Chromatin associated protein, PC4 in genome organization and breast cancer manifestation

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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To

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Declaration

I hereby declare that this thesis entitled "**Chromatin associated protein, PC4 in genome organization and breast cancer manifestation''** is an authentic record of research work carried out by me under supervision of Prof. Tapas K. Kundu at Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and this work has not been submitted elsewhere for the award of any degree.

In keeping the general practice of reporting scientific observations, due acknowledgements have been made wherever the work is based on the finding of other investigators. Any omission, which might have occurred by over sight or misjudgment is deeply regretted.

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Certificate

This is to certify that the work described in this thesis entitled, **"Chromatin associated protein, PC4 in genome organization and breast cancer manifestation'',** is the result of investigation carried out by Ms. Sujata Kumari, at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (Deemed University), Bangalore, India, under my supervision, and the results presented in this thesis have not previously formed the basis for award of any other diploma, degree or fellowship.

Date: **Prof. Tapas K. Kundu**

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INTRODUCTION

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1.1 Eukaryotic chromatin

The eukaryotic genome possesses hierarchical order of chromatin organization. The state of chromatin and its transcriptional competency is dictated by epigenetic bookmarks. Accessibility of DNA within chromatin plays a pivotal role in fundamental processes of life such as transcription, replication, recombination and repair. There appears to be integrating complex signalling networks associated with these events, where multi-protein complexes associate with specific genomic locations in structurally ordered and time-dependent fashion. Non-histone chromatin associated proteins (CAPs) are the major class of proteins, which influence the intrinsic dynamics of chromatin fibres. CAPs impart their own topological affects and thus regulate the accessibility of the underlying DNA. These CAPs also interact with other proteins and effect chromatin organization and gene expression. Post-translational modifications of these proteins are known to modulate their functions. Studies have shown

that altered expression of some non-histone proteins is associated with developmental abnormalities and diseases.

1.1.1 Eukaryotic genome organization and chromatin compartmentalisation

Higher order chromatin organisation based on chromatin fibres and loops: The eukaryotic genome organization is fairly complex and dynamic in the limited concise of nucleus. Compartmentalization (spatial as well as temporal) and compaction of DNA in the nucleus is the characteristic feature of a eukaryotic cell. The DNA helix is folded hierarchically into several layers of higher-order structures, which eventually form a chromosome (Woodcock, 2006). DNA packaging in chromatin occurs in several steps: rolling of DNA onto nucleosomes (a nucleoprotein complexes consisting of 147 bp of DNA wrapped around an octamer of core histone proteins) (Richmond et al, 1984); compaction of nucleosome fibre with formation of the so-called 30 nm fibre; folding of the latter into the giant (50–200 kbp) loops, fixed onto the nuclear matrix. Physiologically, chromatin is not just a DNA- histone complex, rather, it is a dynamic organization of DNA associated with histone, histone interacting non-histone proteins and a small amount of RNA. The dynamic, temporal, and spatial organization of chromatin emerges as a central determinant of genome functions of the eukaryotic cell. Essential functions of the genome, such as transcription, replication, and DNA repair, are controlled by a variety of molecular mechanisms each involving dynamic interplay between multiple protein factors and specific genomic loci in structurally ordered and time-dependent fashion. Cytologically defined largescale chromatin domains exist, that are mainly subdivided into heterochromatin and euchromatin. Euchromatin is correlated with the bulk of transcribed chromatin, which is in an “open” conformation. In contrast, heterochromatin is characteristically silenced chromatin loci, correlated with more condensed

chromatin. However, this correlation of chromatin structure and gene function is circumscribed. Findings by Gilbert et al, 2004, suggest that the ability of genes to be activated is not necessarily lost in more compact chromatin fibres (Gilbert et al, 2004). Conversely, inactive genes can stay inactive close to active genes in an open chromatin environment as well. In these cases, gene activity is regulated by other factors such as covalent modifications of histones or DNA rather than its genomic location (Spector, 2004). Nonhomogeneous clustering of active sites of transcriptiom mediated by both RNA polymerase II and III in mammalian cells have been revealed and are termed as "transcription factories" (Pombo et al, 1999; Jackson et al, 1998). Distantly separated active genes can colocalize to these sites, resulting in activation or suppression of their transcription (Osborne et al, 2004). The concept of transcription factories is newly emerging and it has been shown that each factory contains only one type of RNA polymerase, and factories may be enriched in specific transcription factors involved in the transcription of specific groups of genes (Bartlett et al, 2006).

Higher order organisation based on CT-IC model: Salient features of higher order organization based on CT-IC model are: (a) Individual chromosomes occupy discrete territories (called as CT, chromosome territories) in the cell nucleus (Cremer et al, 1993); (b) CTs are irregularly shaped, posses variable chromatin densities and different regions of a metaphase chromosome occupy distinct subregions within CTs (Dietzel et al, 1998; Teller et al, 2011); (c) Chromatin bulk of one CT is separated from the other, however, DNA segments harbouring active genes form large chromatin loops towards periphery of respective CTs (Foster and Bridger, 2005); (d) There does not exist a fixed pattern of neighbouring CTs in the cell nucleus (Bolzer et al, 2005); (e) The radial organisation of chromatin domains (CDs) based on gene density is observed. Gene rich, early replicating chromosomes and gene dense segments are enriched in the nuclear interior whereas gene poor, transcriptionally repressed

and late replicating chromosomes occupy nuclear periphery or around nucleoli (Ku¨pper et al, 2007).

Interesting observations have been made with chromatin arrangement in cycling cells. Live cell imaging revealed that CT arrangement is stably maintained during interphase (Ma et al, 1998; Walter et al, 2003). Interestingly, chromatin arrangement was entirely changed during mitosis resulting in daughter nuclei with different pattern of chromatin when compared with parent nucleus. Major chromosomal rearrangement was found during prometaphase (Strickfaden et al, 2010; Cvackova et al, 2009). Transcriptional activity is correlated with movement of gene loci. There exists topological level of gene regulation where gene loci associated with heterochromatic regions or nuclear lamina move away from these sites upon transcriptional activation (Lanctot, 2007). Nevertheless, few genes near nuclear lamina associated heterochromatic regions are found to be active (Kumaran and Spector, 2008; Deniaud and Bickmore, 2009; Peric-Hupkes et al, 2010).

According to CT-IC model, chromatin domains (CDs) with DNA content in order of 1 Mb constitute the fundamental structural unit of CT organisation which on the other hand are predictated to be made of a series of compact chromatin loops of DNA (in order of 100 Kb) (Berezney, 2002; Lieberman-Aiden et al, 2009; Mirny, 2011). CDs are highly dynamic and harbour both transcriptionally silent and competent chromatin. Interior of chromatin domain is more condensed and made up of transcriptionally silenced genes whereas periphery of CDs has a layer of decondensed chromatin called as perichromatin region (PR) (Fakan and Driel, 2007). With transcriptional activation or replication signal, respective chromatin segments move to the PR and after completion of transcription or replication, these fall back to the CDs interior. PR has been shown to be associated with machinery for transcription, splicing, DNA replication and repair (Markaki et al, 2010; Rouquette et al, 2010). This model postulates existence of interchromatin compartment (IC) which is described as a three dimensional

network starting from nuclear pore and spreading into the nuclear space. IC is chromatin free region present in between CTs as well as CDs within it. IC diameter varies and could be detrimental for direct contacts between PRs from different CDs. IC may represent platform for the export of ribonucleoproteins (Mor et al, 2010) (Figure 1.1).

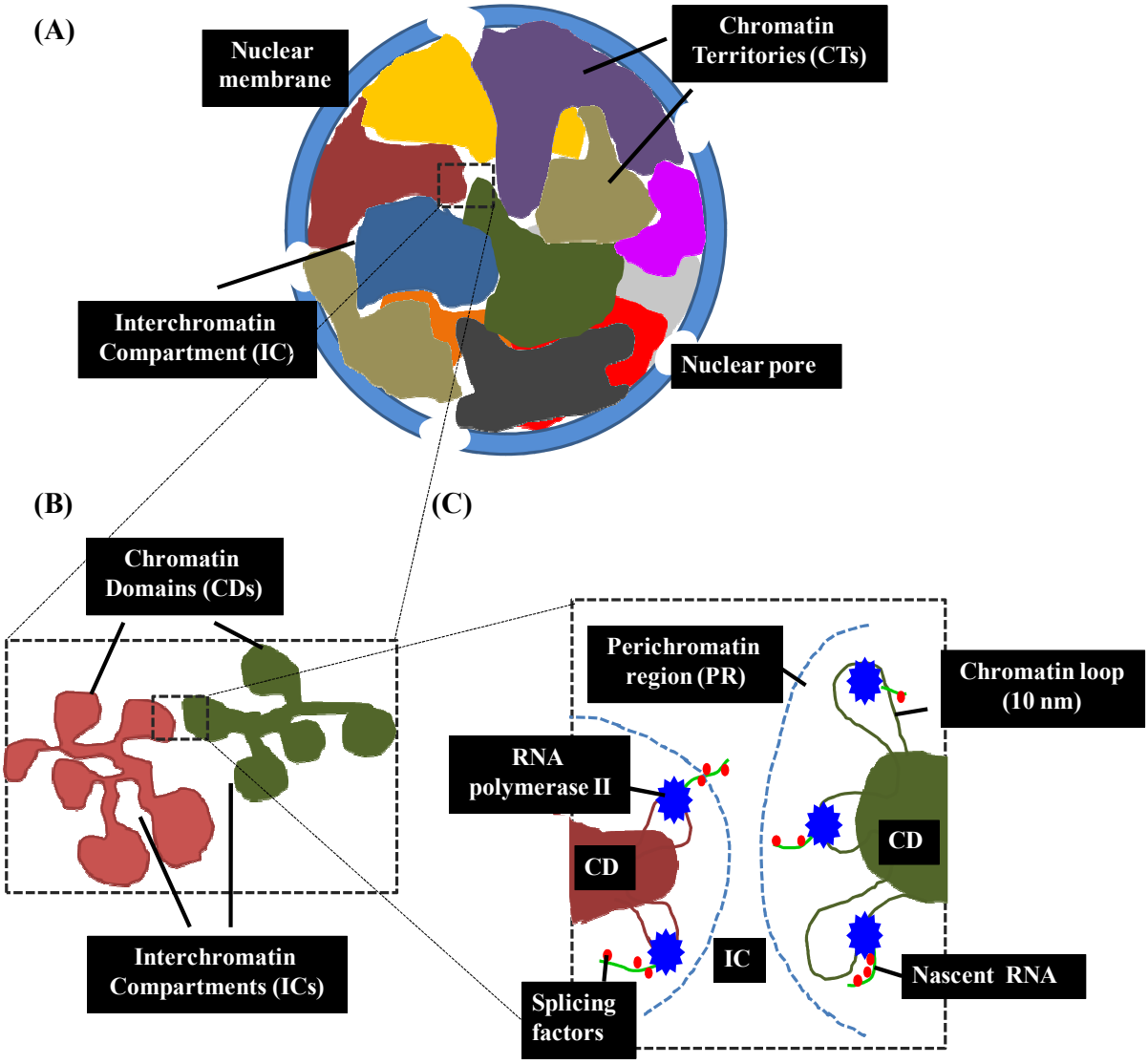


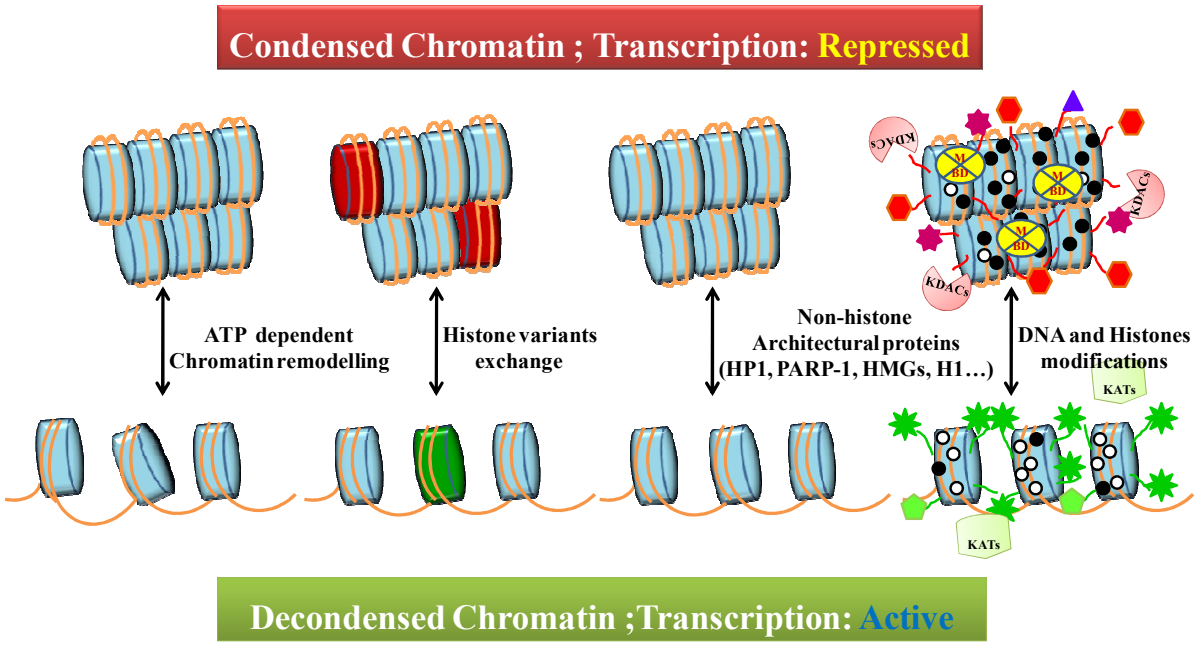
Figure 1.1 CT-IC model for eukaryotic chromatin organisation and topography of transcription. (A) Schematics of nuclear architecture on the basis of chromosome territory-interchromatin compartments. (B) Enlarged view of parts of two chromatin territories showing chromatin domains as their building blocks. (C) Nuclear topography of RNA polymerase II producing nascent RNAs on the decondensed 10 nm chromatin loops.

1.1.2 Chromatin dynamics and epigenetic regulation of gene expression

Covalent modifications of protein and nucleic acid components of chromatin, by small chemical groups (like acetyl, methyl, phosphate etc.) are often encountered *in vivo*. Such heritable change in the genome without changing underlying DNA sequence is known as "epigenetics". Specific epigenetic signature marks functional state of the chromatin. Molecularly, inactive and active chromatin domains can be differentiated by the presence of specific post-translational modifications of histone and non-histone proteins (reviewed in Kouzarides, 2007). Some modifications (like acetylation, methylation) neutralize the positive charge of histones, and thus could affect the structure of chromatin. It has been proved beyond doubt that it is not a single modification, rather the combination of modifications, which indicate the transcriptional state of the chromatin. The enormous combinatorial potential of these modifications can be read out by proteins that bind to specific modifications (Turner, 1993; Strahl and Allis, 2000). The genome-wide mapping studies of histone modifications revealed enrichment of acetylated histones H3, H4, H2A (Davie et al, 1978), at actively transcribed chromatin regions. Additionally, mono-, di-, or trimethylation of H3K4, H3K36 trimethylation, and monomethylation of H3K9, H3 K27, and H4K20 are also seen at transcriptionally active sites. In contrast to this, trimethylation of H3K9, H3K27, and H3K79 is linked with repression (Barski et al, 2007). The tri-methylation of H3K9 is a constitutive heterochromatin mark. Additionally, DNA methylation and incorporation of histone variants add to the complexity of the epigenetic regulation (Figure 1.2).

Chromatin is often viewed as a barrier of protein access to DNA. At the chromatin level, regulation of gene expression involves two major steps: unfolding of chromatin and activation of promoters. ATP dependent chromatin remodelers participate actively in folding and unfolding of the chromatin fibres. Opening of chromatin domains occur during gene activation as evidenced by transition from a DNase resistant to DNase-sensitive configuration

of chromatin. As mentioned earlier this process involves acetylation of histones by histone acetyltransferases (HATs). Local acetylation of histones inside the regulatory sequences may trigger the progressive acetylation. Mostly, these HATs are part of complexes which are recruited to the promoters of active genes. Promoter regions of active genes are nucleosome free or low nucleosome containing for efficient formation of RNA polymerase pre-initiation complex (Razin et al, 2007 and references within). Apart from Promoters, Operators and Enhancers are two more specific DNA sequences that determine the level of gene expression. Operator sequences are recognized by repressor proteins which upon binding inhibit transcription. Activators bind to enhancer regions, which are mostly away from the promoter and stimulate transcription. Chromatin undergoes bending and looping to facilitate interaction of enhancers bound activators with transcription machinery at the promoter and stimulate transcription.



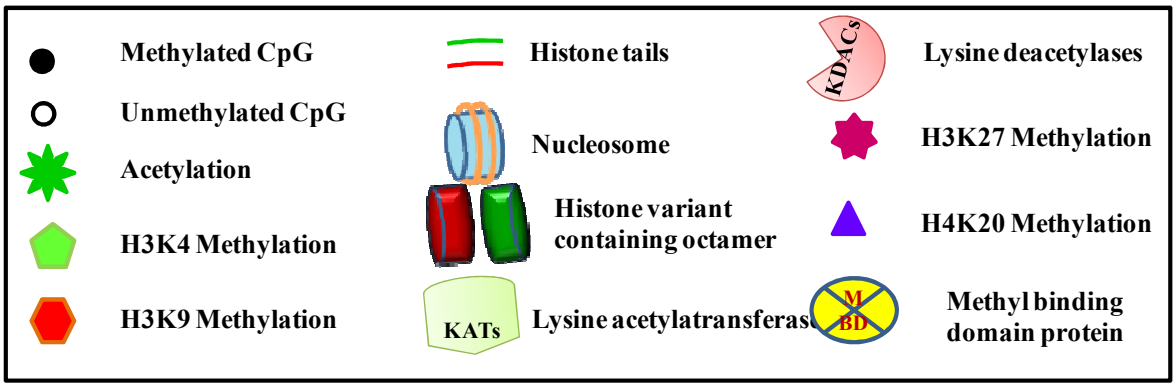


Figure 1.2 Chromatin dynamics regulate transcription. Diverse classes of regulators that affect reversible folding and unfolding of chromatin are shown. Chromatin structure has profound impact on the gene expression. The condensed chromatin does not allow access to the transcription machinery whereas active transcription has been associated with the decondensed chromatin.

1.2. Non-histone Chromatin Associated Proteins (CAPs): The Architectural proteins

CAPs are defined as proteins (excluding the histones), that isolate together with DNA in purified chromatin or chromosomes. These proteins are generally multifunctional which play structural, enzymatic, and regulatory roles in the chromatin complex. CAPs are considerably more complex than histones. Chromatin isolated from different sources show varied number of CAPs (individual polypeptide chains). CAPs possess many physical and biological properties that contrast sharply with those of histones. Several subclasses of non-histone chromatin proteins have now been isolated and characterized. In contrast to that of histones, synthesis of these proteins generally occurs throughout the cell cycle (Cave, 1968; Stein and Baserga, 1970; Cross, 1972). Histones have very long half-lives, on the order of that of the DNA (Bloch et al, 1967; Byvoet, 1966; Hancock, 1969). CAPs possess shorter half-lives, in general similar to those of the cytoplasmic proteins, although, exceptions to this are noted in particular circumstances (Appels and Wells, 1972; Appels and Ringertz, 1974; Garrard and Bonner, 1974). The CAPs show limited tissue specificity unlike histones. Analyses indicate

that the bulk of the non-histone chromosomal proteins are common to all tissues, although quantitative variations are observed across tissue samples. Recent advances in the field have uncovered various biological processes performed by these proteins. Non-histone proteins generally are multifunctional, involved in processes like chromatin organisation, gene expression, DNA repair, epigenetic marks maintenance and cell-cycle regulation. Their importance in transcription regulation has been revealed by large number of studies by different groups.

Possible mechanisms by which these proteins regulate eukaryotic transcription can be broadly classified as following: (a) CAPs themselves could be the components of transcription machinery; (b) Being chromatin components these proteins affect the chromatin folding and thus accessibility to factors involved in transcription; (c) Most of the CAPs are DNA binders which allow them to participate in the DNA transition ability during transcription. Their interaction with DNA may create steric hinderance for any fundamental cellular processes including transcription. Furthermore, CAPs serve as architectural protein altering the topology of the chromatin. Chromatin fibre bridging (Sir3p, Tup1, MENT etc.) and other non-histone proteins (H1, HMGs, PC4, HP1, MeCP2, PARP1 etc) have been shown to mediate chromatin compaction or decompaction, through their direct interaction with core-histones and/or DNA. Increasing number of evidences suggest that small RNAs are also the component of differentially compacted chromatin. Histone chaperones are a class of proteins which are not as abundant as those of CAPs, but are component of highly compact chromatin. Most of the CAPs are subjected to different post-translational modifications, which most often regulate their functions.

1.2.1 Chromatin folding and CAPs

The primary 'beads on string' structure of chromatin need further hierarchical folding for three dimensional organization and functional regulation of chromatin. In the following section, different modes of chromatin folding brought about by diverse classes of CAPs is discussed.

