. Subsequently cells were harvested and then resuspended in cold homogenization buffer (10 mM Tris HCl pH 7.5, 10% Glycerol, 0.1% NP0, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 500 mM NaCl, 15 mM Imidazole and 50 μg/ml of each of the protease inhibitors Leupeptin and Aprotinin). Cells were homogenized using a Dounce's homogenizer (Wheaton) and a tight pestle for 30 mins at 4°C. The cell lysate was centrifuged 11000

rpm for 15 mins at 4°C. The supernatant was bound to Ni-NTA agarose (Novagen) beads for 2 hrs at 4°C in an end-to-end shaker. The beads were washed 5 times in wash buffer (10 mM Tris HCl pH 7.5, 10% Glycerol, 0.2%NP40, 2 mM β Mercaptoethanol, 2 mM PMSF, 300 mM NaCl, 15 mM Imidazole). The beads were packed in a USB column (Catalogue No. 13928) and the protein was eluted with the elution buffer (10 mM Tris HCl pH 7.5, 10% Glycerol, 0.1% NP0, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 200 mM NaCl, 250 mM Imidazole and 50 μg/ml of each of the protease inhibitors Leupeptin and Aprotinin). The protein was checked in a 12% SDS PAGE, aliquoted and stored at -80°C.

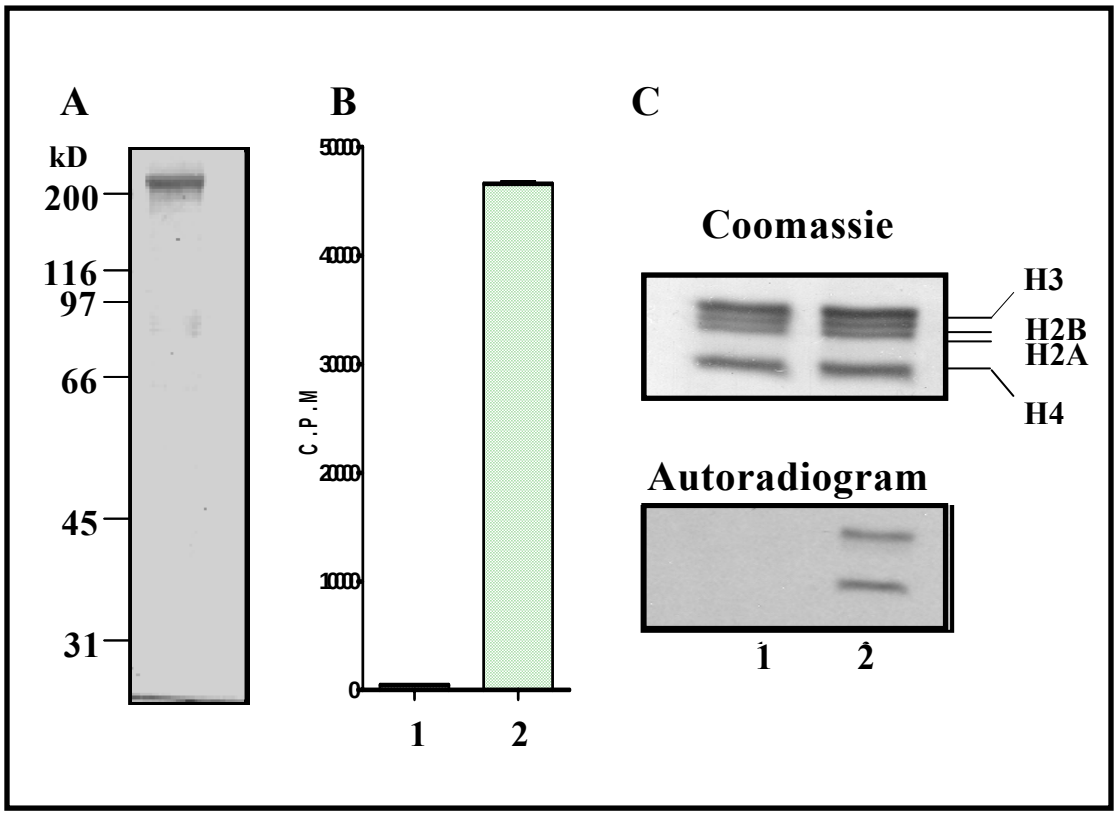


Figure 2.13- (A) Purified protein profile of HAT p300. (B) Filter binding assay for testing the activity of the purified core histones and p300. Reactions 1, 2- core histones incubated with 3[H] Acetyl CoASH in absence or presence of p300. (C) Gel HAT assay for assessing the activity of the purified core histones and p300.

2.2.3 Antibody raising

2.2.3.1 Raising polyclonal antibody against PC4 and affinity purification by protein G sepharose

Untagged recombinant PC4 was expressed in *E. coli* and purified by Heparin Sepharose followed by Phosphocellulose column. Regular immunization schedules were followed to raise the antibody against full length PC4 in rabbit. Briefly 100 μg PC4 in PBS (172 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4) was emulsified with equal volume of Freund's Complete Adjuvant and injected subcutaneously into rabbit. This was followed by first booster doses after 2 weeks when 50 μg PC4 in PBS was emulsified with equal volume of Freund's Incomplete Adjuvant and similar subcutaneous injection was given. After 3 weeks, the second booster was given with 50 μg PC4 in PBS emulsified with equal volume of Freund's Incomplete Adjuvant injected subcutaneously. After 3 weeks of second booster heart puncture was done to collect the immunized blood and serum was extracted from it. The antibody titre was checked after the first injection as well as booster doses. The rabbit polyclonal antibody was purified by Protein – G Sepharose and used for the immunofluorescence experiment as well as other biochemical studies. Preimmune Serum (PIS) was used as negative control. The antibody dilution was 1:1000, which recognized purified PC4 protein as well as PC4 from HeLa and 293 whole cell lysate as well as in brain tissue samples.

The specificity of PC4 antibody was verified by western blotting using HeLa whole cell extract HeLa nuclear extract. The specificity of the PC4 antibody was further confirmed by doing competition assays where anti-PC4 polyclonal antibodies was preabsorbed with purified PC4 protein and checked in western blotting analysis using HeLa whole cell extract and HeLa nuclear extract. The control experiment was similarly done with anti-PC4 polyclonal antibodies preabsorbed with BC100 buffer.

2.2.3.2 Preparation of PC4 depleted Nuclear Extract

HeLa nuclear extract was prepared as described elsewhere (Dignam et al, 1983). Briefly Hela cells were lysed by a hypotonic buffer treatment, followed by centrifugation to separate the cytosolic S100 extract and nuclear pellet.

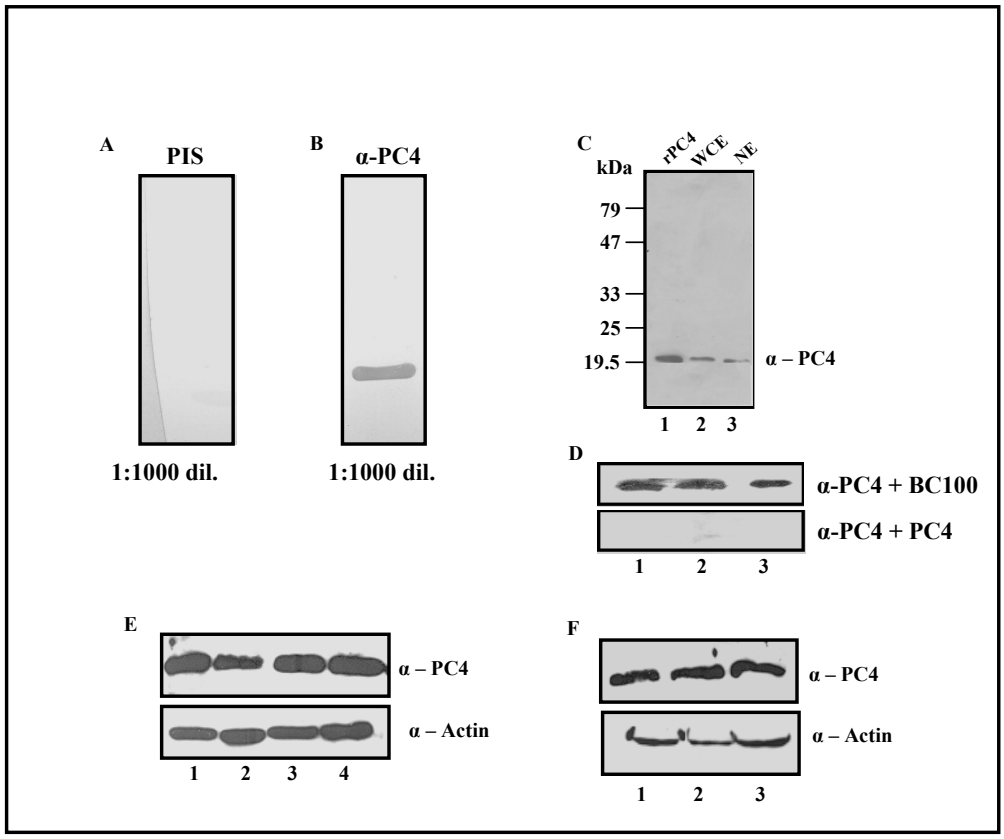


Figure 2.14- Specificity of the polyclonal α-PC4 antibody. 1:1000 dilution of α-PC4 (A) antibody was suffice to pick up 200 ng of PC4 protein. PIS (1:1000 dilution) was used as the negative control (B). (C) The antibody was tested for its specificity in recognizing PC4 protein from HeLa whole cell extract (lane 2) and HeLa nuclear extract (lane 3). Recombinant PC4 protein was used as the positive control (lane1). (D) For the competition assays recombinant PC4 (lane 1), whole cell extract (lane 2) and Nuclear extract (lane 3) were used. (E) HeLa, HEK293, MCF7, U87 MG whole cell extracts were tested with α-PC4 antibodies, where α-Actin was used as the loading control. (F) Tissue lysates- whole brain (lane 1), white (lane 2) and gray matter (lane 3) were also tested with α-PC4 antibodies, where positive control was α-Actin.

The nuclear pellet was subsequently homogenized in a low salt buffer, with dropwise addition of high salt buffer to it with constant stirring. This was followed by centrifugation to separate the nuclear extract (supernatant fraction) and chromatin (nuclear pellet) fraction. The commercially available polyclonal PC4 antibody (N17) was used to immunodeplete PC4 from HeLa nuclear extracts. 5 µg of the antibody was incubated with 30 µl Protein G Sepharose beads (Amersham Pharmacia) in 300 µl PBS at 4˚C for 12 hrs in an end- to-end shaker. The beads were then washed thrice with cold

PBS and incubated with 20 µl of BSA (20 mg/ml) at 4˚C for 20 mins in an end- to-end shaker, for preventing nonspecific binding. The beads were then washed thrice with cold PBS and a final wash in BC100. The beads were incubated with 300 µl HeLa nuclear extract of 8 mg/ml concentration, at 4˚C for 2 hrs in an end- to-end shaker. After the binding the beads were pulled down at 3000 rpm for 10 mins at 4˚C. PC4-depleted nuclear extract was aliquoted and stored in -80˚C, after freezing in liquid Nitrogen. The PC4 depletion was further confirmed by western blotting analysis with anti-PC4 antibodies, before proceeding with further experiments.

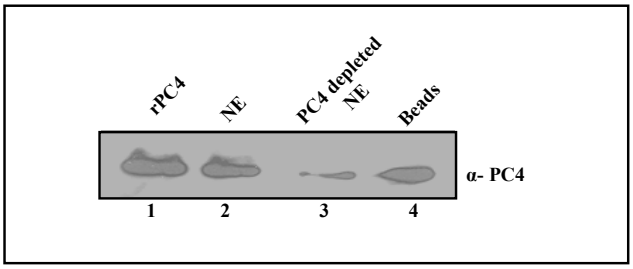


Figure 2.15- Immunodepletion of PC4 from HeLa nuclear extract: Depletion of PC4 from HeLa nuclear extract with α-PC4 antibody was confirmed by western blotting analysis. Lane 1- recombinant PC4, lanes 2, 3- PC4 undepleted and depleted nuclear extract, lane 4- nuclear extract bound protein G Sepharose beads.

2.3 Protocols for various assays

2.3.1 Cell based study:

2.3.1.1 Immunofluorescence and Hoechst staining

Cells were fixed in 2% paraformaldehyde in PBS (172 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4) for 20 mins at room temperature. The cells were permeabilized by 1% Triton X100 in PBS, followed by blocking in 1% FBS. Probing was done with purified polyclonal antibody against PC4 followed by secondary antibody conjugated to Rhodamine. In order to visualize the DNA, the cells were stained with 0.1 µg /ml Hoechst 33258 in PBS. Fluorescence for Rhodamine and Hoechst were visualized by using different filters of the Carl Zeiss microscope (Axioskop 2 plus), the image was captured by AxioCam MRc camera and AxioVision 3.1 software was used, to process the images.

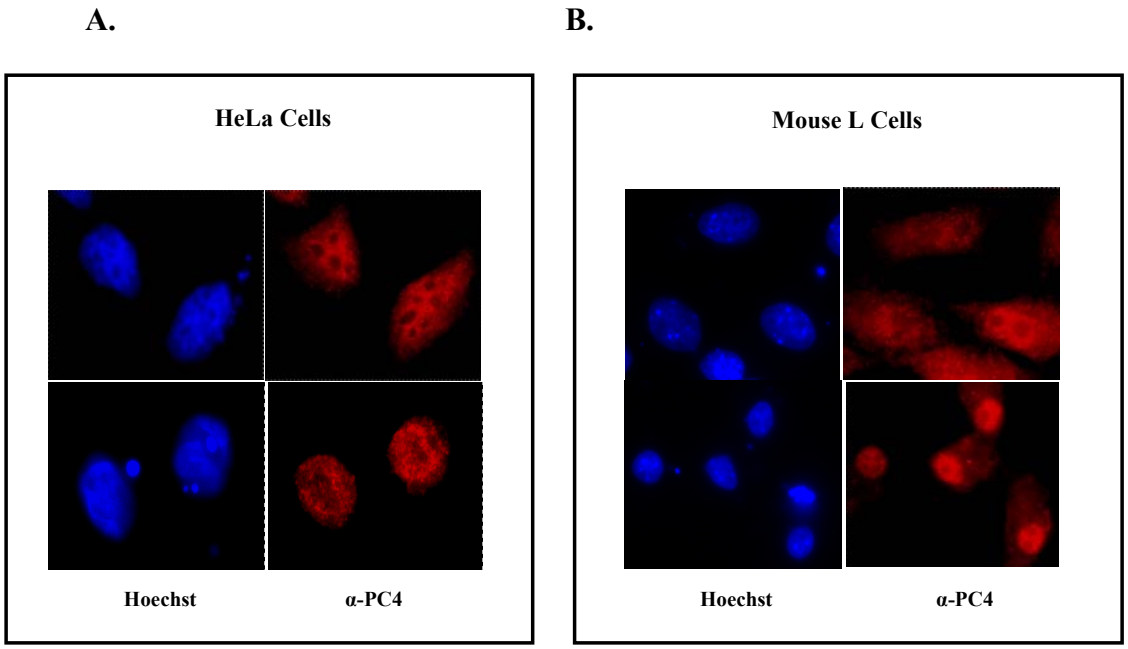


Figure 2.16: Localization of PC4 in HeLa (A) and mouse L (B) cells visualized through Immunofluorescence: PC4 antibody stained and Hoechst counterstained nuclei visualized through immunofluorescene.

2.3.1.2 Chromosome spreads and immunostaining

The HeLa and mouse L cells were cultured as monolayers on poly L-Lysine coated glass coverslips in DMEM medium. Condensed mitotic metaphase chromosomes (after Nocodazole treatment) 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.

2.3.1.3 Synchronization of cells at different phases of cell cycle

The relative amount of PC4 in the different stages of cell cycle was assessed by immunofluorescence or western blotting analysis. 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. Furthermore the cells were arrested in G1/S phase of cell cycle by a double Thymidine (2mM) (Sigma) and Hydroxyurea (2mM) (Sigma) block. On the

other hand, Nocodazole (Sigma) treatment, leading to a pre-metaphase arrest was done for a period of 16hrs.

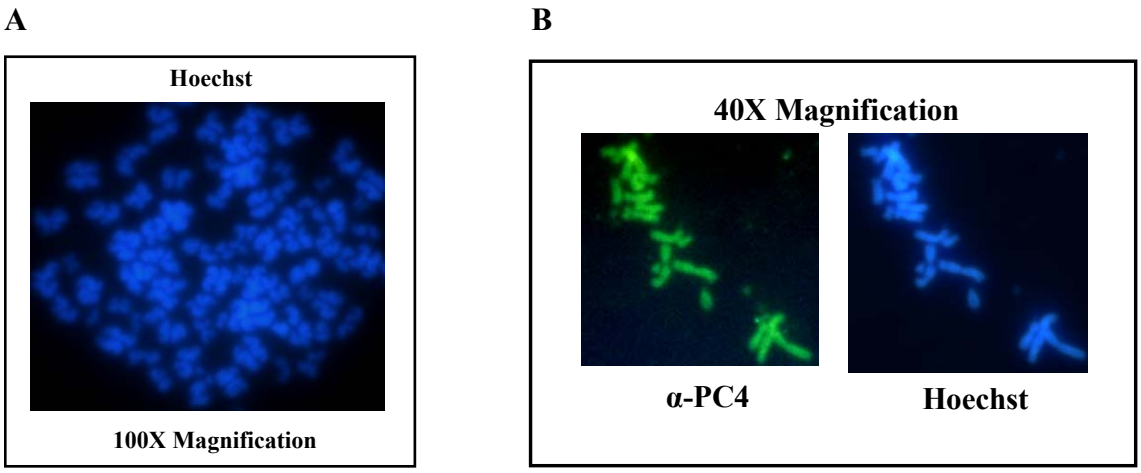


Figure 2.17: (A) A metaphase spread visualized through Hoechst staining. Chromosomes isolated from nocodazole treated metaphase arrested cells were stained with Hoechst and visualized. (B) Immunostaining to visualize the localization of PC4 in metaphase chromosome spreads. After the preparation of metaphase spreads chromosomes were stained with anti-PC4 antibodies and counterstained with Hoechst.

2.3.1.4 Fluorescent Activated Cell Sorting (FACS)

HeLa cells were transfected with pGShin2 (vector) (Kojima et al, 2004) or PG7 (PC4 siRNA cloned into pGShin2 plasmid). Propidium Iodide (PI) staining was done as described elsewhere (Einarson et al, 2004). Briefly cells were harvested by mild trypsinization (0.25%) followed by centrifugation at 2000 rpm for 10 mins at 4^{O}C. Cells were washed with cold PBS by centrifugation at 2000 rpm for 10 mins at 4^{O}C. Cells were fixed in cold 70% Ethanol which was added dropwise along with mild vortexing. Samples were left for 12 hrs, after which Ethanol was removed followed by two washes in cold PBS. RNase (100 μg/ml) treatment was subsequently given at 37^{O}C/30 mins to ensure only DNA staining. 50 μg/ml Propidium Iodide was added for staining. 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.

2.3.1.5 Transient transfection by Lipofectamine 2000 reagent

Mammalian cells are seeded, and grown overnight in 10% FBS supplemented DMEM medium (without Penicillin-Streptomycin-Amphotericin B). Prior to transfection, the medium was replaced with fresh DMEM without antibiotic ad FBS. The amount of constructs taken was in accordance with manufacturer's protocol, maintaining 1:1 ratio of μg of DNA: μl of Lipofectamine 2000 (Invitrogen). For siRNA trasfections, a non-specific plasmid DNA was used along with the siRNA for enhancing the transfection efficiency. The constructs and Lipofectamine was incubated for a period of 20 mins to ensure Lipofectamine-DNA complex formation, as per the manufactuer's protocol. After 6 hrs the medium was replaced by 10% FBS supplemented DMEM medium (without antibiotic) and grown for a period of 24 hrs.

2.3.1.6 Differential cell permeabilization assays

Hela 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, histone H3 and HSC70.

Briefly, adherent cells were washed three times with ice-cold Transport Buffer TB (20 mM Hepes pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT) or TB with 0.1% NP40 or 40 μg/ml digitonin. Supernatants (S) were recovered and preserved. The cell remnants (P) were incubated for 10 mins at 37°C with TB buffer supplemented with 0.1% NP40, 10 mM MnCl2, 20 μg/ml DNaseI. The supernatants and cell remnants were analyzed by western blotting, using antibodies against PC4, HMGB1 and Histone H3.

In order to explore the difference in the strength of association of the proteins across the Mitotic and Interphase stage of cell cycle, the adherent cells were washed three times with ice-cold Transport Buffer TB (20 mM Hepes pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT) or TB with 0.2% NP40. The supernatant (S) and pellet (P) fractions were analyzed as before.

2.3.2 Protein-protein interactions:

2.3.2.1 Ni-NTA pull down assay

The histone interaction ability of PC4 was 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 (composition mentioned previously) containing 150 mM KCl supplemented with 30 mM imidazole at $4^{O}C$ for 2.0 hrs. The beads were washed five times (1 ml each) with the incubation buffers. The Ni-NTA agarose pull down complex 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.

2.3.2.2 GST pull down assay

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 (Kundu et al, 2000). Briefly interactions were set with 1 μg individual deletion mutants, 200 ng native PC4 in presence of 150 mM NaCl. For scoring the interaction, GST-pull down complex was probed with anti-PC4 antibodies.

1 μg PC4-GST was put for interaction with whole cell extract and the pull down complex was analyzed by western blotting using anti-histone antibodies.

2.3.2.3 *Immunoprecipitation assay*

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 probability of PC4 interaction with the centromeric histone H3 variant CENP-A was verified by immuno pull down assays (by anti-HA-antibody) using the whole cell extract prepared from the HeLa cells transfected with HA-CENP-A mammalian expression construct. Histone H4 was used as a positive control.

2.3.3 Chromatin-Protein interactions:

2.3.3.1 Body labeling of Oligonucleotide fragment

For mononucleosome assembly 160 bp radiolabelled DNA fragment was generated from pGUB vector using specific primers by PCR using α^{32}[P] dATP as radioactive label. The radiolabelled DNA fragment was subsequently purified by gel extraction.

2.3.3.2 In vitro reconstitution of chromatin by Salt Dialysis

a. Mononucleosome assembly

Mononucleosome assembly was carried out by salt dialysis method (Mutskov et al, 1998) using a 160 bp body labeled DNA fragment and core histones. Initial reaction was set with a core histone (7.2 μg) : DNA (0.2 μg) ratio of 36 in presence of sonicated sperm DNA (3 μg) and 2X Initial dilution buffer (1X- 10 mM Tris HCl pH 8, 1mM EDTA, 2 M NaCl, 10 mM β-Mercaptoethanol, 1 mg/ml BSA). The assembly reaction was carried out at 37°C for 30 mins. Step dialysis was subsequently carried out with buffer (10 mM Tris HCl pH 8, 1mM EDTA, 10 mM β-Mercaptoethanol and 0.03 mM PMSF) containing decreasing concentration of NaCl (1.2, 1.0, 0.8, 0.6 M) for a period of 2-3 hrs for buffer at 4°C. Final dialysis was carried out in TE for 12 hrs at 4°C.

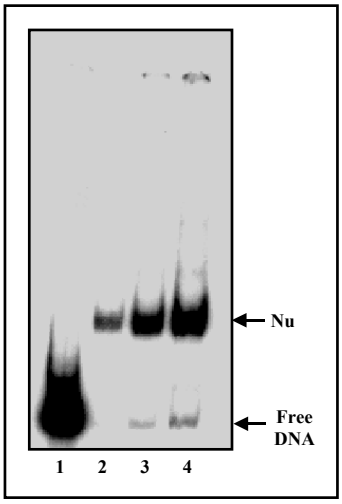


Figure 2.18: *In vitro* reconstituted mononucleosome by salt dialysis. Lane 1- Labeled DNA, lanes 2-4- Increasing concentration of assembled mononucleosome (Nu).

b. Chromatin assembly on a 100 kb plasmid using full-length and tail-less histones

The 100 kb chromatin was reconstituted using plasmid DNA and highly purified HeLa core histones. In brief equal amounts (0.5 μg) of the purified DNA and the histone

octamer were mixed in Hi-buffer [10 mM Tris HCl (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 (0.5µg) to tail-less histones (0.365 µg) was altered to 1.37: 1.

2.3.3.3 Purification of mononucleosomes by Sucrose density gradient centrifugation

The assembled mononucleosomes were purified by sucrose density gradient centrifugation (5-25%). Individual sucrose solutions were prepared in NTE (10 mM NaCl, 10 mM Tris HCl pH 7.4, 1 mM EDTA) buffer containing 0.4 mM PMSF and 0.5 mg/ml BSA. The ultracentrifugation was carried out for 18 hrs for 32000 rpm at 4°C in a SW60Ti rotor.

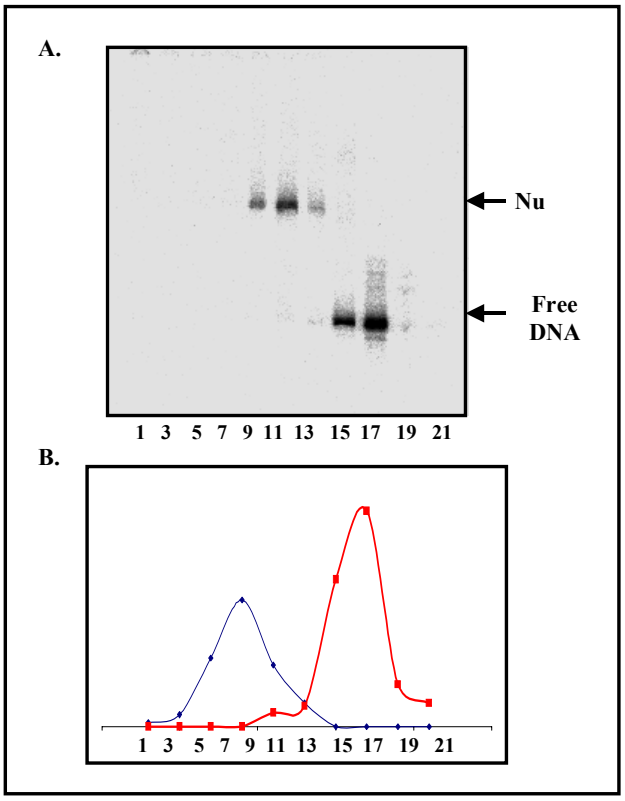


Figure 2.19: (A) Sucrose density gradient purification of assembled mononucleosomes from free DNA. (B) Densitometric scan showing distinct separation of DNA and mononucleosomal peaks.

2.3.3.4 Isolation of HeLa chromatin

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 $MgCl_2$), 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.

2.3.3.5 Histone H1-stripping of chromatin

The HeLa nuclei were isolated and subjected to Micrococcal Nuclease (MNase) digestion as described above. The digested nuclei were spun down and the pellet was resuspended in TE. The suspension was subjected to H1 stripping using CM Sephadex C-25 (Pharmacia) in TE buffer supplemented with 350 mM NaCl for 12 hrs**.**

2.3.3.6 Sucrose gradient fractionation of chromatin fragments

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 result section.

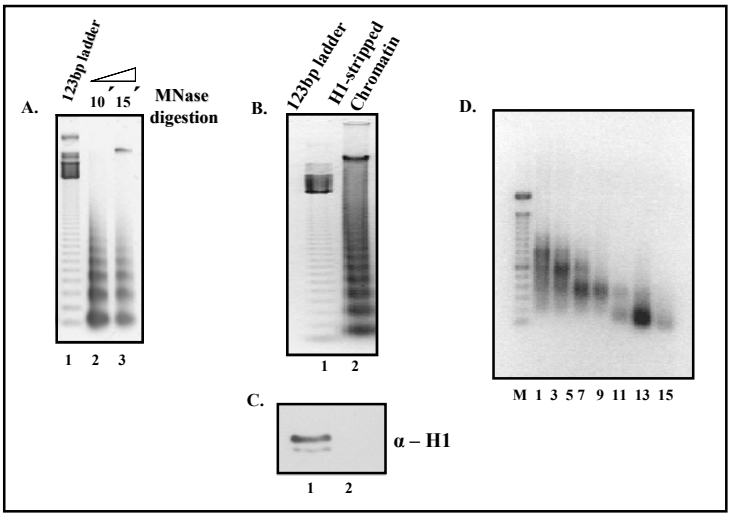


Figure 2.20: Preparation of native and histone H1-stripped chromatin: (A) Lane 1- 123 bp ladder, lanes 2-3 chromatin subjected to increased time point of MNase digestion. (B) Lane 1- 123 bp ladder, lane 2-Histone H1-stripped chromatin. (C) Western blotting analysis with α-H1 antibody ensuring depletion of histone H1 from prepared H1-stripped chromatin. Lane 1- native chromatin, lane 2- histone H1-stripped chromatin. (D) Analyzing the fractions collected after sucrose density gradient centrifugation with MNase digested native chromatin. M- 100 bp ladder, lanes 1-15 alternate fractions collected after sucrose density gradient centrifugation.

2.3.3.7 Electrophoretic mobility shift assays

The body labeled DNA fragments and the *in vitro* reconstituted mononucleosomes (equivalent to 25 ng of DNA) were incubated with different concentrations of PC4 for 30 minutes at 30^0C in a buffer containing 20 mM HEPES KOH, pH-7.5, 2.0 mM β-Me, 0.1 mM PMSF, 0.2 mg/ml BSA, 5% glycerol, 1 μM ZnCl2, 1 mM EDTA, 100 mM NaCl and 0.1% NP40. The reaction mixtures were then resolved on a 4.5% native polyacrylamide gel at 130 V in TBE buffer (45 mM Tris-Borate and 1 mM EDTA) for 3 hours. The gels were dried and autoradiogrammed.

2.3.3.8 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.

In order to compare the kinetics of chromatin compaction by PC4 as compared to histone H1, the incubation times were altered from 15-60 mins. For estimating the concentration dependence on the extent of chromatin compaction, 90 mins time point of incubation was carried out. For studying the functional interaction between PC4 and histone H1, a sub-stoichiometric amount of PC4 was incubated with H1 for a period of 15 mins to study the effect of PC4 on the chromatin compaction by H1. In order to study the effect of H1 on PC4 mediated chromatin compaction, sub-stoichiometric amount of H1 was incubated with PC4 for a period of 90 mins.

2.3.3.9 Atomic Force Microscopy

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 $MgCl_2$, 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 ug/ul) in the fixation buffer for 30 min as described above.

2.3.3.10 Micrococcal Nuclease accessibility assay

The chromatin was isolated from untransfected, scRNA transfected and siRNA transfected HeLa cells. The amounts were normalized for each set (~1 μg DNA) and subjected to partial MNase digestion (0.01 U/μl) in nuclei digestion buffer (10% glycerol, 10 mM Tris-HCl pH 8, 3 mM CaCl2, 150 mM NaCl, 0.2 mM PMSF) for 10 mins. The reaction was stopped using 10 mM EDTA. Individual reactions were treated with Proteinase K followed by Phenol- Chloroform extraction. Subsequently RNase treatment was carried out followed by reextraction with Ph-OH-CHCl3. The DNA was precipitated by Ethanol and analyzed on a 1% agarose gel. Similar MNase digestions were also carried out at three different time points (5, 10 and 15 mins) with the chromatin isolated from siRNA and scRNA transfected HeLa cells to study the alteration in chromatin accessibility by MNase as a function of time.

2.3.4 Knockdown of gene expression:

2.3.4.1 siRNA

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(UCUGCUGCUGCUGCUCUGU) dTT 3′ "**) were synthesized. As a control we used the scrambled RNA with the sequence – sense: "**5′r(GAAAGGCAACGACGGACAC)dTT3′";** antisense: **"5′r(GCGAACACUAACGUACCUCAU)dTT3′"**). HeLa cells were transfected with siRNA and scrambled RNA using Lipofectamine 2000 Plus (Invitrogen) according to the manufacturers 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.

2.3.4.2 Vector based system

Silencing was also done using a vector-based system where PC4 siRNA (sense: 5′GATCCCCACAGAGCAGCAGCAGCAGATTCAAGAGATCTGCTGCTGCTGCTCTGTTTTTT3′; antisense: 5′AGCTAAAAAACAGAGCAGCAGCAGCAGATCTCTTGAATCTGCTGCTGCTGCTCTGTGGG3′) was cloned in tandem with a GFP expression cassette into pGShin2 plasmid (Kojima et al, 2004) a kind gift from Dr**.** Shin-ichiro KOJIMA.

2.3.5 Global gene expression analysis:

2.3.5.1 Microarray

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.

2.3.5.2 Validation of Microarray data by Real Time PCR

Validation of Microarray after knocking down PC4 was carried out by Real Time PCR analysis of candidate upregulated and downregulated genes. The total RNA was isolated from untransfected, PC4 siRNA and scRNA transfected HeLa cell. Corresponding cDNA

was made from each set. PCR amplification was carried out with gene specific primers, using Actin as the control in each step. Subsequently Real Time PCR analysis was carried out using iQ SYBR Green Supermix (Biorad) (2X stock contains- 100 mM KCl, 40 mM Tris HCl pH 8.4, 0.4 mM each dNTP, 50U/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20 nM Fluorescein and stabilizers), respective gene specific primers and prepared cDNA (used as template). The PCR cycle was set in a Real Time PCR machine, where comparative analysis was carried out in quantitative manner. The threshold cycle Ct values of the tests were normalized with respect to β-Actin and the fold change was plotted.

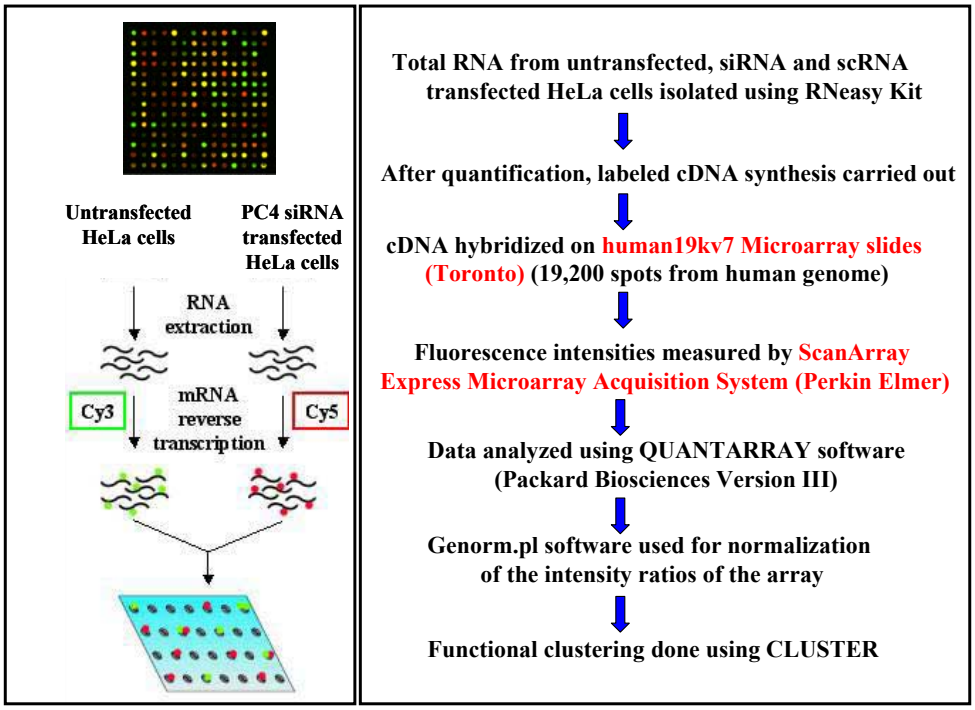


Figure 2.21: Scheme enumerating the microarray analysis performed after knocking down PC4 gene expression.

2.3.6 In vitro covalent modifications of proteins:

2.3.6.1 In vitro phosphorylation

1 µg PC4 or His6-PC4 was incubated with the enzyme 1µl Casein Kinase II (20 mU) at 30ºC/30 mins in 2X Phospho buffer (1X composition: 50 mM Hepes-KOH, pH-7.6, 125

mM NaCl, 10 mM MgCl2, 6% Glycerol, 5 mM DTT, 0.5 mM PMSF) and γ 32P ATP. To check for phosphorylation the reaction mixture was then loaded onto a 15% SDS PAGE, dried and exposed to X-ray films.

For mass phosphorylation of PC4 or His6-PC4 (2.5 μg) used in different assays, similar incubations of proteins were carried out with 1μl Casein Kinase II (20 mU), 2X Phospho-buffer and 200 mM cold ATP at 37ºC/30 mins, followed by replenishment with the enzyme and ATP for 3 times after every 30 mins and an incubation for 90 mins after that until complete saturation (~90%). As controls mock phosphorylation reactions were set with the same components except the enzyme CKII, and similar protocol was followed. The confirmation of phosphorylation was also done by western blotting analysis using anti- phosphor Serine anti bodies.

2.3.6.2 In vitro acetylation

Filter binding assay: 1 μg PC4 or His6-PC4 or histones was incubated with the enzyme 1μl p300 (giving ~ 8000 Counts/ μl) at 30ºC/30 mins in 2X HAT buffer (1X composition: 50 mM Tris-HCl pH 8, 10% Glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA), 10 mM Na-Butyrate and 0.5 μl of 3.3 Ci/mmol of 3H Acetyl CoASH. The reaction mixture was then spotted on a Whatman P-81 filter paper. The radioactive counts were recorded on a Wallac 1409 liquid scintillation counter.

Gel Assay: The reaction was carried out as described above, followed by TCA precipitation of the proteins using 25 % TCA on ice for 30 mins. This was followed by acetone wash of the TCA precipitated protein pellet, drying and loading onto a 15% SDS PAGE. The gel was visualized by Coomassie staining followed by Fluorography, using the Amplify solution (Amersham Pharmacia) for 30 mins. The gel was then dried and exposed to X-ray films for a period of 3-6 days at -80ºC before developing.

For mass acetylation of PC4 or His6-PC4 (2.5 μg) used in different assays, similar incubations of proteins were carried out with 1μl p300 (giving ~ 8000 Counts/ μl), 2X HAT buffer, 10 mM Na-Butyrate and cold 1.68 mM Acetyl CoASH, at 37ºC/30 mins, followed by replenishment with the enzyme and Acetyl CoASH for 3 times after every 30 mins and an incubation for 90 mins after that until complete saturation (~90%). As controls mock acetylation reactions were set with the same components except the

enzyme p300, and similar protocol was followed. The confirmation of acetylation was also done by western blotting analysis using anti- acetylated Lysine anti bodies.

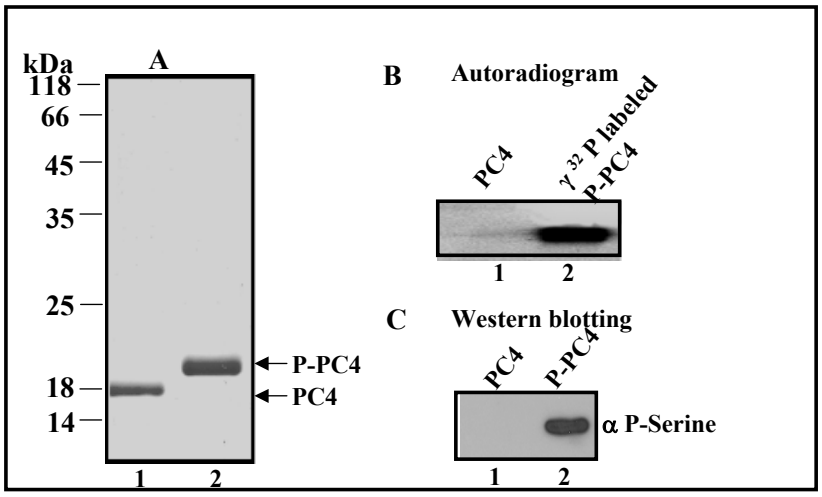


Figure 2.22: *In vitro* phosphorylation of PC4 by Casein Kinase II. (A) Coomassie gel to visualize P-PC4 (lane 2) in comparison to PC4 (lane1). (B) Phosphorylation reactions were carried out with γ32P labeled ATP, such that labeled phosphate incorporation could be studied by autoradiography (lane 2). (C) Western blotting analysis was also done with α P-Serine antibodies, which could specifically recognize phosphorylated form of the protein (lane 2).

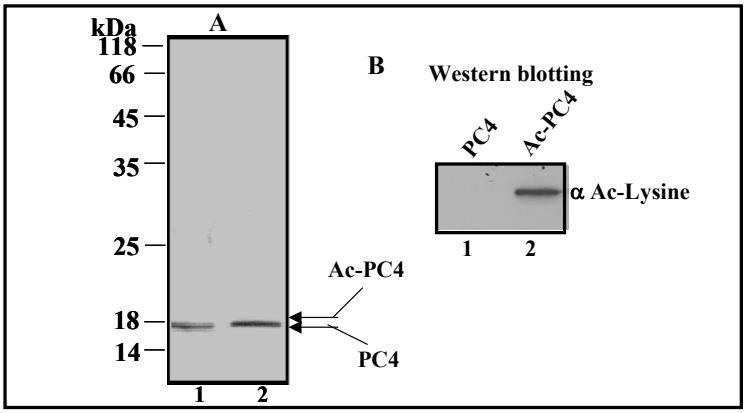


Figure 2.23: *In vitro* acetylation of PC4 by HAT p300. (A) Coomassie gel to visualize Ac-PC4 (lane 2) in comparison to PC4 (lane 1). (B) Western blotting analysis by α-Acetylated Lysine to probe for Ac-PC4 (lane 2).

2.3.7 In vivo acetylation status of PC4

HeLa cells were transfected with FLAG-PC4 in presence or absence of 100 nM Trichostatin A and 5 mM Na-Butyrate for a period of 24 hrs. In order to see whether

p300-specific acetylation of PC4 also occurs *in vivo*, in another experimental set Curcumin (a p300- specific HAT inhibitor) was added after FLAG-PC4 transfection. Whole cell extracts were prepared in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% Na-Deoxycholate, 1% SDS, 1mM EDTA, 50 µg/ml of Aprotinin and Leupeptin), followed by binding with M2-Agarose beads at 4ºC for 12 hrs. The immune complex was pull down, washed with RIPA buffer and loaded onto a 12% SDS PAGE and subjected to western blotting analysis using anti-Lysine antibodies.

2.3.8 In vitro Transcription

The *in vitro* transcription assay was scored using a 395 bp sequence devoid of any G residues in the sense strand, incorporated into a pG5ML array plasmid. The plasmid has 5S rDNA as nucleosome positioning sequence flanking on either side of the G-less transcription cassette, which is also under the control of 5 Gal4 (activator) DNA binding sites followed by AdML (Adenovirus Major Late) promoter. The transcription is carried out with ATP, CTP and α32P UTP and 3'O-Methyl GTP (a GTP analog lacking 3' OH, which leads to chain termination). RNase T1 is used to cleave the transcript after G-specific residues, further restricting the chain elongation.

For the transcription reaction 30 ng DNA or chromatin- template was incubated with 50 ng Gal4-VP16, in a buffer containing 4 mM Hepes (pH 7.8), 20 Mm KCl, 2 mM DTT, 0.2 mM PMSF, 10 mM Na-Butyrate, 0.1 mg/ml BSA, 20% Glycerol. Indicated amounts of proteins were incubated at 30˚ C for 30 mins. PC4- depleted HeLa nuclear extract, was added to this reaction and incubated for 20 mins at room temperature (DTT was added after an incubation of 10 mins), to initiate pre-initiation complex formation. Transcription reaction was subsequently started after the addition of NTP-mix consisting of 12 mM ATP, 12 mM CTP, 0.5 mM UTP, 2mM 3'O-Methyl GTP and 15 µCi α32P UTP, for a period of 30 mins at 30˚ C. This was followed by incubation with RNaseT1 for a period of 15 mins at 30˚ C, and subsequently terminating the reaction with the addition of stop buffer (20mMTris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1% SDS and 0.025 ng/µl tRNA. The radiolabelled transcript was extracted with Ph-OH-$CHCl_3$, EtOH precipitated, dried and the pellet was dissolved in the Transcription loading buffer (8M Urea, 0.005% Bromophenol Blue and Xylene cyanol) and analysed on a 5% Urea PAGE. Gels were

dried and analysed by Phosphorimager (Fuji BAS system) and analyzed by Image Quant software.

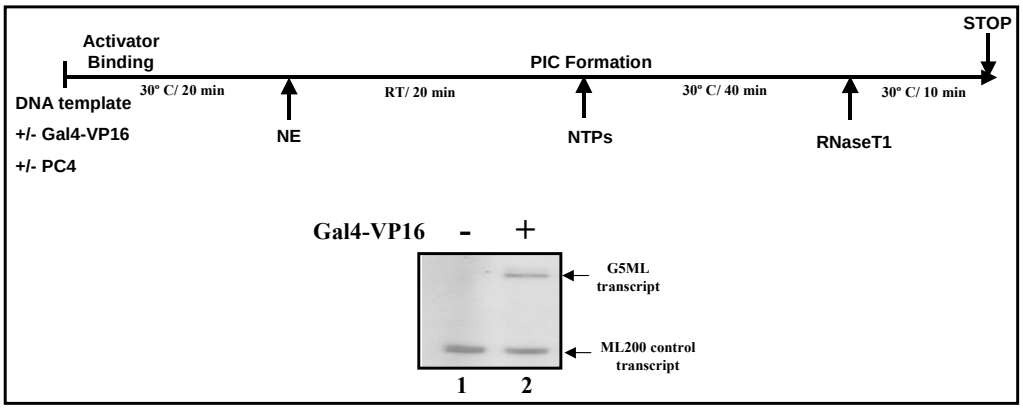


Figure 2.24: Scheme enumerating scoring Transcription from a DNA template in an activator-dependent manner. Gal4-VP16 dependent transcription scored from G5ML array (lane 2). ML200 was used as the loading control.

RESULTS

3.1 PC4, a nonhistone chromatin associated protein:

3.1.1 Association of PC4 with the oligonucleosomes

The human transcriptional coactivator PC4 is a highly conserved nuclear protein, which plays diverse roles in cellular function. Based on its cellular (nuclear) abundance, nonspecific double stranded DNA binding and ability to activate p53 function (like HMGB1), we speculated that PC4 may be a multifunctional chromatin-associated nonhistone protein. To examine the association of PC4 with the chromatin HeLa nuclei was partially digested with micrococcal nuclease (MNase) and the resultant chromatin fragments were fractionated on sucrose density gradient. The fractionated nucleosomal fragments were analyzed on a 1% agarose gel (Figure 3.1 A), to detect the presence of nucleosomal DNA in a particular fraction. The same fractions were also subjected to immunoblotting (Figure 3.1 B), to analyze the proteins associated with the nucleosomal

fragments. The results show that PC4 is only present in the fractions where nucleosomes are detected, as validated by the presence of core histone H3 or linker histone H1. To confirm the proper fractioning of the nucleosomal fragments and associated proteins, we also subjected the fractions to immunoblotting analysis using HMGB1 monoclonal antibody (Figure 3.1 B, panel IV). As reported previously (Falciola et al, 1997), HMGB1 was distributed over all the fractions, unlike PC4. Significantly, the general transcription factor TFIIA was present in the non-chromatin fraction (Figure 3.1 B, panel III, lane 16) but not in the chromatin fractions (Figure 3.1 B, panel III, lane 1-15), 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.

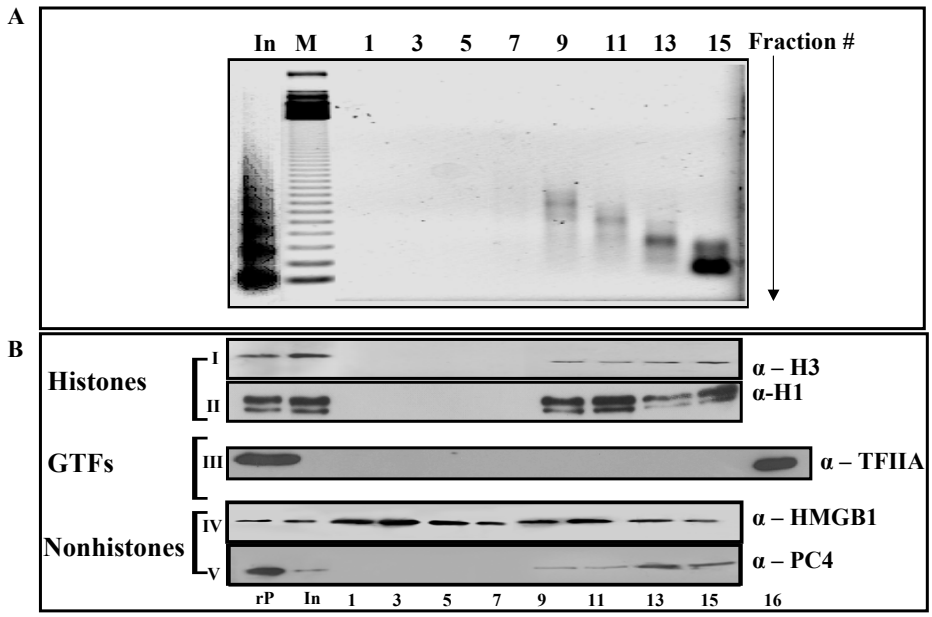


Figure 3.1- PC4 cofractionates with HeLa Nucleosomes in Sucrose gradient: (A) HeLa nuclei were partially digested with micrococcal nuclease (MNase) and fractionated on a 15-40% sucrose gradient. Individual fractions were deproteinised, and the alternative fractions were resolved on a 1 % agarose gel and visualized by ethidium bromide staining. (B) Corresponding fractions were analyzed by western blotting for the presence of histone H3 (I), histone H1 (II), TFIIA (III), HMGB1 (IV) and PC4 (V) using respective antibodies. Lanes 1- 15 are chromatin fractions and lane 16 corresponds to the non-chromatin fraction. The lane In stands for input control and the lane rP stands for the corresponding recombinant proteins.

3.1.2 Distribution of PC4 throughout the different stages of cell cycle

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 (Figure 3.2, panel II), whereas the cytosolic protein HSC70 was found in cytosolic fractions (Figure 3.2, panel III) 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 (Figure 3.2, panel I, compare lanes 1 and 3 vs lanes 2 and 4).

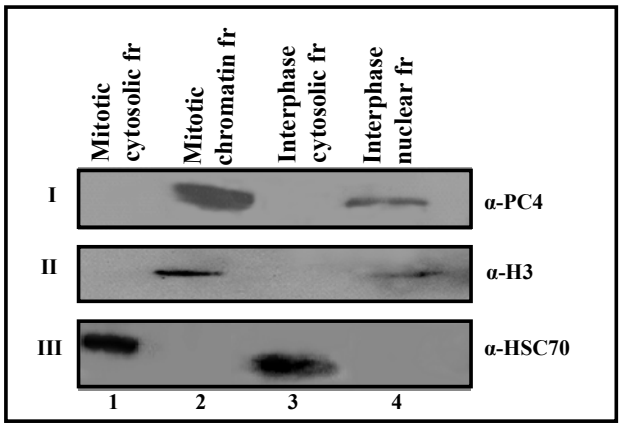


Figure 3.2- Subcellular localization of PC4 visualized by western blotting analysis: HeLa cells were synchronized with the treatment of 500 ng/ml nocodazole for 16 hrs. Mitotic and interphase nuclear pellet was separated from cytosolic fraction. The individual nuclear and cytosolic fractions were then subjected to Western blotting using antibody against PC4 (I), histone H3 (as a nuclear marker) (II) and HSC70 (as a cytosolic marker) (III).

In order to visualize the chromatin association of PC4, immunofluorescence localization of PC4 was performed in HeLa cells using affinity purified highly specific polyclonal PC4 antibody (Figure 2.14, Materials and Methods). The results show a predominant localization of PC4 in the nucleus. The nuclear association of PC4 was further investigated during the mitotic division of HeLa cells. As it has been depicted in Figure

3.3 A, PC4 was found to be associated with the chromosomes throughout the different stages of mitosis, indicating its association with individual metaphase chromosomes.
The relative amount of PC4 in the different stages of cell cycle was also assessed, biochemically (Rodriguez et al, 2000). 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 (Figure 3.3 B, panel I, 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 (Figure 3.3 B, panel I, lane 5). On the other hand, pre-metaphase arrest done with Nocodazole treatment showed a large amount of PC4 present in the Mitotic stage as compared to Interphase (Figure 3.3 B, panel I, 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.

3.1.3 Affinity of PC4 to the chromatin

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 (Figure 3.4, lanes 3 and 4) and digitonin (Figure 3.4, lanes 5 and 6). NP40 is a stronger detergent as compared to digitonin (Diaz and Stahl, 1989). Treatment of the cells with only buffer was taken as the control (Figure 3.4, lanes 1 and 2). Though digitonin treatment could not dissociate PC4 from the chromatin (Figure 3.4, lanes 5 and 6), the stronger detergent NP40 could release some amount of PC4 in the supernatant (Figure 3.4, 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) (Figure 3.4, 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 (Figure 3.4, 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.

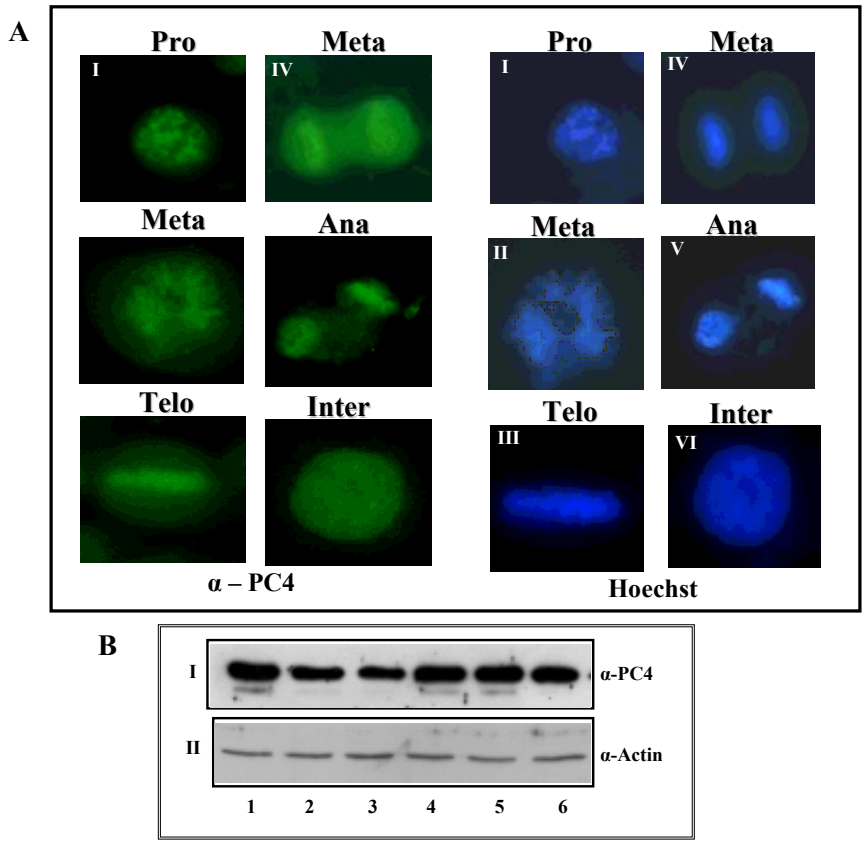


Figure 3.3- Distribution of PC4 throughout the different stages of cell cycle: (A) HeLa cells were fixed and stained for PC4 with the purified polyclonal antibody against PC4 followed by FITC conjugated secondary antibody and for DNA with Hoechst. Representative cells at different stages during mitosis: 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 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).

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 (Figure 3.5). 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 (Figure 3.5, 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 (Figure 3.5), as treatment with 0.2% NP40 could not mobilize these proteins.

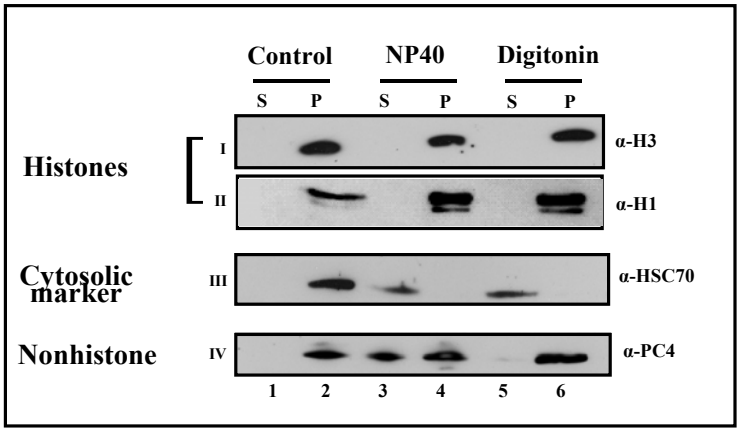


Figure 3.4: Relative affinity of PC4 to the chromatin: The cells were incubated in a buffer with 0.1% NP40 or 40 µg / ml digitonin. After the incubation the supernatants (S) and the remnants of permeabilised cell pellets (P) were analyzed by western blotting using antibodies against PC4 (IV), histone H3 (I), histone H1 (II) and HSC70 (III).

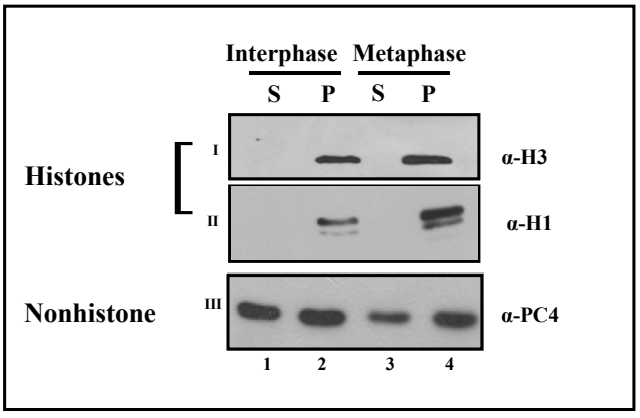


Figure 3.5- Comparative affinity of PC4 to the chromatin during Interphase and Mitotic stages of cell cycle: Comparative affinity of PC4 to the chromatin during Interphase and Mitotic stages of cell cycle. The Mitotic and Interphase stage cells were incubated in a buffer with 0.2% NP40. After the incubation 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 (panel III), histone H1 (panel II) and histone H3 (panel I).

3.1.4 Broad distribution of PC4 on the metaphase chromosome arms

To find out the chromosomal distribution of PC4, chromosome spreads were made from metaphase arrested mouse L cells and probed with the PC4 antibody. Significantly, it was found that PC4 is distributed throughout the entire chromosome arms in mouse L-cells (Figure 3.6) in a punctate manner without any apparent chromosome specificity. Interestingly, PC4 is not associated with the chromatin in the centromeric region (Figure 3.6 merge and also indicated by arrows)**.**

3.1.5 Stoichiometry of PC4 binding to the nucleosomes

Since PC4 was found to be a nonhistone chromatin-associated protein having significant strength of association to the chromatin, the stoichiometry of PC4 binding to the nucleosome was an important aspect to be addressed. This was done both *in vitro* and *in vivo* context.