1.2.2 Linker histone H1: The linker histones are involved in higher order chromatin organization. H1 interacts with the nucleosomes, and limits its mobility. Histone H1 restricts access of regulatory proteins, chromatin remodeling factors and chromatin modifying enzymes to their chromatin binding sites. The globular domain of linker histone binds simultaneously to the central portion of nucleosomal DNA, which is close to the dyad axis of the symmetrical core particle, and to one (incoming or outgoing) of the DNA helices. The C-terminal domain has a major impact on the linker DNA conformation and chromatin condensation. DNA linkers are brought together by the linker histone C-terminal tail to form a stem structure of ~30 bp (~10 nm). The length of the MNase protected DNA is 168 bp as in chromatosome (The particle containing 168 bp of DNA, the histone octamer and a molecule of linker histone) because the stem structure does not provide additional protection against MNase cleavage (Simpson, 1978; Hamiche et al, 1996). Till date, chromatosome crystal structure has not been solved. Thus lots of ambiguity is still there regarding location of H1 in nucleosome. H1 subtypes exhibit different affinities for chromatin and different abilities to promote chromatin condensation, as studied with the atomic force microscopy. According to this criterion, H1 subtypes H1.1 and H1.2 can be classified as weak condensers, H1.3 mediated intermediate condensation and strong condensers are H1.0, H1.4, H1.5 and H1x (Clausell et al, 2009).

1.2.3 HMG proteins: There is a repertoire of non-histone proteins binding to nucleosome competing and/or cooperating with linker histones for chromatin binding sites. Among them, HMGs (High Mobility Group proteins) are established chromatin architectural proteins. HMGs are subdivided into 3 families namely HMGA, HMGB, and HMGN by the virtue of having characteristic functional sequence motif: 'AT-hook', for HMGA family; 'HMG-box', for HMGB family; while HMGN family has 'nucleosomal binding domain'. HMG proteins are highly mobile within the nucleus affecting multiple DNA-dependent activities by modulating the structure of chromatin and orchestrating the efficient engagement of other proteins in such vital nuclear activities. HMGNs differ from other HMGs in their ability to recognise generic structural features of the 147 base pair nucleosome core particles, rather than purified DNA or histones. Binding of HMGN to the nucleosome stabilizes their structure (Bustin and Reeves, 1996). HMGN family proteins are capable of dimerizing. The binding of HMGNs to chromatin is cell cycle dependent (Cherukuri et al, 2008). In interphase cells, only one type of HMGN homodimer, either HMGN1 or HMGN2, and never mixtures of HMGN1/2 proteins localize to the nucleosomes (Postnikov et al, 1997; Postnikov et al, 1995). In metaphase cells, only monomeric proteins associate with chromatin but with low binding affinity. The conserved AT-hook containing HMGA recognises structure rather than nucleotide sequence of the DNA and binds preferentially to the minor groove of short stretches of A/ T rich B-form DNA (Reeves and Nissen, 1990). HMGA binds to nucleosomes via AT-hooks and induces localized changes in the rotational setting of DNA on the surface of reconstituted core nucleosomal particles. The mechanism resembles that of chromatin remodelling but differs in being restricted in rotational degree and not involving ATP hydrolysis (Banks et al, 1999; Reeves and Wolffe, 1996). HMGA proteins can bend, straighten, unwind and supercoil DNA substrates *in vitro* in an energy independent manner. Physiological significance of this property of HMGA is seen as its ability to participate in

enhanceosome formation and the regulation of gene expression *in vivo* (Reeves and Beckerbauer, 2001). HMGB, yet another member of HMG family binds to the minor groove of DNA with limited or no sequence specificity (Thomas JO, Travers, 2001). DNA bending and the recognition of distorted DNA structures are the two main ways by virtue of which HMGBs function as chromatin architectural proteins. HMGB and other DNA bending proteins produce an allosteric transition structure that promote the recognition and binding by other proteins. Both HMGA and HMGB proteins have high affinity for distorted DNA structures such as four-way junctions, bulges, kinks and modified DNA containing cisplatin adducts (Pil and Lippard, 1992).

1.2.4 Heterochromatin protein 1: Eukaryotic chromatin has been compartmentalized into densely packed heterochromatin and relatively loosely organized euchromatin. Different non-histone proteins are involved in differential arrangement of chromatin in these distinct domains. In a classical experiment Jamal Tazi and Adrian Bird had shown that active chromatin domain differs from inactive one in three respects: histone Hl is present in very low amounts; histones H3 and H4 are highly acetylated; and nucleosome-free regions are present (Tazi and Bird, 1990). They defined first the molecular basis of the difference between the two domains of the chromatin. HP1 (Heterochromatin Protein 1) is well-characterized member of the CAP family having established role in formation heterochromatization. HP1 family of proteins possess three distinct domains: Chromodomain, Chromoshadow domain and Linker domain. Chromodomain is responsible for HP1 binding to di- and trimethylated lysine 9 of histone H3 which are epigenetic marks for silenced genes (Bannister et al, 2001; Lachner et al, 2001). The chromodomain provides hydrophobic pocket and thus, the appropriate environment for docking onto the methylated residue (Nielsen et al, 2002; Jacobs and Khorasanizadeh, 2002). Localization of HP1 appears to be isoform-specific: in mammalian cells, HP1α and HP1β are mainly enriched in heterochromatin

regions, whereas, HP1γ is found both in heterochromatin and euchromatin (Minc et al, 2000). Studies have implicated involvement of RNA in targeting HP1 to pericentric heterochromatin. In another study, MeCP2 (methyl DNA binding protein) was found to interact with HP1γ during myogenic differentiation. HP1γ was relocalized to the heterochromatin and found associated with MeCP2 to stabilize transcription repression during differentiation (Agarwal et al, 2007).

1.2.5 Other CAPs: MENT (Myeloid and Erythroid Nuclear Termination stage-specific protein) heterochromatin protein is found to be concentrated in compact heterochromatin foci (Grigoryev and Woodcock, 1998). MENT is another class of chromatin architectural protein which belongs to the Serpin protein family of serine protease inhibitors. MENT causes chromatin condensation both *in vivo* and *in vitro*. The structural analysis indicates at least two distinct DNA-binding sites in MENT. Furthermore, it is present with the stoichiometry of two MENT per nucleosome. (McGowan et al, 2006). MENT binds to both DNA and core-histones in the nucleosomes. MENT appears to promote chromatin condensation by bringing linker DNA entry/exit regions in close proximity. MENT is also known to form protein bridges between chromatin fibres through its oligomerization. These modes of chromatin organization by MENT proteins might be critical for heterochromatin formation (Springhetti et al, 2003).

DEK is another non-histone protein which binds to DNA based on its structure and not the underlying sequence (Waldmann et al, 2003). It is an abundant nuclear protein found in all multicellular organisms (animals, plants, fungi), as well as in some single-cell organisms such as *Trypanosoma*. DEK prefers to bind to supercoiled over relaxed DNA and to cruciform over linear DNA. It alters DNA topology by inducing positive supercoils into closed circular DNA. The interaction of DEK with histone H2A/H2B dimers is necessary for the DEK-mediated change in topology of nucleosomal DNA (Alexiadis et al, 2000). There

are two DNA binding modules in DEK and both are needed together for high affinity binding. Several DEK molecules can cooperate to link separate DNA sites on the same or on different DNA strands. CK2 mediated phosphorylation reduces the affinity of DEK for DNA *in vitro* and stimulates DEK–DEK interactions (Kappes et al, 2004) (Figure 1.3). *In vivo*, however, phosphorylated DEK remains on chromatin throughout the cell cycle probably tethered by un- or underphosphorylated form of DEK. It can oligomerize and thereby alter chromatin architecture in a phosphorylation-dependent manner.

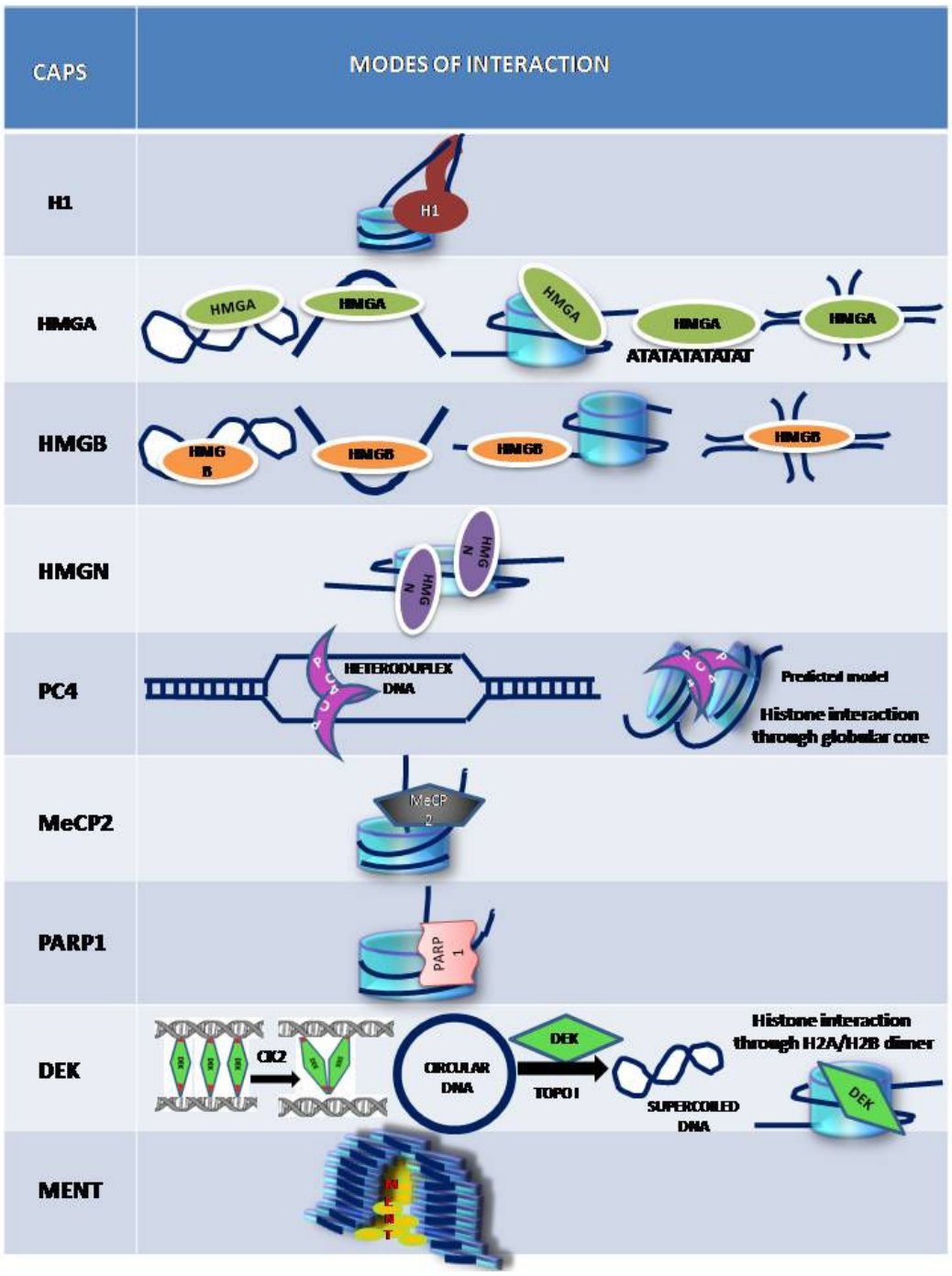


Figure 1.3 A summary of the binding substrates and modes of interaction of different CAPs in the chromatin. CAPs such as linker histone H1, MeCP2, PARP1, HMGN bind at entry/exit site of nucleosome core particles and stabilize the chromatin fibre. HMGs, PC4 and DEK interact with different kinds of DNA substrates, as well as core-histones. DEK and MENT are chromatin bridging proteins. MENT protein oligomerizes and form protein bridges between the chromatin fibres.

1.2.6 Crosstalk among CAPs

Binding of a protein to the entry/exit site of the nucleosome is very crucial for the stability of nucleosome, and the chromatin compaction ability. A new terminology - chromatosome-like particle (CLiP), is given to define any nucleosome, in which a protein is bound at the entry/exit site of the nucleosome. In addition to the classical linker histone H1, several other proteins are known to bind to the DNA entry/exit site. The protein accessibility in nucleosomal DNA is controlled at various levels. First level is specified by the strength of binding of the particular protein at the DNA entry/exit site of the nucleosome. That would influence the dynamics and stability of a CLiP and thus, the accessibility of nucleosomal DNA to regulatory factors. Proteins like linker histones prevent any spontaneous motions of nucleosomal DNA that is; they either permanently lock the nucleosome gate or close it, depending stability of the binding. Other chromatin binding proteins (HMG1/2, PARP1) enable some motions to occur, thereby permitting protein-factor access to nucleosomal DNA. MeCP2 binds to the entry/exit site, possibly in direct competition with H1 and locks the nucleosomal gate (Nikitina et al, 2007).

Second level of control is through exchange of one protein by other at the nucleosome entry/exit site. Direct competition among these proteins by binding directly to each other has been implicated to affect their interaction with the nucleosome. PARP-1-dependent PARylation (Poly-ADP Ribosylation) affect chromatin binding of various non-histone proteins. PARP-1 exclude H1 from the promoters of some PARP-1- regulated genes, possibly by competing with H1 for binding to nucleosomes or by PARylating it (Krishnakumar et al, 2008). It has been seen during estrogen-induced transcription of *TFF1* gene, PARP-1 not only promotes the removal of H1, but also increases the levels of a chromatin architectural protein, HMGB1, which on the other hand, enhances transcription (Ju et al, 2006). Also, PARP-1-dependent PARylation of an abundant and ubiquitous non-histone protein, DEK,

causes enhancement of transcription by promoting the release of DEK from the chromatin (Gamble and Fisher, 2007). HMG group of proteins also compete with H1 histones and influence its binding kinetics to the nucleosome (Catez et al, 2004). Each of the three HMG subfamily proteins (HMGA, HMGB, HMGN) weaken the interaction of H1 with chromatin. Post translational modification of H1 and interaction with other structural protein components of chromatin further modulate histone H1 exchange kinetics. The phosphorylation of H1 by CDK2 (modification important for efficient cell cycle progression), disrupts its interaction with HP1α. It seems that phosphorylation of H1 provides a signal for the disassembly of higher order chromatin structures during interphase, by reducing the affinity of HP1α for heterochromatin. Protein exchange is also brought about by protein chaperones or chromatin remodelers. For example, linker histone chaperone Nap1 binds to linker histones releasing them from their nucleosome binding sites. It also directly interacts with HMGB1 and HMGB2 (Zlatanova et al, 2007). Chromatin assembly factor 1 (CAF-1) binds to HP1α (Murzina et al, 1999; Thiru et al, 2004) to promote its delivery to heterochromatic sites during replication. Furthermore, regulation of such histone chaperons through their PTMs and/or interacting partners affects their ability in deposition, removal andr exchange of chromatin binding proteins.

Different CAPs share common mode of interaction with the chromatin components. Many four way junction (4WJ) binding proteins can substitute for each other in a variety of (often unrelated) functions. Such mutual complementation between structurally and functionally unrelated proteins suggests that DNA binding property of these proteins plays a crucial role and not the proteins that are functionally important. Crosstalk among CAPs occurs at various levels. The temporal expression and post-translational modifications of CAPs influence their functions. Presently, we have very limited knowledge about crosstalk among CAPs.

Emerging studies indicate a perplexed relationship among these proteins, which is not yet fully interpreted.

1.2.7 CAPs in transcription regulation

By sharing their participation with transcriptional machinery, CAPs contribute to transcription regulation. A very well known transcriptional co-activator of activator dependent, RNA polymerase II mediated transcription is positive coactivators 4 (PC4). PC4 is a highly abundant, nuclear, DNA binding protein which facilitates activator-dependent transcription by RNA polymerase II by several folds. PC4 binds to TBP and TFIIA components of the basal transcription machinery and a wide variety of transcriptional activators (Ge and Roeder, 1994). *In vitro* studies indicate that PC4 actually facilitates formation of minimal pre initiation complex (PIC) formation. By virtue of its interaction with DNA template and multiple PIC machineries, PC4 help in recruitment of general transcription factors to the core promoter. In this regard, role of PC4 is analogous to those of HMGB and HMGA proteins. Importantly, phosphorylation renders PC4 incapable of interacting with DNA and activators (Kretzschmar et al, 1994; Kaiser et al, 1995; Ge et al, 1994). Data suggests that TAFII250 and TFIIH can phosphorylate PC4 in the PIC dislodging latter from the PIC and alleviating the PC4 induced block and initiation of an active PIC. Physiologically, PC4 is an activator of p53 function (Banerjee et al, 2004) and induces p53 mediated gene expression and apoptosis. Interestingly, expression of PC4 is also controlled by p53, which establishes the first known positive loop for p53 activation (Kishore et al, 2007). PC4 possess an intrinsic DNA-bending ability as well. The DNA-bending ability of PC4 significantly contributes to the recruitment of p53 to p53-responsive promoters *in vivo***.** In an another example, HMGB interacts with TATA binding protein (TBP) to form a stable HMG-1/TBP/TATA complex, which is proposed to inhibit the assembly of the PIC on gene

promoters (Das and Scovell, 2001). TFIIA then binds to TBP and displace HMGB1 from the inhibitory HMGB1/TBP/TATA complex, thus alleviating HMGB1 mediated transcriptional repression (Dasgupta and Scovell, 2003). HMGB1 can also bend promoter DNA, thus increasing TBP affinity for the TATA box. The recruitment of other GTFs like TFIIB, TFIIA and RNA polymerase II then follows with increased efficiency. HMGB proteins facilitate stable binding of other transcription factors (including p53, all class I steroid receptors, TBP, RAG1, HOX and POU proteins, several NF-κB subunits etc.) to their DNA recognition sites and then get dissociated from the ternary complex (reviewed in Agresti A, Bianchi ME, 2003). In the case of p53, HMGB first binds and bends the DNA, presents it to p53 which then binds tightly to its pre-bent recognition sequence and HMGB leaves the complex.

Some CAPs control expression of certain genes by becoming part of heterochromatin machinery. Recent studies have shown that PC4 is involved in the maintenance of epigenetic state of the chromatin and thereby, gene expression. In agreement, genome is found dramatically hyperacetylated upon PC4 silencing. Furthermore, knocking down PC4 gene expression leads to a significant increase in H3K4 tri-methylation and H3K9, 14 acetylation, which are indicative of a transcriptionally active state of the chromatin. On the other hand, there is decrease in H3K9 dimethylation upon silencing PC4 arguing the possibility of global chromatin opening in absence of PC4. It was found that silencing of PC4 causes over-expression of neuronal-specific genes in non-neuronal cells (HeLa, 293T) (Das et al, 2010). Silencing of neural genes in non neuronal cells is achieved by recruitment of HP1 onto H3K9-dimethylated marks (mediated by G9 methyltransferase) at the promoter (Sampath et al, 2007). Remarkably, PC4 interacts with REST/NRSF, CoREST and HP1α thus, participating in the repression of neural specific gene expression (Das et al, 2010; Schoenherr and Anderson, 1995; Lunyak et al, 2002) (Figure 1.4A). Similar evidences are seen in the

study where interaction between HMGB2 and SP100 facilitates the maintenance of heterochromatin with HP1 (Lehming et al, 1998).

The linker histone H1 has long been known as general transcription inhibitor probably due to hindering access of transcription machinery by its higher-order chromatin folding ability. Its preferential binding to methylated DNA links with the deacetylation of histones and yields effective gene repression through stabilization of folded chromatin domains. But recent studies done on the expression analysis in H1 null chicken cells showed decreased transcription, in multiple genes. It suggests that H1 is involved in transcriptional activation as well (Hashimoto et al, 2010). Its role in transcriptional activation could be attributed to its effects on nucleosome positioning. Remarkably, results suggest that individual subtypes may have non redundant functions in the control of specific gene expression in addition to their fundamental contribution in the organization of chromatin fibre. Linker histone H1 also modulates activity of histone modifying enzymes and thus the transcription output. In a study, it was found that linker histones, H1 and H5, specifically inhibit the acetylation of mono- and oligonucleosomes and not that of free histones or histone-DNA mixtures. The inhibition is probably due to steric hindrance of H3 by the tails of linker histones (Herrera et al, 2000).

HMGs have a general transcription-favourable activity but they can promote repression of some genes. HMGs bring about expression modulation of the specific genes, either by binding directly to nucleosomes (in the case of HMGNs) or by organizing complexes of transcription factors and cofactors (HMGAs), or both (HMGBs). HMGNs affect transcription from chromatin template basically by following mechanisms: By competing with H1 for chromatin binding sites; and at least in some cases relaxing the wrapping of DNA over histone octamer and activating transcription which is otherwise repressed. By locally affecting specific histone modifications HMGN1can be either inhibitory or activating on

transcription of specific genes. HMGN can enhance activity of some histone modifiers like PCAF while inhibits activity of others such as MSK1 and RSK2 (Lim et al, 2005; Postnikov et al, 2006). By inhibiting ATP-dependent remodeling chromatin activity, HMGNs can drive transcription in either direction. A study showed that both HMGN1 and HMGN2 could suppress ATP-dependent nucleosome remodeling by ACF and BRG1 (Rattner et al, 2009). Also, HMGNs facilitate binding of regulators to the chromatin. Studies have shown that incorporation of HMGN2 into chromatin template facilitates the ability of GAL4-VP16 to activate transcription (Paranjape et al, 1995) (Figure 1.4B).