A) PC4 binds to reconstituted mononucleosomes

The mononucleosomes assembled on a 160 bp labeled DNA fragment was purified and incubated with increasing concentration of PC4. The PC4-nucleosome complex was analyzed on a 4.5% native gel and visualized by autoradiography (Figure 3.7). Detailed densitometry analysis led us to conclude that stoichiometry of PC4 binding to mononucleosomes is as follows-

a) PC4: Nucleosomes = 1.93 (considering PC4 as a monomer)

b) PC4: Nucleosomes = 0.96 (considering PC4 as a dimer, which is its predicted *in vivo* oligomeric status.

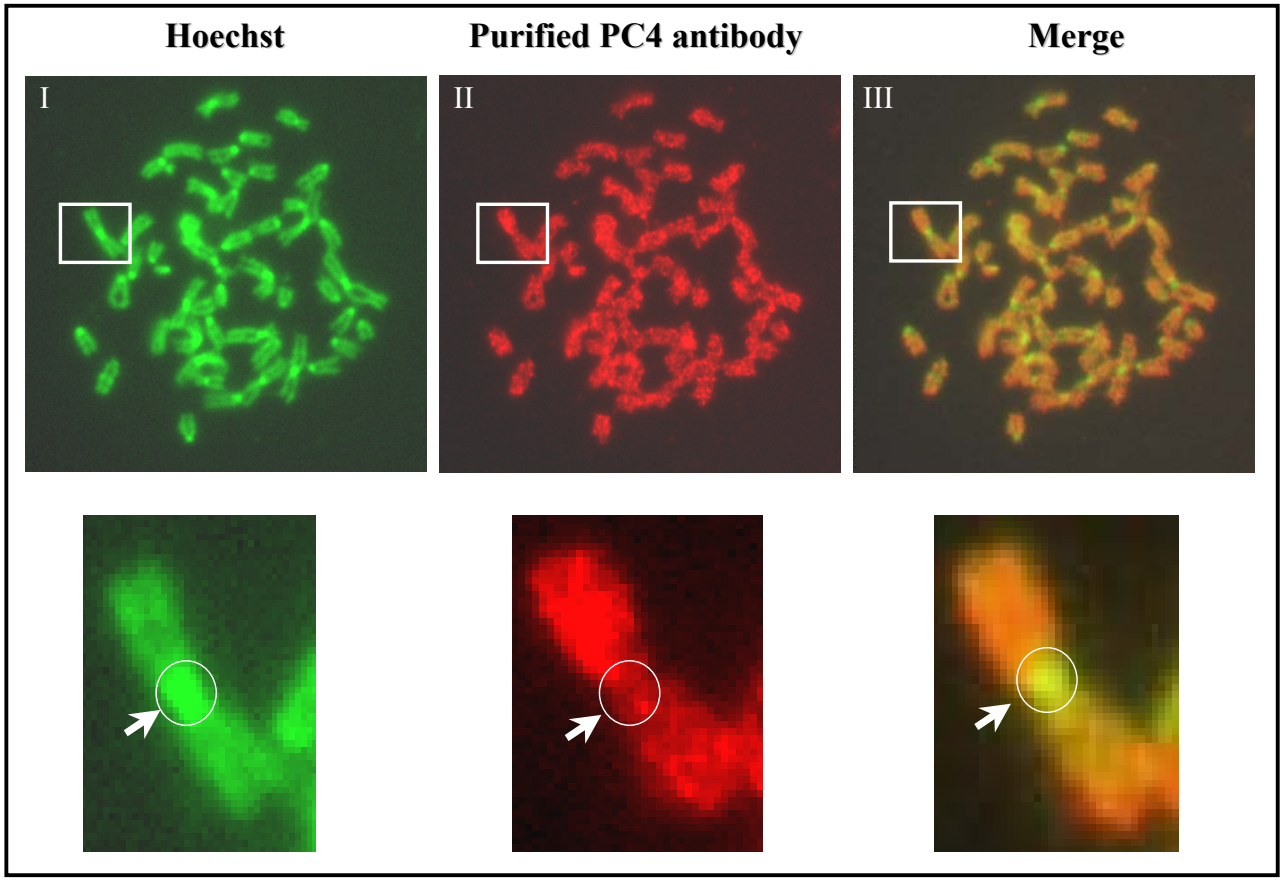


Figure 3.6- Distribution of PC4 on mitotic chromosomes: The condensed mitotic metaphase chromosomes from mouse L cells were spread on a slide and stained with Hoechst for DNA (I). The chromosomes probed with purified polyclonal antibody against PC4, followed by secondary antibody conjugated to rhodamine (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.

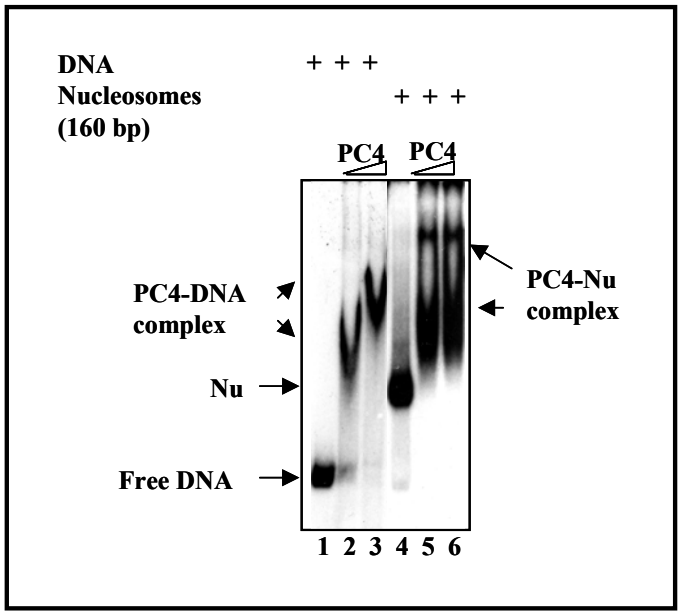


Figure 3.7- Stoichiometry of PC4 binding to *in vitro* reconstituted mononucleosomes: Binding of PC4 to the *in vitro* reconstituted mononucleosomes. Lane, 1- DNA, lanes, 2,3- DNA incubated with increasing concentration of PC4, lane, 4- mononucleosomes, lanes, 5,6- mononucleosomes incubated with increasing concentration of PC4.

B) PC4 binds to native nucleosomes

In order to study the interaction of PC4 with sucrose density gradient purified mononucleosomes (Figure 2.20, lane 13, Materials and Methods), GST pull down experiments were performed. PC4-GST could efficiently pull down the nucleosomal histones as visualized by western blotting analysis with anti-H3 antibodies (Figure 3.8). This indicates that PC4 effeciently interacts with native nucleosomes.

The amount of PC4 and core histones associated with a sucrose density gradient purified chromatin fraction (tetranucleosomes) (Figure 2.20, lane 1, Materials and Methods) was estimated by western blotting analysis, probing with anti-PC4 and anti-H3 antibodies followed by densitometric scanning. The stoichiometry of PC4 binding to nucleosomal histones was estimated. Calculating the number of molecules of PC4 binding to the mononucleosomes indicated that,

a) PC4: Nucleosomes = 2.2 (considering PC4 as a monomer)

b) PC4: Nucleosomes = 1.1 (considering PC4 as a dimer, which is its predicted *in vivo* oligomeric status),
which corroborated with the in vitro results.

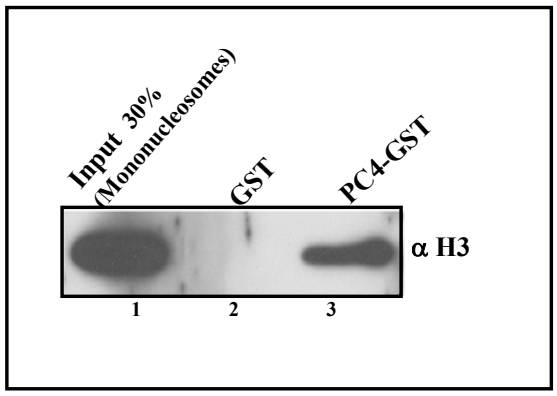


Figure 3.8- PC4 interacts with native HeLa mononucleosomes: GST pull down assay was done with PC4-GST and sucrose density gradient purified mononucleosomes. The interaction was probed by western blotting analysis by probing with α- H3 antibodies. Lane 1 mononucleosomes, lane 2 mononucleosomes incubated with GST and lane 3 mononucleosomes incubated with PC4-GST.

3.2 Functional consequence of chromatin association by PC4:

3.2.1 Histone interaction ability of PC4

A) *In vivo* interactions:

The stable association of PC4 to the chromatin could occur through its non-specific DNA binding ability (Kumar et al, 2001), interaction with bookmarked general transcription factors (Segil et al, 1996; Chen et al, 2002; Christova and Oelgeschlager, 2002), other nonhistone chromatin-associated proteins (Kriaucionis and Bird, 2003; Li et al, 2002) 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 (Stros and Kalibalova, 1987; Nielson et al, 2001). 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 (Figure 2.14, Materials and Methods). M2-agarose beads pulled down FLAG-

tagged PC4, 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 (Figure 3.9, lane 2). Furthermore, the PC4-GST construct could also pull down the core histones (Figure 3.9, lane 4) from the whole cell extract, but not the GST alone (Figure 3.9, lane 3).

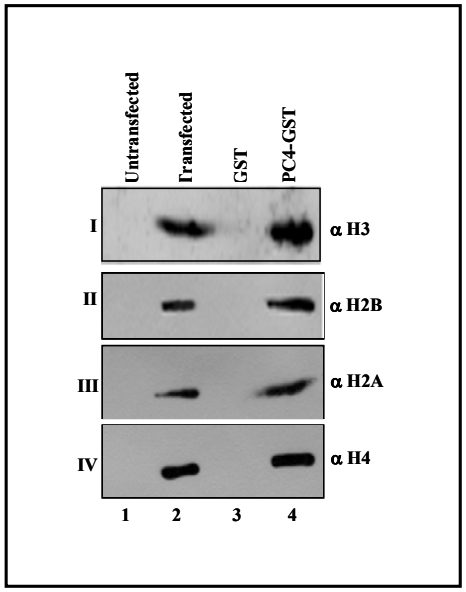


Figure 3.9- PC4 interacts with histones *in vivo*: To find out the histone interaction ability of PC4 *in vivo*, HeLa cells were transfected with FLAG- PC4 (F-PC4) mammalian expression construct. The expressed F-PC4 was pulled down by M2-Agarose beads, and the complex was subjected to western blotting 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) *In vitro* interactions:

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 (Figure 3.10, panel II and III, lane 3). The amount of histones H2A and H4 pull down by PC4 was found to be almost negligible, as compared to H3 and H2B (Figure 3.10, compare lane 3 of panels I and IV vs II and III). These data indicates 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 (Figure 3.10, panel V, lane 3).

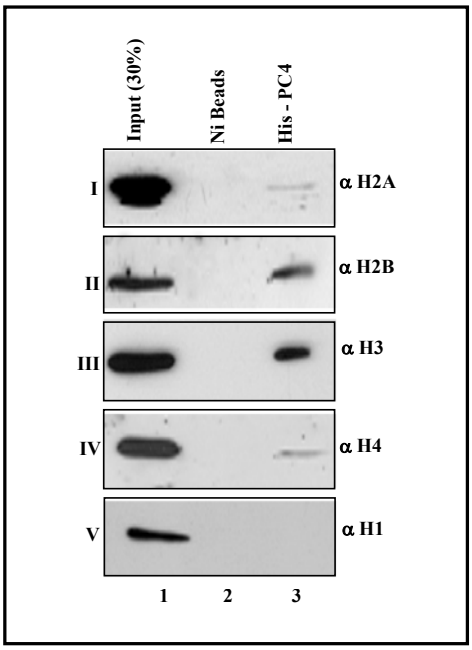


Figure 3.10- PC4 interacts with histones *in vitro*: The *in vitro* interactions were assessed by incubating 1 µg of His_6-PC4 bound to Ni-NTA beads with 200 ng of individual recombinant core histones (H2A, H2B, H3 and H4) and the linker histone H1. The complexes were pull down and analyzed by western 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) Strength of PC4-histone interaction:

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 (Figure 3.11, compare lane 4 vs 5).

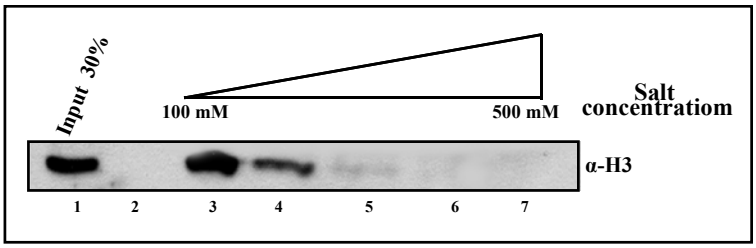


Figure 3.11- The strength of interaction of PC4 with the core histones: The strength of interaction of PC4 with the histones was checked by stringency washes with the buffer containing increasing concentration of salts, 100 (lane 3), 200 (lane 4), 300 (lane 5), 400 (lane 6) and 500 (lane 7) mM.

D) Interaction of PC4 with histone H3 variant CENP-A:

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 was 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 (Figure 3.12, panel II, lane 2), while PC4 did not show any detectable interaction with CENP-A (Figure 3.12, panel I, lane 2).

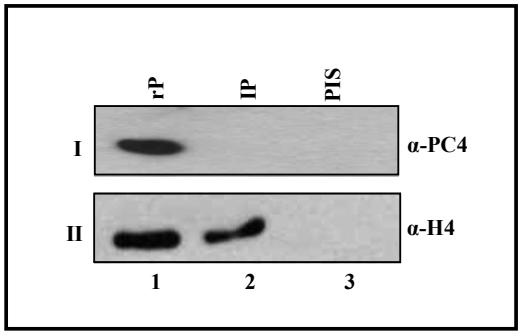


Figure 3.12- PC4 does not interact with centromeric histone variant CENP-A: HA tagged CENP-A construct was transfected into HeLa cells, the expressed protein was pull down by anti-

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.

Taken together these results suggest that PC4 binds to the chromatin through a preferential interaction with core histones H2B and H3 but not the centromeric variant of histone H3, CENP-A.

3.2.2 PC4 induces chromatin compaction

A) PC4 can condense the histone H1-stripped chromatin like histone H1:

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 fibre. 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 (Figure 3.14 A). 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 (Catez et al, 2004) (Figure 3.14). In order to visualize the ability of PC4 to compact a chromatin fragment of defined length, sucrose density gradient purified pentanucleosomes were taken as substrate and circular dichroism spectroscopic studies were carried out. Like native chromatin, PC4 could condense purified pentanucleosomal fragments also (Figure 3.13).

B) PC4 does not condenses the DNA, unlike the chromatin:

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 histone-DNA association) for PC4 to induce the chromatin compaction (Figure 3.14).

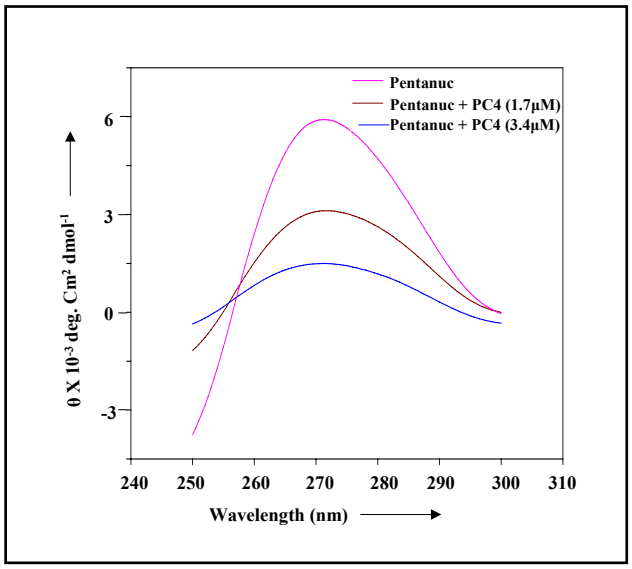


Figure 3.13- Circular Dichroic spectral studies with purified pentanucleosome: Purified pentanucleosomes were taken for circular dichroic spectral studies. Increasing concentration PC4 could condense the chromatin spectra to a greater extent.

A. **B.**

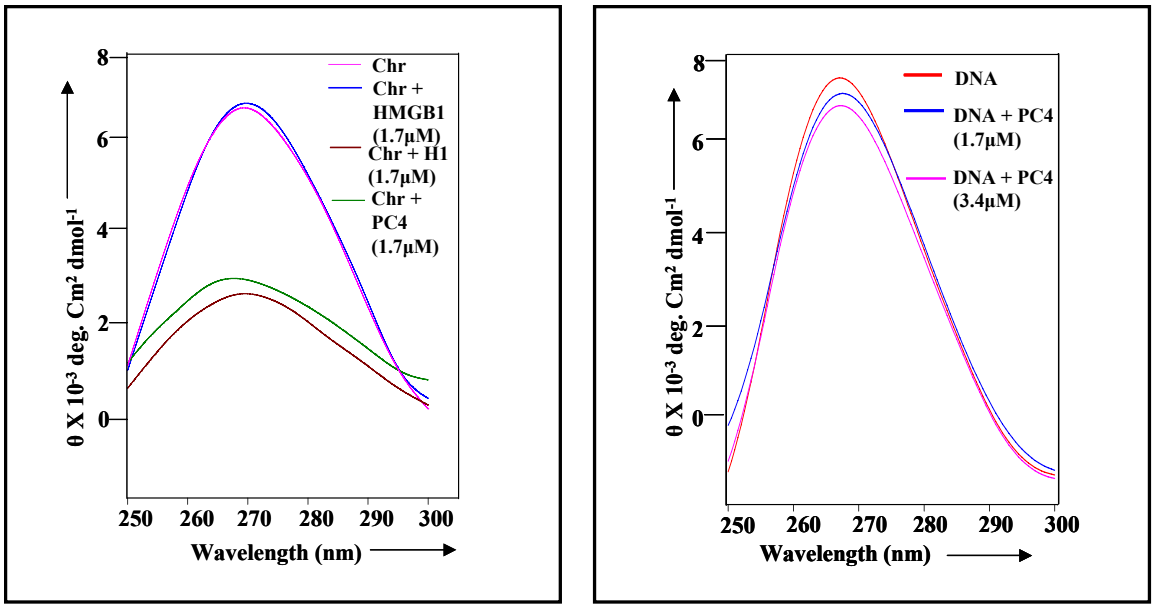


Figure 3.14- A. PC4 induces chromatin compaction: Circular dichroic (CD) spectra of histone H1 stripped chromatin incubated with PC4, H1 and HMGB1. **B. PC4 does not induce compaction of chromosomal DNA:** CD spectra of DNA incubated with increasing concentrations of PC4.

C) Atomic Force Microscopy of PC4-mediated chromatin compaction:

In order to visualize the PC4-mediated chromatin condensation, we subjected the 100 kb reconstituted chromatin (Figure 3.15 A) with either PC4 (Figure 3.15 B) or H1 (Figure 3.15 C) complexes to Atomic Force Microscopy (AFM). Significantly, though histone H1 induced the formation of expected higher ordered fiber structure (Figure 3.15 C), incubation of the reconstituted chromatin with PC4 led to the formation of distinct compact globular structure (Figure 3.15 B). In agreement with the circular dichroism spectroscopic data, addition of recombinant PC4 to the purified DNA (Figure 3.15 D and E) had no visual effect on the folding of the DNA molecules.

Control experiments for CD and AFM were performed with purified proteins. The molar ellipticity values of PC4/H1/HMGB1 of the CD spectra followed the baseline curve in the scanned wavelength range of 250-300 nm (Figure 3.16A). Atomic Force Microscopic imaging of purified PC4 or histone H1 was performed (Figure 3.16 B). Globular proteins having a Molecular Weight ~20 kDa should have ~3.6 nm diameter. The diameter of PC4 as visualized from frequency distribution curves corresponded to ~3.6 nm. The diameter of histone H1 was smaller than 3.6 nm, possibly because of the shape of the protein.

Thus both AFM and CD results indicate that PC4 can indeed cause a structural alteration of chromatin. The CD spectroscopic analysis shows that PC4 can condense the chromatin structure as visualized by the alteration of mean residue ellipticity values. However the direct visualization of structural alteration could be monitored by AFM. AFM images showed that PC4 could compact the 100 kb chromatin fragment to distinct globular structures. Interestingly, AFM images also gave an indication that the mechanism of PC4 mediated chromatin folding is different as compared to histone H1. While H1 induced chromatin compaction lead to the formation of ordered fibre structure, PC4 could form a distinct globular structure as visualized in case of many other nonhistone chromatin condensing proteins (like PcG) (Francis et al, 2004).

D) Comparative study of the chromatin compaction by PC4 and histone H1 in a dose dependent manner:

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 (Figure 3.17 A-B).

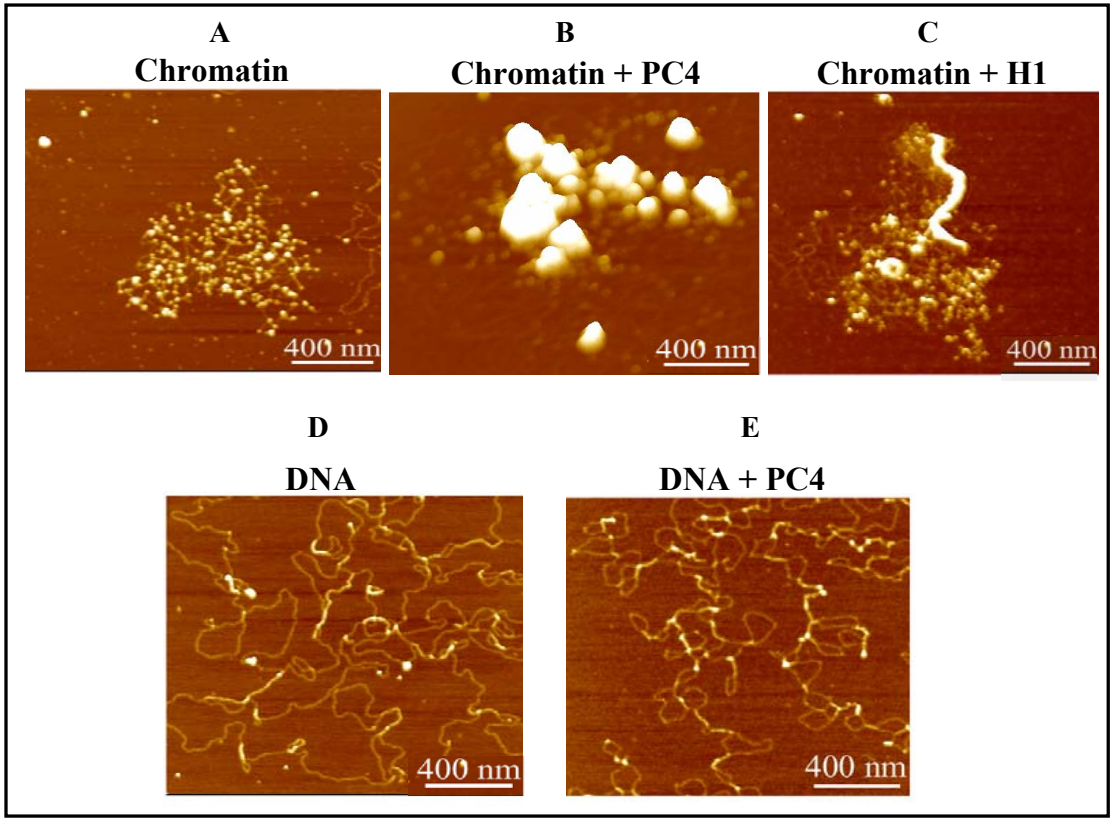
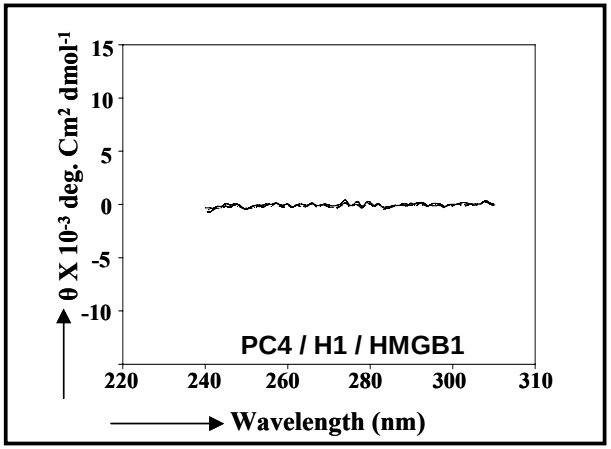


Figure 3.15- **PC4 condenses the chromatin fiber into a distinct globular structure:** AFM images of the 106 kbp reconstituted chromatin fibers incubated with histone H1 and PC4. The molar ratio of histone H1 (or PC4) to the histone octamer was 1:1. Upon 60 mins incubation on ice the complexes were fixed by 0.3 % glutaraldehyde, mounted on mica and observed under AFM. The 106 kb plasmid DNA similarly incubated with or without PC4 at the same ratio and processed for AFM imaging (see methods).

A



B

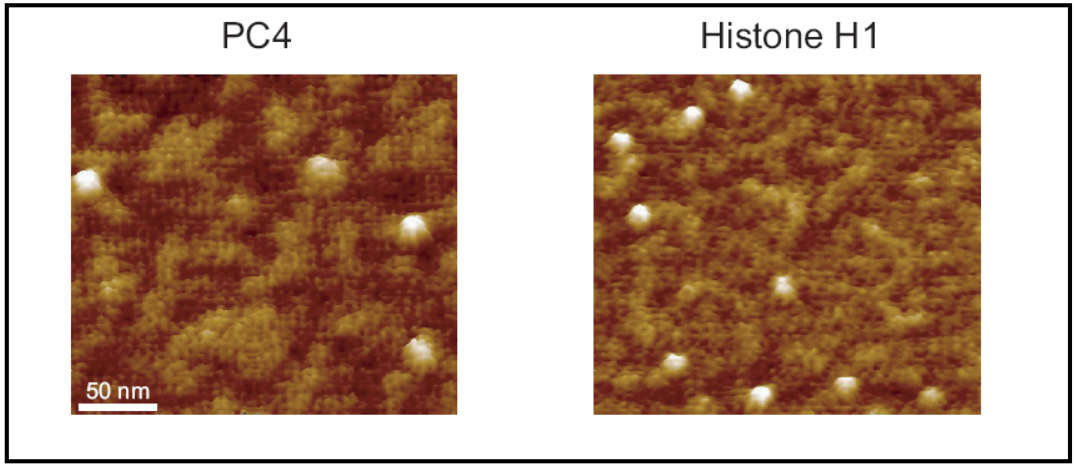


Figure 3.16- Circular Dichroism spectroscopic (A) and Atomic Force Microscopic (B) study was carried out with purified proteins PC4 and histone H1.

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 (Figure 3.17 A vs. B).

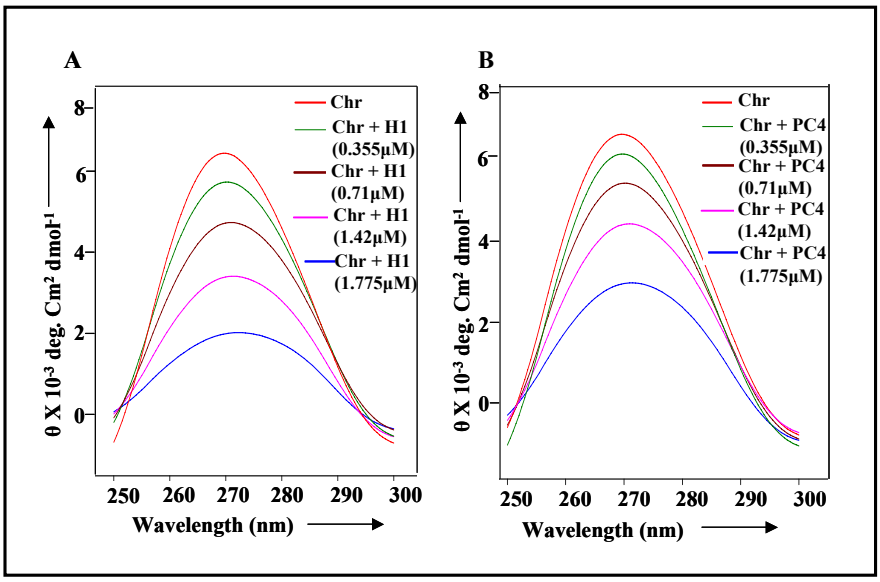


Figure 3.17- Comparative dose dependent condensation of chromatin by histone H1 and PC4 visualized by Circular Dichroism spectroscopy: Effect of increasing concentration of histone H1 (A) and PC4 (B) on the circular dichroic spectra of histone H1 stripped HeLa chromatin.

The alteration in mean residue ellipticity vs. the molar concentration ratios (PC4/H1: core octamer) has been plotted in Figure 3.18.

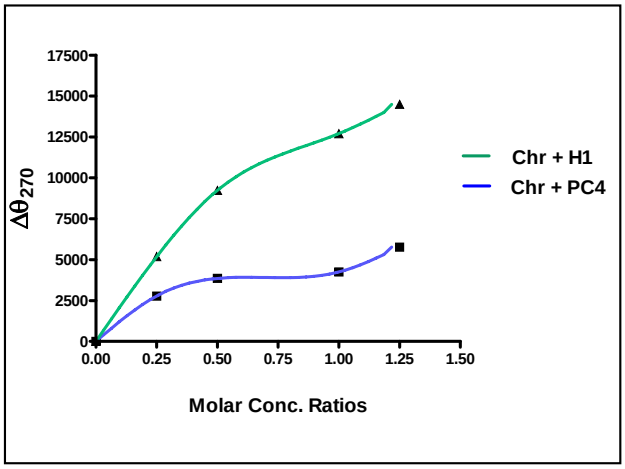


Figure3.18- Alteration of mean residual ellipticity value as a function of molar concentration ratios: Chromatin compaction by H1 or PC4 was quantified by plotting the

changes of mean residual ellipticity with respect to molar concentration ratios. The results indicate although PC4 can compact chromatin, the efficacy is less as compared to histone H1.

The results indicate that both PC4 and linker histone H1 can alter the mean residue ellipticity values. However histone H1 is found to be more efficient as compared to PC4 in this regard.

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) (Figure 3.19, I or II) showed that PC4-mediated chromatin globule formation is achieved optimally at the equimolar ratio of core histone and PC4 (Figure 3.19, I C). Further increase in the concentration of PC4 did not increase the size of the globule (Figure 3.19, I C vs D). However, when core histone: histone H1 molar ratio was increased to 1:1.25, a highly folded fiber structure could be observed (Figure 3.19, II C vs D).

E) Kinetics of chromatin compaction by PC4 and histone H1:

Both PC4 and linker histone H1 showed distinct chromatin compaction function. In order to compare the rates of chromatin compaction by these two proteins kinetic analysis was carried out. We have monitored 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 (Figure 3.20 A) and AFM images (Figure 3.21 II A). 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 (Figure 3.20 B and 3.21 I B).

The rate of change of the mean residual ellipticity with time 341.97 deg $cm^2dmol^{-1}min^{-1}$ for histone H1 and 143.6 deg $cm^2dmol^{-1}min^{-1}$ for PC4 suggesting that though both histone H1 and PC4 induce chromatin condensation, the type and mode of actions are distinctly different (see discussion).

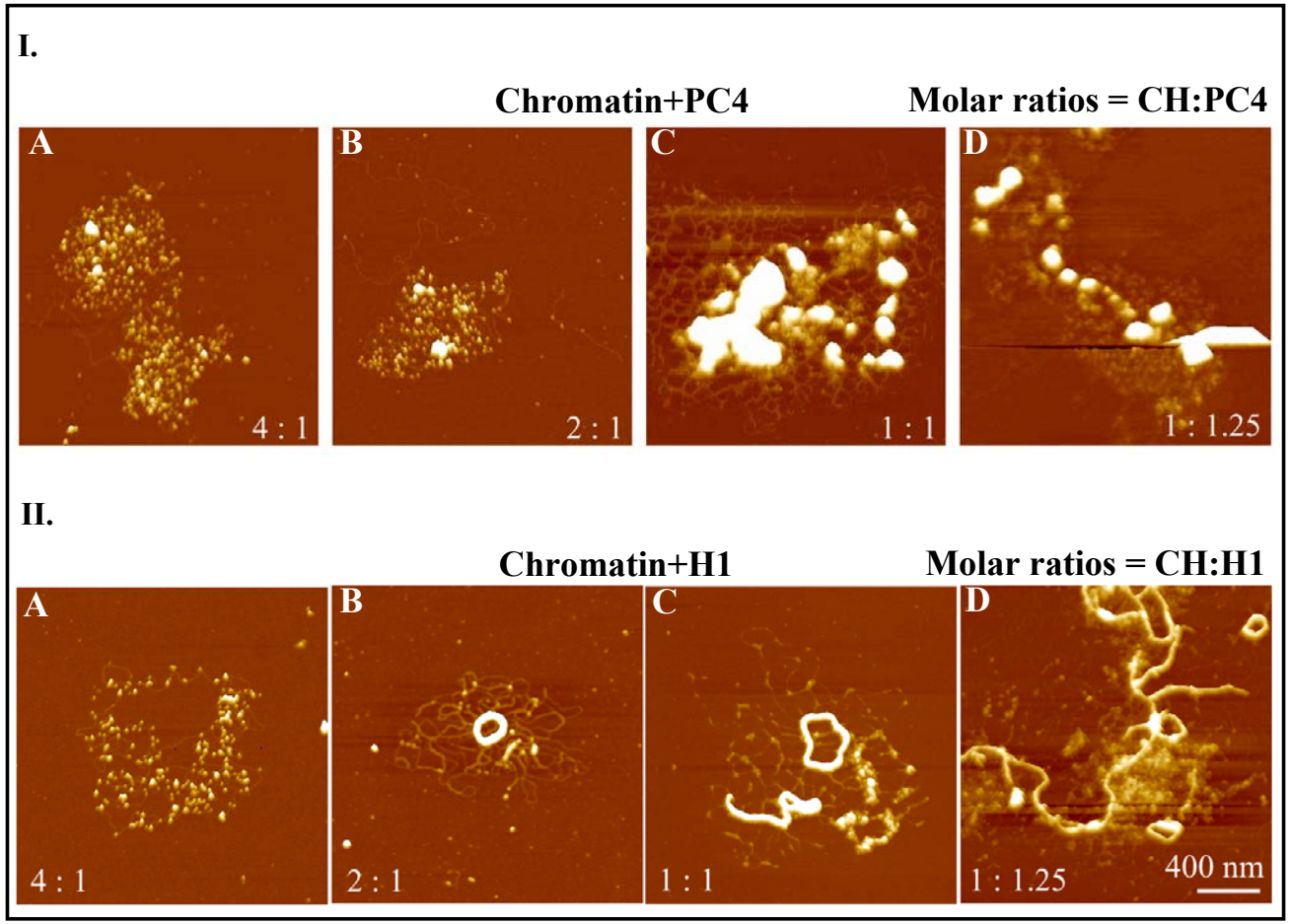


Figure 3.19- Visualizing the dose-dependence on chromatin condensation by PC4 and histone H1: AFM images of the chromatin incubated with various amount of histone H1 (II) or PC4 (I). PC4 or H1 were mixed with the reconstituted chromatin in 50 mM NaCl; at the molar ratios of the histone octamer to PC4 or H1 of 4:1, 2:1, 1:1, and 1:1.25, respectively.

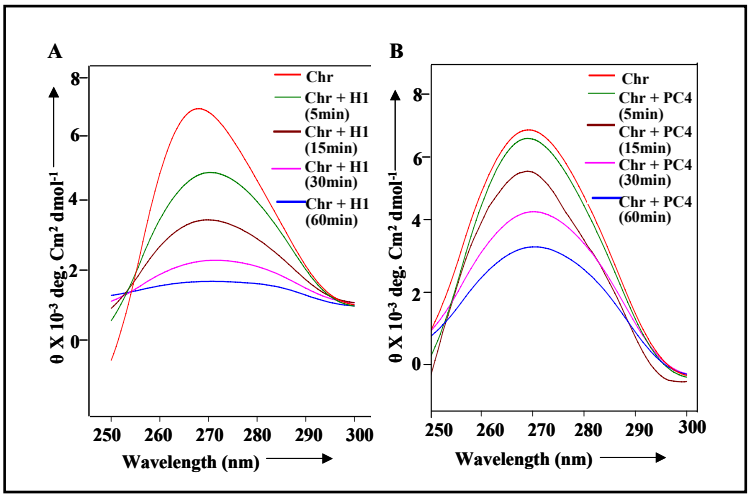


Figure 3.20- Comparative kinetics of chromatin compaction by histone H1 and PC4: Histone H1 (A) and PC4 (B) were incubated with H1 stripped HeLa chromatin at different time points (5, 15, 30 and 60 mins) and subjected to circular dichroism spectroscopy.

3.2.3 Functional interaction between PC4 and linker histone H1, two distinct chromatin condensers

We have found that both PC4 and linker histone H1 can compact the chromatin. In order to address the functional interaction between these two proteins, two different experimental schemes were followed:

(I) In order to study the effect of H1 on PC4-mediated chromatin compaction, H1 stripped chromatin was incubated with sub-optimal amount of H1 along with increasing concentrations of PC4 and incubated at 25°C for 60 mins. CD spectra were subsequently recorded for each of these reactions.

(II) The reverse effect of PC4 on H1-mediated chromatin compaction was assessed by adding sub-optimal amount of PC4 along with increasing concentrations of H1 onto the H1-stripped chromatin and subsequently incubated at 25°C for 15 mins. CD spectral recording were done as before.

The alteration of mean residual ellipticity (θ_{270}) in scheme I could be attributed to the effect of H1 on PC4 mediated chromatin compaction. Similarly the effect of PC4 on H1 mediated compaction could be scored through scheme II.

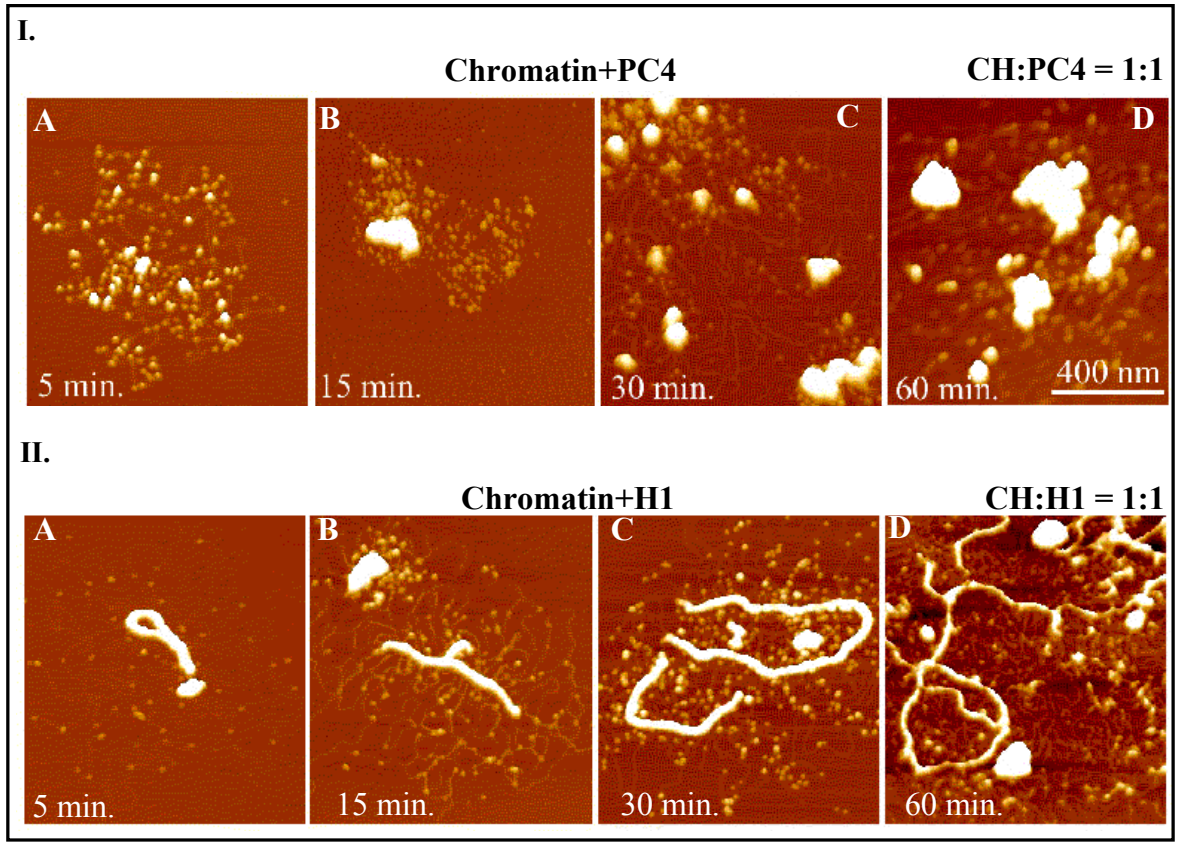


Figure 3.21- Visualizing the kinetics of chromatin condensation by PC4 and histone H1: In order to visualize the time dependent condensation brought about by PC4 the salt dialyzed reconstituted chromatin and H1 (II) or PC4 (I) were mixed at the 1:1 molar ratio of the histone octamer to PC4 or H1. After keeping on ice for 5 min, 15 min, 30 min, and 60 min, they were fixed by 0.3 % glutaraldehyde and observed under AFM.

A set of ten different concentrations of PC4 with a fixed sub-saturated amount of H1 was taken for scheme I, and vice versa for scheme II. We observe an alteration of mean residual ellipticity (θ270) values more prominent in case of scheme I but not in case of scheme II. This was true for all the ten experimental sets. However for simplicity we have represented here the mean residual ellipticity values for three such concentration ranges for each of scheme I (Figure 3.22 A) and scheme II (Figure 3.22 B).

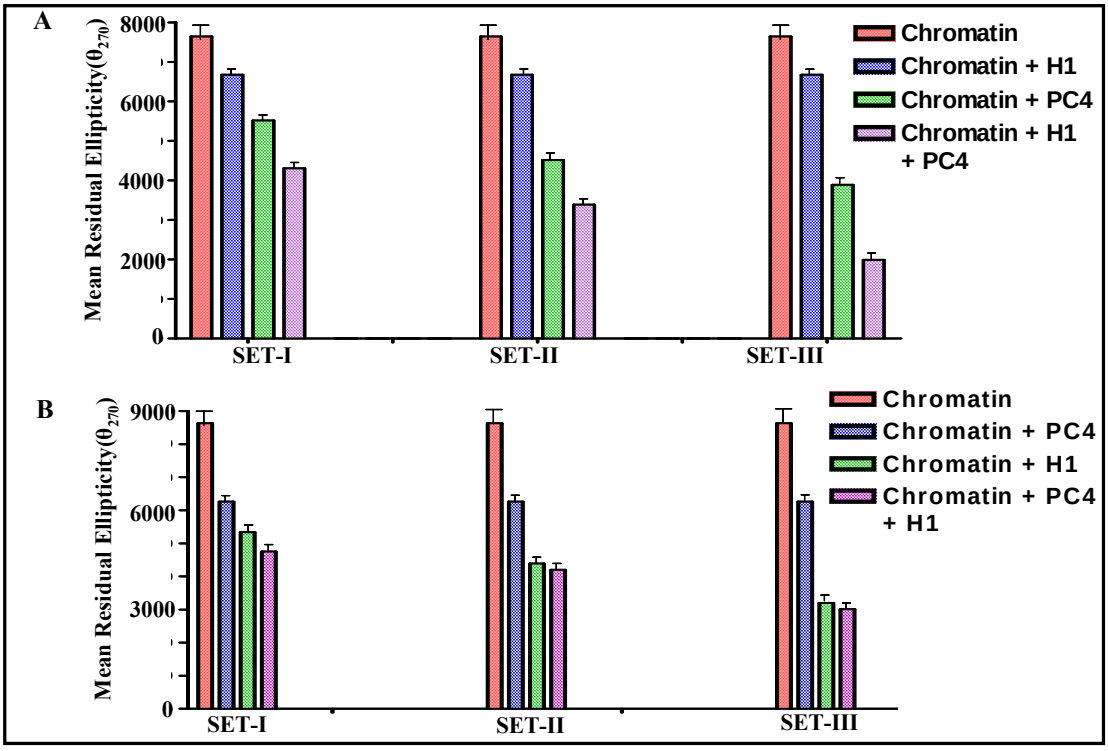


Figure 3.22- Plotting of mean residual ellipticity from Circular Dichroic Spectroscopic studies across two previously mentioned experimental schemes. A Presence of sub-stoichiometric amount of histone H1 assists PC4 mediated chromatin compaction. In set I-III, the extent of compaction executed by only PC4 is enhanced in presence of sub-stoichiometric histone H1 (compare green vs purple bar). B. Presence of sub-stoichiometric amount of PC4 does not assist H1-mediated chromatin compaction. In set I-III, the compaction executed by only H1 does not alter significantly in presence of sub-stoichiometric amount of PC4 (compare green vs purple bar).

The quantitative estimation of alteration of θ_{270} as a function of molar concentration ratios (concentration of PC4/H1: core octamer) has been represented in Discussion (Figure 4.5).

Furthermore, Atomic Force Microscopic visualization was carried out to monitor the propensity of globular structure or fibre structure formation in presence of both H1 and PC4. The detailed analysis of the functional interactions between PC4 and histone H1 (as per the different molar ratios) facilitating globular or fibre structure of chromatin is summarized in Table 3.1.

Table 3.1: Functional interaction between PC4 and linker histone H1 visualized by Atomic Force Microscopy.

CH:PC4	CH:H1	Globular Structure	Fiber Structure
4:1		+	
2:1		++	
1:1		++++	
	4:1		+
	2:1		+++
	1:1		++++
4:1	2:1	+++	+
4:1	1:1	++	++++
1:1	4:1	+++	+
2:1	4:1	+++++	+
2:1	2:1	+++++	+

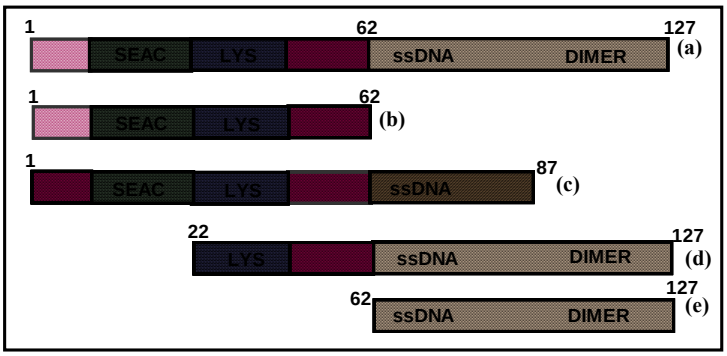
Taken together these results show that H1 assists PC4 mediated globular structure formation, but PC4 does not assist fibre structure formation by H1. This shows a unique complementarity between the two proteins involved in distinct pathways of compaction.

3.3 Functional correlation between histone interaction and chromatin condensation ability of PC4:

3.3.1 Mapping the domain of PC4 involved in interaction with the core histones and in chromatin compaction

PC4 interacts with core histones H3 and H2B *in vitro* (Figure 3.10) and induces chromatin condensation (Figure 3.14 A). 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 studied the histone interaction ability of different deletion constructs of PC4 (1-62, 1-87, 22-127, 62-127) (Figure 3.23 A). It was found that except PC4 (1-62), all the other PC4 deletion mutants could interact with both the core histone H3 and H2B (Figure 3.23 B).

A.



B.

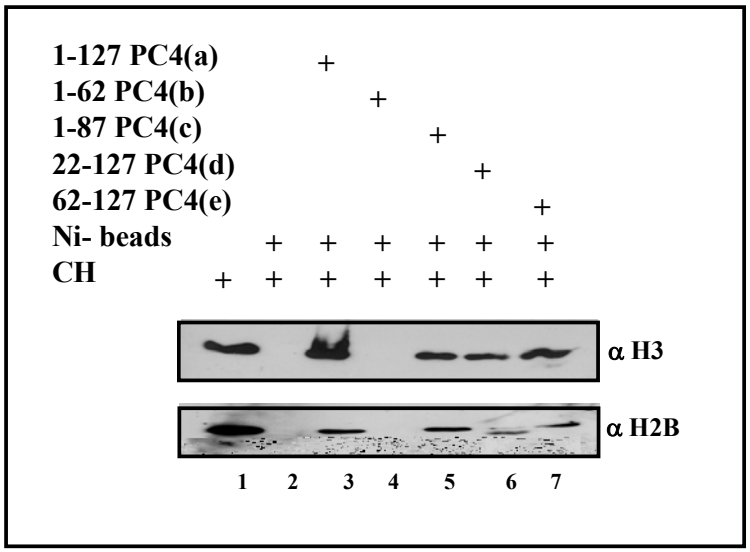


Figure 3.23- Histone interaction ability of PC4 deletion mutants: 1 µg of His_6 tagged PC4 deletion constructs 1-62, 1-87, 22-127 and 62-127 were incubated with HeLa core histones and the complexes were pulled down and analyzed by western blotting using antibodies against histone H3 and H2B.

CD spectroscopic analysis also showed that except PC4 (1-62), all the other mutants could induce chromatin compaction with varying ability (Figure 3.24).

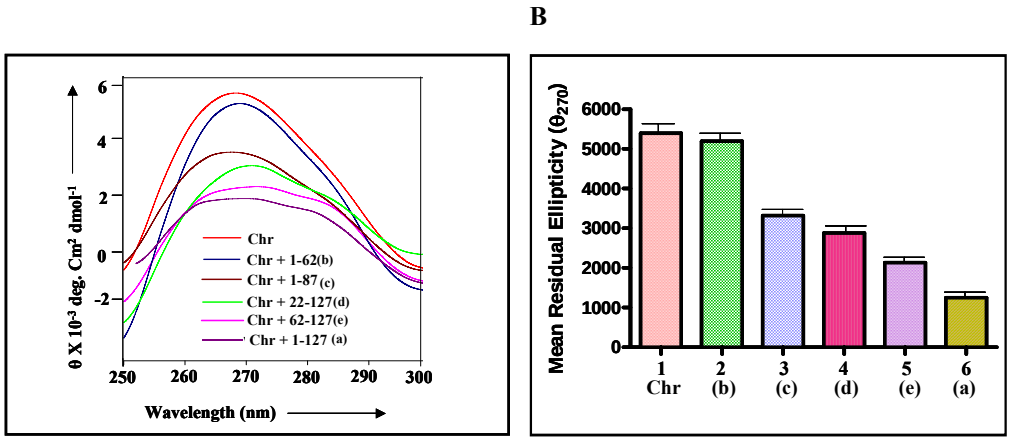


Figure 3.24- Chromatin condensing ability of PC4 deletion mutants: Effect of PC4 deletion mutants (1-62, 1-87, 22-127 and 62-127) on Circular dichroic spectra of histone H1 stripped chromatin.

Table 3.2: Histone interaction ability and chromatin condensation functions of PC4 deletion mutants.

PC4 Deletions	Histone interaction	Chromatin condensation
1-62	-	-
1-87	+	+
22-127	+	++
62-127	+++	+++
1-127	++++	++++

3.3.2 Histone interaction is essential to induce chromatin compaction

Based on these results an internal deletion construct of PC4, PC4 Δ62-87 was used to study the histone interaction and chromatin compaction by PC4 (Figure 3.25 A). As expected PC4 Δ62-87 could not interact with core histones H3 and H2B (Figure 3.25 B, panels I and II, lane 3). These deletion mutants of PC4 were then used in the CD spectroscopic analysis. Interestingly, it was observed that PC4 Δ62-87 (Figure 3.25 C)

could not induce chromatin condensation 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 (Figure 3.25 D), the addition of PC4 Δ62-87 showed negligible effect on the reconstituted chromatin images (Figure 3.25 D), suggesting that PC4 induce the chromatin compaction through the direct interactions with the core histones.

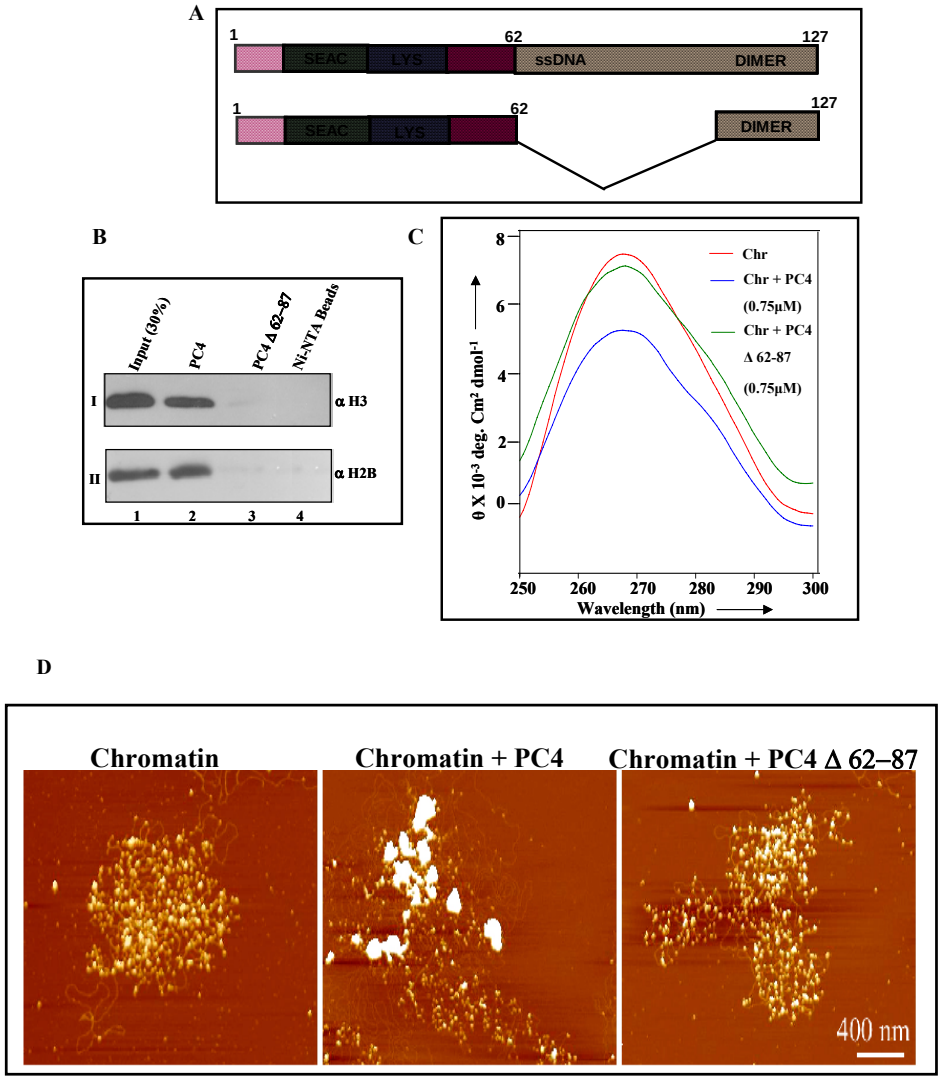


Figure 3.25- Histone interaction ability is essential for chromatin condensation by PC4: Full length and mutant PC4 were incubated with core histones and analyzed by western blotting with antibodies against histone H3 (A, panel I) and H2B (A, panel II). (B) Comparative analysis of chromatin condensing ability of PC4 and PC4 Δ62-87 visualized through CD spectroscopy. (C) AFM images of the reconstituted chromatin with PC4 and histone interaction deficient PC4 mutant. 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.

3.3.3 Finer mapping of histone interaction and chromatin compaction domain of PC4

In order to fine map the histone interaction ability of PC4, different deletions of PC4 spanning the stretch of 62 to 87 amino acids – PC4 (1-62), PC4 (1-67), PC4 (1-72), PC4 (1-77), PC4 (1-82), PC4 (1-87) (Figure 3.26 A) were incubated with the core histones. Except PC4 (1-62) all the other deletions could interact with the histones (Figure 3.26 B). Interestingly, except PC4 (1-62) all the other deletions could induce chromatin compaction ability to differential extent (Figure 3.27 A and B).

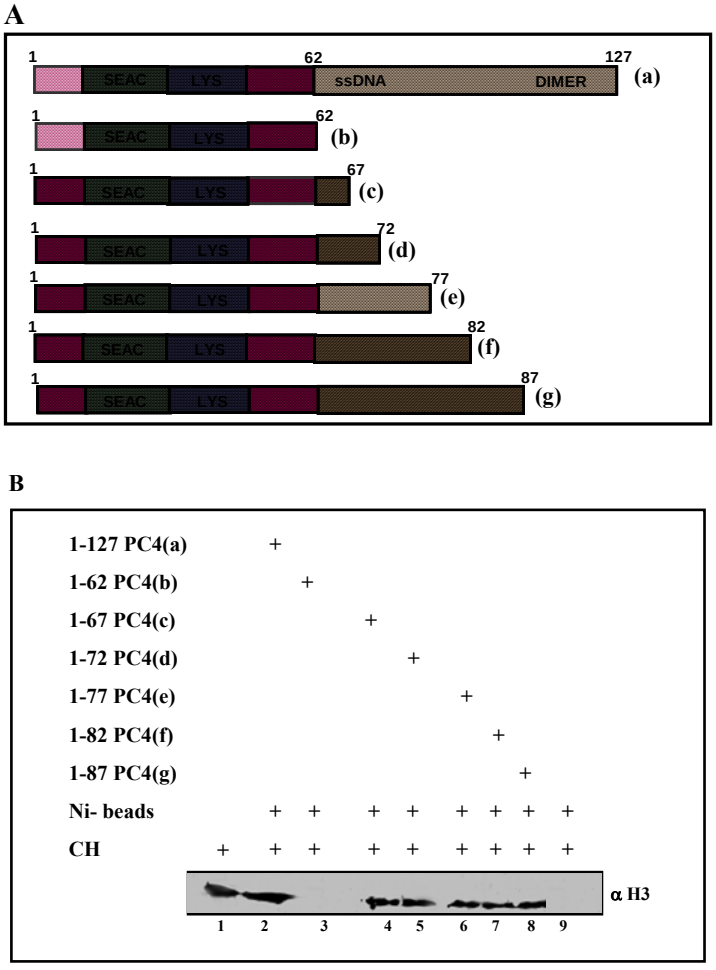


Figure 3.26- Finer mapping of histone interaction functions of PC4: 1 µg of His_6 tagged PC4 deletion constructs 1-62, 1-67, 1-72, 1-77, 1-82 and 1-87 were incubated with HeLa core histones and the complexes were pull down and analyzed by western blotting using antibodies against histone H3 and H2B.