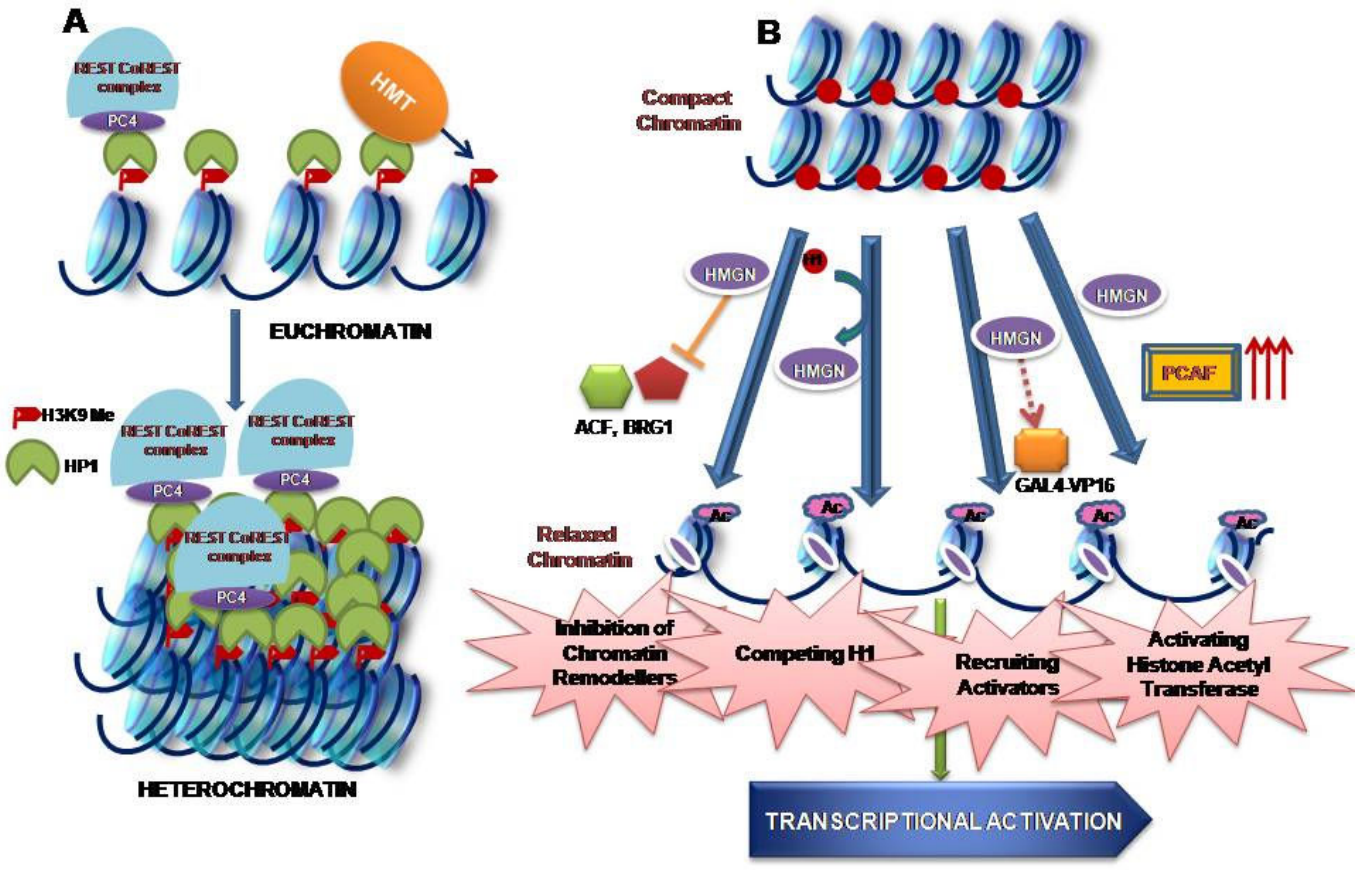


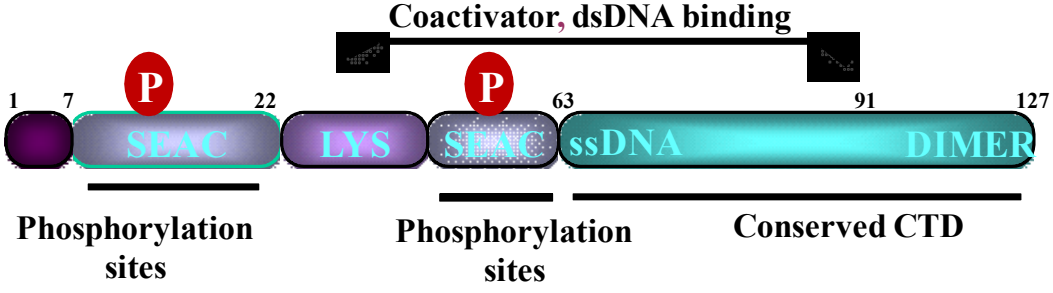
Figure 1.4 Model for transcription regulation by CAPs. (A) Model showing interplay between PC4 and HP1 at the promoter of neuronal genes in non-neuronal cells. PC4 interacts with REST/NRSF, CoREST and HP1α thus, participates in the repression of neural specific gene

expression. (B) Schematic showing the possible mechanisms through which HMGNs affect transcription from the chromatin template.

1.3 Human positive coactivator 4 (PC4)

The human positive coactivator 4, PC4 is a highly abundant, nuclear protein. PC4 is a small protein with molecular weight of 14 kDa known to be involved in several cellular functions. PC4 is highly conserved across species which argues its essentiality. In human, PC4 consists of 127 amino acids having unstructured N-terminal domain (1-62) and highly structured C-terminal domain (62-127). N-terminal domain contains two serine rich acidic regions (SEAC) separated by lysine rich (LYS) region. SEAC region is predicted to undergo phosphorylation (Figure 1.5A). The crystal structure of PC4 C-terminal domain (at 2.8Å resolution) revealed dimeric structure with two ss DNA binding channels running in opposite direction to each other (Brandsen et al, 1997) (Figure 1.5B). PC4 also has double stranded DNA binding domain which overlaps with its coactivation domain (22-87). Human positive coactivator, PC4, was first purified from the upstream stimulatory activity (USA) fraction and was shown to enhance the activator-dependent RNA polymerase II mediated transcription upto ~85 fold. Apart from its role in transcription, lately PC4 has been implicated in several other important cellular phenomena like replication, repair, chromatin organization and tumor suppression.

(A)



(B)

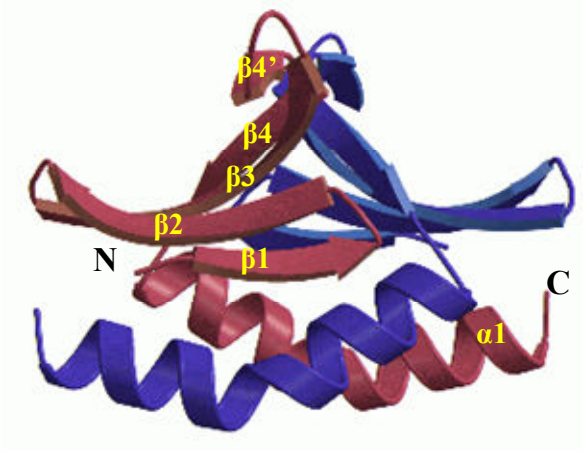


Figure 1.5 Domain structure of PC4. (A) Domain organization of PC4 protein is shown. The N-terminal of PC4 has SEAC regions which are sites for phosphorylation (P), while, C-terminal contains ss DNA binding and dimerization domain. (B) Ribbon diagram of CTD of PC4 is shown with monomers in red and blue. Indicated are alpha helices (α) and beta sheets (β) of the dimer (Taken from Brandsen et al, 1997).

PC4 protein is highly conserved across different organisms. Homologues of PC4 are identified in rat, mouse, yeast (Sub1), Arabidopsis, Caenorabhditis, Zebra fish, chicken and Drosophila. Table 1.1 summarizes percent identity and other gene and protein features of the homologues.

Organism	Uniprot	Protein	Identity	Mass (Da)	Size	Chromosome No.	Gene names
***H. sapiens* (Human)**	P53999 (TCP4_HUMAN)	RNA Polymerase II Transcription Coactivator P15, PC4, P14, SUB1 homolog	100%	14,395	127	5	**SUB1** PC4 RPO2TC 1
A. thaliana	O65154 (KIWI_ARATH)	RNA polymerase II transcriptional coactivator KIWI	35%	12,083	107	4	**KIWI**
C. elegans	Q94045 (TCP4_CAEEL)	Putative RNA polymerase II transcriptional coactivator	37%	14446	124	-	ORF names: T13F2.2

D. melanogaster	Q9VLR5 (TCP4_DROME)	RNA polymerase II transcriptional coactivators, p11	32%	12,189	110	2L	**Ssb-c31a**
***M. musculus* (Mouse)**	P11031 (TCP4_MOUSE)	RNA Polymerase II Transcription Coactivator P15, PC4, P14, SUB1 homolog, Single-stranded DNA-binding protein p9	93%	14427	127	15	**Sub1** Pc4 Rpo2tc1
***R. norvegicus* (Rat)**	Q63396 (TPC4_RAT)	RNA Polymerase II Transcription Coactivator P15, PC4, P14, SUB1 homolog	93%	14,441	127	2	**Sub1** Pc4 Rpo2tc1
S. cerevisiae	P54000 (SUB1_YEAST)	Sub1p	47%	33,102	292	XIII	**SUB1**, TSP1
***D. rerio* (Zebrafish)**	Q504B1 (Q504B1_DANRE)	Uncharacterized protein	80%	13,876	123	5, 10	ORF names:zgc: 109973
***G. gallus* (Chicken)**	Q5ZK63 (TCP4_CHICK)	Activated RNA polymerase II transcriptional coactivator p15, SUB1 homolg	89%	14,237	126	Z	**SUB1** RPO2TC 1

Table 1.1 Different homologues of PC4.

1.3.1 Post-translational modifications of PC4

The post-translational modifications of PC4 have been shown to render distinct functional fates. The acetylation and phosphorylation are two well-studied post-translational modifications of PC4. The functional outcomes of PC4 acetylation and phosphorylation are antagonistic in nature. Acetylation enhances double stranded DNA binding ability of PC4, while its phosphorylation abolishes it (Kumar et al, 2001). Remarkably, phosphorylation negatively regulates the acetylation of PC4, while sustaining the reverse reaction feasible. (Kumar et al, 2001). The acetylation of PC4 is reported *in vitro* and *in vivo* (unpublished

data). Lysine acetyltransferase KAT3B (p300) acetylates PC4 *in vitro*, but there has been no report till date about deacetylation of PC4. The acetylation of PC4 enhances p53 functions like DNA binding to its cognate sites and p53 mediated apoptosis. PC4 contains large number of phosphorylating serines and can be phosphorylated by casein kinase II (CKII) and protein kinase C (PKC) *in vitro*. There are seven predicted phosphorylation sites by CKII in N-terminal of PC4 protein (Ge et al, 1994). Deletion mutant and mass spectrometric studies indicated that *in vivo* hyperphosphorylation of PC4 could be mainly mediated by CKII (Ge et al, 1994). However, TFIIH and TAFII250 subunit of TFIID can also phosphorylate PC4 in the PIC complex. Interestingly, phosphorylation of PC4 negatively regulates its transcription coactivation function (Ge et al, 1994). Thus post-translation modifications dependent distinct regulation is critical in understanding the multifunctionality of PC4.

1.3.2 Role of PC4 in transcription

The first functional role of PC4 was discovered in RNA polymerase II mediated eukaryotic transcription. PC4 cofactor, purified from the upstream stimulatory activity (USA) fraction of HeLa nuclear extract could activate GAL4-AH dependent transcription upto 85 folds *in vitro* in conjunction with TFIID and other general factors. PC4 could also facilitate activation in response to different activation domains fused to minimal DNA binding domain of GAL4. Results revealed that PC4 acts as a general coactivator that mediate direct interactions between general transcriptional machinery and upstream transcriptional activators (eg. p53, VP16, Tat, CTF1, NFκB, Sp1, BRCA1, AP2) to stimulate transcription activation (Banerjee et al, 2004; Ge and Roeder, 1994; Haile and Parvin, 1999; Kannan and Tainsky, 1999; Holloway et al, 2000; Liao et al, 2011). PC4 interacts with free or DNA bound TFIIA and TBP complex but not with TBP alone (Ge and Roeder, 1994). PC4 binds to the promoter in

the presence of TFIID and TFIIA and subsequently initiates activator dependent transcription (Kaiser et al, 1995). Interestingly, in contrast to its activating property in activator dependent RNA polymerase II transcription, PC4 is reported to repress basal transcription initiation in the absence of TAFs (TBP associated factors) and TFIIH (Malik et al, 1998). Furthermore, both TFIIH and $TAF_{II}250$ can phosphorylate PC4 in preinitiation complex (PIC) and reverse PC4 inhibition. PC4 effectively inhibits transcription in regions containing melted or unpaired ds DNA (Werten et al, 1998). PC4 mediated transcription repression was shown to be relieved by ERCC3 helicase activity of TFIIH (Fukuda et al, 2003). Role of PC4 has also been reported in different stages of RNA polymerase II transcription beyond transcription initiation. The phosphorylation of RNA polymerase II, a crucial event in course of transcription progression is influenced by PC4. Human PC4 acts as a substrate specific inhibitor of largest subunit of RNA polymerase II phosphorylation by cdk7, cdk1 and cdk2 (Schang et al, 1999). PC4 also interacts with CstF64, indicating its role in polyadenylation and subsequent transcription termination (Calvo and Manley, 2001). DPE (downstream core promoter element), a diverse transcriptional module of RNA polymerase II requires both PC4 and CKII enzyme apart from TFIID and GTFs (general transcription factors) for transcription. Interestingly, PC4 was not required for DCE (downstream control element) dependent transcription. Thus, PC4 has selective role to coordinate transcription from specific downstream promoter element (Lewis et al, 2005).

Apart from RNA polymerase II transcription, role of PC4 and its yeast homolog Sub1 has also been implicated in RNA polymerase III transcription. Human PC4 was found in human holo TFIIIC complex along with DNA topoisomerase I. DNA topoisomerase I and PC4 could extend TFIIIC interactions to the downstream promoter regions and promote multiple round of transcription by RNA polymerase III. Thus, TFIIIC mediated efficient reinitiation and accurate termination of RNA polymerase III is enhanced and extended by human PC4 and

DNA topoisomerase I (Wang and Roeder, 1998). Consistently, yeast homolg Sub1 associates with RNA polymerase III transcribed genes. Sub1 interacts with TFIIIB and TFIIIC components and stimulates transcription initiation and reinitiation in an *in vitro* reconstituted polymerase III transcription system (Tavenet et al, 2009).

1.3.3 Role of PC4 in chromatin compaction

Recently, a novel function of human coactivator 4, PC4 as a chromatin organizer was discovered. PC4 is tethered to the chromatin and was found exclusively associated with the nucleosomal fraction but not in nonchromatin fraction as evident from sucrose density gradient-fractionated HeLa chromatin. PC4 was found associated to chromatin throughout the cell cycle stages. It was shown that PC4 is broadly distributed on metaphase chromosome in punctuate manner except at the centromeric region. Stable association of PC4 with the chromatin could be through its interaction with different components of chromatin. Apart from its interaction with DNA, general transcription factors or nonhistone chromatin associated proteins; PC4 directly interacts with all core histone proteins with preference towards histones H3 and H2B *in vitro*. PC4 dock to globular domains of histones H3 and H2B but not their tail regions. No interaction of PC4 with centromere specific histone, CenH3 was observed explaining absence of PC4 in the centromeric region. Histone interaction ability rendered PC4 to induce chromatin condensation. PC4 was able to compact H1 stripped HeLa chromatin *in vitro*. Interaction with the core histones is crucial for PC4 mediated chromatin condensation since histone interaction deficient PC4 mutant could not condense H1 stripped HeLa or *in vitro* reconstituted chromatin (Das et al, 2006). Transient silencing of PC4 in HeLa cells caused decompaction of chromatin as visualised by atomic force microscope. Global gene expression analysis upon transient PC4 silencing showed

around 128 upregulated genes as compared to 49 downregulated genes emphasizing majorily repressive role of PC4 *in vivo*. Recent results indicate that PC4 might participate in heterochromatinization thereby regulating a subset of genes (Das et al, 2010).

1.3.4 PC4 in other chromatin template phenomena

Besides transcriptional regulation and chromatin organization, PC4 plays important role in other cellular processes like replication, DNA repair and tumor suppression.

Replication: PC4 influences SV40 origin of replication depending on the concentration of HSSB or RPA, a trimeric single stranded DNA binding protein. PC4 interacts with the replication protein RPA on ssDNA and corroborates T-antigen mediated unwinding of DNA. (Pan et al, 1996). The C-terminal of PC4 interacts with adeno associate virus type–II (AAV-II) replication protein and regulates AAV-II gene expression (Muramatsu et al. 1998; Weger et al, 1999).

Repair: PC4 renders prevention from mutagenesis and killing by oxidative DNA damage by virtue of its ss DNA binding activity. The multifunctional human DNA repair protein, XPG binds to and enhances interaction of PC4 to bubbled DNA. Hydrogen peroxide sensitivity and peroxide induced hypermutability of Sub1 null cells can be rescued by human PC4 suggesting human protein has similar function (Wang et al. 2004). Recent findings have demonstrated human PC4 as an activator of nonhomologous end joining (NHEJ) and double strand break repair activity (Batta et al, 2009). PC4 enhances joining of non-complementary DNA ends thereby stimulating double strand break rejoining *in vivo*. Consistent with these findings, it was found that there is rapid and transient accumulation of PC4 at DNA damaged

sites in the mammalian cells. Although, different mobility and binding kinetics of PC4 and replication protein A (RPA) suggested their distinct functions in DNA repair, requisite of single stranded DNA binding domain for PC4 recruitment to damaged site indicates its similar role in DNA repair as that of RPA (Mortusewicz et al, 2008).

Tumor suppression: AP2-α negatively regulates its transcriptional activity in *ras* transformed cell line. PC4 physically interacts with AP2-α through its C-terminal domain and inhibits AP2 transcriptional self interference thereby, acting as a putative tumor suppressor (Kannan and Tainsky, 1999). The ability of PC4 enhance the function of a tumor suppressor protein, p53 *in vivo* could also be interpreted as tumor suppressor activity of PC4 (Banerjee et al, 2004). Figure 1.5 summarizes the different functions performed by PC4.

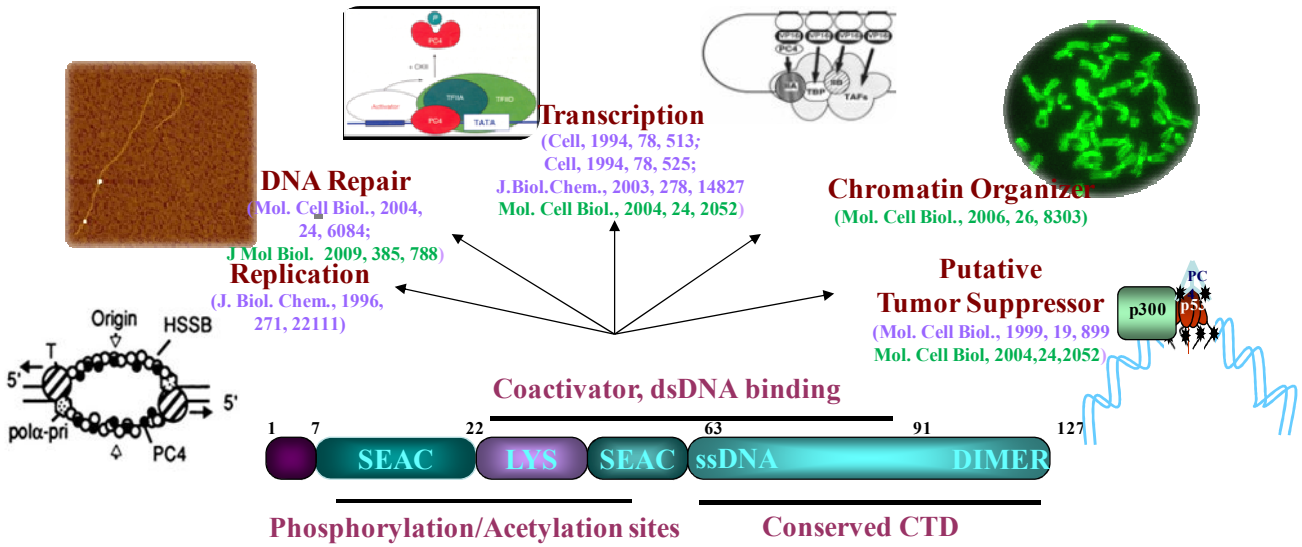


Figure 1.6 Model for PC4 as a multifunctional protein. Domain organization and related functions of PC4 has been represented. The N-terminal of PC4 is unstructured and has two serine rich (SEAC) and one lysine rich (LYS) regions. The amino terminal undergoes post-translational modifications and has several phosphorylation and acetylation sites. The C-terminal domain (CTD) of PC4 is structured

and highly conserved. The carboxy terminal contains single stranded DNA binding (ssDNA) and dimerization (DIMER) domains. The figure also lists out the different known functions of PC4.

1.4 Tumorigenesis

1.4.1 Cancer: The hallmarks

Cancer is a very complex process and cannot be acknowledged merely by sustained proliferation of a cell population. Cancer is composed of multiple distinct cell types where contribution from tumor associated stroma is increasingly appreciated during last decade. The active participation of tumor microenvironment argues that traits of cancer and its manifestation cannot be solely understood by analysing cancer cell genomes. Decades of research has attempted to characterize complexity of the cancer and placed it under six hall marks: Sustaining proliferative signalling, Evading growth suppressors, Resisting cell death, Enabling replication immortality, Inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2000). Furthermore, additional hallmarks were added to the existing original six hallmarks: the ability to reprogramming energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011) (Figure 1.6).