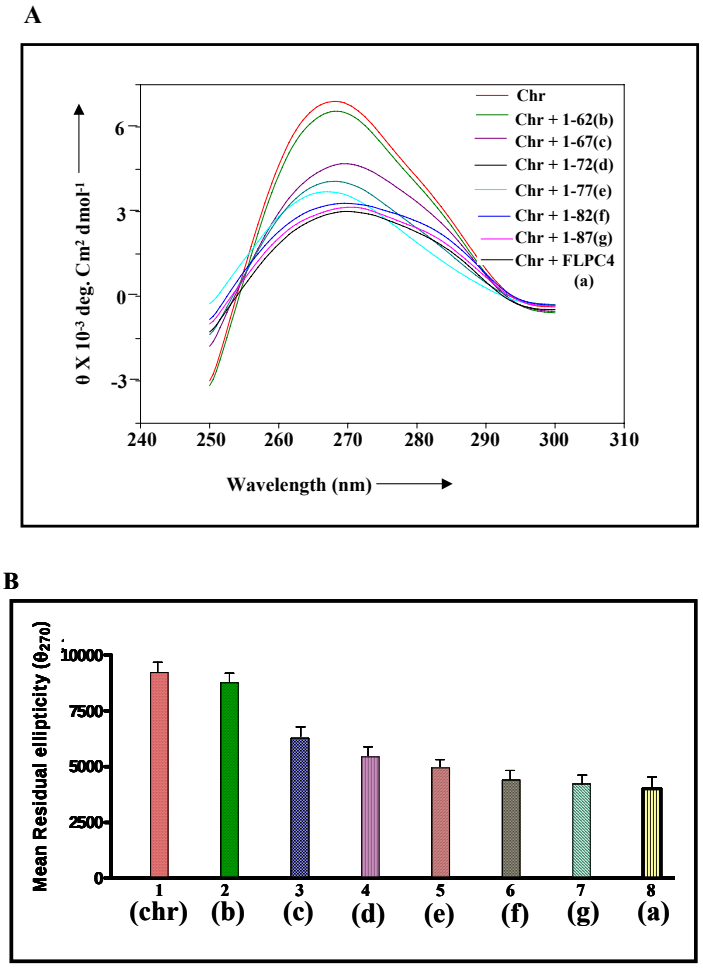


Figure 3.27- Finer mapping of chromatin condensing ability of PC4 deletion mutants: : Effect of PC4 deletion mutants (1-62, 1-67, 1-72, 1-77, 1-82 and 1-87) on Circular dichroic spectra of histone H1 stripped chromatin.

Table 3.3: Finer mapping of histone interaction ability and chromatin condensing functions of PC4.

PC4 Finer Deletions	Histone interaction	Chromatin condensation
1-62	-	-
1-67	++	+
1-72	++	++
1-77	++	++
1-82	++	++
1-87	+++	+++
1-127	++++	++++

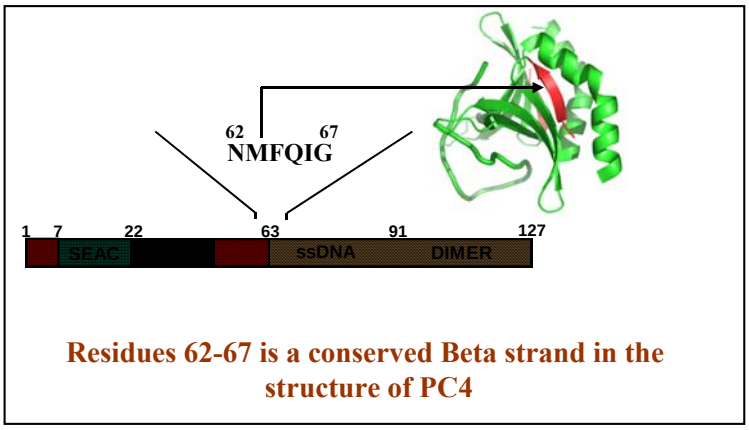


Figure 3.28- Amino acids 62-67 of PC4 is involved in interaction with histones: Detailed domain analysis indicated that residues 62-67 of PC4 are critical for interaction with core histones.

3.3.4 Mapping the domain of histones involved in interaction with PC4

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 were constructed (Figure 2.12, Materials and Methods). 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) (Figure 3.29, A and B, 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, (Figure 3.29 A and B, compare lanes 2 vs 4) indicating that the N-terminal tail rather plays a negative role in this phenomenon.

3.3.5 Role of flexible histone tails in PC4-mediated chromatin compaction

PC4 interacts with the tail-less globular domains of histones (H3 and H2B) quite efficiently (Figure 3.30) 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 (Francis et al, 2004). In agreement with the histone interaction data we observed that PC4 could efficiently condense the chromatin, reconstituted with the tail-less histones (Figure 3.30). Thus, for the PC4-mediated chromatin compaction the flexible N-terminal tails of histones may not be essential.

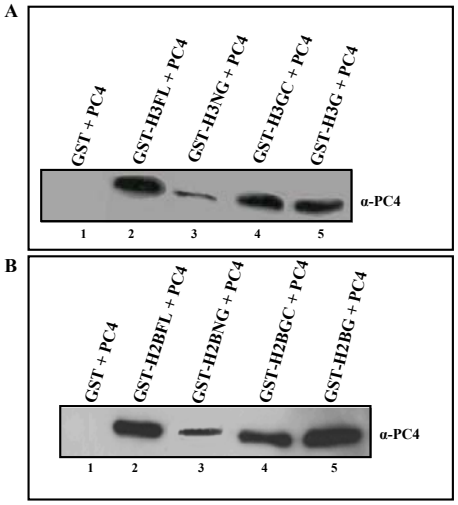


Figure 3.29- Mapping the domain(s) of core histones H3 and H2B involved in the interaction with PC4: Different deletion mutants were subjected to GST-pull down followed by western blotting analysis using anti-PC4 polyclonal antibodies. PC4 incubated with GST (lane 1), FL (Lane 2), NG (lane 3), GC (lane 4), G (lane 5) domains of the deletion mutants of H3 (panel I) and H2B (panel II).

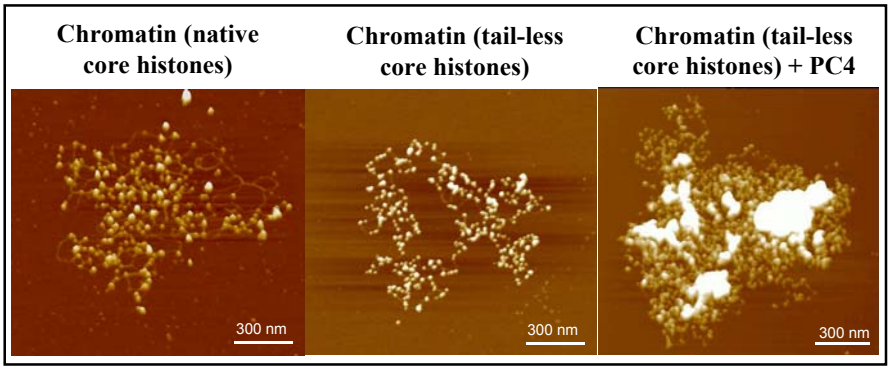


Figure 3.30- PC4-mediated compaction of chromatin assembled with tail-less histones: AFM images of the reconstituted chromatin with wild type, tail-less histones and the effect of adding PC4 to the chromatin reconstituted with tail-less histones. The molar ratio of the histone octamer to PC4 was 4:1.

3.4 Effect of PC4 silencing in chromatin organization, gene expression and cell cycle progression:

3.4.1 Visualizing the chromatin architecture upon PC4 knock down

A) Alterations in chromatin compactness upon knocking down PC4:

PC4 expression was silenced by siRNA (Figure 3.31) or vector based system (Figure 3.32), as described in Materials and Methods section.

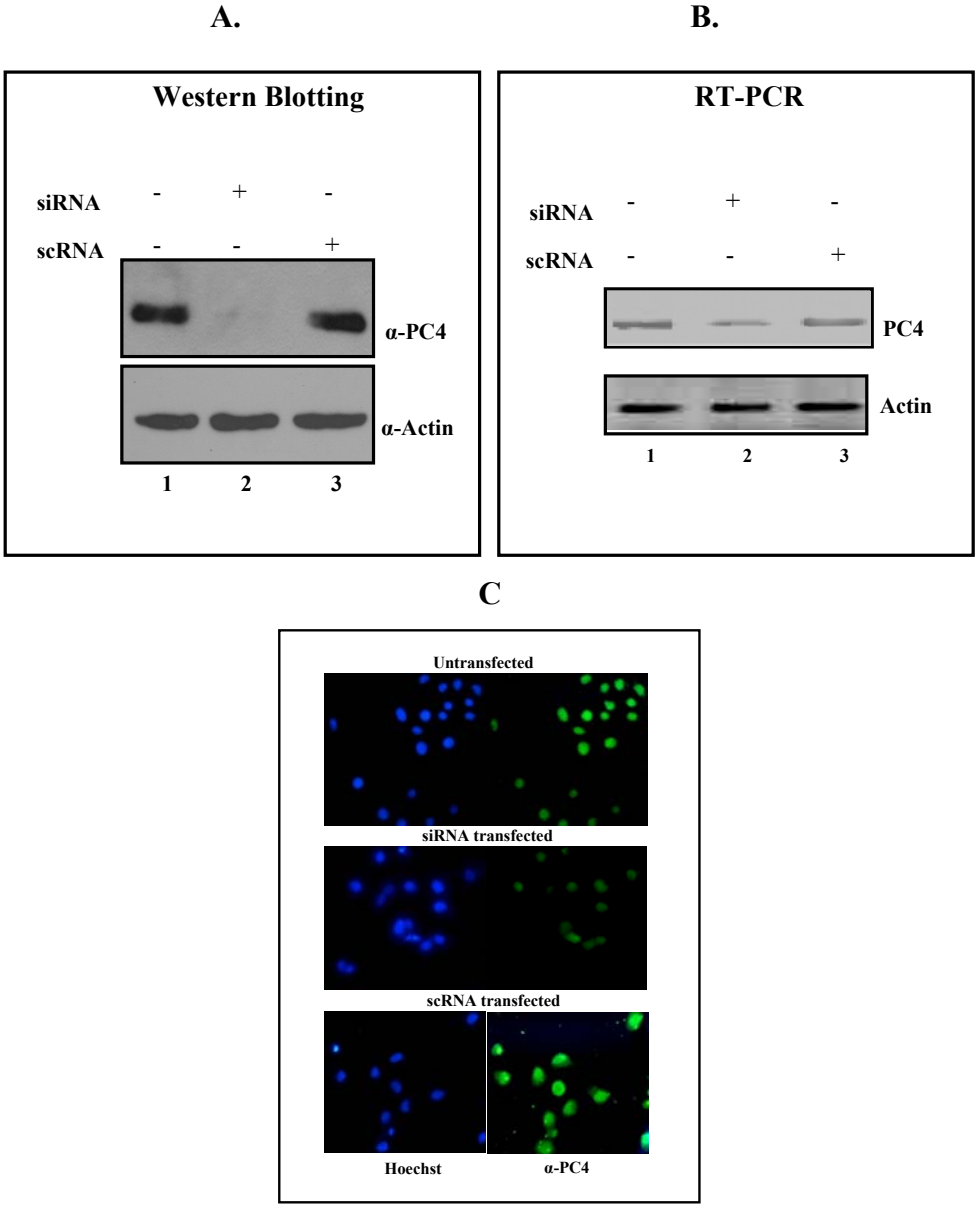


Figure 3.31: Knocking down of PC4 by designed synthetic siRNA. Silencing of PC4 expression by siRNA visualized by Western blotting (A), RT-PCR (B) and Immunofluorescence (C). In

Western blotting (A) and RT-PCR (B), lane 1-Untransfected, lane 2- siRNA transfected, lane 3-scRNA transfected. In imunofuorescence (C) α-PC4 and counterstain Hoechst was used.

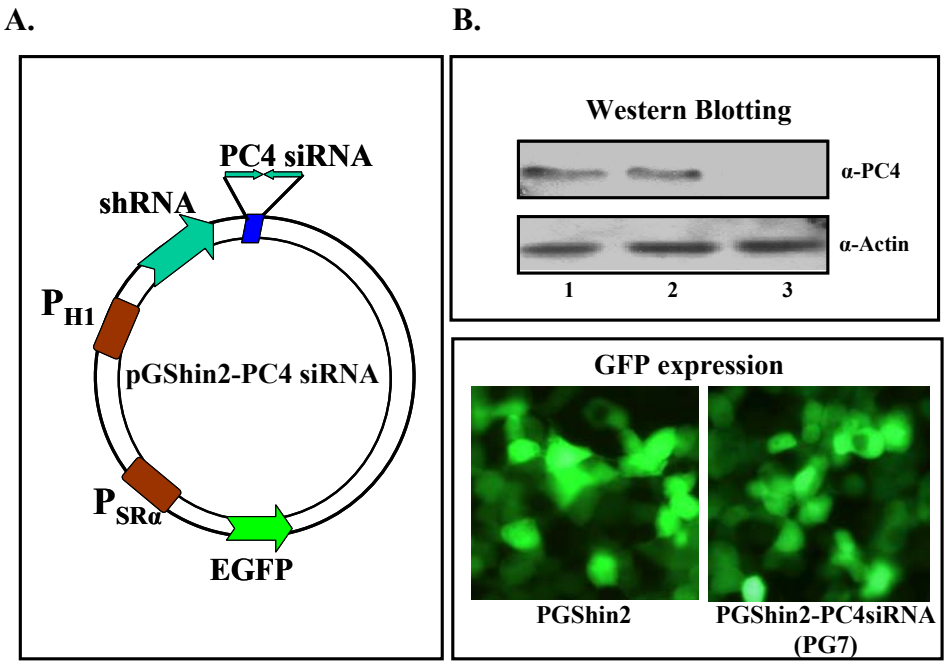


Figure 3.32: Silencing of PC4 gene expression by the vector-based system. (A) pGshin2 vector map having GFP in the expression cassette, where PC4 siRNA was cloned. (B) Western blotting and visualization of GFP expression after transfection of pGShin2 or PG7. Lysates prepared from untransfected (lane 1), pGShin2 transfected (lane 2) and PG7 transfected (lane 3) cells were subjected to western blotting analysis with α-PC4, where α-Actin was used as loading control.

In order to visualize the alteration of chromatin architecture upon silencing PC4 gene expression, shRNA vector mediated silencing of the PC4 gene expression was done. Subsequently Hoechst staining, followed by confocal microscopic imaging (Figure 3.33) of the control (vector transfected) and knock down of PC4 (PG7 transfected) showed differential density of compaction of chromatin DNA (Figure 3.33 compare panel A vs B). The PC4 knock down cells lost most of the densely packed chromatin (Figure 3.33 panel B). Further we also observed a significant reduction in the number of metaphase plates upon silencing PC4 gene expression in comparison to the control.

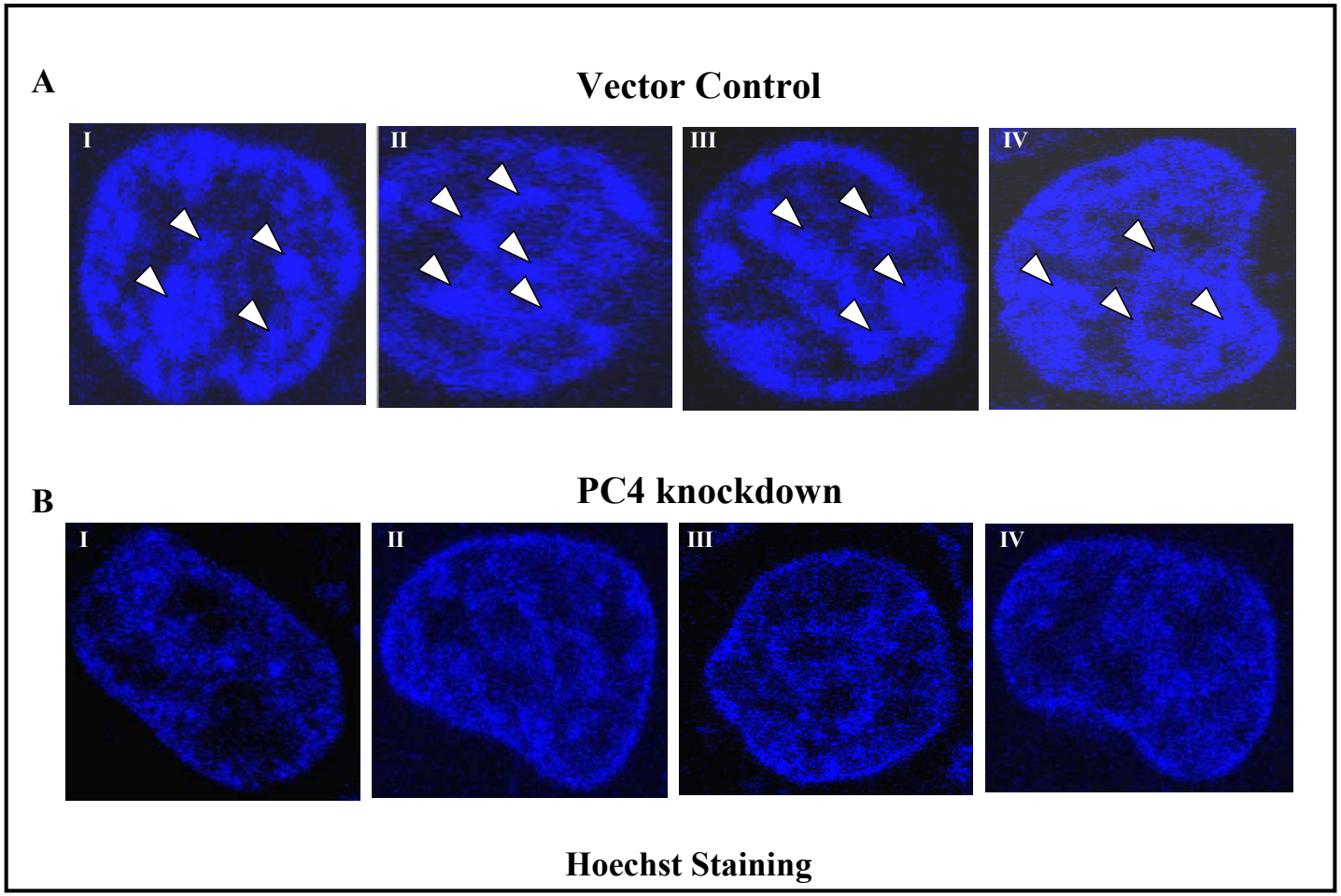


Figure 3.33- Alterations in the nuclear architecture upon knocking down PC4: Hoechst staining images of vector control (panel A) and PC4 knockdown (panel B) HeLa cells.

In order to validate the chromatin condensation by PC4 *in vivo*, PC4 expression was also 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 (scRNA). We investigated the effect of PC4 repression on the global chromatin folding in human cells by the MNase accessibility assay (Figure 3.34). The equal amount of chromatin used in the experiment was confirmed by western blotting using antibodies against different core histones and histone H1 (Figure 3.34 C). 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 (Figure 3.34 A).

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 (Figure 3.34 B).

B) Alterations in the levels of epigenetic markers upon silencing PC4:

Interestingly, we observe a distinct change in the levels of epigenetic markers upon PC4 knockdown. There is an increase in H3K9, 14 acetylation, H3K4 methylation levels, whereas there is a drop in H3K9 methylation levels as visualized by western blotting analysis (Figure 3.35) and immunofluorescence (Figure 3.36), which indicate a more open chromatin structure upon PC4 knockdown. For these experiments the vector based system, harboring PC4 siRNA was used. The GFP positive cells were analyzed by immunofluorescence analysis, for assessing the levels of different active and repressed chromatin marks in comparison to the vector transfected cells. It was found that upon knocking down PC4 the levels of active marks H3K9,14 acetylation and H3K4 methylation are enhanced (Figure 3.35, panel I, II and Figure 3.36, panel I, II), while the H3K9 methylation levels are repressed (Figure 3.35, panel III and Figure 3.36, panel III), indicating a more open and transcriptionally amenable chromatin architecture.

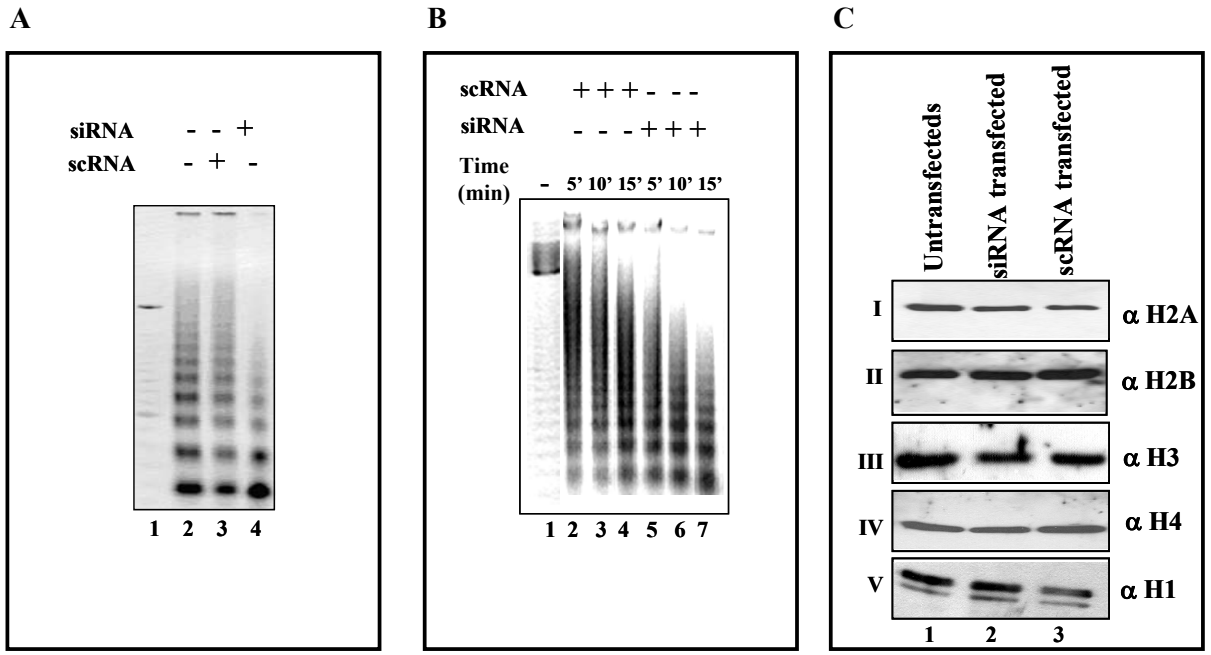


Figure 3.34- MNase accessibility of chromatin isolated after knocking down PC4: (A) After knocking down of PC4, chromatin was isolated from untransfected (lane, 2), scRNA transfected (lane, 3) and siRNA transfected (lane, 4) HeLa cells and were subjected to partial MNase digestion and analyzed on a 1% agarose gel. (B) 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. (C) In order to ensure the equal amount of chromatin taken for the MNase assays profile of core and linker histones were analysis.

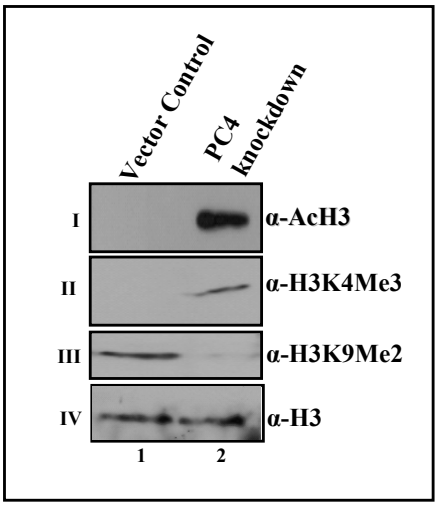


Figure 3.35- Alteration of the levels of epigenetic marker upon silencing PC4 as visualized by western blotting analysis: Increase in the levels of Ac-H3 (panel I), H3K4Me3 (panel II) and decrease in the level H3K9Me2 (panel III) was observed upon silencing PC4 gene expression by western blotting analysis.

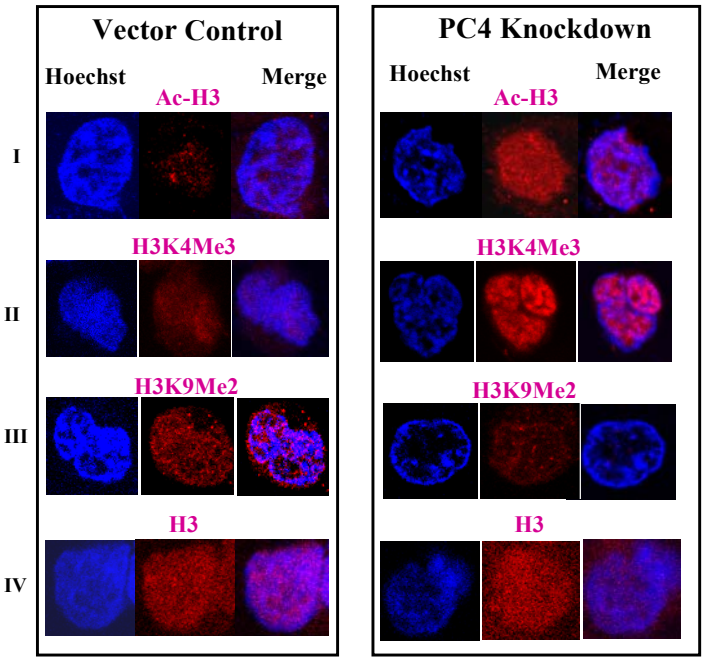


Figure 3.36- Alteration of the levels of epigenetic marker upon silencing PC4 as visualized by immunofluorescence: Similar increase in Ac-H3 (panel I), H3K4Me3 (panel II) levels and decrease in H3K9Me2 (panel III) level was confirmed by immunofluorescence upon silencing PC4.

3.4.2 Global gene expression upon PC4 knockdown

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 (Figure 3.37 A). The extensive table with all of the differentially expressed genes grouped into functional groups is represented in Table 3.4. The control experiment was carried out with the untransfected HeLa cells and scrambled RNA transfected HeLa cells did not show any differential regulation. 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 (Figure 3.37 B). On the other hand S100A11 gene expressionwas reduced upon PC4 siRNA transfection, as compared to the scRNA transfected cells (Figure 3.37 C). 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.

Out of 128 up-regulated genes 19 genes code for transcription factors from diverse families- like TAF4B is involved in initiation of gene transcription by RNA pol II, Pax8 plays an important role in foetal development and cancer, CDC14B dephosphorylates tumor suppressor protein p53, and regulates the function of p53, UBE3A is a negative regulator of p53 and TSC2 is a tumor suppressor. 16 of the identified genes code for signal transduction pathways like G-protein signaling (SNX4), WNT gene family

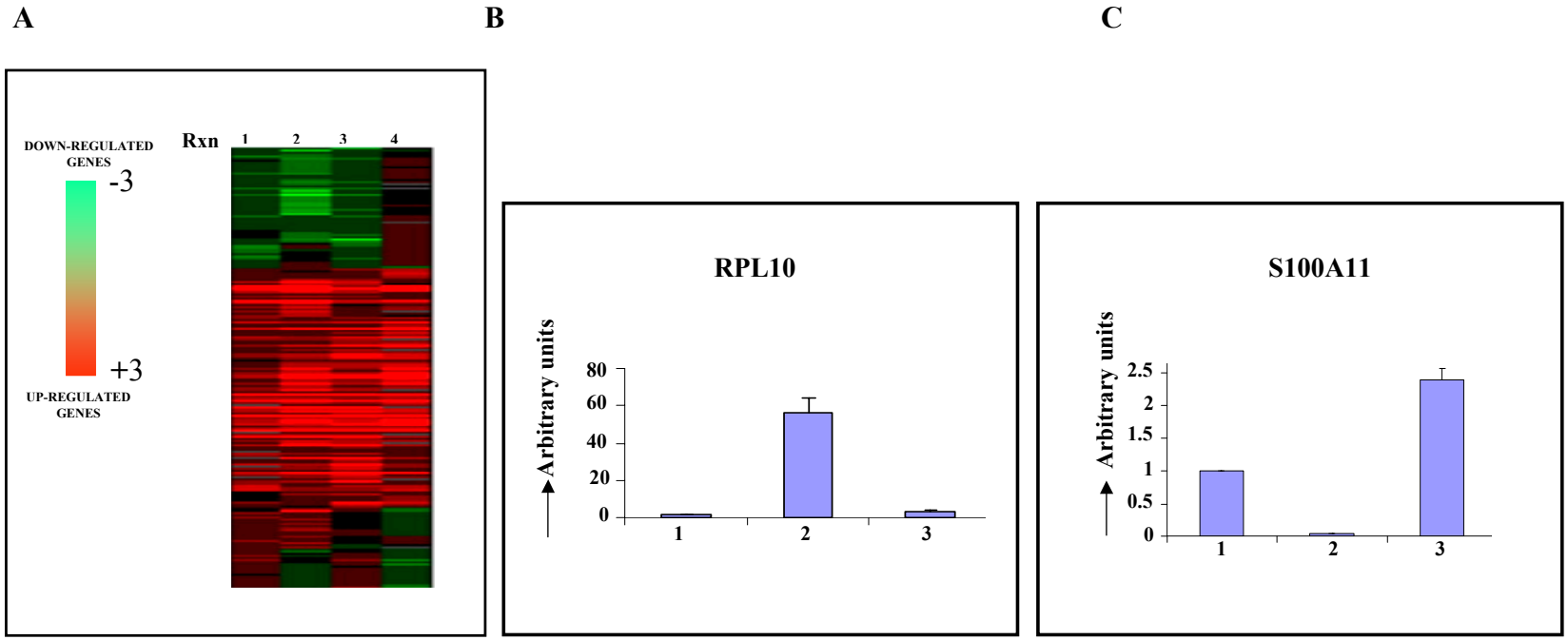


Figure 3.37- (A) Microarray analysis of gene expression upon PC4 knock- down by siRNA: Microarray analysis of gene expression upon PC4 knock- down by siRNA. Hierarchical clustering of the gene expression profiling data obtained by cDNA microarray analysis of siRNA-mediated PC4 knock down HeLa cells. Lanes 1-3, forward reaction and lane 4, dye swap. **(B, C) Validating the microarray result by real time PCR analysis:** 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.

(WNT5B), MAK3K7IP1 regulating MAK3K mediator of signaling pathways induced by TGF beta, interleukin-1 and WNT 1. There are 6 genes associated with protein biogenesis like RPS28, RPL32, RPL12, and RPL10. In addition, STK4 and SAFB are the two chromatin- associated proteins. STK4 is involved in attaching the base of chromatin loops to the nuclear matrix to serve as a molecular base to assemble a 'transcriptosome complex' in the vicinity of actively transcribed genes. SAFB on the other hand can phosphorylate histone H2B that has been correlated with apoptosis, and induces the chromatin condensation.

A very small subset of genes showed down regulation. Of the 49 down regulated genes that we identified only 19 are well annotated. Interestingly, a down- regulated gene SMUG1 contributes to base excision repair (DNA repair). 5 genes are involved in the energy metabolism pathways like GP1, PKM2 (pyruvate kinase), NADSYN (NAD synthetase), DIA1 (cytochrome 5-reductase) and CDC10. 4 genes having functional association with neuronal development like CNTN2 (axon connection in the development of nervous system) are also down regulated. Genes differentially expressed in the PC4 knock down experiment has been summarized in Tables 3.4 and 3.5.

Table 3.4:

Up-Regulated genes

1. Transcription Regulators:

Symbol	Function
PAX8	Critical roles during fetal development and cancer growth. May involve kidney cell differentiation, thyroid development, or thyroid dysgenesis.
VIK	This gene encodes a zinc finger protein. Multiple alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.
WT1	This gene encodes a transcription factor that functions in kidney and gonad proliferation and differentiation. Mutations in this gene can be associated with the development of Wilms tumors in the kidney or with abnormalities of the genitourinary tract.
MBNL2	This gene encodes a C3H-type zinc finger protein, which is similar to the Drosophila melanogaster muscleblind B protein required for photoreceptor differentiation.
TAF4B	TATA-binding protein associated factors (TAFs) participate, which is involved in the initiation of gene transcription by RNA polymerase
MEF2C	RNA polymerase II transcription factor activity - muscle development - neurogenesis -
ZNF219	Nucleus - regulation of transcription, DNA-dependent - transcription factor activity - zinc ion binding
MEF2C	RNA polymerase II transcription factor activity - muscle development - neurogenesis -

Symbol	Function
	nucleus - regulation of transcription,
CIC	DNA binding - regulation of transcription
CHURC1	Development - positive regulation of transcription - transcriptional activator activity - zinc ion binding
ZFX	DNA binding – DNA binding - metal ion binding - nucleus - regulation of transcription - regulation of transcription, DNA-dependent - transcription coactivator activity - transcription regulator activity - zinc ion binding
SETBP1	DNA binding - nucleus - regulation of transcription, DNA-dependent
DDX54	A member of the DEAD box protein family interacts in a hormone-dependent manner with nuclear receptors and represses their transcriptional activity.
SUI1	Regulation of translation - regulation of translational initiation - response to stress – has translation initiation factor activity
SETBP1	DNA binding - nucleus - regulation of transcription, DNA-dependent
PPP1CA	A catalytic subunit of protein phosphatase 1 (PP1). PP1 is an important regulator of cardiac function. Mouse studies also suggest that PP1 functions as a suppressor of learning and memory.
PTRF	rRNA binding –nuclear protein involved in transcription termination

2. Signal Transduction pathways:

Symbol	Function
SNX14	A member of the sorting nexin family whch contains a phox (PX) domain involved in intracellular trafficking. The protein also contains a regulator of G protein signaling (RGS) domain.
KREMEN1	A component of a membrane complex that modulates canonical WNT signaling through lipoprotein receptor-related protein 6 (LRP6). It contains extracellular kringle, WSC, and CUB domains.
WNT5B	A member of the WNT gene family consisting of structurally related genes, which encode secreted signaling proteins. These proteins have been implicated in oncogenesis and in several developmental processes,
MAP3K7IP1	A regulator of the MAP kinase kinase kinase MAP3K7/TAK1, which is known to mediate various intracellular signaling pathways, such as those induced by TGF beta, interleukin 1, and WNT-1.
PTPNS1	A member of the signal-regulatory-protein (SIRP) family, and also belongs to the immunoglobulin superfamily. Participates in signal transduction mediated by various growth factor receptors.
DSG2	Desmoglein 2 is a calcium-binding transmembrane glycoprotein component of desmosomes in vertebrate epithelial cells.
NR2F2	Ligand-regulated transcription factor activity - lipid metabolism - nucleus - regulation of transcription from Pol II promoter - signal transduction - steroid hormone receptor activity -
PL6	guanyl-nucleotide exchange factor activity - integral to membrane - signal transduction
PTN	Cell proliferation - cytokine activity - extracellular space - growth factor activity - heparin binding - neurogenesis - positive regulation of cell proliferation -
PLA2G4B	arachidonic acid metabolism - calcium ion binding - calcium-dependent phospholipase A2 activity -

Symbol	Function
STK3	ATP binding - ATP binding - apoptosis - cytoplasm - magnesium ion binding - nucleus - positive regulation of apoptosis -
LIMS1	Involved in integrin signaling through its LIM domain-mediated interaction.
DISP2	One of two human homologs of Drosophila dispatched.
NRP1	A membrane-bound coreceptor to a tyrosine kinase receptor for both vascular endothelial growth factor and semaphorin;
MAPK4	A member of the mitogen-activated protein kinase family phosphorylates nuclear targets.
CALB1	Calbindin is a calcium-binding protein The neurons in brains of patients with Huntington disease are calbindin-depleted.

3. Chromatin Associated:

Symbol	Function
STK4	Similar to the yeast Ste20p kinase, which acts upstream of the stress-induced mitogen-activated protein kinase cascade. Also induces the chromatin condensation
SAFB	A DNA-binding protein with high specificity for scaffold or matrix attachment region DNA elements (S/MAR DNA). Involved in the regulation of the HSP 27 transcription and also can act as an estrogen receptor corepressor. And is a candidate for breast tumorigenesis.

4. Protein Biogenesis:

Symbol	Function
RPL32	The protein belongs to the L32E family of ribosomal proteins.
RPL12	The protein belongs to the L11P family of ribosomal proteins.
RPL10	The protein belongs to the L10E family of ribosomal proteins.
RPS28	The protein belongs to the S28E family of ribosomal proteins.
RBX1	An evolutionarily conserved protein that interacts with cullins. And plays a unique role in the ubiquitination reaction to catalyze ubiquitin polymerization.
SF3B14	A 14 kDa protein subunit of the splicing factor 3b complex.

5. Ras, p53 and tumor associated:

Symbol	Function
CDC14B	A member of the dual specificity protein tyrosine phosphatase family shown to interact with and dephosphorylates tumor suppressor protein p53
RAPGEF1	A human guanine nucleotide releasing protein for Ras protein. They mediate binding events that control the activity and localization of many proteins involved in the transmission of signals from the cell surface to the nucleus.
RASSF2	A protein that contains a Ras association domain.. The specific function of this gene has not yet been determined..
UBE3A	An E3 ubiquitin-protein ligase, is maternally expressed in brain and biallelically expressed in other tissues. Maternally inherited deletion of this gene causes Angelman Syndrome,
TSC2	Mutations in this gene lead to tuberous sclerosis complex. Its gene product is believed to be a tumor suppressor and is able to stimulate specific GTPases..

6. Disease associated:

Symbol	Function
COL5A1	An alpha chain for one of the low abundance fibrillar collagens. Mutations in this gene are associated with Ehlers-Danlos syndrome, types I and II.

Symbol	Function
FSTL1	A protein with similarity to follistatin, an activin-binding protein and is thought to be an autoantigen associated with rheumatoid arthritis.
FLII	A protein with a gelsolin-like actin binding domain and an N-terminal leucine-rich repeat-protein protein interaction domain and gene is located within the Smith-Magenis syndrome region on chromosome 17.
AMPH	A protein associated with the cytoplasmic surface of synaptic vesicles. patients with stiff-man syndrome who were also affected by breast cancer are positive for autoantibodies against this protein..
KRT10	A member of the type I (acidic) cytokeratin family,. Mutations in this gene are associated with epidermolytic hyperkeratosis.
MSX1	Also called HOX7, this gene is deleted in patients with Wolf-Hirschhorn syndrome. This is a candidate gene for human cleft palate.
ACAA1	Acetyl-Coenzyme A acyltransferase (ACAA1) operates in the beta-oxidation system of the peroxisomes. Deficiency of this enzyme leads to pseudo-Zellweger syndrome

Table 3.5:

Down-Regulated genes

1. Energy pathways:

Symbol	Function
DIA1	Two forms of NADH-cytochrome b5 reductase are generated from tissue-specific alternative transcripts differing in the first exon. Deficiency of DIA1 has been associated with methemoglobinemias.
CDC10	a protein that is highly homologous to the CDC10 protein of S. cerevisiae.and is predicted to play a similar role to that of its yeast counterpart.
GPI	A multifunctional phosphoglucose isomerase involved in glycolysis in the cytoplasm and as a neurotrophic factor for spinal and sensory neurons. Outside the cell it functions.
PKM2	This protein with pyruvate kinase activity interacts with thyroid hormone, and thus may mediate cellular metabolic effects induced by thyroid hormones.
NADSYN1	NAD synthetase (EC 6.3.5.1) catalyzes the final step in the biosynthesis of NAD from nicotinic acid adenine dinucleotide (NaAD)

2. Cell Growth and differentiation:

Symbol	Function
TSPAN-2	A member of the transmembrane 4 superfamily, also known as the tetraspanin family. Mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility.
S100A11	a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. This protein may function in motility, invasion, and tubulin polymerization. Altered expression of this gene has been implicated in tumor metastasis.

3. Nervous system associated:

Symbol	Function
CNTN2	A glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that may play a role in the formation of axon connections in the developing nervous system. It may also be involved in glial tumorigenesis

Symbol	Function
MGST1	Catalyzes the conjugation of glutathione to electrophiles and the reduction of lipid hydroperoxides. Localized to the endoplasmic reticulum and outer mitochondrial membrane where it is thought to protect these membranes from oxidative stress.
RHO	Gene encoding the rod photoreceptor-specific protein rhodopsin. transmembrane protein which, when photoexcited, initiates the visual transduction cascade.
HTR6	G-protein signaling, coupled to cyclic nucleotide second messenger|histamine receptor activity|integral to plasma membrane|rhodopsin-like receptor activity|synaptic transmission

4.DNA repair:

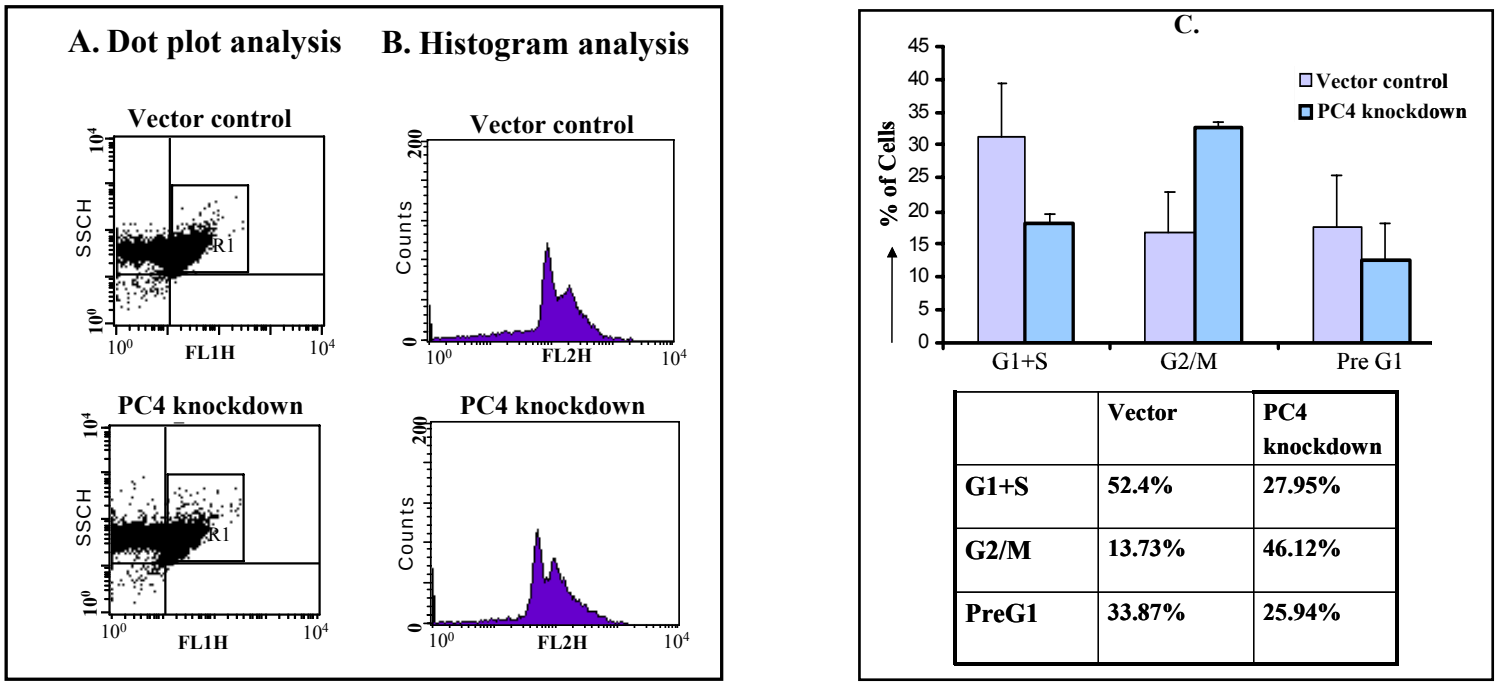
Symbol	Function
SMUG1	uracil glycosylase contributing to base excision repair

(Marked in italics are the genes that have been validated. Marked in bold are important cell cycle regulatory genes)

3.4.3 Effect of PC4 silencing on cell cycle

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. The vector-based shRNA knocking down system was used to probe into the role of PC4 in cell cycle.

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 (Figure 3.38 A). 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 (Figure 3.38 B). 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 (Figure 3.38 C), 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.



	Vector	PC4 knockdown
G1+S	52.4%	27.95%
G2/M	13.73%	46.12%
PreG1	33.87%	25.94%

Figure 3.38- Knocking down PC4 cause G2/M cell cycle checkpoint arrest: (A, B) 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 (A) and Histogram analysis (B) of pGShin2 and PG7 transfected cells are represented. (C) The difference in G1 + S, G2/M and PreG1 cell population in vector control and PC4 knockdown HeLa cells have been shown in a bar graph.

3.5 Mechanism of functional switch of PC4- addressing the role of posttranslational modifications:

Diverse activity of multifunctional protein is predominantly regulated by their posttranslational modifications. Significantly, PC4 gets phosphorylated and acetylated. Although functional consequences of phosphorylation have been enumerated partially, very little is known about acetylation. There are seven *in vivo* phosphorylation sites of PC4 located in the N-terminal Serine rich acidic domain (SEAC). Phosphorylation negatively regulates the coactivator function of PC4, its interactions with the activator and TATA box- binding protein / TFIIA (Ge et al, 1994). Phosphorylation also inhibits double stranded DNA binding but not the single stranded DNA binding (Kretzschmar et al, 1994). Recently it has been reported that PC4 can be acetylated specifically by p300 *in vitro* and phosphorylation inhibits the acetylation (Kumar et al, 2001). Acetylation significantly enhances the DNA binding ability of PC4. These observations lead us to investigate the role of posttranslational (acetylation and phosphorylation) modifications of PC4 in the histone interacting ability and thereby chromatin organization.

The *in vivo* acetylation status of PC4 has been investigated. For this purpose a mammalian expression construct of PC4 containing FLAG epitope tag was made. HeLa cells were transfected with the construct and the expression of FLAG epitope tagged PC4 was confirmed by western blotting using anti-FLAG antibodies. In order to find out whether PC4 is acetylated *in vivo*, the FLAG-PC4 protein was pulled down by M2-agarose beads and subjected to western blotting analysis. It was found that anti-acetylated Lysine antibody could efficiently detect the M2-agarose pulled PC4 in a western blotting analysis (Figure 3.39, lane 1). Addition of Histone Deacetylase inhibitors, Trichostatin A (TSA) and Sodium Butyrate (NaBu) in cell culture system further enhanced the acetylation of PC4 whereas the presence of p300 HAT activity specific inhibitor Curcumin drastically reduced the amount of acetylated PC4 (Figure 3.39, compare lanes 1 vs 2 and lanes 1 vs 3). These results confirm that indeed PC4 is a bonafide substrate of p300 HAT activity *in vivo*.

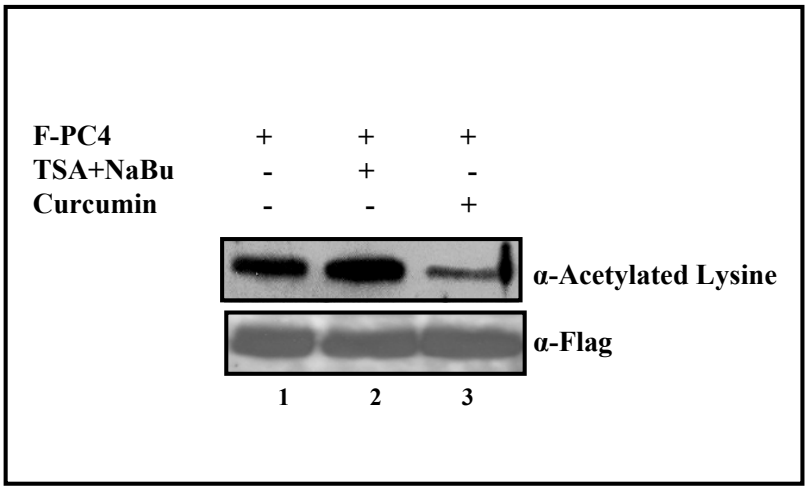


Figure 3.39- PC4 gets acetylated *in vivo*: HeLa cells were transfected with FLAG-PC4 construct and grown in presence of 100 nM TSA and 5 nM NaBu or 100 μM Curcumin. Using M2-agarose Flag-PC4 was pull down and western blotting was done using α-Acetylated Lysine or α-Flag antibody. Lane 1, cells transfected with Flag-PC4; lane 2, after transfection cells treated with TSA and NaBu; lane 3, after transfection cells treated with Curcumin.

3.5.1 Posttranslational modification of PC4 alters its dynamic interaction with chromatin

Posttranslational modifications regulate the functional dynamicity of PC4. Thus the role of posttranslational modifications of PC4 in histone interaction and chromatin compaction was an important issue to be addressed. Significantly, we have found that the acetylated PC4 does not interact with the histone H3 whereas the phosphorylated PC4 shows a strong interaction with the histone H3 (Figure 3.40 A, lanes 4 vs 5). The interaction affinity of phosphorylated PC4 seems to be stronger than the unmodified protein (Figure 3.40 A, lanes 3 vs 5). Mock phosphorylation or acetylation does not cause any alteration in histone interaction ability of PC4 (Figure 3.44 B, lanes 3 vs 4 vs 5).

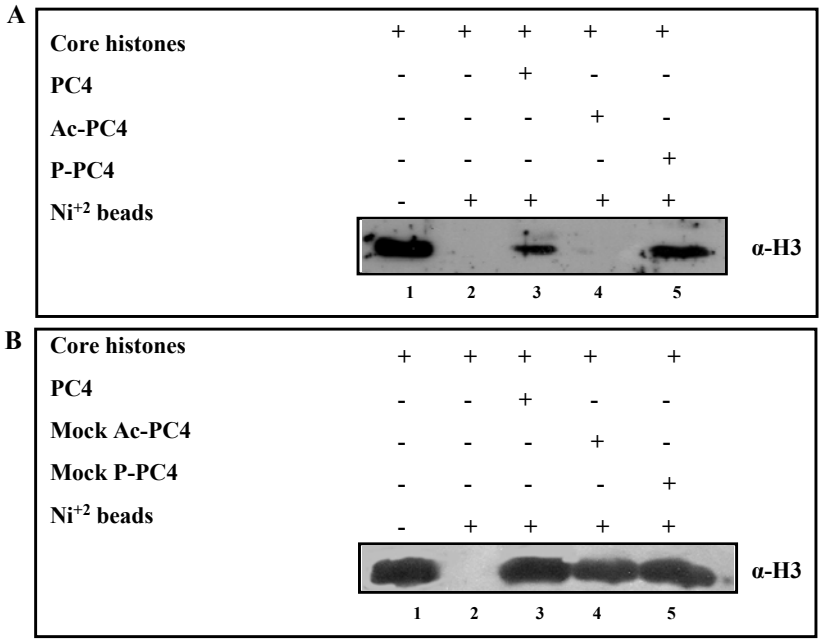


Figure 3.40- Phospho-PC4 shows stronger histone interacting ability as compared to Acetylated-PC4: (A) 1 µg of His_6-PC4 or *in vitro* acetylated or phosphorylated PC4 was incubated with HeLa core histones in presence of 150 mM NaCl and the complexes were pull down with Ni-NTA agarose and analysed by western blotting using α-H3 antibody. Lane 1, core histones; lane 2, core histones incubated with Ni-NTA agarose; lanes 3-5, core histones incubated with His6 PC4, acetylated His6 PC4 and phosphorylated His6 PC4 respectively. (B) Similar interactions of His_6-PC4 and mock acetylated or mock phosphorylated PC4 proteins and core histones were performed. Lane 1, core histones; lane 2, core histones incubated with Ni-NTA agarose; lanes 3-5, core histones incubated with His6 PC4, mock acetylated His6 PC4 and mock phosphorylated His6 PC4 respectively.

Interaction with the core histones was found to be essential for the PC4-mediated compaction (globule formation) of chromatin. In agreement with the histone interaction data, it was found that unmodified PC4 and phosphorylated PC4 could condense the chromatin very efficiently but the acetylated PC4 did not show any chromatin condensing ability (Figure 3.41 A, C and Figure 3.42 A, C). However PC4 subjected to mock (without PC4) acetylation or phosphorylation reactions could not alter the mean residual ellipticity (θ_{270}) values (Figure 3.41 B and Figure 3.42 B).

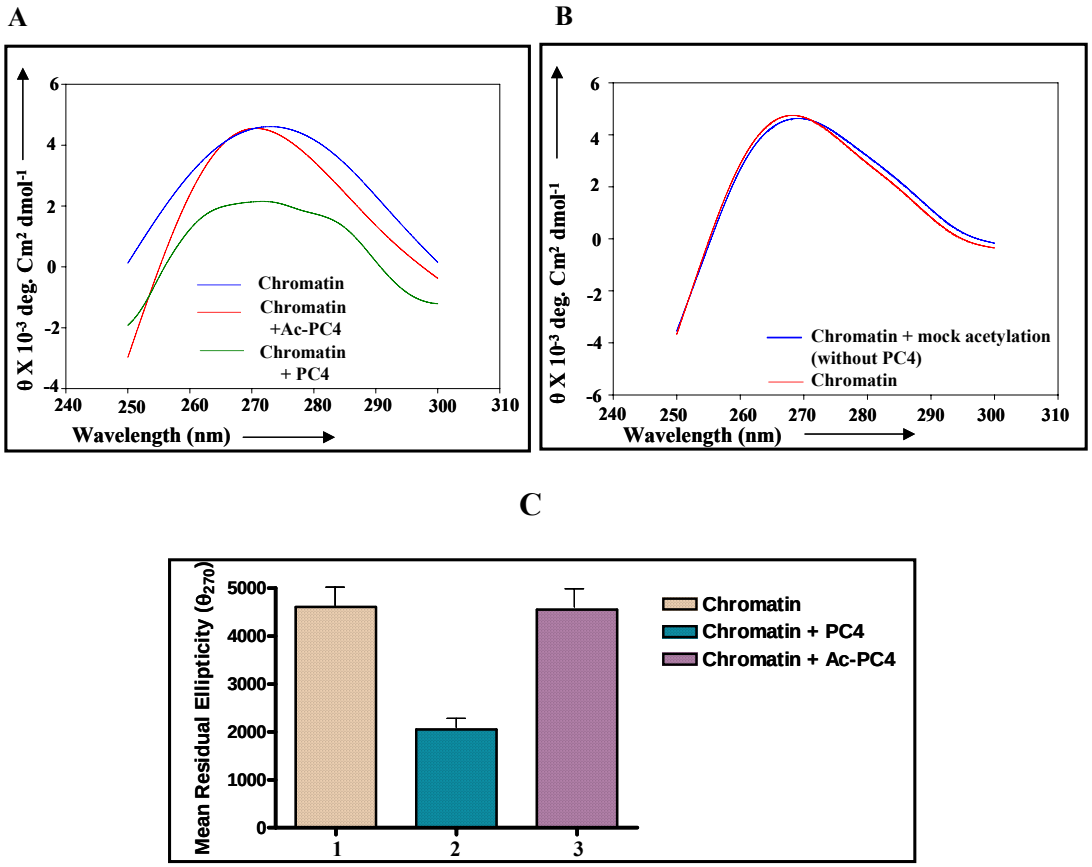


Figure 3.41- Acetylated PC4 does not induce chromatin compaction as compared to native PC4: *In vitro* acetylated PC4 was incubated with H1-stripped chromatin and circular dichroic spectrum was recorded as before with proper buffer controls. (A) CD spectra of chromatin incubated with PC4 or Ac-PC4. (B) Chromatin incubated with buffer used for the acetylation reaction. (C) The mean residue ellipticity was plotted for the corresponding spectra in

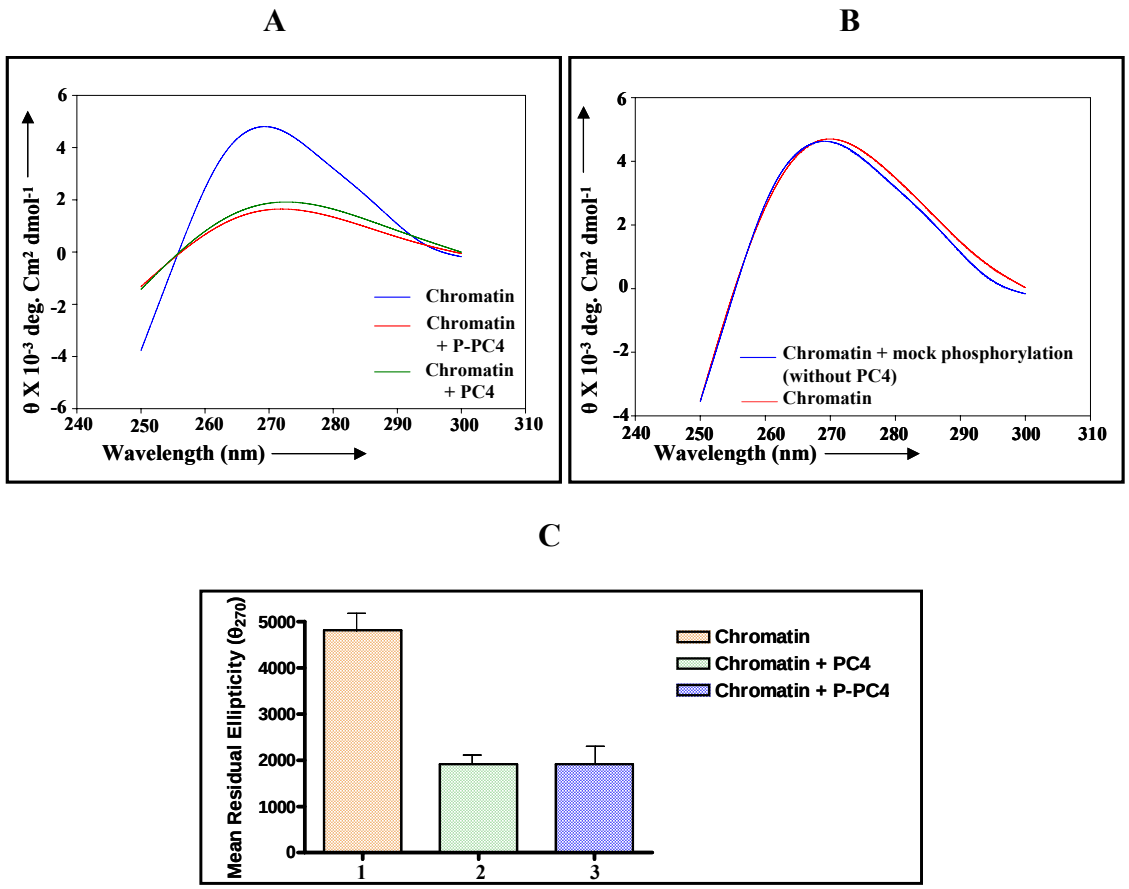


Figure 3.42- Phospho-PC4 can induce chromatin condensation alike unmodified PC4: *In vitro* phosphorylated PC4 was incubated with H1-stripped chromatin and circular dichroic spectrum was recorded as before with proper buffer controls. (A) CD spectra of chromatin incubated with PC4 or P-PC4. (B) Chromatin incubated with buffer used for the phosphorylation reaction. (C) The mean residue ellipticity was plotted for the corresponding spectra in A.

However the difference in the chromatin condensing abilities of PC4 and P-PC4 were not very distinct at a 60 mins time point of incubation. Assuming that 60 mins incubation lead to a complete saturation of chromatin condensing functions of PC4 and P-PC4, the alteration in the molar ellipticity values of these two proteins was studied in a time. dependent manner. Although 15 mins of incubation could not cause a significant alteration of the mean residue ellipticity (θ_{270}) values of PC4 and P-PC4 (Figure 3.43 A, B), 30 mins of incubation showed that P-PC4 was a better chromatin condenser as compared to unmodified PC4 (Figure 3.43 C, D).