Sustaining proliferative signalling: Cancer cells suffer from the chronic proliferation, which unlike normal cells perturb the homeostasis of cell number and tissue architecture. The abrupt mitogenic signalling in cancer cells has been extensively studied which revealed multiple strategies cancer cells opt for sustain their proliferative signalling namely, stimulating autocrine proliferative signal by producing growth factor ligands themselves or stimulating tumor associated stroma cells to secrete growth factors (Cheng et al, 2008; Bhowmick et al, 2004). Cancer cells overexpress receptor proteins thus making themselves hyper-responsive

to the available growth factor ligands. Cancer cells are also capable of bypassing growth factor dependency for their proliferative signal by altering the receptor protein structure in such a way that receptor can be stimulated even in the absence of growth factor ligand. Somatic mutations encompassing growth factor mediated signalling cascade in some cancers result in the activation of downstream pathways (Davies and Samuels, 2010). Several cancer cells are found having defective negative regulators of proliferation signals. Loss of PTEN phosphatase in several cancers prevents it to counter phosphoinositide 3-kinase (PI3-kinase) thus, activating PI3K signalling and promote tumorigenesis (Jiang and Liu, 2009; Yuan and Cantley, 2008).

Evading growth suppressors: In addition to growth stimulatory signals, cell harbours negative regulators as well, to ensure controlled cell proliferation. Such regulators termed as tumor suppressors, are generally hijacked or corrupted by cancer cells. Retinoblastoma-associated (RB) and TP53 are very important proteins of the two complementary cellular regulatory circuits. RB serves as a critical gatekeeper of cell cycle progression and TP53 govern the decision of cell fate. Several cancers lack RB and/or TP53 proteins resulting in uncontrolled cell proliferation. Yet another regulation of cell proliferation employed by normal cells, is the contact inhibition mediated by cell to cell contact of dense population propagating in two dimensional cultures. *In vitro* culture of the cancer cells have shown abolishment of contact inhibition with not very definite mechanisms known. Few studies have indicated the involvement of NF2 gene implicated as tumor suppressor in one form of human neurofibromantosis (Curto et al, 2007; Okada et al, 2005). In another example, suppression of LKB1, the epithelial polarity protein, destabilizes epithelial integrity and the cells thereby, overcome inhibition over mitogenic effect of Myc oncogene (Shaw, 2009, Partanen et al, 2009, Hezel and bardeesy, 2008).

Resisting cell death: Apoptosis or programmed cell death serve as a natural barrier for any abnormal growth and in this context the cancer development. Stress that signal activation of apoptotic pathway involves elevated level of oncogene signalling and DNA damage, the traits commonly seen during cancer development. The loss of critical damage sensor, TP53, in cancer cells disable the apoptosis inducing circuits. Alternatively, the cancer cells elevate expression of antiapoptotic regulators (Bcl-2, Bcl-x_L) through downregulating proapoptotic factors (Bax, Bim, Puma, Noxa) to overrule apoptosis. Other forms of cell death operating in a cell include autophagy and necrosis. Autophagy has been detected in cancer cells possibly induced by nutrient starvation and certain cytotoxic drugs. Studies have shown conflicting observations towards tumor cell survival and tumor cell death via autophagy (White and Dipaola, 2009; Amaravadi and Thompson, 2007). Reversible dormancy in cancer cells has also been documentated via autophagy (Lu et al, 2008). Necrosis is often seen in the core of a tumor tissue. Necrotic cells releases proinflammatory signals into surrounding tissue microenvironment which attracts immune inflammatory cells, which in turn are tumor promoting (Grivennikov et al, 2010; White et al, 2010, Galluzzi et al, 2008).

Enabling replicative immortality: Immortalization is the key feature of a cancer cell that is, acquiring unlimited replicative potential without undergoing senescence or cell death. With continuous propagation of non-immortalized cells, telomeres end progressively get eroded eventually losing the ability to protect chromosomal end fusion. The chromosome fusion otherwise, results in unstable dicentric chromosomes threatening the cell survival. The enzyme, telomerase adds telomeric repeat sequence to the telomere ends and thus protects it from eroding. Telomerase is almost absent in normal non-immortalized cells whereas it is

significantly upregulated in human cancers countering the progressive telomere erosion, thereby explaining partly atleast, the immense replication potential of tumor cells.

Inducing angiogenesis: Tumor tends to develop vasculature for their nutrient and oxygen sustenance. Angiogenesis inducer, vascular endothelial growth factor–A (VEGF-A) gets upregulated in tumorigenic environment in presence of hypoxia and oncogene signalling (Ferara, 2009; Mac Gabhann and Popel, 2008). Matrix metalloproteases secreted by tumor cells aid in the release and activation of VEGF-A (Kessenbrock et al, 2010) which eventually promote angiogenesis. Tumor specific vasculature is different from the normal ones in their forms, blood flow, auxillary cells coverage etc (Nagy et al, 2010; Raza et al, 201, Baluk et al, 2005).

Activating invasion and metastasis: Tumor cells arising from epithelial tissues dislocate from their primary location and move to secondary, manifested by local invasion and distant metastasis. Invasion and metastasis are multistep processes and discussed in section 1.4.2. Key regulators of the primary neoplasia to metastases reprogramming events are cell adhesion molecules, E and N-cadherin, matrix metalloproteases (MMPs) and diverse transcriptional factors regulating these regulators. Emerging evidences suggest significant crosstalk between cancer and stromal cells occurring during invasion and metastasis. For example, stromal mesenchymal stem cells (MSCs) respond to signals released by cancer cells and secrete CCL5/RANTES to stimulate invasion (Karnoub et al, 2007).

Reprogramming energy metabolism: Cancer cell exhibits anomalous cell energy metabolism, first observed by Warburg (Warburg 1956a, 1956b) which is popularly known as Warburg hypothesis. The cancer cell limits their energy metabolism mainly to glycolysis even in

presence of oxygen, rather than pyruvate mediated energy production in mitochondria as seen in the normal cells. This phenomenon is known as aerobic glycolysis. Glycolysis seems highly efficient in energy production in cancer cells, partly due to upregulation of glucose transporters, GLUT1, thus increasing glucose uptake and utilization in the cancer cells (DeBerardinis et al, 2008; Hsu and Sabatini, 2008). Hypoxic conditions, prevalently observed in many tumors, upregulate glucose transporters and several enzymes of glytolytic pathway, (Semenza 2010a; Jones and Thompson, 2009) thus, explaining high rate glycolysis in the tumors. Mutant TP53 and activated oncogenes like Ras, Myc is also shown to be involved in upregulating glycolysis. Some tumors contain two symbiotically functioning subpopulations of tumor cells; one utilizing glucose and secrete lactate whereas, cells of other subpopulation metabolize lactate for their energy production through Kreb cycle (Kennedy and Dewhirst, 2010; Feron 2009; Semenza, 2008).

Evading immune destruction: Cancer cells are capable of blinding immune system, which forms the defence module of the body. They somehow, seem to escape the vigilance of the different arms of immune system or dilute their killing extent. The increased incidence of tumor formation in immunocompromised individuals or various experimental models, validate the concept of paralyzed immunological response during tumor development (Vajdic and Leeuwen, 2009; Teng et al, 2008; Kim et al, 2007). Cancer cells secrete immunosuppressive factors like TGF-β and deactivate cytotoxic lymphocytes (CTLs) and NK immune cells (Yang et al, 2010; Shields et al, 2010). Cancer cells also recruit immunosuppressive regulatory T cells and myeloid derived suppressor cells to counter the action of CTLs (Mougiakakos et al, 2010; Ostrand-Rosenberg and Sinha, 2009).

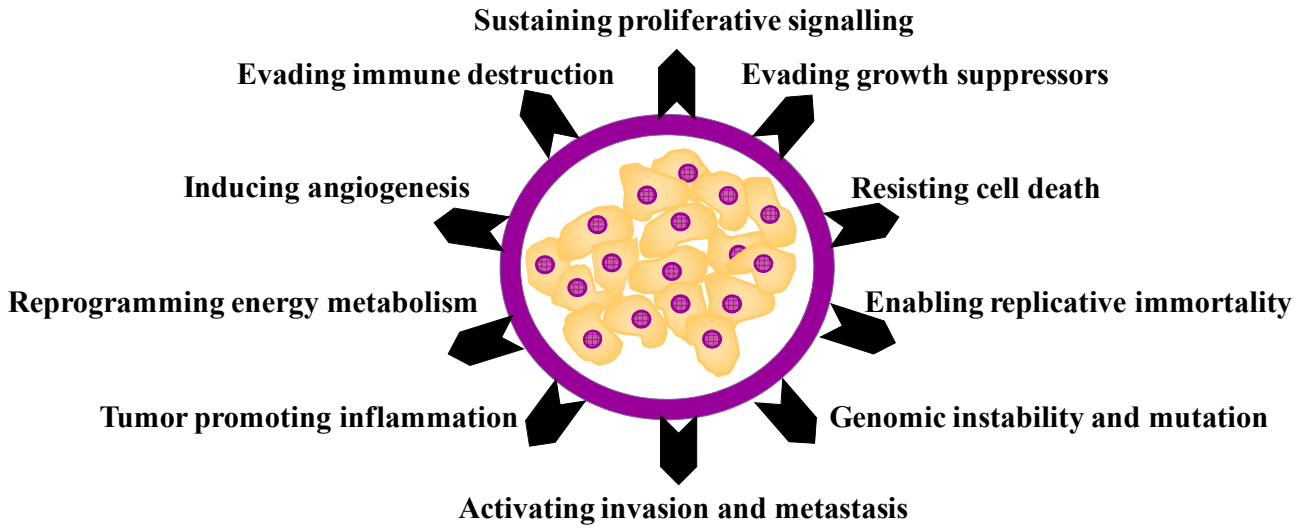


Figure 1.7 Hallmarks of cancer. Figure showing current view of extended hallmarks and characteristics of cancer (Adapted from Hanahan and Weinberg, 2011).

1.4.2 Invasion and metastasis are multistep process

Multiple reprogramming events occur in the process of invasion and subsequently, distant metastasis of tumor. Such events result in a series of cellular and biological changes in cancer cells namely: local invasion into stroma, intravasion into nearby blood vessels, transit and survival in circulation, extravasation at distant site, micrometastasis in the form of small group of cancer cells and finally colonization with the formation of macroscopic tumor (Figure 1.7).

Epithelial-mesenchymal transition (EMT): The primary carcinomas arising from epithelial tissues, when observed to progress to higher pathological stages, were seen to undergo change in shape and detachment from other cells during the process. They follow local invasion and eventually metastasis. The event is characterized by the loss of E-cadherin activity either by lower transcript expression or mutational inactivation (Berx and Roy, 2009; Cavallaro and Christofori, 2004). The dysfunction of E-cadherin is one of the key changes in

the process of epithelial to mesenchymal transition (EMT). Conversely, N-cadherin, involved in the cell migration during embryogenesis was found upregulated in many cancers. A set of transcription factors orchestrating EMT has been identified, which includes Snail, Slug, Twist and Zeb1/2. These factors solicit change of epithelial cell into spindle shaped morphology, enhance cell motility, increase expression of matrix metalloproteinases, repression of E-cadherin, and increased resistance to apoptosis - all traits associated with invasion and metastasis of the cancer cells (Peinado et al, 2004). Increasing evidences suggest that apart from cancer cells themselves, EMT signal are evoked by microenvironment cues as well (Hlubek et al, 2007).

Apart from mesenchymal type of invasiveness elicited by EMT, tumor also presents two other modes of invasion: collective invasion and amoeboid invasion (Friedl and Wolf, 2008, 2010). Collective invasion is characterized by the movement of small groups of cancer cells in mass. Such cancers rarely show any metastatic behaviour. Amoeboid form of invasion lack the ability of basement membrane degradation as seen in mesenchymal and collective invasion. Instead, cells show plasticity in their cellular morphology to squeeze out through the existing opening in the extracellular matrix (Madsen and Sahai, 2010; Sabeh et al, 2009).

Mesenchymal-epithelial transition (MET): For the cells that have undergone EMT, invaded and survived the circulation and extravasate to colonize the secondary site, must be able to revert back to an epithelial state. The new tumor colonies formed exhibit similar histopathology as that of primary tumor. The process involved genetic changes driving cells to a more epithelial state. MET is likely to get influenced by differing signals in the new microenvironment (Zeisberg et al, 2005).

Metastatic colonization: After dissemination of tumor cells from primary tumor to secondary site, tumor cells need to adapt to foreign microenvironment for successful colonization.

Colonization is not always successful and doenot always follow dissemination (Talmadge and Fidler, 2010; McGowan et al, 2009; Townson and Chambers, 2006). Micrometastases after exavasation, sometimes undergo dormancy. It is believed that atleast in some types of cancers, primary tumor releases suppressive factors that prevent micrometastases to colonize. Such primary tumors when are excised result in explosive increase in the metastatic growth (Demicheli et al, 2008; Folkman, 2002). Unsuccessful colonization after dissemination can be attributed to poor adaptation to the foreign microenviernment. New tissue site may harbour anti growth signals (Barkan et al, 2010) or tumor suppressive immune cells (Teng et al, 2008; Aguirre-Ghiso, 2007) thus presenting a non permissive tumor microenvironment. In addition, micrometastases also suffer from lack of angiogenesis and/or activated autophagy driving them towards reversible dormancy. With change to permissive microenvironment, such dormant cells resume their proliferation state and develop into tumor (Kenific et al, 2010; Lu et al, 2008).

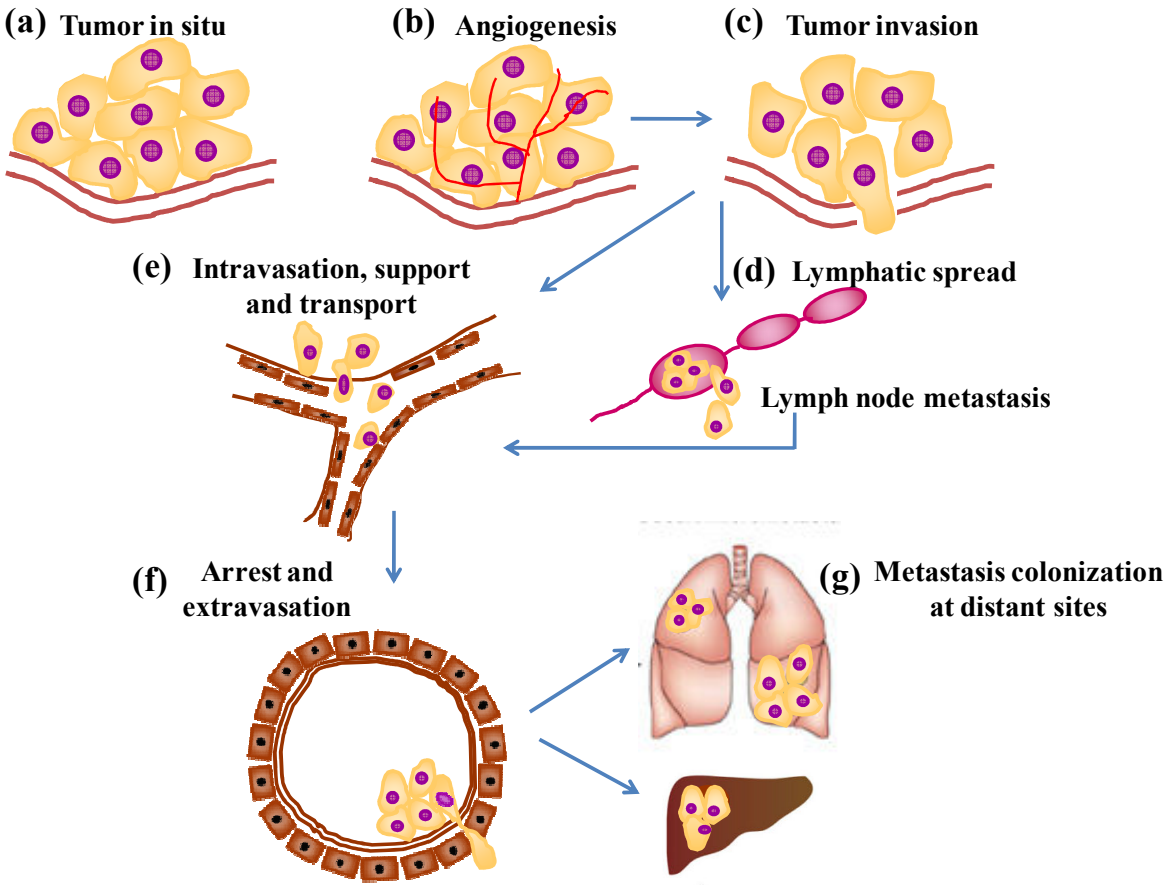


Figure 1.8 Schematics demonstrating key steps during tumor invasion and metastasis. (a) Primary tumor is surrounded by an intact basement membrane. (b) Extensive angiogenesis is required for the survival of cancer cells. (c) Cancer cells invade through basement membrane by detaching themselves from neighbouring cells destructing extracellular matrix and basement membrane. (d) Metastasizing cells enter to neighbouring lymphatics, or (e) directly enter the circulation. (f) Tumor cells get transported to distant places through circulation, upon survival and arrest of tumour cells extravasation follows. (g) Metastatic colonization occurs at distant place from primary tumor site.

1.4.3Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) consists of a family of proteinases comprising of 28 enzymes, that are structurally similar. (Page-McCaw et al, 2007). MMPs belong to the metzincin superfamily, which are zinc dependent endopeptidases having a conserved

methionine reside in their active site (Stocker et al, 1995). Based on their substrate preference and domain organization, MMPs are grouped into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs and others. The MMP family shares a conserved domain structure, consisting of an auto-inhibitory prodomain, a catalytic domain and the haemopexin-like C-terminal domain. The pro-domain harbors a cryptic cysteine residue that keeps the enzyme catalytically inactive by interacing with the zinc in the active site. The catalytic domain is attached to the C-terminal domain by a flexible unstructured hinge or linker region, which is approximately 75 amino acids long. The C-terminal domain has a four-bladed β-propeller structure which provides a large flat surface, thought to be involved in protein-protein interactions. Presence of haemopexin-like C-terminal domain determines substrate specificity and is the site for interaction with TIMPs (tissue inhibitor of metalloproteinases). All MMPs are synthesized as inactive form (zymogen) and need extracellular signals for their activation. The pro-domain is removed or destabilized in order for MMPs to recognize the substrate and cleave. All MMPs consists of basic features that make up the minimal domain. Additionally, MMPs are further divided into eight structural groups (Figure 1.8). MMP7 and MMP26 consist of minimal domain structure. Among eight groups, five are secreted and three of them are bound in the plasma membrane, also known as the membrane-tethered MMPs (MT-MMPs) (Egeblad and Werb, 2002). MMP1, MMP3, MMP8, MMP10, MMP12, MMP13, MMP19, MMP20, MMP21, MMP27, and MMP28 harbour hemopexin domain that has been characterized as a mediator of protein-protein interaction, substrate recognition, activation of the enzyme, protease localization, internalization and degradation (Overall, 2002; Page-McCaw et al, 2007). MMP21 is the sole member that contains a vitronectin-like insert. MMP11 closely resembles MMP21 but contains a furin recognition motif between the prodomain and the catalytic motif that facilitates its intracellular activation by serine proteinases. MMP2 and MMP9 contain

fibronectin type II repeats present between the catalytic and zinc-containing domains that allows for gelatin substrate recognition. MT-MMPs are classified on the basis of their linkage with the plasma membrane. MMP14, MMP15, MMP16, and MMP24 are tethered to plasma membrane through a transmembrane domain, whereas, MMP17 and MMP25 are linked by a glycosylphosphatidylinositol (GPI) linkage. All MT-MMPs contain a furin-like recognition element. The type II transmembrane MMP has only one member, MMP23, which contains an N-terminal signal anchor that targets it to the cell membrane, a cysteine array and immunoglobulin (Ig)-like domain (Egeblad and Werb, 2002) (Figure 1.8).

Collectively, MMPs are able to degrade almost all extracellular membrane (ECM) components. A major protein component of the basement membranes, type IV collagen, has been shown to be degraded by MMP2 and MMP9 in several studies. Nevertheless, MMPs target several non-ECM proteins as well. MMPs enhance cell proliferation by releasing heparin binding epithelial growth factor, insulin-like growth factor, and fibroblast growth factor from the cell surface. Conversely, by releasing and activation of ECM sequestered TGFß by MMPs can lead to inhibition of cell proliferation (Yu and Stamenkovic, 2000). Likewise, MMPs play contrary roles in cell survival mechanism through release of Fas ligand from the cell surface, which can either enhance or interfere with the cell survival. MMP14 and MMP1 have also been implied in enhancing cancer cell migration. MMP14 has shown to be crucial for tumor cell invasion in most *in vitro* and *in vivo* studies (Rowe and Weiss, 2009). It has been implicated in driving single-cell and subsequent collective cell migration and invasion (Wolf et al, 2007). Subsequent studies identified a set of three membrane anchored proteases, MMP14, MMP15, and MMP16 responsible for invasive pseudopodia, and transmigration (Hotary et al, 2006). High expression of MMP-10 is significantly correlated with the invasiveness and metastasis in head and neck squamous cell carcinoma (HNSCC) cases. In support of this, ectopic overexpression of MMP-10 favoured the invasion

of HNSCC cells, and conversely, knockdown of MMP-10 suppressed the invasion (Deraz et al, 2011). Furthermore, MMP9 dependent tumorigenic activities like migration, invasion, and angiogenesis of breast carcinoma cells are dependent on cholesterol levels in lipid rafts (Raghu et al, 2010). E-cadherin, a key adhesion protein, which is lost during onset of EMT is cleaved by MMP3 and MMP7, driving cells towards EMT and subsequently invasion and distant metastasis (Noe et al, 2001). MMPs inhibitors reduce angiogenesis (Martin et al, 1999; Li et al, 2001; Gatto et al, 1999) signifying their role in vasculature. MMP2-deficient mice have been shown to downregulate angiogenesis process (Itoh et al, 1998). MMP9 enhances the bioavailability of VEGF and thus, triggers angiogenesis (Bergers et al, 2000). Consistently, MMP9 and MMP14 null mice exhibit impaired vessels formation during development (Vu et al, 1998; Zhou et al, 2000). MMPs are capable of cleaving plasminogen to generate angiostatin (Dong et al, 1997) which possess potent antiangiogenic and antitumor activities (O'Reily et al, 1994; O'Reily, 1997; Kim et al, 2000). Taken together, the MMP proteins can regulate multiple processes, leading to tumor initiation and progression of cancer.

Interestingly, most of the MMPs in tumors are produced by neighbouring stromal cells rather than the cancer cells themselves. Cancer cells stimulate fibroblasts by producing a cell surface glycoprotein, extracellular matrix metalloproteinase inducer (EMMPRIN), to produce MMP1, 2, 3, and MMP14 (Yan et al, 2005). MMPs are regulated at both transcriptional and translational levels. MMP's promoters have been validated to reveal a variety of functional cis-elements including AP1, Sp1, Sp3 and other GC binding proteins (Clark et al, 2008; Angel et al, 1987). MMP14 has a proximal Sp1 site, deletion of which, reduced promoter activity indicating crucial role of Sp1 in driving MMP14 transcription. (Lohi et al, 2000). MMP genes including MMP9 (He, 1996; Bond et al, 1998) and MMP1 (Vincenti and Brinckerhoff, 2007) are reported to regulated by NFkB transcription factor. Additionally,

recent studies have indicated a novel role for E2F transcription factors in regulation of few MMPs namely, MMP2, MMP9, MMP14, and MMP15. It was also reported that 23 human MMP family members contain putative E2F binding sites (Johnson et al, 2011). The most studied endogenous MMP inhibitors are the tissue inhibitor of metalloproteinases (TIMP) family. TIMPs show tissue specific expression and the increased levels of TIMP1 and TIMP2 have been identified in malignant stromal tissue. In addition, MMPs are regulated through zymogen activation, inhibition of active enzymes by specific inhibitors, localization within or outside the cell and finally through degradation by proteases (Clark et al, 2008).

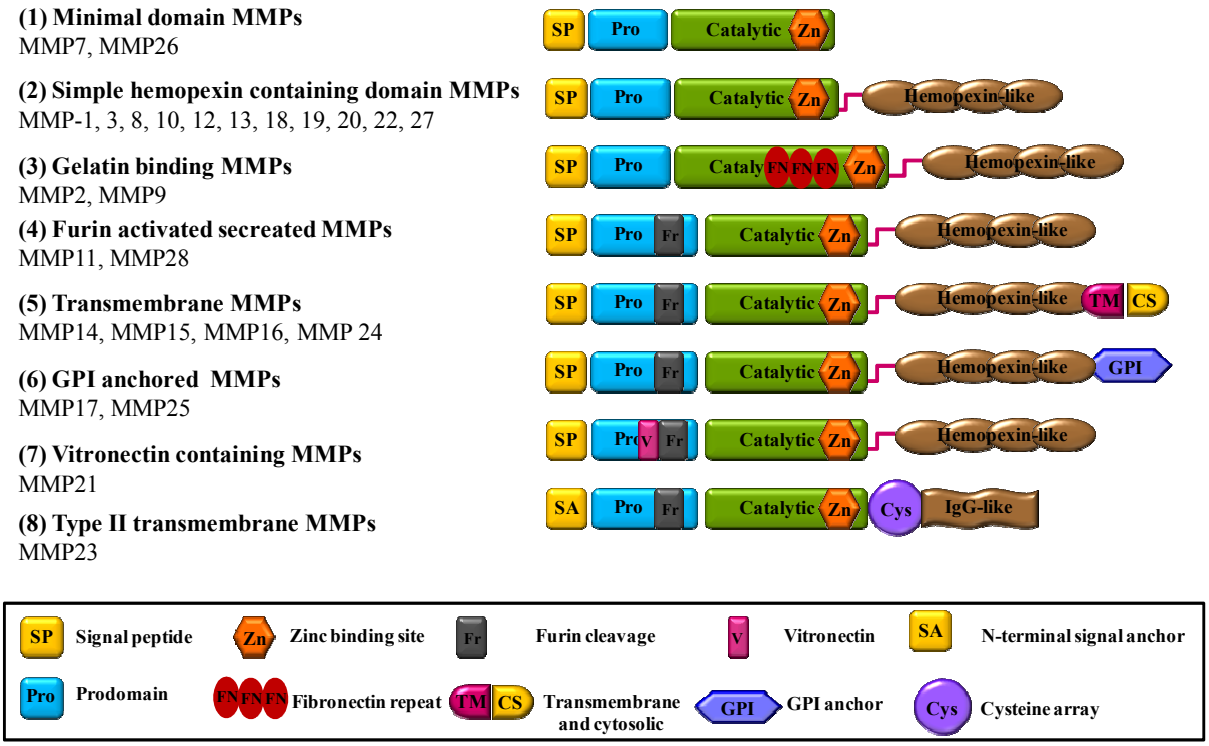


Figure 1.9 Different classes of MMPs. Domain structure of mammalian MMPs has been shown. All MMPs possess minimal domain consisting of signal peptide, a prodomain and zinc binding site containing catalytic domain. MMP23 contains a B-terminal signal anchor in place of signal peptide. Except for minimal domain containing MMP7 and MMP26, other MMPs have extra domain composition as illustrated in the figure.

1.4.4 Cancer cell nuclear structure and genome

Altered nuclear architecture: Tumor cells have abnormal nuclear structure, which includes change in nuclear size and shape, chromatin texture and organization, number and size of nucleoli. Nuclear structure and its spatial arrangement are largely maintained by nuclear matrix. The nuclear matrix is a ribonucleoprotein network consists of large number of proteins and RNAs. Changes in nuclear matrix protein composition affect nuclear functions like DNA organisation, gene expression, DNA replication etc and are implicated as tumor markers (Coffey, 2002; Nickerson, 1998). Some of the nuclear matrix proteins exihibit tumor specific expression, such as PC1, which is found only in nuclear matrix of prostate tumor cells (Partin et al, 1997). An abundant nuclear matrix protein, nuclear mitotic apparatus protein (NUMA) fuses with retinoic acid receptor-α (RARα) in acute promyelocytic leukaemia (APL) and exihibits similar oncogenic effects in transgenic mice (Sukhai et al, 2004).

Nuclear lamina, is a proteinaceous layer inside the nuclear envelope made of lamins and associated proteins. Lamin proteins are often mutated or deleted in several human degenerative diseases and cancers (Ostlund and Worman, 2003; Mounkes et al, 2003; Broers et al, 1993). Alteration in lamin function and structure causes change in nuclear shape in these diseases. One possible explanation for the association of lamin dysfunction or altered nuclear shape in malignant transformation could be impacting lamina-chromatin domains interaction at nuclear periphery. In the most cell types, there is transcriptionally silent heterochromatin at nuclear periphery called as the perinuclear heterochromatin (Brown et al, 1997). Lamins interact with the chromatin directly or through other proteins (Zastrow et al, 2004; Lee et al, 2001). Nuclear shape alterations are often associated with change in the

heterochromatin organization as seen in several carcinomas. Possibly, changes in nuclear lamina and shape alter spatial chromatin organization and gene positioning thereby, affecting gene expression pattern, which favours transformation.

Nucleoli, the largest structure in the nucleus, are often altered in tumor cells. Specifically, nucleoli become larger and prominent (Frost, 1986). For example, bigger nucleoli are diagnostic marker of large cell lung carcinoma. Large nucleoli are partly explained by higher cell proliferation rate occurring during cancer manifestation. Nevertheless, exceptions of inconspicuous nuclei are found in rapidly growing cervical intraepithelial tumor. Recent evidences suggest cancer nucleolus as hub of several tumor suppressors and oncoproteins such as p53, MDM2, p19, B23 and MYC (Zaidi, 2007; Derenzini, 2009). Tumor suppressors like p53 and Rb act as repressors of ribosomal genes (Zhai and Comai, 2009; Budde and Grummt, 1999; Voit et al, 1997). In case of loss of activity of p53 and Rb proteins in several cancers, there is loss of control to the overexpression of ribosomal genes thereby causing nucleolar enlargement. There is emergence of perinucleolar compartment (PNC), closely associated with nucleolus in transformed cells. PNCs are rarely seen in primary cells. PNCs vary in size and its prevalence is correlated with progression of breast cancer (Wang, 2003). PNC formation might be related to nucleolar changes seen during malignancies as evident from its close association with nucleolus.

The promyelocytic leukaemia (PML) body is yet another nuclear structure found altered in several cancers. PML bodies are most extensively studied in APL patients where loss of PML bodies occurs due to formation of PML-RARα fusion protein (Koken et al, 1994; Dyck et al, 2004; Daniel et al, 1993; Weis et al, 1994). PML expression is altered in solid tumors as well. Where overexpression of PML is seen in hepatocellular carcinoma, larynx and thyroid carcinoma, kaposi's carcinoma and other epithelial carcinomas (Terris et al, 1995; Chan et al, 1998; Koken et al, 1995), loss of PML expression is observed in central nervous system

(CNS) carcinoma, germ cell tumors, prostate adenocarcinoma, colon adenocarcinoma, breast carcinoma, lung carcinoma etc (Gambacorta et al, 1996; Gurrieri et al, 2004; Zhang et al, 2000). Loss of PML expression has been correlated with invasiveness of the malignancies.

Abnormal nuclear chromatin: Spatial organization of chromosome territories when compared in normal and cancer cell lines, no gross alterations in the spatial organization of chromatin domains were observed. The difference in the radial distribution of gene poor chromosome 18 and gene rich chromosome 19 chromatin, is found in both normal and malignant cell types except for partial loss of radial chromatin order in tumor cell nuclei. In majority of tumor cell lines, a higher fraction of nuclei with an inverted position of chromatin territory (CT) #18- and CT #19-specific material, i.e., a CT #18 located more interior than a CT #19 was observed (Cremer et al, 2003). Moreover, chromosomal translocation associated malignancies rely on close proximity of the chromosomal loci involved, emphasizing detrimental role of genome spatial organization in such cancers.

The change in chromatin texture in tumor cells is of diagnostic importance. Tumor cells are seen with two important chromatin configurations namely chromatin coarsening and exaggerated open chromatin, which corresponds to increase or decrease of heterochromatin aggregates respectively. Chromatin coarsening is characterized by larger and angular aggregates of heterochromatin shown to be induced by activated HRAS (Fischer et al, 1998). Coarsening of the chromatin correlates with the increasing metastatic potential of the tumor. Exaggerated open chromatin characterized by loss of heterochromatin aggregates is typical of several tumor types. Such chromatin alteration is shown to be mediated by expression of RET/PTC oncogene, also known to alter nuclear shape (Fischer et al, 1998). The mechanisms through which HRAS and RET/PTC oncogenes operate is unknown but are predicted to modulate epigenetic machineries. Figure 1.9 represents the differences in nuclear structure between normal and cancer cell and lists out common chromosomal aberrations found in

cancers. Table 1.1 summarizes altered nuclear characteristics and their association with respective cancers.

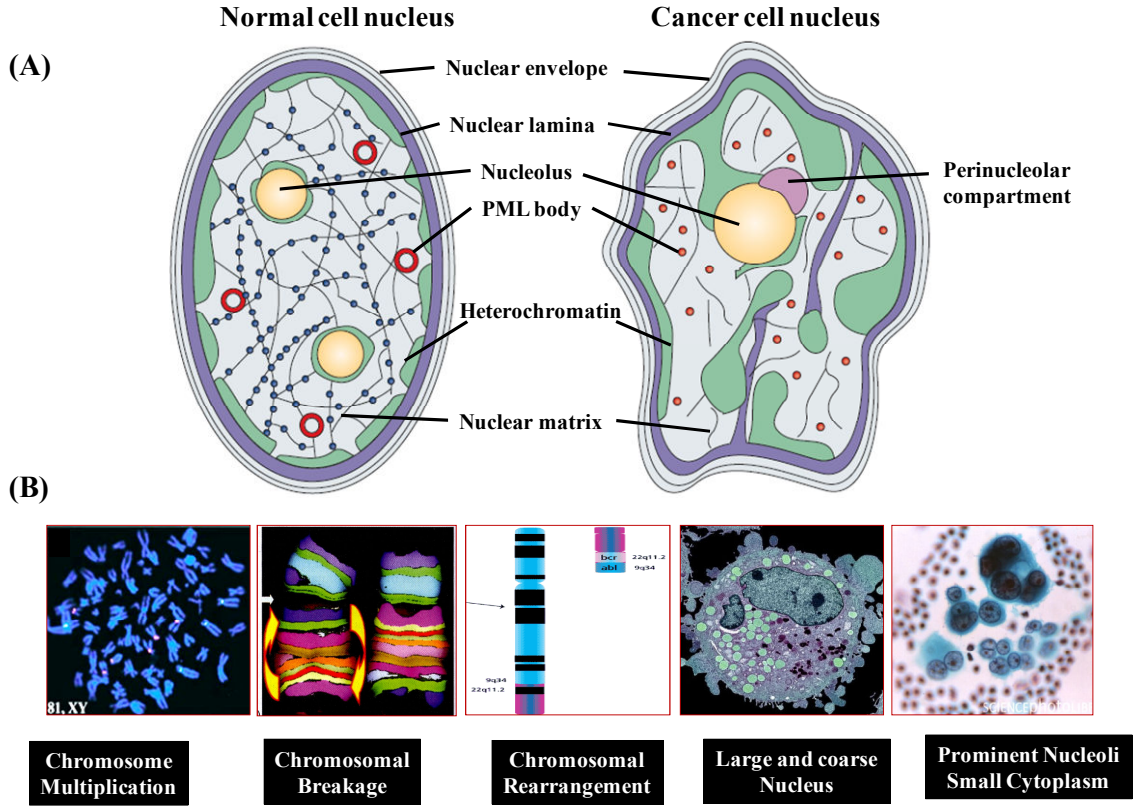


Figure 1.10 Altered nuclear structure and chromatin aberration of a cancer cell. (A) Comparison of nuclear structure and substructures between a normal and cancer nucleus. Nuclear shape, lamina and heterochromatin distribution become irregular in a cancer cell. Nucleolous is generally enlarged and prominent with appearance of perinucleolar compartment in cancerous state. Composition of nuclear matrix and PML bodies is altered in cancer (Taken from Zink et al, 2004). (B) Cancer cell suffers from genetic instabilities as exemplified here by indicated chromatin aberrations in a cancer cell.

Nuclear characteristic	**Cancer type**
Alterations in nuclear shape	
Pliable nuclei with frequent crush artifacts	Small-cell lung carcinoma
Furrows and long crevices	Papillary thyroid carcinoma; Langerhans-cell histiocytosis; urothelial tumours, including Brenner tumour of the ovary; granulosa-cell tumour of ovary; follicular lymphomas

Multiple lobes (nucleus subdivided into several lobes)	Some adenocarcinomas
Folded, wavy, indentations	Many kinds of cancer
Changes in Chromatin	
Coarse chromatin (Granular, aggregates of heterochromatin)	Many forms of cancer and precancerous changes, including follicular thyroid adenoma
Irregular aggregates of heterochromatin	Wide range of cancers
Dispersed heterochromatin	Small-cell lung carcinoma
Loss of heterochromatin aggregates	Papillary thyroid carcinoma; wide range of other carcinomas
Nucleolar alterations	
Larger nucleoli	Wide range of cancers
Inconspicuous (obscure) nucleoli	Precancerous squamous lesions of the cervix; small-cell anaplastic lung carcinoma
Change in number and size of nucleoli	Wide range of cancers

Table 1.2 Altered nuclear characteristics and their association with cancers (Taken from Zink et al, 2004).

Genome instability and mutation: Tumor development involves acquiring of several unique characters in cancer cells, violating or modifying regulators of cellular processes and corrupting the existing system of the normal cell. Such an accomplishment largely depends on guidance from the cancer cell genome. Certain mutant genotypes confer selective advantage to cancer cells enabling them to grow and thrive against all the barriers posed by tissue microenvironment. For e.g mutation of TP53 helps cancer cells to compromise the surveillance system of genomic integrity thus bypassing the senescence or apoptosis of genetically damaged cells (Jackson and Bartek, 2009; Sigal and Rotter, 2000). Regulation of tumor suppressors involved in DNA maintenance activities also occur through non-

mutational changes employing epigenetic mechanisms (DNA methylation and histone modifications) (Berdasco and Esteller, 2012; Esteller, 2007; Jones and Baylin, 2007). Cancer cells accumulate mutations through enhanced sensitivity to mutagenic agents and/or compromising one to several components of genomic maintenance machinery. In addition to acquiring mutations, cancer genome reveals immense instabilities in terms of altered gene copy number, loss of telomeric DNA, karyotypic instabilities involving chromosomal amplification, deletion and rearrangement (Artandi and DePinho, 2010). Recent findings have revealed that somatic mutation rates in cancer cells are highest in heterochromatic regions (as observed by H3K9me3, H3K9me2 and H4K20me3 heterochromatic marks) and lowest in accessible euchromatic regions (Bockler and Lehner, 2012).Such an association was consistent with diverse tissue type, mutation type and genomic regions with diverse gene densities strengthing the emerging concept of chromatin organization as determinant of regional mutation rates in cancer cells.

1.5 Aim and scope of the study

The eukaryotic genome is organized into a highly complex nucleoprotein structure, chromatin. Chromatin is highly ordered, compact and dynamic in nature which is comprised of DNA, histones, several nonhistone proteins and RNAs. The dynamicity of chromatin is key to the essential cellular processes like transcription, replication and repair. Hence, there are regulatory machineries which facilitate dynamic folding and unfolding of chromatinised genome. The dynamic chromatin organization is regulated by different groups of factors: (i) histone and nonhistone modifying enzymes; (ii) ATP-dependent chromatin remodeling enzymes and (iii) histone chaperons. Chromatin modifying enzymes bring about modification of histone tails as well as other chromatin components like nonhistone chromatin associated proteins (CAPs) and DNA, thereby fine tune the process of gene expression intricately. Although, the primary nucleosomal structure of chromatin, bead on a string is composed of DNA, wrapped around a core histone octamer, a diverse group of nonhistone proteins dynamically interact with the nucleosomal histones and DNA and confer the essential fluidity to the chromatin. This facilitates all the DNA template phenomena in the cell. These proteins are post-translationally modified by different enzymes which add further diversity and regulation in their function. Human transcriptional coactivators, PC4 was found to be one of the highly abundant chromatin associated protein which play a significant role in the chromatin compaction as well as gene expression (Das C et al. 2006, Das C et al., 2010). It is involved in the diverse DNA-templated phenomena including transcription, replication and repair. PC4 is known to function as a transcriptional coactivator having mostly positive and a few examples of negative regulatory functions. PC4 is found to be post-translatioanlly modified with distinct functional consequences. Phosphorylation of PC4 at N-terminal SEAC domain mediated by Casein Kinase II (CKII) is found to inhibit its transcription coactivation ability presumably due to the inhibition of DNA binding by the coactivator. There are seven

CKII phosphorylation sites 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. PC4 gets acetylated in vitro by the lysine acetyl transferase p300 (KAT3B). The acetylation of PC4 enhances its double stranded DNA binding as well as DNA bending ability. (Kumar B R et al. 2001; Batta K and Kundu T K 2007). Phosphorylation of PC4 inhibits its acetylation; however, acetylation does not affect the phosphorylation *in vitro*. Presumably, phosphorylation and acetylation of PC4 serve as distinct functional signals.

At this juncture of understanding of PC4 functions, a mechanistic question was raised regarding the dual functions of PC4: an organizer of the chromatin and a transcriptional coactivator. We hypothesized that the post-translational modifications of PC4, namely phosphorylation and acetylation should be operating the switch of these two opposite functions in terms of chromatin dynamics. We were also interested to investigate the requirement of both these functions of PC4 in cellular physiology by knocking down PC4 gene in a stable cell line model. The drastic features of these stable cell line suggested that PC4 might be essential for the maintenance of cellular integrity and tumor suppression. When we investigated the breast cancer samples, it was found that indeed in majority of the cases, PC4 was downregulated. An effort has been made to understand the link between cellular functions of PC4 and its role in breast cancer manifestation. The summary of these three chapters of the thesis has been described in the following paragraphs.

1. Post translational modifications switch of PC4 functions

PC4 is present in the nucleus and is associated with the chromatin. It localizes to the all chromosomes arms in punctuate manner except for the centromere. PC4 interacts with core-histones and show preferential specificity towards H3 and H2B *in vitro*. It was shown that by virtue of its interaction with core-histones, it compacts the chromatin. siRNA knockdown of

PC4 led to decondensation of chromatin in the nucleus. On the other hand, PC4 has been shown as transcriptional coactivator of RNA polymerase II transcription through its ability to interact with general transcriptional factors (specifically to TFIID-TFIIA) and wide variety of activators. Its role as coactivators for p53 system has been well established in cellular system (Banerjee S et al., 2004; Batta K and Kundu T K 2007). Present thesis has dealt with mechanism of dual functions of PC4. It has been found that PC4 gets predominately acetylated by p300 (KAT3B) and acetylation site mapping showed lysines 53 and 68 to be preferred acetylating sites for p300. Acetylated PC4 specific polyclonal antibodies were generated and used as tool to investigate *in vivo* scenario of PC4 acetylation. Cellular localisation studies using these antibodies revealed uneven occupany of acetylated PC4 in the chromatin. The acetylated PC4 was not detected in chromatin during mitosis. Reversibilty of the acetylation of PC4 was explored and it was found that SIRT2, a class III HDAC member, deacetylates PC4. Role of PC4 acetylation in transcription was investigated utilizing p53 dependent transcription system. Lack of acetylation significantly compromised the transcriptional activation ability in the given context, whereas lack of phosphorylation did not show any significant change. Phosphorylated PC4 on the other hand showed enhanced core-histones interation and thus better chromatin compaction ability *in vitro* than unmodified or acetylated PC4. Furthermore, PC4 could interact with linker histone H1 only when it was phosphorylated. Taken together, these data suggest that acetylated PC4 is the predominant form involved in transcription regulation whereas phosphorylated PC4 through its interaction with core-histones and linker histones is involved in the chromatin condensation.