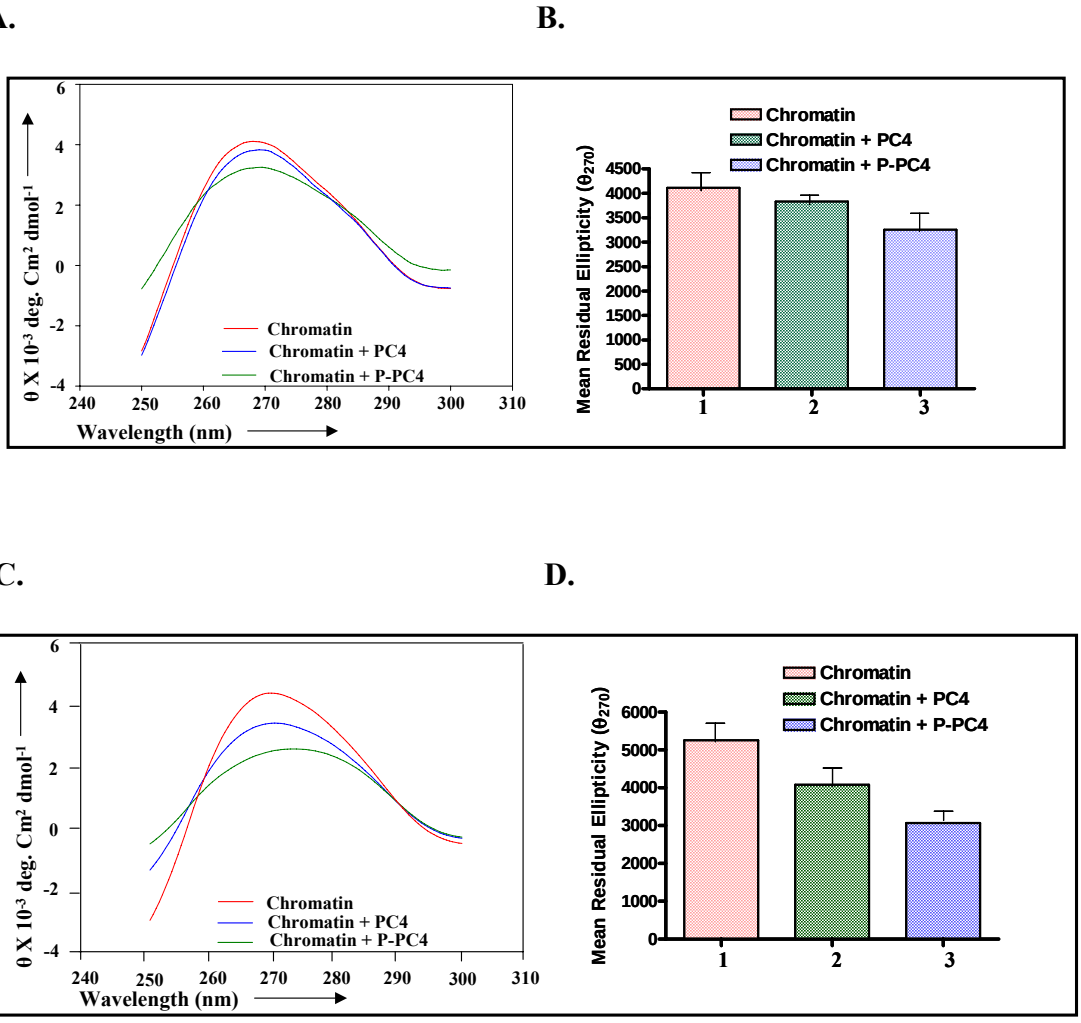


Figure 3.43- Phospho-PC4 is a better chromatin condenser as compared to unmodified PC4: Comparative analysis of PC4 and P-PC4 induced chromatin compaction was compared at 15 mins (A and B) and 30 mins (C and D) time point of incubation. While 15 mins time point of incubation did not show a significant alteration of mean residue ellipticity values of PC4 as

compared to P-PC4 (compare panel B, bar 2 vs 3), 30 mins time point of incubation could show a prominent alteration (compare panel D, bar 2 vs 3).

The alteration in the mean residue ellipticity value as a function of time has been plotted in Figure 4.9 (Discussion). Thus the circular dichroic spectral analysis of PC4- chromatin complexes suggests that posttranslational modifications of PC4 induce its chromatin compaction function.

3.5.2 Interaction of PC4 with hyperacetylated histones and chromatin compaction studies with hyperacetylated chromatin substrate

Acetylation of histone is the epigenetic mark for an active chromatin. Hyper acetylation of histones provides the cellular signal of an open chromatin structure where the assembly of the transcription machinery takes place. PC4 has been shown to compact chromatin depending upon its histone interaction ability. This indicated that its interaction should be weaker with hyperacetylated chromatin substrate, which cannot be compacted into a higher order chromatin structure. It was also essential to study its ability to interact with hyperacetylated chromatin in order to further establish the fact that the mechanism of chromatin condensation is through the histone interaction ability of PC4.
Although PC4 interacts with histones quite efficiently, it does not preferentially interact with hyperacetylated histones (histones isolated after treatment with HDAC inhibitors 100 nM TSA and 1 mM NaBu). *In vitro* histone interaction studies were performed with PC4-GST and equivalent amount of core histones or hyperacetylated core histones. It was found that in comparison to core histones, interaction with hyperacetylated core histones is much weaker, indicating hyperacetylated histones is not the preferred docking site of PC4 (Figure 3.44). This necessitated the investigation of condensing function of PC4 with hyperacetylated chromatin substrate. Hyperacetylated chromatin was isolated after similar treatment with HDAC inhibitors (100 nM TSA and 1 mM NaBu). When such a hyperacetylated chromatin was taken as the substrate PC4 could not induce chromatin compaction (Figure 3.45). This indicates that PC4 mediated chromatin compaction occurs to a lesser extent in active chromatin.

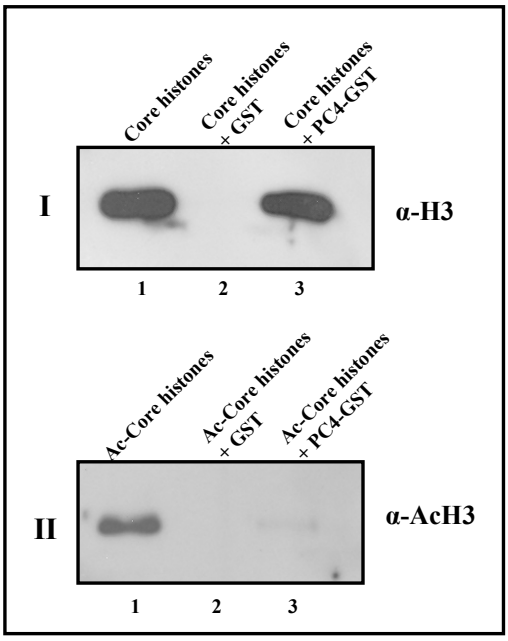


Figure 3.44- Interaction of PC4 with acetylated histones: PC4-GST is subjected to interaction with Acetylated core histones (panel II) in comparison to only core histones (panel I). Lane 1- core histones (panel I) or acetylated core histone (panel II) input, lanes 2, 3- interactions of core histones (panel I) or acetylated core histones (panel II) with GST or PC4-GST.

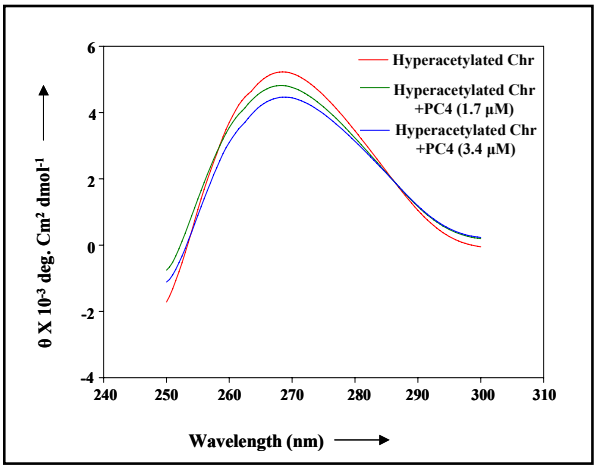


Figure 3.45- Chromatin compaction studies of PC4 with hyperacetylated chromatin: TSA and NaBu treated hyperacetylated chromatin was subjected to Circular Dichroic spectroscopic studies with increasing concentration of PC4. Hyperacetylated chromatin template is not a preferred for PC4-mediated chromatin compaction.

3.5.3 Effect of PC4 modifications in Transcription

Human transcriptional coactivator PC4 is known to stimulate the activator-dependent transcription. However the current work indicates a completely different functional attribute of this transcriptional coactivator. Transcriptional activators/coactivators have been earlier reported to cause chromatin decompaction (like c-Myc, HMGB1-see introduction). However in the present study chromatin compaction function of PC4 has been brought into focus. Since posttranslational modification regulates PC4 function, it was intriguing to address its role in this context. Earlier results have underscored the role of PC4 phosphorylation showing that it is inhibitory to its coactivator function (Ge et al, 1994). In the present study the role of phosphorylation has been elucidated as a positive signal that activates chromatin-organizing function of PC4. However it is seen that acetylation does not favour this process. Thus in order to address the functional consequence of PC4-acetylation we have explored its role in transcription.

In vitro DNA transcription assay was carried out with PC4-depleted HeLa nuclear extract (Figure 2.15, Materials and Methods), which was used as the source of GTFs. PC4. Acetylated-PC4 (Ac-PC4) or Phosphorylated-PC4 (P-PC4) was supplemented in the transcription reaction. As already reported Phosphorylation of PC4 was found to inhibit transcription (Ge et al, 1994). Interestingly, acetylation was found to activate the transcription similar to unmodified PC4 as represented in Figure 3.46.

These results establish that posttranslational modification of PC4 act as a functional switch between transcriptional coactivator and chromatin condensing protein.

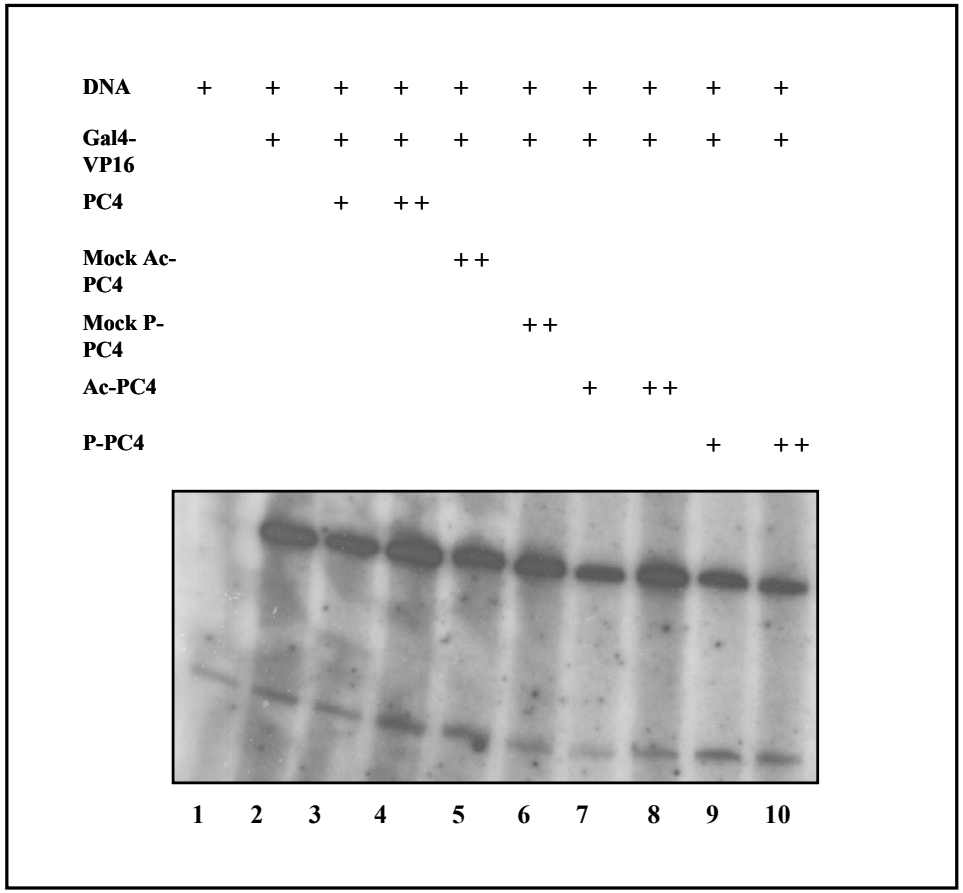


Figure 3.46- **Acetylated PC4 activates transcription unlike Phosphorylated PC4:** Lane1, without Gal4-VP16; Lane 2, Gal4-VP16 dependent transcription; Lane 3, 4-Gal4-VP16 dependent transcription in presence of increasing concentration of PC4; Lane 5, 6- Addition of mock acetylated and mock phosphorylated PC4 in Gal4-VP16 dependent transcription; Lanes 7, 8 and lanes 9, 10- Increasing concentration of Acetylated PC4 and Phosphorylated-PC4 in Gal4-VP16 dependent transcription respectively.

3.6 Summary of the results

Human transcriptional coactivator PC4 is a multifunctional, highly abundant nuclear protein. The present study investigates whether PC4 is a bona fide nonhistone component of chromatin. PC4 is a present associated to the chromatin throughout the different stages of cell cycle with a reasonably moderate strength of interactions. The punctate distribution of PC4 throughout the metaphase chromosome arms suggests its involvement in chromatin organization. In agreement with the speculation, we have observed that PC4 is a chromatin condensing protein, and the histone interaction ability of PC4 is essential to induce chromatin compaction. However, the mechanism of PC4-mediated chromatin compaction is distinct from that of linker histone H1. While histone H1 can form ordered fibre structure, PC4 induce the formation of globular structure as visualized by AFM. Interestingly, the linker histone H1 can assist PC4-mediated globular structure formation, but PC4 does not favour the H1 mediated chromatin fibre. The physiological significance of PC4 induced chromatin compaction is reflected from the siRNA-mediated and vector-based knockdown studies. Silencing PC4 leads to the loss of compact chromatin organization as visualized by MNase accessibility assays and Hoechst staining followed by confocal microscopic imaging of the nuclei lacking PC4. There is also an alteration of the epigenetic markers profile towards a more open chromatin conformation upon knocking down of PC4. In agreement with these results silencing of PC4 leads to an upregulation of global gene expression and also a G2/M cell cycle checkpoint arrest. The mechanism of functional dynamics of PC4 from a transcriptional coactivator to a chromatin condensing protein is achieved through its posttranslational modification status. Phosphorylation of PC4 enhances its histone interaction and consequent chromatin condensing ability. Acetylated PC4, on the other hand, could not interact with histones and condense the chromatin, but could activate Gal4-VP16-dependent transcription. Thus posttranslational modification dependent distinct functional switch converts a transcriptional coactivator to a chromatin condensing protein. The global significance of chromatin-association of PC4 has been discussed in the next chapter.

DISCUSSION

Multifunctional, highly abundant, nuclear proteins are often associated with chromatin having distinct functional consequences (Bustin and Reeves, 1996; Kraus and Lis, 2003; Maison and Almouzni, 2004). 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 (Pan et al, 1996), repair (Wang et al, 2004) and the proper termination of multiple rounds of Pol-III transcription (Wang and Roeder, 1998). 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 also caused a G2/M checkpoint arrest indicating its function in cell cycle progression. In an attempt to visualize this functional dynamicity of PC4 we find that post-translational modification of PC4 acts as a functional switch from a

transcriptional coactivator to a chromatin condensing protein. Interestingly, we observe that Phospho-PC4 which shows an ability to interact with the core histones can compact the chromatin structure, where as acetylated- PC4 does not do so. The acetylated PC4 can show enhanced Gal4-VP16 dependent transcriptional activation, which probably explains how the functional dynamics of PC4 is achieved from a transcriptional coactivator to a chromatin condensing protein.

4.1 Association of PC4 with the chromatin

PC4 was distributed throughout the different stages of cell cycle with slightly varying amounts (as visualized biochemically and through immunofluorescence analysis) (Figure 3.3, Results section). It showed a predominant nuclear distribution (Figure 3.2, Results section). Furthermore, PC4 was associated with the oligo-nucleosomal fractions of sucrose gradient, and it was completely absent in any other fractions where nucleosome could not be detected (Figure 3.1, Results section). In contrast to PC4, HMGB1, another multi-functional chromatin associated protein was found to be present in a broad range of fractions irrespective of the presence of the nucleosomal DNA as reported earlier (Falciola *et al*, 1997). The stable association of PC4 to the chromatin was further confirmed by the fact that on treatment with a weak detergent digitonin, which can permeabilize the cell- membrane, but not the nuclear- membrane (Diaz and Stahl, 1989) PC4 remained associated to the chromatin (Figure 3.4, Results section). Furthermore, on treatment with NP40, a stronger detergent capable of permeabilizing all the cellular membranes, substantial amount of PC4 was still found to be associated with the chromatin. In case of HMGB1, though digitonin treatment could release the protein from chromatin to a lesser extent, exposure to NP40 lead to the dissociation of more than 70% HMGB1 (Falciola *et al*, 1997). 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 core histone H3 or linker histone H1. The treatment of NP40 is almost ineffective to mobilize core histone H3 or linker histone H1 from

the chromatin (Figure 3.4, Results section). Histone H1 has higher relative affinity to chromatin as compared to HMGB1 (~20 times more) (Ura *et al*, 1996) or PC4. The mechanism of high affinity association of PC4 to the chromatin is yet to be elucidated.

The strength of interaction of PC4 with the chromatin across the stages of cell cycle (mitotic vs. interphase) was an important issue to be investigated. It was found that upon treatment with 0.2 % NP40 to the cells synchronized in mitotic and interphase stages of cell cycle led to only a partial release of PC4 from the chromatin (Figure 3.5, Results section). This establishes that the strength of association of PC4 to the chromatin does not alter with the stages of cell cycle. Since, PC4 acts as a transcriptional coactivator and also a chromatin organizing protein, the strength of association of PC4 to the chromatin is expected to be in a moderate range. This optimization would be essential for the dynamicity of the protein from a coactivator to a chromatin organizer.

Interestingly, we observed a unique pattern of distribution of PC4 in the mitotic chromosomes. The punctate appearance of PC4 on the chromosome arms differs from other nonhistone chromatin associated proteins (Figure 3.6, Results section). The HMGN (especially N2) proteins appear as small speckles, distributed throughout the entire non-nucleolar space in the cells that are highly active in transcription. In the less transcriptionally active cells, HMGNs are often clustered into large aggregates (Alfonso et al, 1994). Interestingly, unlike PC4, HMGN does not get associated with the chromatin in either metaphase or anaphase. On the other hand immunofluorescence studies show that HP1 is present as uniformly painted. All the three subtypes of HP1 (α, β and γ) are localized to the centromeric heterochromatin during interphase, whereas only HP1-α remains localized to the centromere during the metaphase (Hayakawa et al, 2003). While MeCP2 is found to be concentrated in the pericentromeric heterochromatin (Nan et al, 1996), histone H1 is diffusely distributed throughout the nucleoplasm concentrated in 20-40 nuclear foci indicating its dynamic localization. These nuclear foci are essentially heterochromatinised, but H1 is completely absent from centromeric heterochromatin (Misteli et al, 2000). These observations suggest

that the distribution and appearance of PC4 on chromatin is not similar to other chromatin-associated proteins. Further investigation is necessary to find out the partners of PC4 on the chromatin surface. Nevertheless the data regarding the stable and regular association of PC4 with the chromatin, definitely suggest a significant role of PC4 in chromatin organization.

4.2 Mechanism of PC4-mediated chromatin compaction

The association of PC4 with the chromatin could be through its ability to bind DNA or interact with core histones or both. The other possible docking centers for PC4 in the chromatin (particularly during mitosis) could be the general transcription factors involved in bookmarking, like TBP, TAFs and TFIIB. TBP in association with several TAFs remain associated to promoters after transcriptional silencing and are incorporated into the higher ordered chromatin structure during mitosis (Segil *et al,* 1996; Christova and Oelgeschlager, 2002). Apart from TBP, a relatively lesser amount of TFIIB is also shown to be associated to the mitotic chromosome, but not TFIIA (Christova and Oelgeschlager, 2002). Since PC4 does not interact with TBP, TFIID or TFIIB alone, the possibility of PC4 getting locked by these general transcription factors on the chromatin surface is remote.

We have shown that PC4 interacts with core histones in vivo (Figure 3.9, Results section) with a distinct preference for H3 and H2B (Figure 3.10, Results section). The strength of interaction of PC4 with core histones was found to be quite stable till 150 mM salt concentration (Figure 3.11, Results section). This was again directly correlated with the affinity of PC4 to the chromatin and was indicative of the dynamicity of the protein. This ability to interact with the core histones is also retained in nucleosomal context. Most of the other chromatin-associated proteins also interact with the core histones directly (McBryant et al, 2003; Nielsen et al, 2001; Reeves, 2001; Stros and Kalibalova, 1987; Verreault, 2000) except HMGN2, which does not interact with the free histones (Bustin, 2001). However, core histone interacting proteins generally interact with either H2A-H2B dimer or H3-H4 tetramer or with all the four core histones in solution (McBryant et al,

2003; Verreault, 2000; Stros and Kolibalova, 1987). The interaction of PC4, specifically with H3 and H2B is unique. Possibly oligomeric PC4 (tetramer) interacts with H3 and H2B and in the nucleosomal context confer stable association with the chromatin. Most of the other chromatin-associated proteins also interact with the core histones directly (McBryant et al, 2003; Verreault, 2000; Stros and Kolibalova, 1987; Nielsen et al, 2001; Reeves, 2001) except HMG17, which does not interact with the free histones (Bustin, 2001). Cross-linking studies show that HMGB1 can interact with the H2A-H2B dimer as well as H3-H4 tetramer (Stros and Kolibalova, 1987). Histone H1 also interacts with the core histones through the C-terminal histone fold domain of histone H2A and structured domain of histone H1 (Zlatanova and van Holde, 1996). However, it is not known whether interaction with the core histones is important for the chromatin association and consequent function of these proteins.

In order to understand the molecular mechanism of chromatin- association of PC4, it was necessary to determine the stoichiometry of PC4–core histone (or mononucleosomes) interaction. The stoichiometry was found to be 1.93 (in vitro) and 2.2 (in vivo) considering PC4 as a monomer. However, considering the predicted oligomeric status of PC4 as a dimer, the stoichiometries are 0.96 (in vitro) and 1.1 (in vivo). This can be directly correlated to its chromatin compacting ability, as one molecule of PC4 can directly interact with two mononucleosomal units. Apart from this primary level of interaction, there may be several secondary stabilizing forces which leads to PC4- mediated chromatin organization. The plausible interaction of PC4 with the nucleosomal histones is represented in Figure 4.1.

The domain of PC4 involved in the histone interaction was mapped. The stretch of amino acids from 62-87 was found to be essential for interacting with the histones. A finer mapping showed that a stretch of 5 amino acid residues (62-67) of PC4 is critical for such interaction. Interestingly, this 5 residue is a unique Beta sheet in the structure of PC4. The interaction of PC4 with the core histones is represented in Figure 4.2. Functionally interacting domain of core histone H3 and H2B was subsequently mapped. The globular domain of histone H3 or H2B was

found to be involved in interacting with PC4 (Figure 3.29, Results section) as shown in Figure 4.3.

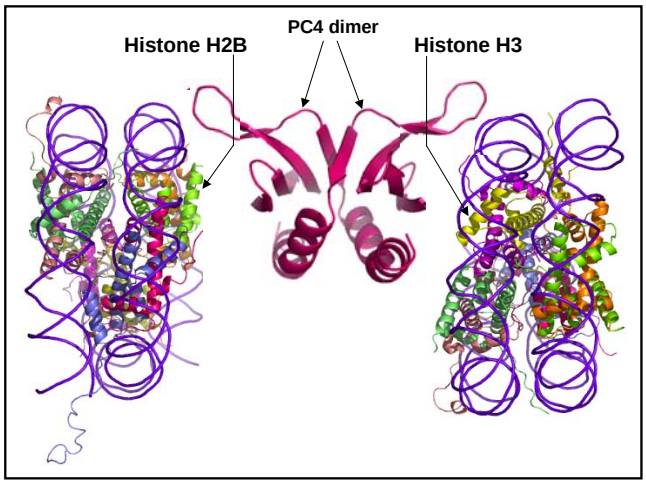


Figure 4.1 – The plausible interaction of PC4 with nucleosomal core histones is represented: PC4 dimer bridges the interaction between two nucleosomal histones (histone H3 and H2B).

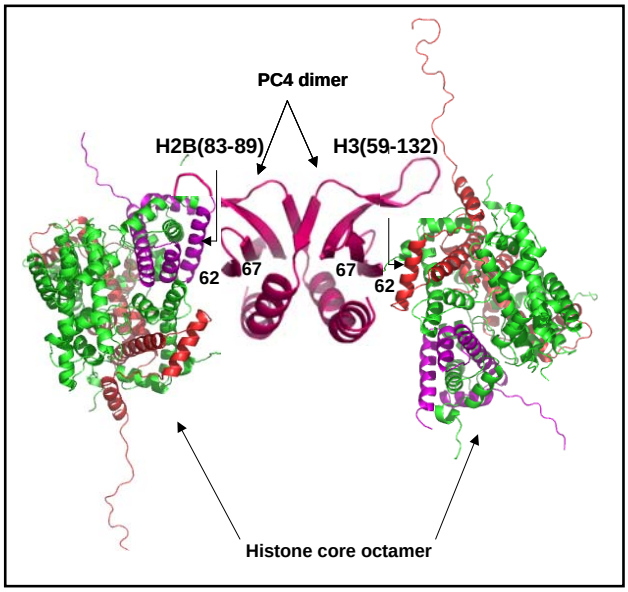


Figure 4.2 Finer mapping of PC4 – core histone interaction: 62-67 amino acids of PC4 (ß sheet) interacts with core histones H2B and H3.

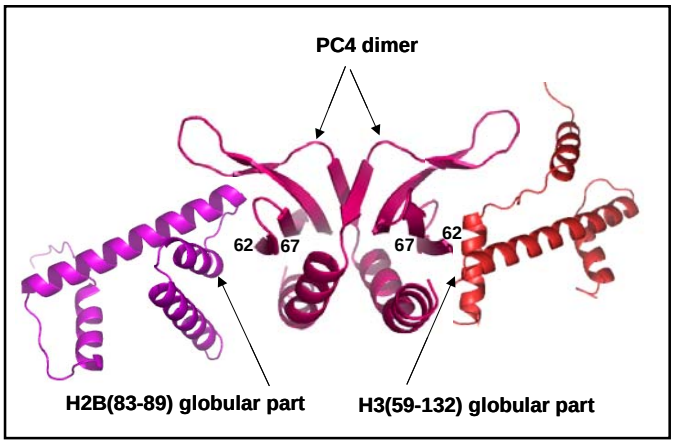


Figure 4.3– Representing the interactions between dimeric PC4 and histone H2B and histone H3 monomeric units: Residues 83-89 of H2B monomer and 59-132 of H3 monomer interacts with 62-67 of PC4 present as a dimer.

The N-terminal tails of the histones were found to have an inhibitory effect on the interaction with PC4 (Figure 3.29, Results section). In case of the polycomb group of protein PRC1, the bridging of nucleosomes is also found to be independent of histone N-terminal domain (Shao et al, 1999). Functional importance 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) (Figure 3.30, Results section). 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. Presence of PC4 throughout the chromosome arms with the exception of centromeric region (Figure 3.6, Results section), 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 (Smith, 2002) 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 containing the histone variants CENP-A.

A functional consequence of the histone interaction ability of PC4 is its chromatin organizing ability. Presumably, acetylated histone is not the preferred interacting partner for PC4 and thus showed very minimal interaction (Figure 3.44, Results section). Since, acetylated histones are epigenetic marks for open chromatin, in vivo PC4 might not bind to the active chromatin, through the histones.

4.3 Implication of chromatin organization by PC4

By employing circular dichroism spectroscopy (which measures the conformational change of the chromatin/DNA) (Khadake and Rao, 1995; Liao and Cole, 1981), visualization of chromatin compaction (upon ectopic addition of PC4) by AFM we have shown that indeed PC4 stimulates the chromatin condensation. The CD spectral data showed that PC4 folds the histone H1 stripped chromatin to a comparable extent as that of histone H1 (Figure 3.17, Results section), but with a slightly lesser efficacy (Figure 3.18, Results section). A comparative analysis of the kinetics of chromatin compaction by PC4 to that of histone H1 showed that H1 executes a faster rate of chromatin condensation compared to PC4 (Figure 3.20). The rate of change of molar ellipticity values has been plotted for PC4 and linker histone H1 (Figure 4.4). The values are 341.97 deg $cm^2dmol^{-1}min^{-1}$ for histone H1 and 143.6 deg $cm^2dmol^{-1}min^{-1}$ for PC4, indicating that histone H1 can compact ~ 2.5 times faster as compared to PC4. This directly indicates that the mechanisms of PC4 or H1-mediated chromatin compaction are distinctly different.

In order to understand the mechanism of PC4 function in the chromatin context it was necessary to study the functional interaction of PC4 with histone H1. Circular Dichroism spectroscopic analysis was carried out and the alteration in the mean residual ellipticity values between chromatin incubated with PC4 with or without a sub-stoichiometric amount of H1 and vice versa was measured for each experimental set as represented in Figure 3.22, Results section. The mean residual ellipticity difference vs. molar concentration ratios (concentration of PC4 or

histone H1: core octamer) has been plotted (Figure 4.5). These results clearly indicate that, while histone H1 can aid in the process of chromatin compaction mediated by PC4, the reverse does not happen.