2. Role of PC4 in maintenance of chromatin architecture

In order to investigate the physiological implications of PC4 chromatin compaction ability, PC4 knockdown stable cell line was generated. Studies done with the knockdown cell line revealed altered nuclear architecture in absence of PC4. Knockdown cells exhibited increased

transcriptional activation associated histone modification marks. PC4 knockdown also led to the loss of distinct heterochromatin foci which indicated more loose or decondensed chromatin. Functional implications of PC4 knockdown were scored in terms of cell division. It was found that PC4 knockdown induces cell cycle defects at different levels. Interestingly, PC4 knockdown cells showed increased tendency towards tumorigenicity.

3. Role of PC4 in breast cancer manifestation

Chromatin organizational irregularities have been reported in several pathological conditions. Cancer is one of the conditions which are correlated with abnormal chromatin architecture. Cancer is often associated with variable and complex genome constitution and reported to have genomic instabilities. The fact, PC4 being important player in maintenance of chromatin architecture tempted us to explore its role in a cancer model. We examined PC4 expression in breast cancer samples. Majority of samples examined showed significant decrease in PC4 expression both at protein and transcript level. The down regulation was observed in cases irrespective of grades, stages and receptor types present. Breast cancer cell lines based *in vitro* experiments revealed that PC4 knockdown led to upregulation of different effector molecules like matrix metalloproteinases and mesenchymal markers, which in turn presumably, facilitate the tumor progression.

In conclusion, this research work has led to the elucidation of first acetylation-phosphorylation switch for the dual function of a chromatin protein, PC4. Studies using PC4 knockdown stable cell line reveal that PC4 is very essential for maintenance of chromatin architecture as events related to cellular homeostasis like chromosome segregation, cytokinesis, and heterochromatin formation were severely affected with PC4 silencing. Absence of PC4 induces pathological conditions as exemplified by breast cancer manifestation. Present study elicits several speculations which need to be addressed. It would

be interesting to explore acetylation of PC4 in p53 mediated transcription regulation under genotoxic insult. PC4 being an important factor controlling chromatin architecture, it is unknown how PC4 is involved in chromatin three dimensional folding and heterochromatin maintenance. Possible mechanisms, which cause downregulation of PC4 expression in breast cancer are yet to be explored.

Chapter 2

MATERIALS AND METHODS

Chapter Outline:

2.1 General Methods

2.2 Cloning

2.3 Protein/Enzyme purification

2.4 Protocols for different assays and analysis

2.1 General Methods

2.1.1Preparation of bacterial competent cells

E.coli strains BL21 and DH5α were inoculated overnight from respective frozen glycerol stocks in 5 ml Luria Broth (LB; Himedia) media. The overnight culture was streaked on fresh LB-agar plate and incubated at 37°C for 8-10 hours. Single colony from the plate was inoculated in 500 ml of medium A (10 mM $MgSO_4$ and 11 mM glucose in LB) and was grown in 37°C shaker incubator till OD_{600} reached 0.3. Culture was cooled and pelleted at 4°C, 2000 rpm for 10 mins. Pellet was resuspended in 5 ml ice cold medium A and again centrifuged for 10 mins.Finally, pellet was resuspended in 25 ml of storage buffer B (36% glycerol, 12 mM $MgCl_2$, 12% PEG-8000) and 100 µl of cell suspension aliquots were stored at -80°C.

2.1.2 Transformation

Bacterial competent cells were thawed on ice for 10 mins. Approximately 100 ng of plasmid DNA was added to it and was further incubated on ice for 30 mins. A brief heat shock at 42°C was given for 90 seconds and cells were kept in ice for 5 mins. 1 ml LB was added and cells were allowed to grow in 37°C incubator shaker for 45mins-1 hour. Cells were pelleted down and plated on LB agar plates with suitable antibiotic for selection. Plated LB agar plates were kept in 37°C incubator for 8-10 hours.

2.1.3 DNA isolation

Plasmid DNA extraction was done using sigma mini and maxi prep kits according to manufacturer's protocols. Briefly, overnight grown culture was pelleted and resuspended till homogeneity in resuspension buffer. Alkaline lysis buffer was utilized to lyse the cells followed by neutralising the solution. Supernatant obtained was allowed to bind to the column through centrifugation. Following two washes of the column, bound DNA was eluted using TE (10 mMTris-Cl, pH 8.0 and 1 mM EDTA) buffer or water.Plasmid was run in agarose gel to assess its quality ((Figure 2.1).For DNA purification from agarose gel, sigma gel extraction kit was used and protocols were followed as provided by manufacturers.

(A) (B)

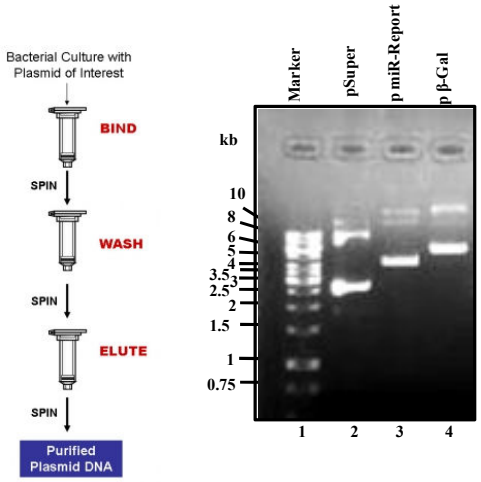


Figure 2.1Plasmid mini prep. (A) Flowchart showing important steps involved in column based plasmid mini prep kit. (B) Ethidium bromide stained 0.8% agarose gel showing plasmid bands isolated and purified from bacterial culture transformed with pSuper.puro, pMIR-REPORT^{TM} Luciferase and pMIR-REPORT^{TM}β- gal control plasmids respectively. These plasmids were further used for cloning miRNAs and PC4 UTR.

2.1.4 Total RNA extraction

Mammalian cells were harvested from the culture dishes by scraping or trypsanisation. Cell pellet was washed with 1X PBS (172 mMNaCl, 2.7 mMKCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4)followed by homogenous resuspension in TRIZOL (Invitrogen) reagent at a ratio of 1 mL per 10 million cells. Cell suspension was centrifuged at 12000 rpm, 4°C for 10 mins. The supernatant was subjected to chloroform extraction thrice and aqueous layer was precipitated by equal volume of isopropanol. Precipitated pellet was resuspended in 70 µLDEPC treated water and was subjected to precipitation with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of isopropanol. Pellet was washed with 70% ethanol, air dried and dissolved in nuclease free water and visualised after running in 1% agarose gel (Figure 2.2).

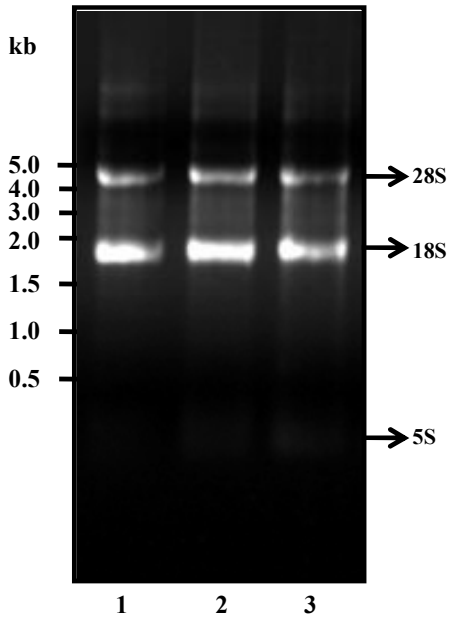


Figure 2.2Total RNA extraction. Ethidium bromide stained 1% agarose gel showing purification profile of total RNA extracted from mammalian HEK 293T cells. RNA was isolated upon p300 siRNA treatment to the cells and was subsequently used for real time PCR using p300 primers.

2.1.5 Whole cell extract preparation

Cells were harvested by scraping or trypsinizingfollowing 1X PBS wash. Pellet was resuspended in 5-10 times packed volumes of cell pellet inRIPA lysis buffer (50 mMTris-Cl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mMNaCl, 1 mM EDTA, 1 mMphenylmethylsulfonyl fluoride (PMSF), 1 µg/ml each of aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mMNaF) or TNN (50 mMTris-Cl, pH 7.4, 100 mMNaCl, 5 mM EDTA, 0.5% NP-40, 200 µM PMSF, 0.5 mMdithiothreitol (DTT), 1 µg/mleach of pepstatinand leupeptin) buffer. Cell suspension was incubated in ice for 3 hours with regular mixing followed by centrifugation at 10000 rpm, 4°C for 15 mins. Supernatant (lysate) was either used immediately or stored at -80°C.

Laemmli buffer: Cells were harvested and centrifuged at 6000 rpm for 5 mins at 4°C. Cell pellet was washed with cold PBS and resuspendedin 10 times packed volumes of cell pellet in laemmli buffer (0.125 mMTris-Cl, pH 6.8, 4% SDS, 20% Glycerol). Samples were boiled for 5 minutes at 90°C and allowed to cool till they attained room temperature. They were then subjected to brief sonication. 5X SDS loading dye was added to samples and boiled for 5 more minutes. These lysates were later used for western blotting.

2.1.6 Nucleic acid estimation

Nucleic acid concentration estimation was done spectrophotometrically by measuring the absorbance at 260 nm wavelength. The concentration was calculated using Beer-Lambert law: A = a(lambda) * b * c

Where, A is the measured absorbance, a(lambda) is a wavelength-dependent absorptivity coefficient, b is the path length, and c is the analyte concentration.

So, concentration calculation: $C = A_{260} X \theta$. C is the concentration of nucleic acid in ng/µl, θ value for DNA is 50 ng/µl, oligonucleotide is 33 ng/µland in case of RNA θ is 40 ng/µl. Furthermore, purity of the samples was verified by A_{260}/ A_{280} ratios.

2.1.7 Estimation of proteins concentration

Biorad protein estimation reagent was used to estimate protein concentration in whole cell lysate using manufacturer's protocols. Known concentrations of BSA were used for plotting standard curve. A linear curve generated by absorbance values of known BSA concentrations was employed to assign concentration values to unknown samples. Recombinant proteins concentration was also estimated by running increasing concentrations of BSA along with protein of interest on SDS PAGE gel.

2.1.8 cDNA synthesis

1 µg of total RNA was used for cDNA synthesis with initial 12 µl reaction volume containing 40 pmoles of oligodT and water. The reaction mixture was 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 0.5 mMdNTP mix, 4 µL 5X first strand synthesis buffer and 1 µL superscript RT enzyme (Invitrogen). Reaction was carried out at 37°C for 50 mins followed by enzyme inactivationat 84-90°C for 15 mins. The cDNA was subsequently used for real time PCR using gene-specific primersand was stored at -20°C.

2.1.9 Western blot analysis

The proteins resolved in a SDS-PAGE gel was incubated for a period of 15 mins in transfer buffer (25 mMTris, 192 mM Glycine, 0.038% SDS and 20% MeOH) along with the methanol activated PVDF membrane of same dimensions as of gel. Proteins were then transferred to the membrane using a semidry western transfer apparatus (Biorad) at 25 V, for appropriate period of time depending upon mobility of the protein on SDS PAGE gel. After transfer, blot was blocked in 5% skimmed milk solution or 3% BSA in PBS for overnight at 4°C or at room temperature for 1 hour.The blot was incubated with primary antibody solution (diluted in 2.5 % milk solution or 1% BSA) for a period of 3 hrs at 4°C (or overnight depending upon the affinity of the antibodies used). Blot was subjected to washing with PBS containing 0.05% tween20. Appropriate HRP conjugated secondary antibody solution was then addedfor a period of 3 hrs at 4°C. After washing of blots, the blot was developed using the Pierce Super Signal West Pico Chemiluminiscence Kit, as per the manufacturer's protocol. The blots were exposed in TMS (Kodak X-Ray films), for different time points and developed using GBX-Developer-Fixer Kit (Premiere Kodak reagents) (Figure 2.3).

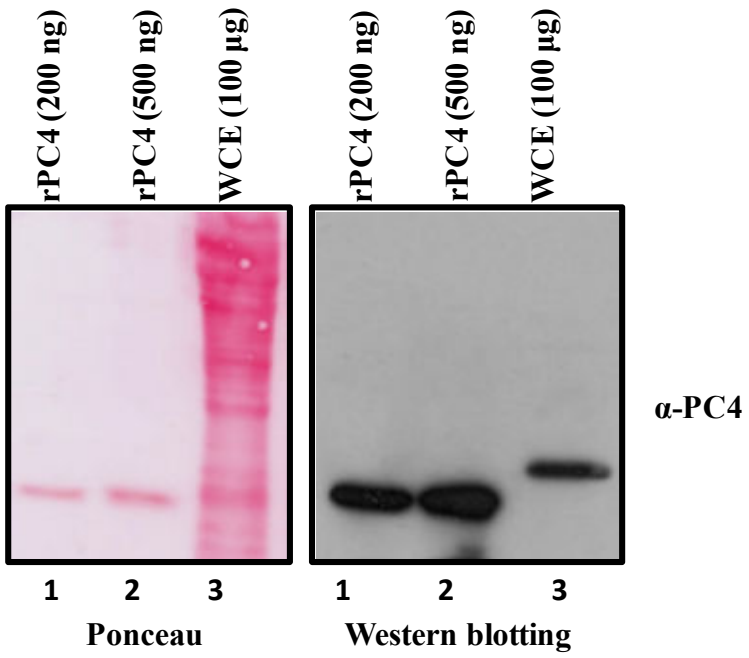


Figure 2.3 Western blot analysis. Ponceau stained PVDF membrane after transfer of recombinant PC4 and HeLa whole cell extract proteins. Same blot was probed with anti PC4 antibodies and HRP conjugated anti rabbit secondary antibodies. Chemiluminiscence was captured on X-ray films.

2.1.10 Real time PCRanalysis

1 µL of respective cDNA was used in PCR reaction mix containing specific gene primers and 2X SYBR green mix (Biorad). SYBR green mix contains SYBR green I dye and required PCR reagents like dNTPs, DNA polymerase and compatible buffers. Reaction tube was put in Corbett real time PCR machine and amplification protocols were followed as provided by manufacturers (Figure 2.4). PCR conditions were standardised for each set of gene primers used. Fold expression change was calculated using ΔΔCt method using either GAPDH or actin gene primers for normalization. Relative fold change = $2^{-\Delta\Delta Ct}$. Specificity and sensitivity of the primers was ascertained by melt curve analysis.

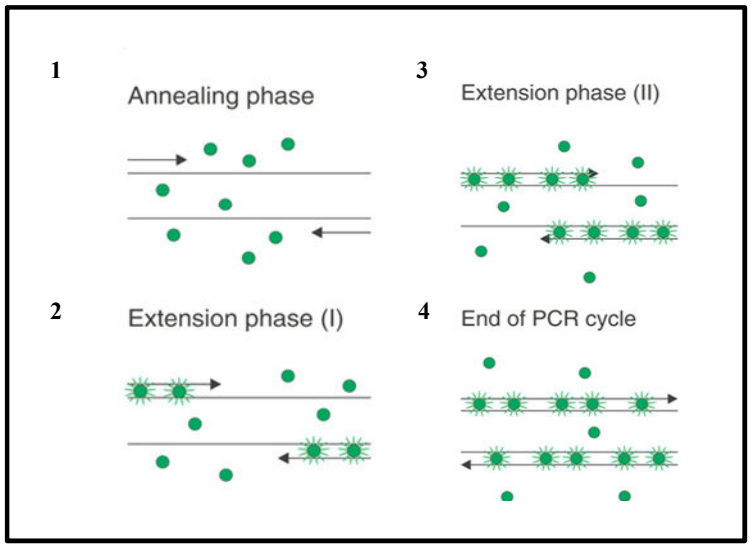


Figure 2.4 Real time PCR using SYBR green chemistry. SYBR Green I binds to double-stranded DNA thereby enhancing its fluorescence enormously. Following primers annealing to the template DNA strand, polymerase will add new dNTPs thus forming double stranded PCR product. Thus, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected. (Adapted from Van der Velden et al, 2003).

2.1.11 Mammalian cell culture

The human cervical carcinoma cell line, HeLa, embryonic kidney cell line HEK293 and 293T were grown in the 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% CO_2 supply and an 80% relative humidity of a CO_2 incubator. Cells were passaged using 0.005% Trypsin-EDTA (Sigma) for 1-3mins followed by inactivation using FBS.

MCF-7, HBL-100 and MDA-MB-231human breast cancer cell lines were cultured in Dulbecco modified eagle medium (DMEM, Cellgro) with 10% foetal bovine serum. The cells were grown at 37°C, 5% CO_2 and an 80% relative humidity in a CO_2 incubator.

2.1.12 Insect cell culture

The insect *Spodopterafrugiperda* 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. The cells were scrapped using a sterile cell scraper and subcultured in 1:3 ratio. These cells were infected with baculovirusescontaing proteins of interest and are subsequently harvested and used for protein purification (Figure 2.5).

(A) Sf21 Cells(B) Sf21 cells infected with p300 baculovirus

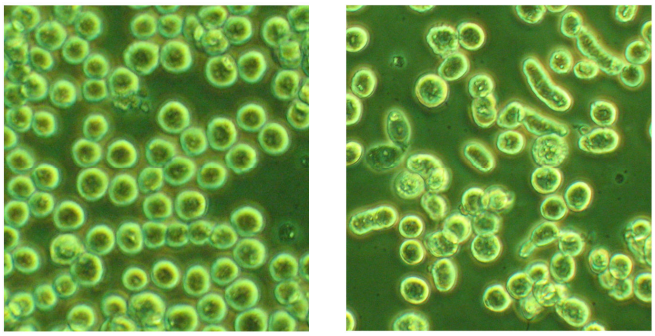


Figure 2.5 Insect cell culture: (A) The insect *Spodopterafrugiperda* ovarian cell line Sf21 grown in TC100 medium. (B) Image showing altered morphology of Sf21 cells upon baculovirus infection.

2.1.13 Agarose gel electrophoresis

Agarose gel electrophoresis was employed for detection, analysis and purification of DNA/RNA samples. Agarose (Sigma) powder (amount depending on percentage of gel required) was added to 1X TBE (0.09 M Tris borate and 0.002 M EDTA) solution and was dissolved by heating in a microwave oven. The nucleic acid samples were mixed with 6X loading buffer to make final concentration as 1X (for 1X- 0.25% Bromophenol Blue, 0.25% Xylene cyanol in 40% Sucrose), loaded on the gel and electrophoresed at 80V in 1X TBE.

Gels were stained in ethidium bromide (10 µg/100 ml water) with gentle rocking, followed by destaining in water. DNA/RNA was visualized in the long wavelength UV lamp, in a gel documentation system (Biorad).

2.1.14 Polyacrylamide gel electrophoresis (PAGE)

SDS-polyacrylamide gel electrophoresis: Proteins of different sizes were resolved according to their molecular weights in an SDS-PAGE. The gel consists of separating layer and the stacking layer. The separating (or resolving) gels were made with different percentages of acrylamide (mostly 30 or 40%), 0.375 M Tris-Cl (pH8.8), 0.1% SDS, 0.1% APS and 8% TEMED. The components of stacking gel included 5% acrylamide 0.375 M Tris-Cl (pH6.8), 0.1% SDS, 0.1% APS and 8% TEMED. Protein samples were heated with 5X SDS sample buffer (for 1X- 50 mMTris-HCl pH 6.8, 100 mM DTT, 0.1% Bromophenol blue, 10% Glycerol), at 90°C for 10 mins and loaded into gel. The gel was electrophoresed in Tris-glycine- electrophoresis buffer (25 mMTris, 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 CH_3COOH).

Native-polyacrylamide gel electrophoresis: Native PAGE was run,in order to visualize DNA-protein complexes. 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.

2.2 Cloning

2.2.1 Generation of acetylation defective point mutants (K-R/A) using PC4-His$_6$ as template in bacterial expressing vector.

Site directed mutagenesis technique was employed to create point mutations replacing lysine with arginine or alanine respectively. QuickChange II XL site directed mutagenesis kit (Stratagene) was used for this purpose. The schematic illustrating protocol of site directed mutagenesis is shown in figure 2.6. Briefly, mutagenic primers were designed and PCR was set up using these. PCR product was incubated with Dpn1 for 1 hour at 37°C to digest the original template DNA (wild type PC4-His$_6$). Transformation of the digested product was done. Colonies were screened for the desired mutation and confirmed by sequencing (Figure 2.7). Mutant PC4-His$_6$ was used as template further for generation of multiple lysine mutants.