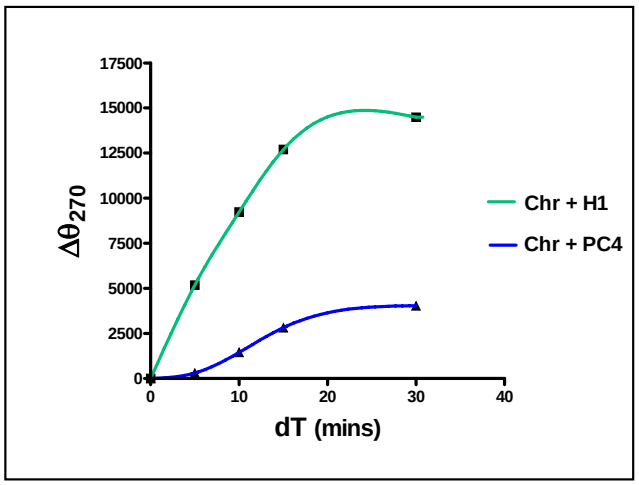


Figure 4.4- Kinetics of chromatin condensation of PC4 as compared to histone H1: The alteration of mean residue ellipticity as a function of time, upon incubating PC4 or histone H1 with chromatin.

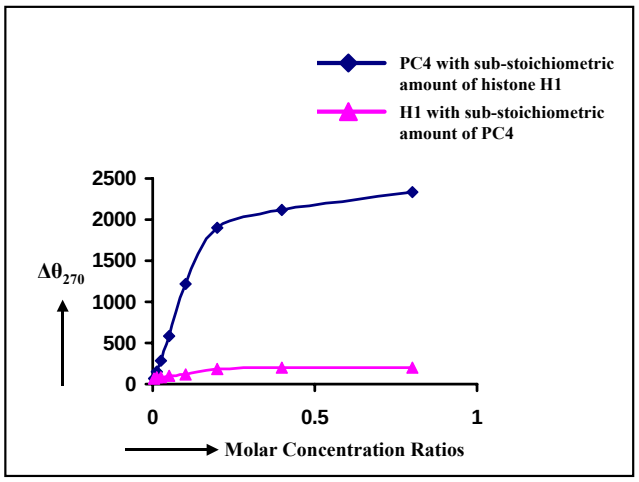


Figure 4.5 - Alterations of mean residual ellipticity values as a function of molar concentration ratios: Sub-stoichiometric amount of H1 assists PC4-mediated compaction till saturation (blue). Sub-stoichiometric amount of PC4 does not assist H1-mediated condensation (pink).

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 (van Holde and Zlatonova, 1996). However, PC4 folds the chromatin into a very distinct type of higher ordered globular structure unlike the linker histone H1 induced folded fiber (compare Figure 3.15, Results section). There are few chromatin interacting proteins that are known to form a compact, globular structure like PC4, which include the Polycomb group of proteins (Francis et al, 2004) and MENT protein (Springhetti et al, 2003). 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. AFM results also showed that histone H1 assisted PC4 mediated globular structure formation, while PC4 did not aid in forming fiber structure by H1 (Table 3.1, Results section). This directly reflects a distinct functional mechanism is operative for each of these two dynamic molecules acting in close association in the chromatin context.

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. Hyperacetylated histones are not the preferred docking site for PC4, and hence it could not compact hyperacetylated chromatin (Figure 3.45 Results section). The alteration of mean residue ellipticity as a function of molar concentration ratios has been represented in Figure 4.6. The results clearly indicate that PC4 does not condense the hyperacetylated chromatin to a great extent. This is another direct evidence showing that the mechanism of chromatin compaction functions of PC4 is mediated through histone interaction. 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.

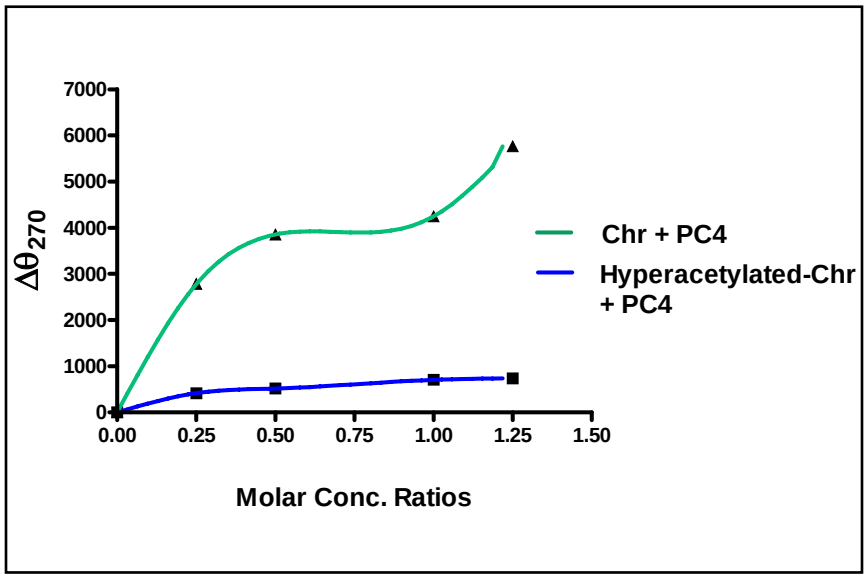


Figure 4.6 Alterations of mean residual ellipticity values as a function of molar concentration ratios: Chromatin and hyperacetylated chromatin were taken as substrates to study the chromatin condensation function of PC4.

Based on the stoichiometry of PC4-nucleosome interaction we propose the following model (Figure 4.7). In vivo oligomeric status of PC4 is a dimer. Each monomer interacts with either a histone H3 or H2B molecule. So as per solenoidal turn PC4 dimer can be positioned as represented in the Figure 4.7.

The expression of PC4 was knocked out efficiently by duplex siRNA or vector based system (PG7) (Kojima et al, 2004) 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.

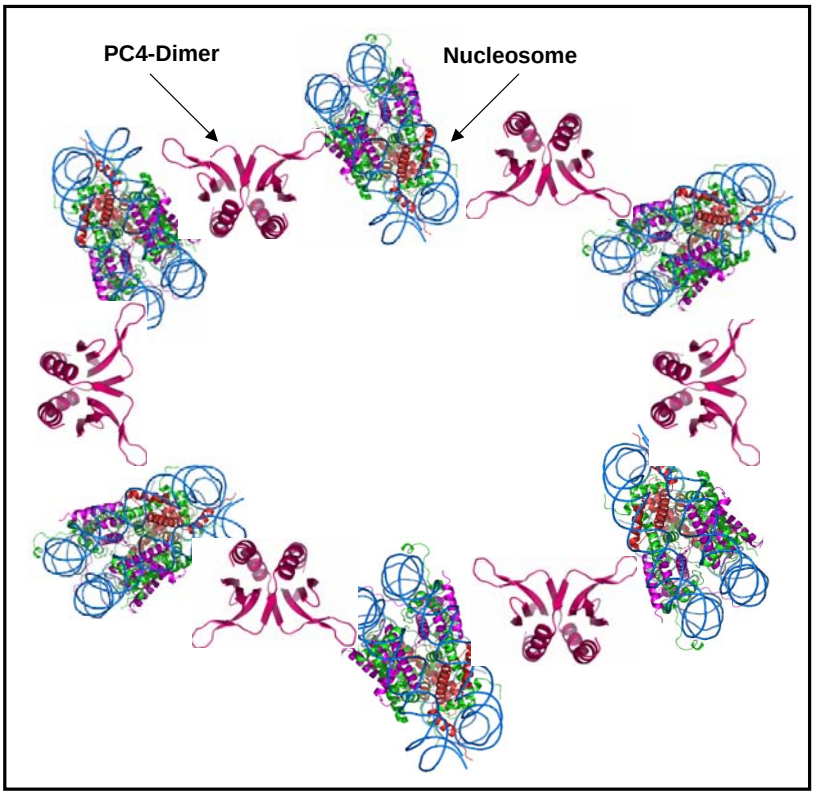


Figure 4.7- Proposed mechanism of oligomeric PC4 mediated chromatin compaction: PC4 may be positioned in between two adjacent nucleosomes bridging the interactions. As per the stoichiometry of interaction a dimer of PC4 interacts with two nucleosomes. Nucleosomal histones involved are histone H3 and H2B of two adjacent nucleosomes. Represented here is the one turn of the solenoidal structure of chromatin showing the primary level of interaction of PC4 with nucleosomes leading to a higher order structure.

This is also evident from the alteration of epigenetic marker profile upon knocking down PC4. The increase in the levels of AcH3 (K9, 14) and H3K4Me3 and decrease in H3K9Me2 upon silencing PC4 reflects a more open chromatin conformation.

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 3.7 and Table 3.4 and 3.5 (Results section), clearly indicates that by siRNA-mediated knockdown of PC4 the number of genes that are upregulated is 3-fold

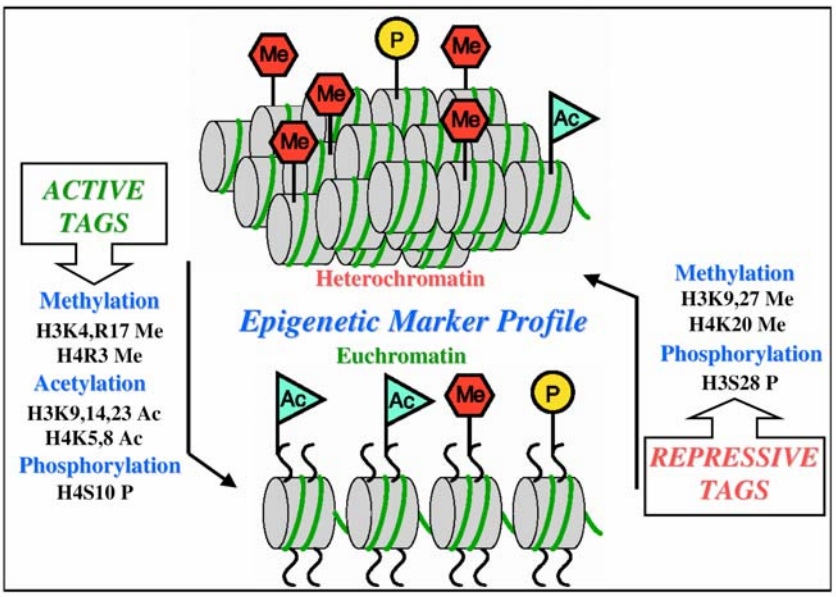


Figure 4.8- Epigenetic markers as key signatures marking active euchromatin and repressed heterochromatin.

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 (Fukuda et al, 2003; Wu et al, 1998), 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) (Fan et al, 2005) 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 (Baum et al, 1997). 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 (Oesterreich et al, 2000). 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.

4.4 One protein multiple functions: a unique switch

Posttranslational modification of core histones and chromatin-associated nonhistone proteins is one of the most important factors to control the fluidity of the chromatin (Agresti and Bianchi, 2003; Hampsey and Reinberg, 2003). Among the several modifications, acetylation is most widely characterized for these proteins. Hyperacetylated histones are the diagnostic feature of transcriptionally active relatively open chromatin. Histone H1 prevents the access of specific HATs (like PCAF) to their target acetylation sites and thereby maintains the proper chromatin folding. The perturbation of the linker histone organization in chromatin is a prerequisite for efficient acetylation of the histone tails in nucleosomes (Herrera et al, 2000). PC4 is acetylated by p300, and heavily phosphorylated by Casein Kinase II (Kumar et al, 2001; Ge et al, 1994; Kretzschmar *et al*, 1994). Phosphorylation negatively regulates the acetylation of PC4 (Kumar et al, 2001).

In the mitotic phase most of the proteins are highly phosphorylated and the histone acetylation is also down regulated (Chen et al, 2005). Phosphorylation causes the dissociation of transcription machineries from the chromatin (Segil et

al 1991; Johnson and Holland, 1965; Farber et al, 1972; Martinez-Balbas et al, 1995; Christova and Oelgeschlager, 2002). The strong association of PC4 with the metaphase chromosome tempted us to find out whether phosphorylation of PC4 has any role to play in its histone (the probable interacting partner in the chromatin) interaction ability. We have found that phosphorylated PC4 strongly interacts with the core histones. Interestingly, phosphorylated PC4 does not bind to the double stranded DNA and is incapable of functioning as a transcriptional coactivator (Werten et al, 1998; Ge et al, 1994). Thus it raises a possibility that phosphorylation may act as a regulatory switch for PC4 to function as chromatin organizer and transcriptional coactivator. These assumptions gain further support from our observation that acetylated PC4 could barely interact with the core histones (Figure 3.40, Results section). Previously we have shown that p300 specific acetylation of PC4 enhances its double stranded DNA binding ability (Kumar et al, 2001). Thus acetylation and phosphorylation are two antagonistic modifications regulating the function of PC4. There are some nonhistone chromatin associated proteins, which bind to the chromatin with a greater affinity upon phosphorylation. This includes the multifunctional protein DEK (Kappes et al, 2001). However, to the best of our knowledge, there is no example so far where acetylation-phosphorylation act as a functional switch for a chromatin protein.

If histone interaction is functionally correlated with the chromatin-association of PC4, the post-translational modifications (viz. acetylation and phosphorylation) should affect the PC4-mediated chromatin folding. In accordance with the assumption, we have found that indeed phosphorylated PC4 induces the chromatin condensation as strongly as that of histone H1 (Figure 3.42, Results section), whereas acetylated PC4 has no effect on the ellipticity of H1 stripped chromatin derived spectra (Figure 3.41, Results section). Comparing the kinetics of chromatin compaction showed that Phospho-PC4 is a better chromatin condenser compared to unmodified PC4 (Figure 3.43). The alteration of mean residue ellipticity values of the chromatin spectra induced by PC4 or its modified form has been plotted as a function of time in Figure 4.9. These results argue for

the fact that in the mitotic stage the phosphorylated PC4 strongly interacts with the histones and assist in the chromatin compaction. Presumably the acetylated PC4 do not bind to the chromatin and thereby has no effect on chromatin compaction rather it acts as a transcriptional coactivator.

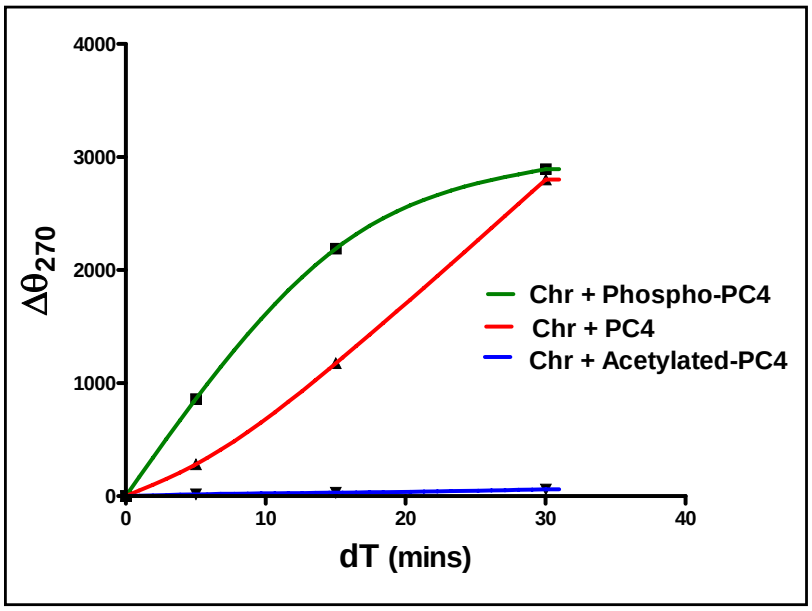


Figure 4.9- Alteration of mean residual ellipticity values by PC4 and its modified forms as a function of time. Phospho-PC4 shows the maximum alteration of θ_{max}, followed by unmodified PC4. Ac-PC4 does not cause any significant change in θ_{max}.

The enhanced transcriptional coactivator function of acetylated PC4 (Figure 3.46, Results section) as compared to phosphorylated PC4 (which is a negative regulator of transcription), clearly indicates that the coactivator function and chromatin organizing function are preferentially executed by two distinct functional modifications of the protein. The summary of the effects of posttranslational modifications regulating the functional switch is represented in Fig 4.10 and Figure 4.11.

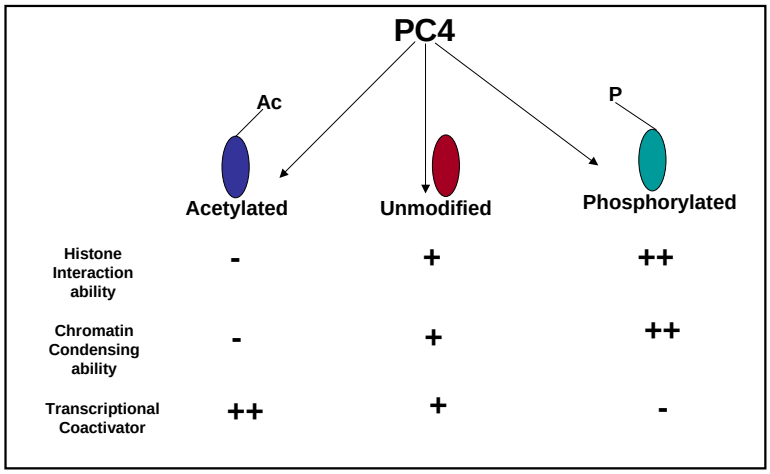


Figure4.10-Summarizing the key events happening with PC4 modifications in chromatin context. Histone interaction, chromatin compaction and transcription coactivator function of unmodified and posttraslationally modified PC4 has been represented.

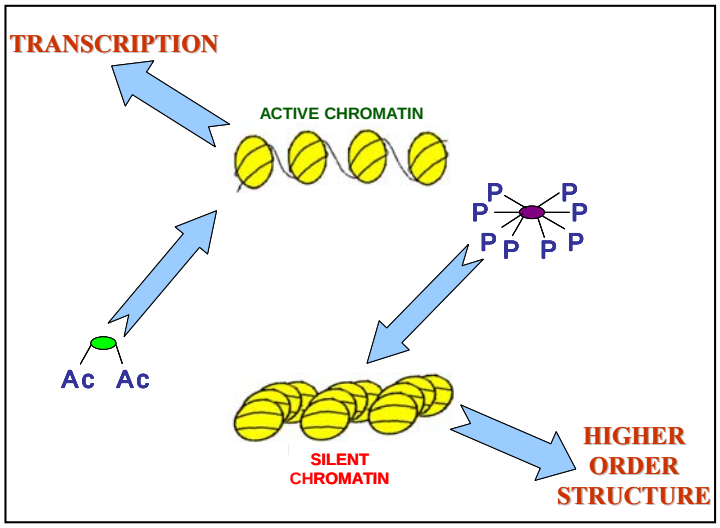


Figure 4.11- Phosphorylation - Acetylation switch model for PC4 function: Acetylated-PC4 binds to the open chromatin activating transcription. Phosphorylated form can bind to chromatin leading to the formation of compact higher order structure.

Based on our results we propose the following model of posttranslational modification dependent functional switch of PC4 (Figure 4.12). In the sequence of events, as schematically represented, we hypothesise an essential role played by a cellular phosphatase. It has been established that phosphorylated PC4 does

not get acetylated (Kumar et al, 2001). Hence for PC4 being converted from a chromatin organizing protein to a transcriptional coactivator, suitable cellular signal should first lead to a dephosphorylation event, followed by its ability to activate transcription upon getting acetylated. However, the functional aspect of a dimodified PC4 (with an acetylation event preceding phosphorylation event), still needs to be explored.

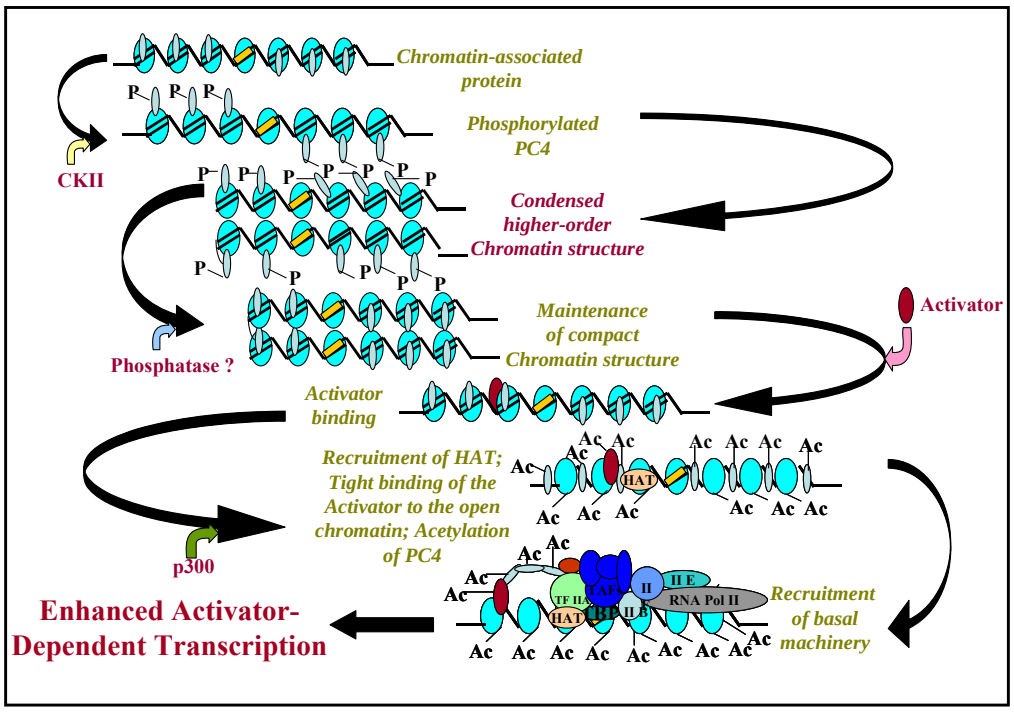


Figure 4.12- Posttranslational modification dependent functional dynamics of PC4 in chromatin context: Schematic representation of the structural alteration of chromatin mediated by posttranslational modifications of PC4. PC4 remains as a chromatin associated protein. Upon phosphorylation by Casein Kinase II, PC4 shows enhanced interaction with core histones and consequent chromatin compaction. In the sequence of events comes a Phosphatase, which can dephosphorylate PC4, still retaining the compact chromatin structure. This is followed by activator binding and recruitment of Histone Acetyl Transferases (p300), which can acetylate the histones and PC4 making the chromatin loose accessible to other transcription factors. Basal machinery components get recruited and acetylated PC4 acts as a bridging molecule between it and the activator leading to an enhanced activator dependent transcription.

4.5 Future perspective

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 in chromatin dynamics. Interestingly, the mechanism of chromatin folding induced by PC4 follows a distinctly different pathway as compared to histone H1. The molecular details of the pathway of chromatin folding mediated by PC4 need to be investigated. Since in physiological condition several factors work in a close interconnected network, it would be interesting to study the functional cooperativity of PC4 and linker histone H1 with other nonhistone chromatin proteins (like HP1, HMGs and PARP1).

Knocking down PC4 causes alteration in the global chromatin architecture and gene expression pattern. The distinct alteration of epigenetic marks, upon silencing PC4, with increased H3K9, K14Ac and H3K4Me3 and decreased H3K9Me2 has a direct correlation with a more open and transcriptionally amenable chromatin structure.

These results indicate a probable role of PC4 in establishment of heterochromatin. There are three subtypes of HP1 (α, β and γ) having differential localization (Nielsen et al, 2001; Gilbert et al, 2003). They are found to be primarily associated with centromeric hetwerochromatin. However, HP1β and in particular HP1γ also localize in the euchromatic sites. HP1γ is found to be associated with transcribed regions of active genes and has been shown to play an important role in transcription memory (Vakoc et al, 2005; Minc et al, 2000). The specificity of HP1α on pericentric heterochromatin has important functional consequence (Gilbert et al, 2003). H3K9Me2 is the docking site of HP1-α whereas HP1-β recognizes H3K9Me3 modification (Peters et al, 2003; Rice et al, 2003), in pericentric heterochromatin. Knock down of PC4 leads to a down regulation of the H3K9Me2 levels indicating a possible role of PC4 in establishing pericentric heterochromatin in an H3K9Me2-dependent manner along with HP1α. Thus under suitable cellular signals coactivator PC4 (most likely in phosphorylated

state) regulates the establishment of heterochromatin thereby preventing expression of a subset of genes. Under specific disease states, PC4 expression might get repressed, thereby leading to the expression of certain virulence genes of heterochromatic origin and consequent disease manifestation. It would be interesting to study the functional interaction of PC4 with HP1 subtypes and other members of the heterochromatin pathway in order to establish the molecular mechanism of such a function.

The role of acetylation and phosphorylation of PC4 as a functional switch between the chromatin associated protein and transcriptional coactivator needs a complete functional dissection. In order to establish the functional states of PC4 at the different stages of cell cycle, CHIP assays with modification-specific (phosphorylation or acetylation) antibodies can be performed. Furthermore, the cellular signal that leads to the conversion of a transcription coactivator to a chromatin organizing protein, in vivo needs to be elucidated.

SUMMARY

Human transcriptional Positive Coactivator 4 (PC4) is a highly abundant, multifunctional nuclear protein, which plays diverse important roles in vital cellular processes including transcription, replication and repair. Furthermore PC4 acts as a putative tumor suppressor probably through its ability to enhance the p53 function and inhibit AP2- mediated self-repression. This functional diversity of PC4 and its similarity to HMGB1 with respect to its DNA binding ability, involvement in p53 induction and cellular abundance prompted us to investigate whether PC4 is a chromatin-associated protein.

In agreement with our speculation we find that PC4 is associated with the nucleosomes (chromatin) throughout the stages of cell cycle. The strength of association of PC4 to the chromatin was determined by the fact that upon treatment with a strong detergent NP40, substantial amount of PC4 remained bound to the chromatin. The distinct and punctate appearance of PC4 on the metaphase chromosomes, without any chromosome bias, suggests its general role in chromatin organization. We have shown that indeed PC4 induce the chromatin condensation through its ability to interact with core-histones (preferentially histone H3 and H2B). The circular Dichroism (CD) spectral data showed that PC4 folds the histone H1 stripped chromatin to a comparable extent as that of histone H1, although the reaction kinetics are different. Interestingly, Atomic Force Microscopy (AFM) of PC4-chromatin complexes showed that, it folds the chromatin into a very distinct type of higher ordered globular structure unlike the linker histone H1 induced folded fibre. Both the CD spectroscopy and the AFM analysis suggest that, H1 assists the globular structure formation by PC4, but PC4 does not contribute to the fibre structure organization of H1. These data indicate a distinct mechanism of chromatin compaction of PC4 as compared to histone H1. 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.

The physiological significance of PC4-mediatd chromatin folding was further investigated by knocking down of PC4 using specific siRNA. Absence of PC4 made the

in vivo chromatin much more accessible to the MNase. Interestingly we observe a distinct change in the levels of epigenetic markers upon PC4 knockdown. There is an increase in H3K9, 14 acetylation, H3K4 methylation levels, whereas there is a drop in H3K9 methylation levels, which indicate a more open chromatin structure upon PC4 knockdown. In agreement with the MNase accessibility and alteration of epigenetic markers upon knocking down PC4, microarray analysis showed upregulation of several genes, suggesting the cellular role of PC4 as a nonhistone chromatin component. Since we visualized a number of cell cycle regulatory genes being affected by global gene expression analysis upon silencing PC4 gene, we wanted to explore the role of PC4 in cell cycle maintenance. Using a vector based system and knocking down PC4 we observed a G2/M checkpoint arrest indicating its function 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. However both the functional attributes are quite contrasting and we find that post-translational modifications of PC4 play the key regulatory role in converting the transcriptional coactivator to a chromatin condensing protein. Remarkably Phospho-PC4, which shows an ability to interact with the core histones, can compact the chromatin structure, whereas acetylated-PC4 can show enhanced Gal4-VP16 dependent transcriptional activation. Taken together these results establish PC4 as a bona fide nonhistone component of chromatin, having post-translational modification dependent distinct functional switch from a transcriptional coactivator to a chromatin organizing protein.

LIST OF PUBLICATIONS

1. Chandrima Das, Kohji Hizume, Kiran Batta, B.R. Prashanth Kumar, Shrikanth S. Gadad, Semanti Ganguly, Stephanie Lorain, Alain Verreault, Parag P. Sadhale, Kunio Takeyasu and Tapas K. Kundu. Transcriptional Coactivator PC4, A Chromatin-associated Protein, Induces Chromatin Condensation. *Mol. Cell. Biol.,* 2006, 22, 8303-8315.

2. **Chandrima Das** and Tapas K. Kundu. Silencing of transcriptional coactivator alters the epigenetic markers and gene expression: Novel role of human transcriptional coactivator PC4 in establishment of heterochromatin. 2006, (Communicated).

3. **Chandrima Das** and Tapas K. Kundu Post-translational modification dependent functional switch of human transcriptional PC4: From a coactivator to a chromatin organizing protein. 2006 (Manuscript under preparation).

4. **Chandrima Das** and Tapas K. Kundu Functional dynamics of chromatin condensation by Linker histone H1 and PC4. 2006 (Manuscript under preparation).

5. **Chandrima Das** and Tapas K. Kundu. Transcriptional regulation by the acetylation of nonhistone proteins in humans- a new target for therapeutics. *IUBMB Life*, 2005, 57, 137-149.

6. Kiran Batta, **Chandrima Das,** Shrikanth S. Gadad, Jayasha Shandilya and Tapas K. Kundu. Reversible acetylation of nonhistone proteins: Role in cellular functions and diseases. In *Chromatin and Disease*, eds: T. K. Kundu and D. Dasgupta, (Springer). 2007, 193–212.

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