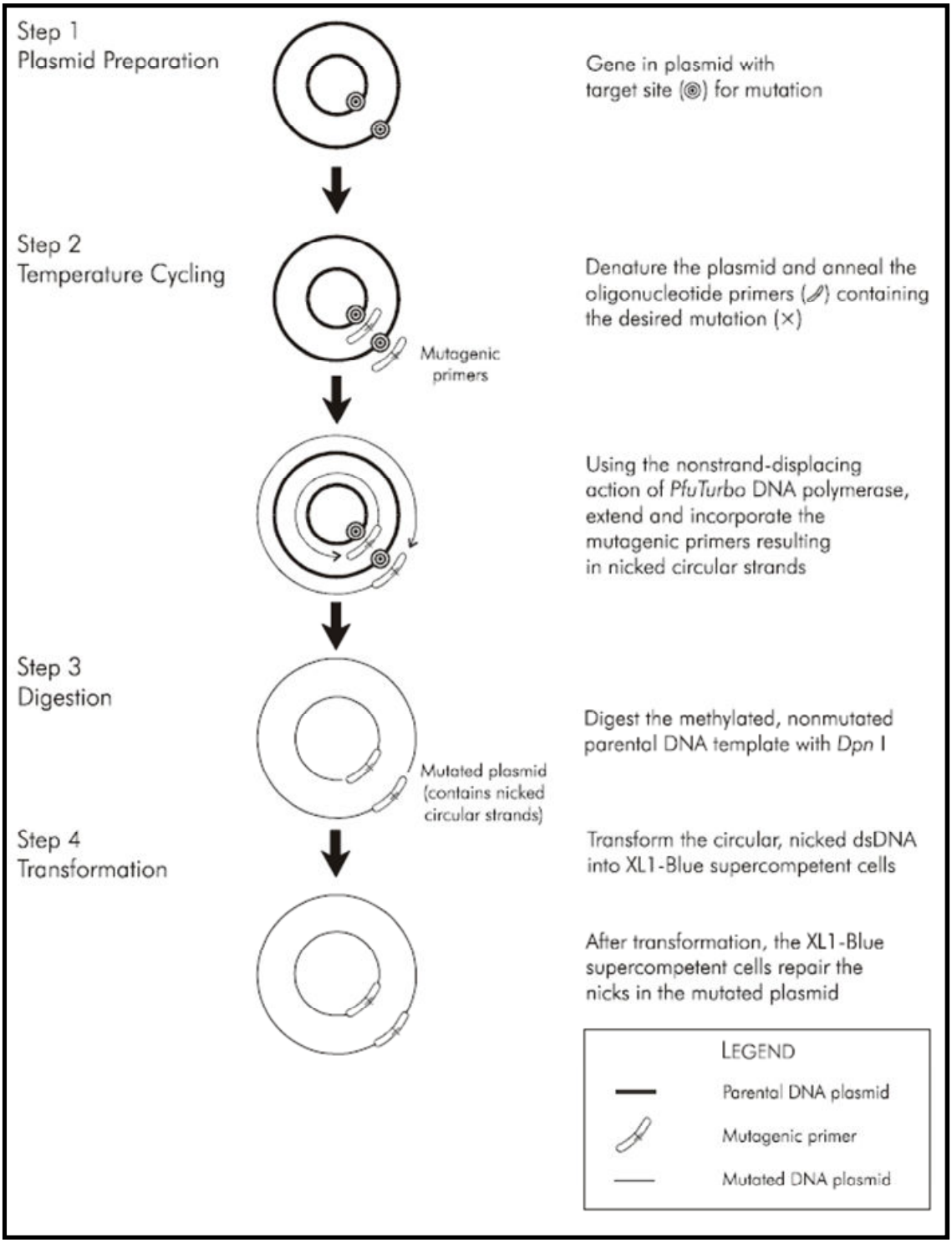


Figure 2.6 Overview of site directed mutagenesis method (Adapted from QuickChange II XL site directed mutagenesis kit, Stratagene).

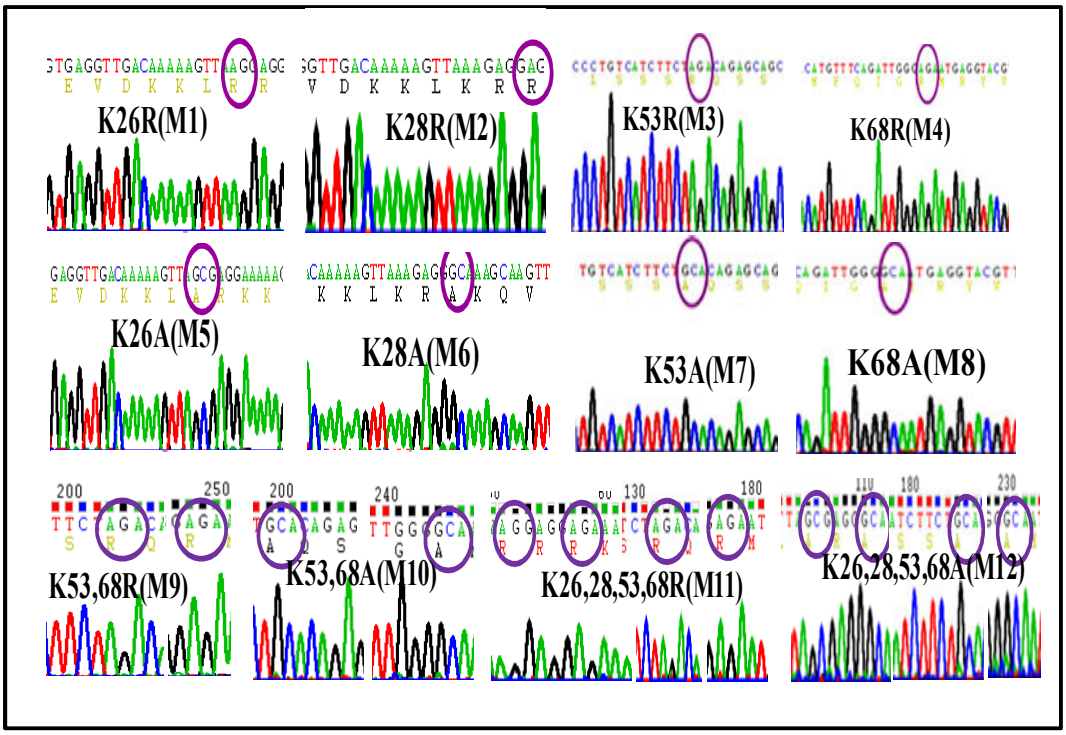


Figure 2.7 Chromatogram presentation of all lysine mutants of PC4 generated through site directed mutagenesis. Blue circle indicates the altered codon to obtain mutant amino acid.

2.2.2 Subcloning of acetylation defective clones in FLAG and GFP tagged mammalian expression vector.

Lysine mutants were purified (Discussed in next section) and checked for its ability to undergo acetylation. Mutants M9 (K53,68R), M11 (K26,28,53,68R) and M12 (K26,28,53,68A) were subcloned in pCMV-FLAG mammalian expression vector (Sigma) in such a way so that the translated proteins had FLAG epitope in their N terminus. Positive clones were confirmed by insert release upon restriction digestion as well as by sequencing. HindIII and SmaI restriction enzymes were used to release M9 insert whereas HindIII and XbaI were used for M11 and M12 (Figure 2.8).

Mutant M9 was also subcloned in pEGFP-N1 vector which results in the expression of GFP tagged protein in mammalian cells. Positive clone was confirmed by insert release using

XhoIand SmaI restriction enzymes as well as by sequencing.Expression of these mutants was also checked in mammalian cells (Figure 2.9).

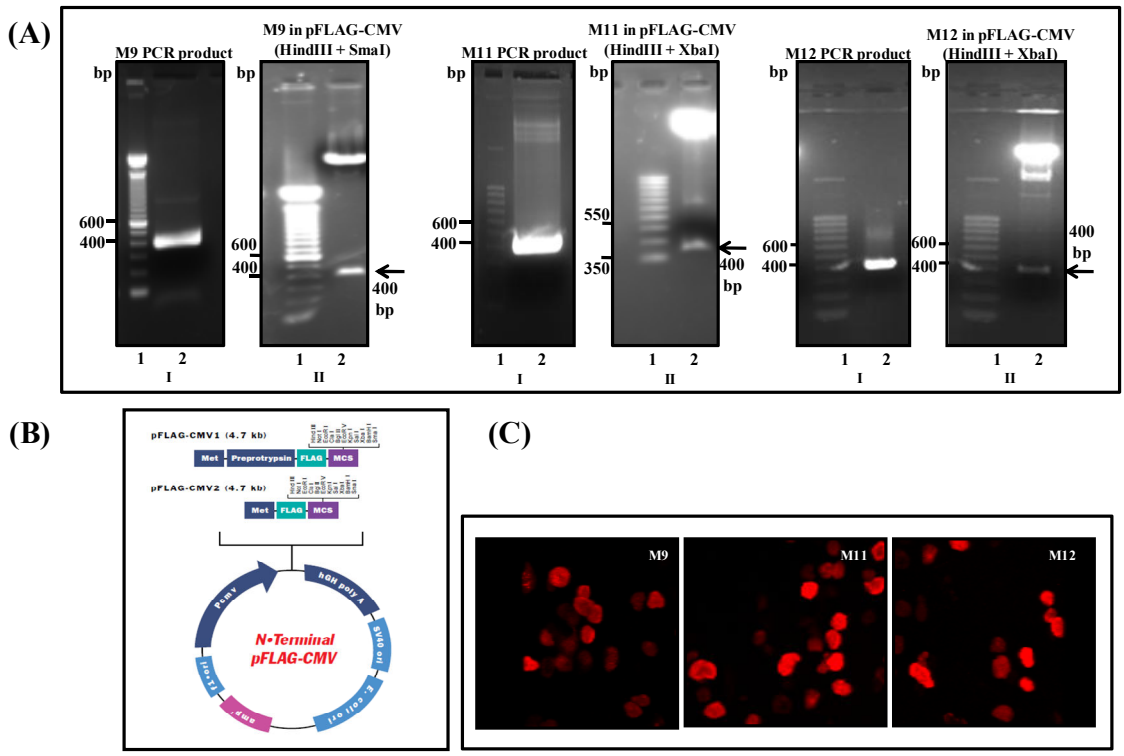


Figure 2.8Subcloning of acetylation defective clones in FLAG tagged mammalianexpression vector. (A) Agarose gels showing PCR amplicons of the indicated mutants to be cloned (Panel I). Positive clones were confirmed by insert release upon restriction digestion using restriction enzymes as indicated (Panel II). (B) Plasmid map of pFLAG-CMV into which subcloning was done. (C) Expression analysis of these subcloned mutants were performed by transfecting these in HEK293 cells followed by immunostaing with anti FLAAG antibodies.

(A) **(B)**

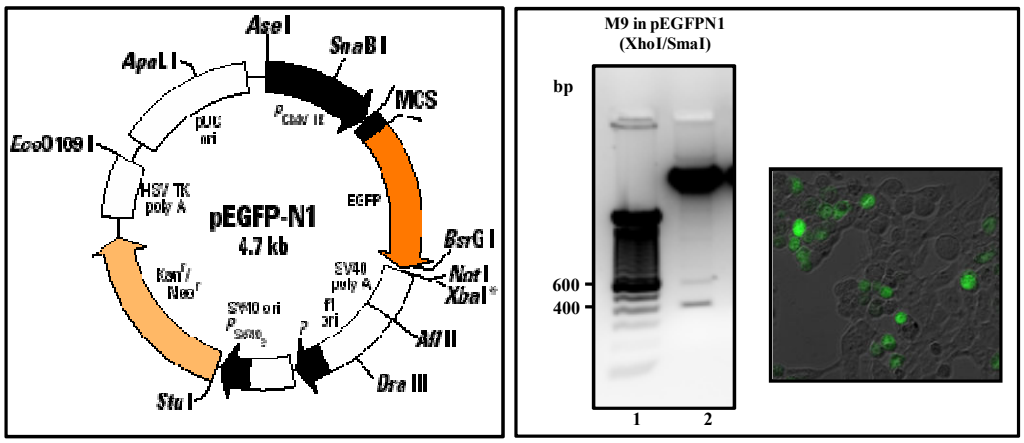


Figure 2.9Subcloning of acetylation defective clone M9 (K 53, 68 R) in GFP tagged mammalian expression vector.(A) Plasmid map of pEGFP-N1 in which mutant M9 was subcloned. (B) Clone confirmation was done by insert release upon restriction digestion using restriction enzymes as indicated (Lane 2). Expression verification of GFP tagged clone was done by transfecting clone into HEK293 cells followed by fluroscence imaging of GFP.

2.2.3 Generation of phosphorylation defective point mutants (S-A/G) of PC4.

Serine residue stretch of PC4 present at its N-terminus was mutated singly or in combination by utilizing site directed mutagenesis technique (QuickChange II XL site directed mutagenesis kit, Stratagene). Serine residues were replaced by either alanine or glycine to generate phosphorylation defective mutants (MTPs). Mutagenic primers were designed and utilized in PCR with wild type PC4 as template DNA. Thus,different combinations of serine mutants were generated. Further, mutants were confirmed by sequencing (Figure 2.10).

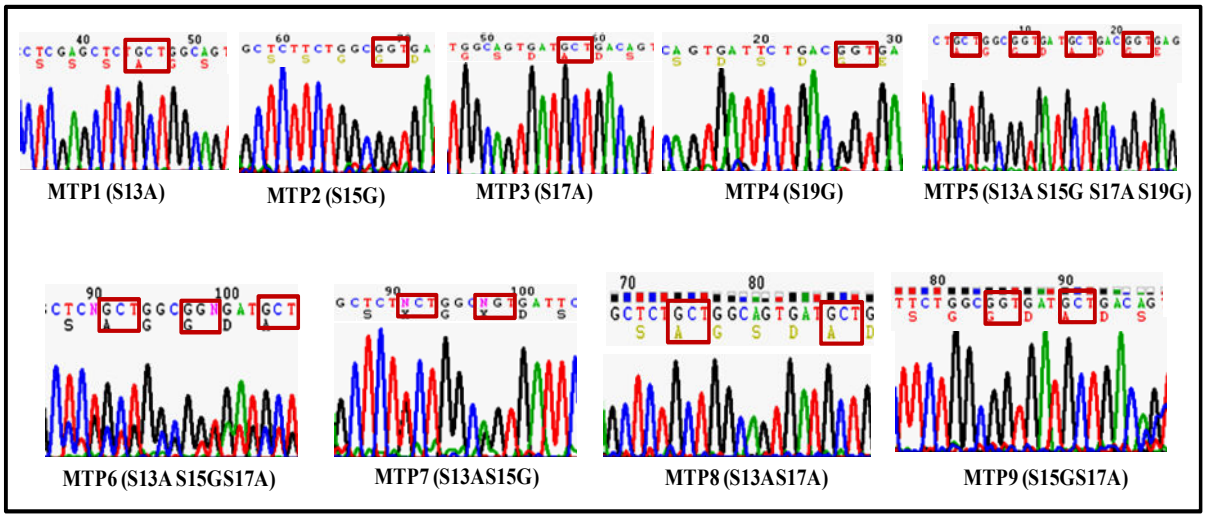


Figure 2.10 Chromatogram presentation of all serine mutants of PC4 generated through site directed mutagenesis. Red box indicates the altered codon to obtain mutant amino acid.

2.2.4 Subcloning of phosphorylation defective point mutants in FLAG and GFP tagged mammalian expression vector.

MTP5 (S13AS15GS17AS19G) was subcloned in mammalian expression vectors for further studies. It was subcloned in pCMV-FLAG (sigma) vector using HindIII and XbaI restriction sites. MTP5 was also cloned in GFP expressing pEGFP-N1 vector within the cohesive sites of XbaI and SmaI. Clones were confirmed by insert release upon restriction digestionmby corresponding restriction enzymes as well as by sequencing. Clones were checked for their expressionin mammalian cells (Figure 2.11).

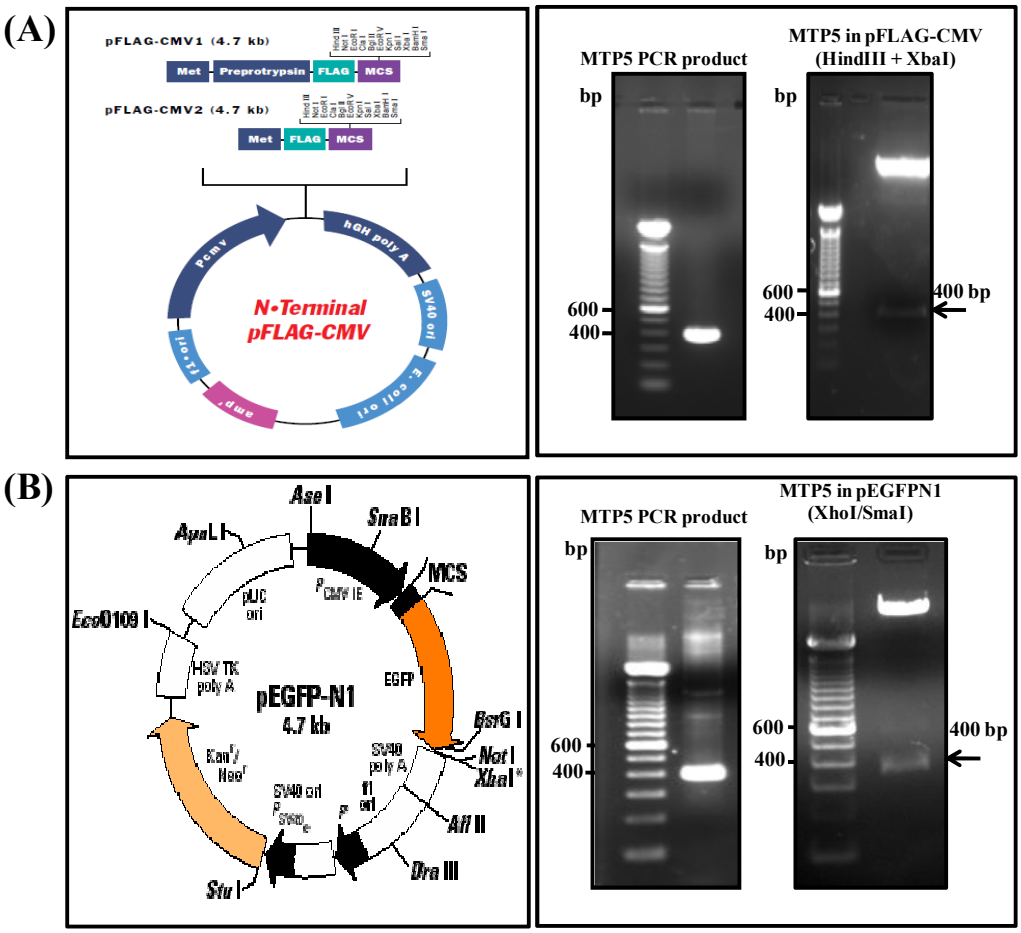


Figure 2.11Subcloning of phosphorylation defective mutant MTP5 in FLAG and GFP tagged mammalian expression vector. MTP5 (S13AS15GS17AS19G) was subcloned in FLAG epitope expressing pFLAG-CMV mammalian vector (A) as well as GFP expressing pEGFP-N1 vector (B).

MTP5 insert was amplified and cloned in these two vectors. Insert release using indicated restriction enzymes were performed for clone confirmation.

2.2.5 Cloning of PC4 (Δ62-67) deletion mutant.

PC4 internal deletion mutant of five amino acids,Δ62-67 were created by overlap extension PCR method. Primers were designed to yield two amplicons with overhangs, N-terminal (1-62) and C terminal (67-127). These two amplicons were subjected to third PCR using DNA polymerase which will extend from both ends to yield full sequence lacking in 5 internal amino acids. The insert was digested with NcoI and XhoI followed by ligation in bacterial expressing pET28b vector. Clones were confirmed by insert release upon restriction digestion and also by sequencing (Figure 2.12).

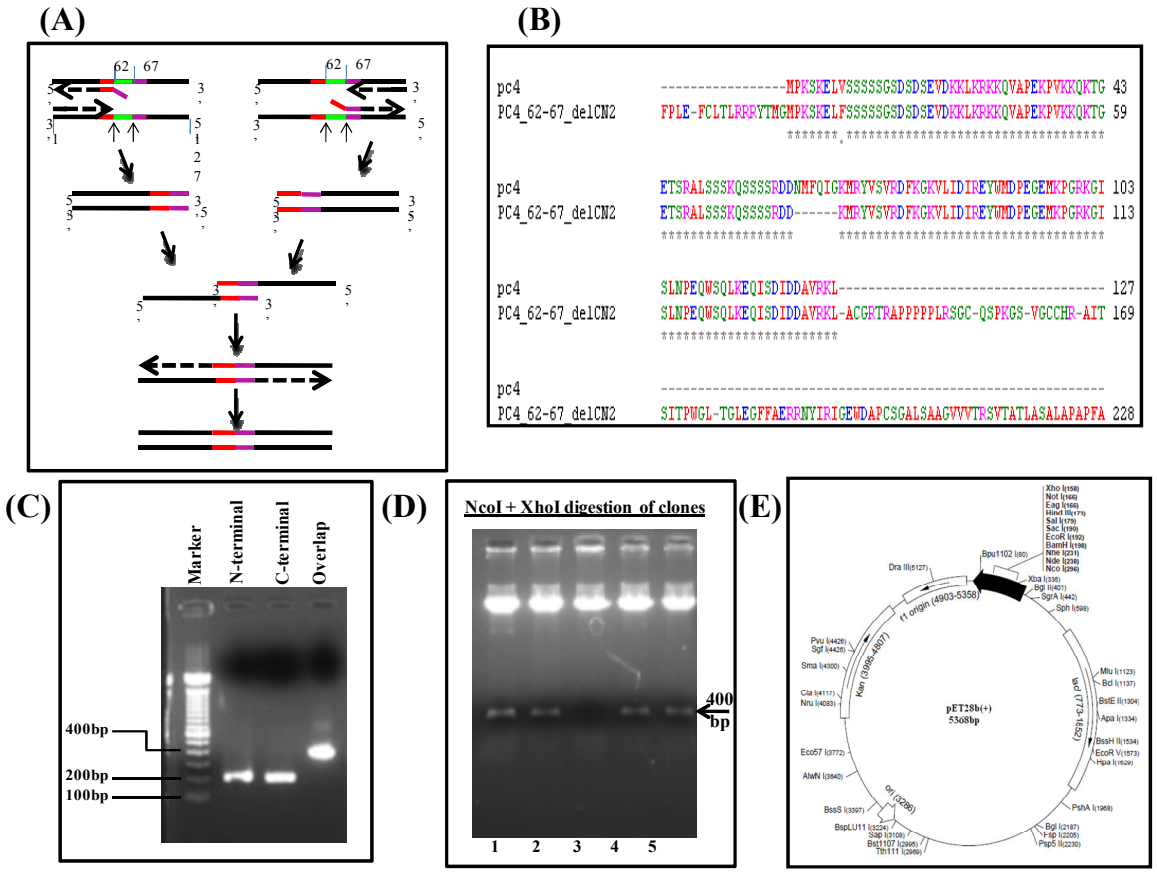


Figure 2.12 Cloning of PC4 (Δ62-67) deletion mutant. (A) Overlap extension PCR method was employed to clone the deletion mutant in bacterial pET28b vector (E). (C) Agarose gel show amplicons of subsequent PCRs and overlap PCR. Clones were confirmed by insert release upon NcoI/XhoI digestion (D) as well as sequence alignment (B).

2.2.6Cloning of PC4 cDNA in mammalian pCDH vector for stable cell line generation.

PC4 cDNA was subcloned into mammalian expression lentivector pCDH-CMV-MCS-EF1-cop-GFP under the restriction sites, XbaI and NotI. FLAG sequence was added to the primer sequence so that the translated protein would have FLAG epitope at its N-terminal. PCR amplicon was gel eluted, purified and digested with above mentioned restriction enzymes. Digested amplicon was ligated with digested vector at 16 °C for 16 hours at molar ration of 3:1. Ligation product was transformed in DH5α and colonies were screened for insert release(Figure 2.13). Positive clone was also confirmed by sequencing. Positive clone was transfected in HEK293 cells to check for its expression. Further, lenticlone was used to generate stable cell line expressing FLAG-PC4.

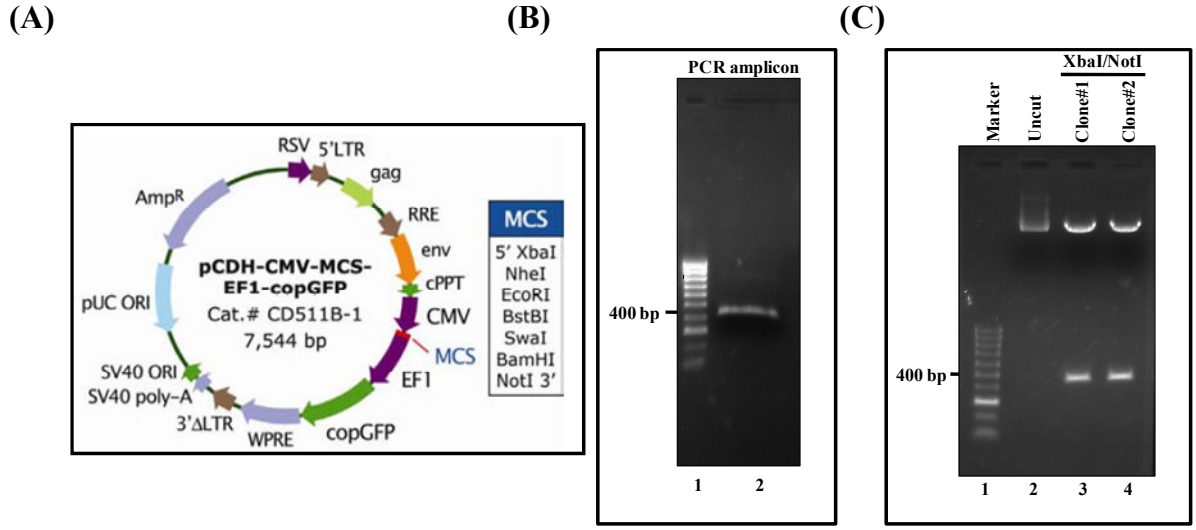


Figure 2.13 Cloning of PC4 cDNA in mammalian pCDH vector. (A, B) PC4 cDNA was amplified and cloned in pCDHlenticlone vector. (C) Clones were screened and verified by insert release upon XbaI/NotI digestion.

2.3 Protein/Enzyme purification

2.3.1 Purification of bacterial expressed untagged and Histidine tagged PC4.

Untagged PC4 purification: A two-column purification protocol was employed to purify untagged recombinant PC4 (Ge and Roeder, 1994) using heparin sepharose followed by phosphocellulose P11 columns. *E. coli* BL21 cells were transformed with the PC4 expression vector in the presence of 100 μg/ml ampicillin.Single colony was inoculated from the transformed plate into 100 ml LB medium containing 100 μg/ml ampicillin and grown overnight at 37°C. The culture was inoculated into 900 ml LB in presence of 100 μg/ml ampicillin and grown till the OD_{600} reached 0.6 at 37°C. The culture was then induced with 0.5 mM IPTG and grown further for 3 hrs at 37°C. The cells were harvested and resuspended in homogenization buffer BC300 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 300 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol) and sonicated with 4 burst of 30 sec at a setting of 7. The cleared supernatant was obtained by centrifugation at 16000 rpm for 30 mins at 4°C. The supernatant was passed through a BC300 pre-equilibrated heparin sepharose column. The column was washed with 2-3 times column volume by BC300 and eluted with BC500 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 500 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). All the eluted fractions were analyzed by 15% SDS PAGE (Figure 2.14A). Fractions majorly containing PC4 protein were pooled and was loaded onto a pre-equilibrated (with BC500) phosphocellulose P11 (Whatman) column. The column was washed with 20-30 ml of BC500 and eluted with BC850 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 850 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). The PC4 protein containing fractions were pooled (Figure 2.14A), dialyzed in BC100 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA,

100 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C.

C-terminal six histidine tagged PC4 (PC4-His_6)purification: *E. coli* BL21 cells were transformed with PC4-His_6 expression vector and subsequently grown in 100 ml LB medium containing 50 μg/ml kanamycin for overnight at 37°C. The overnight 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 mMTris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mMKCl, 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 (Novagen)agarose beads for a period of 3 hrs inan end-to-end shaker at 4°C. The resin was washed eight times with the wash buffer (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 600 mMKCl, 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 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 250 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol), and analyzed in a 15% SDS PAGE. Fractions containing protein of interest were pooled and dialysed in BC100 buffer (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C. Proteins were checked in 12% SDS-PAGE gel (Figure 2.14B)

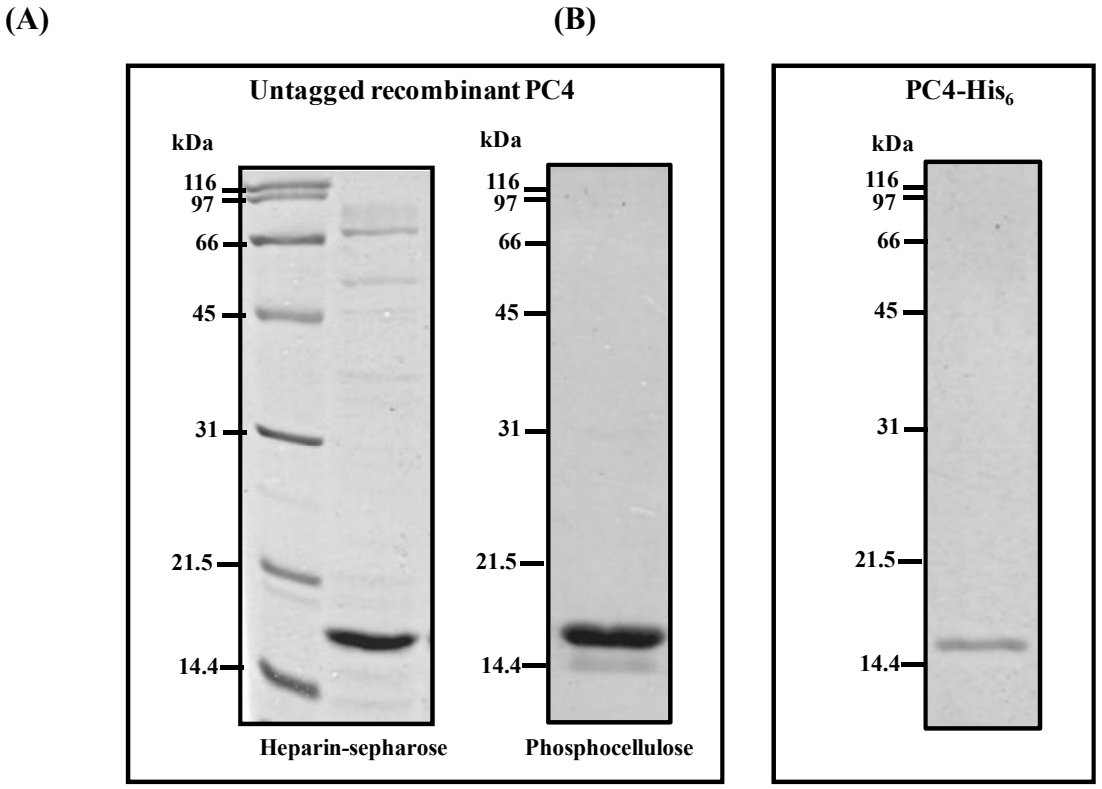


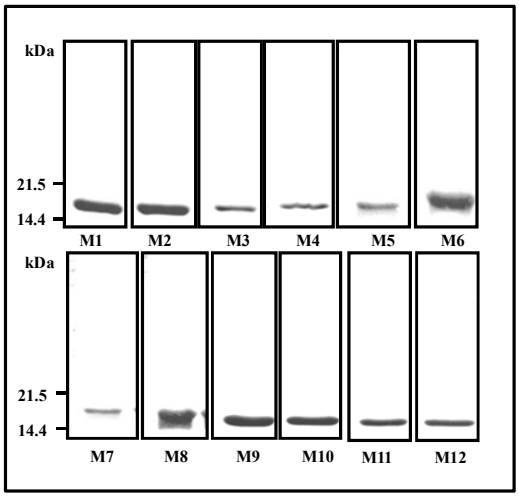
Figure 2.14 Purification of bacterial expressed untagged and Histidine tagged PC4. Purification profile of recombinant untagged PC4 (A) and histidine tagged PC4 (B) from transformed bacterial culture. Untagged PC4 was purified through two columns namely heparin sepharose and phospho cellulose.

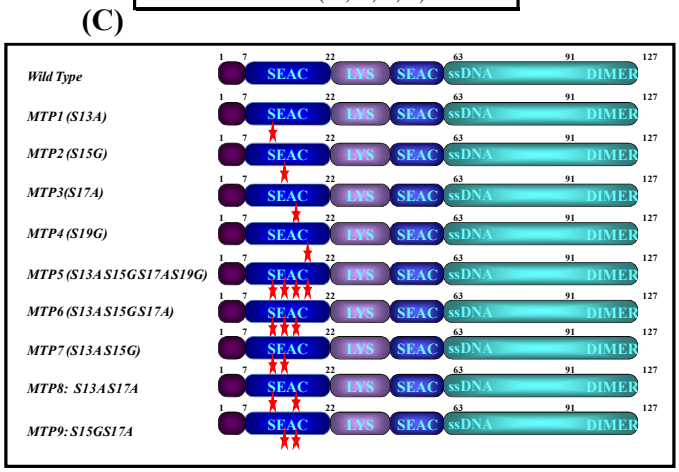
2.3.2 Purification of acetylation and phosphorylation defective PC4 mutants.

Acetylation and phosphorylation defective PC4 mutants were purified till homogeneity using Ni-NTA agarose and heparin sepharose followed by phosphocellulose P11 columns respectively as described in previous section for PC4-His_6 and untagged PC4 (Figure 2.15).

(A)

Name	Mutation(First Generation)
M1	K26R
M2	K28R
M3	K53R
M4	K68R
M5	K26A
M6	K28A
M7	K53A
M8	K68A
	Mutation (Second Generation)
M9	K(53,68)R
M10	K(53,68)A
	Mutation (Third Generation)
M11	K(26,28,53,68)R
M12	K(26,28,53,68)A





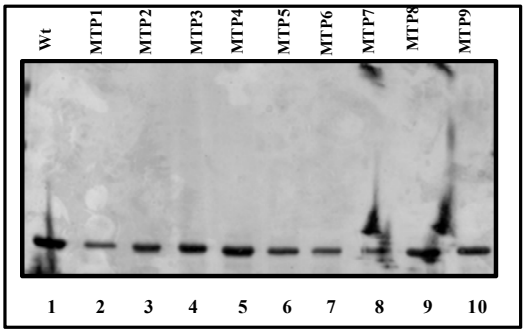


Figure 2.15 Purification of acetylation and phosphorylation defective PC4 mutants. (A, C) Tables showing list of lysine and serine mutants of PC4 indicating the residues mutated. Purification profile of proteins mutated in one or more residues (B: Lysine mutants; D: Serine mutants). All the mutants showed similar mobility in 12% SDS PAGE as of wild type recombinant PC4.

2.3.3 Purification of bacterially expressed histone H3.

Recombinant histone H3 (Xenopus) was purified till homogeneity from inclusion bodies. Purification protocol includes denaturation in 8M urea followed by renaturation (Luger et al, 1997). *E. coli* BL21 cells harboring the expression vector for histone H3 were grown in 100 ml LB medium containing 100 µg/ml ampicillin overnight in 37°C shaker incubator. 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 mMTrisHCl pH 7.5, 100 mMNaCl, 1 mMBenzamidine, 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 mMNaOAc 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 mMTrisHCl 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 2.16). Small volume aliquots of the protein were made and stored at -80°C.

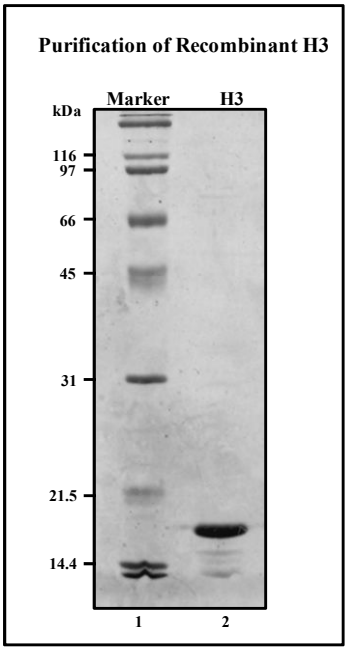


Figure 2.16 Purification of bacterially expressed histone H3. Recombinant bacterially expressed xenopus H3 was purified from inclusion bodies till homogeneity.

2.3.4 Purification of human somatic linker histone variants and their domains.

E. coli BL21(DE3) transformed with C-terminal six histidine tagged different somatic linker histone variants (H1-His_6) and domains (NG: N-terminal+globular domain; G: Globular domain and GC: C-terminal+globular domain) of H1.1 and H1.3 were grown overnight in 37°C shaker incubator. 100 mL of overnight culture was inoculated in 1 litre of LB media containing 50µg/mL kanamycin and grown till O.D600 reached 0.8. The culture was induced with 0.5mM IPTG and grown further at 37°C for 3 hours. Cells were harvested by centrifugation and resuspended in resuspension buffer. Cells were lysed by sonication and lysate was clarified by hard spinning. Cell lysates were precipitated with 5% perchloric acid. The resultant supernatant is again precipitated with 15% trichloric acetic acid followed by one acidified acetone (0.5 mL conc. HCl/100 mL) wash and two acetone wash. Pellet was dried and then dissolved in BC100 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol). Proteins were analysed on 12% SDS PAGE for their purity (Figure 2.17).

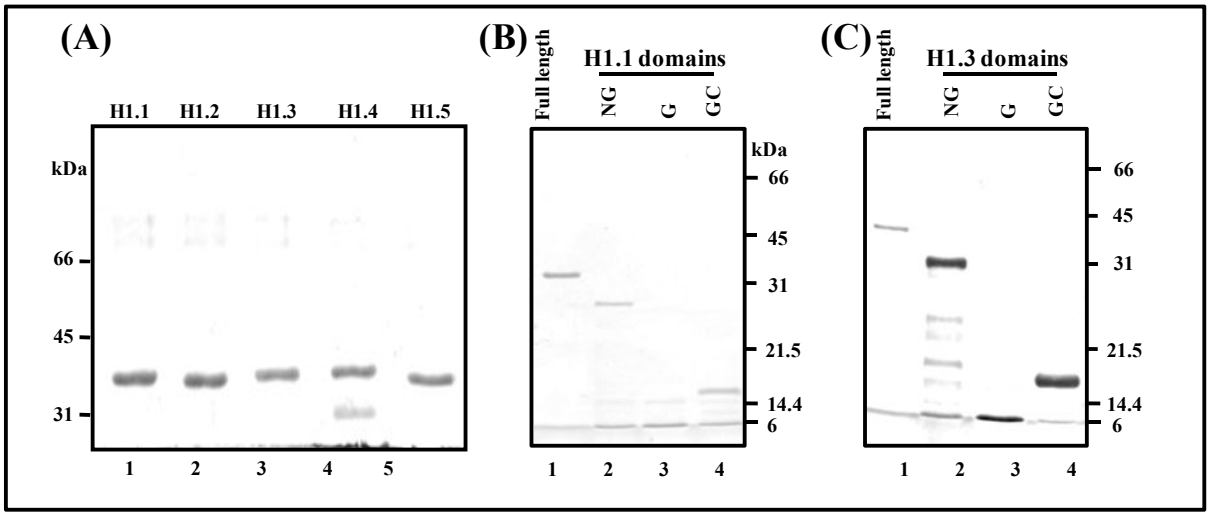


Figure 2.17 Purification of human somatic linker histone variants and their domains.(A)Purification profile of recombinant linker histones somatic variants from transformed bacterial culture. Domains of linker histones H1.1 and H1.3 namely NG (Nterminal and globular), G (Globular) and GC (Globular and C terminal) were purified in similar fashion.

2.3.5 Purification of recombinant full length p300 (KAT3B),lysineacetyltransferasefrom insect Sf21celllines.

p300 (KAT3B) purification: Full length His6 tagged p300 was purified from baculovirus (p300) infected Spodopterafrugiperda (Sf21) insect ovary cell line. The infection time was around 72 hrs. Subsequently cells were harvested and then resuspended in cold homogenization buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.1% NP0, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 500 mMNaCl, 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 3hrs at 4°C in an end-to-end shaker. The beads were washed 8-9 times in wash buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.2%NP40, 2 mM β Mercaptoethanol, 2 mM PMSF, 300 mMNaCl, 15 mM Imidazole). The beads were packed in a USB column (Catalogue No. 13928) and the protein was eluted with the elution buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.1% NP0, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 200 mMNaCl, 250 mM Imidazole and 50 μg/ml of each of the protease inhibitors Leupeptin and Aprotinin). The protein was checked in a 8% SDS PAGE (Figure 2.18A), aliquoted and stored at -80°C.Subsequently, activity of enzyme was verified by *in vitro* HAT assay (Figure 2.18B).

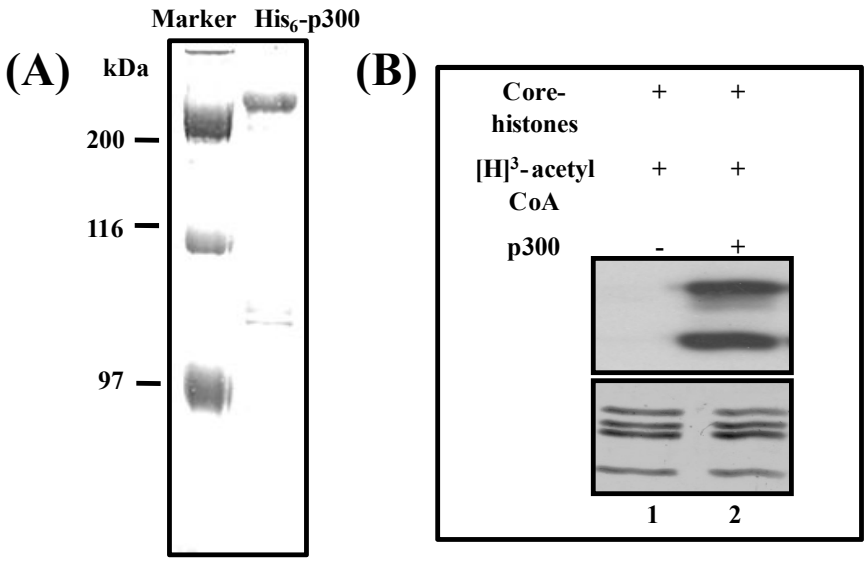


Figure 2.18 Purification of active full length p300 (KAT3B), lysine acetyltransferase expressed as baculoviruses in insect Sf21celllines. (A) Recombinant baculovirus expressed full length p300 was purified from insect Sf21 cells utilizing Ni-NTA affinity pull down protocol. Bead eluted protein was loaded onto 8% SDS PAGE followed by coomassie staining to visualise protein purity. (B) Activity of purified enzyme was checked by gel assay using core-histones and ^{3}H-acetyl CoA (NEN-PerkinElmer) as substrates.

2.3.6 Purification of bacterially expressed GST tagged lysine deacetylases SIRT1and SIRT2.

GST-tagged human SIRT1 and SIRT2 was purified using GST bind resin column. *E. Coli* BL21(DE3) harbouring SIRT1 and SIRT2 expression vectors were inoculated in 100 ml LB containing 100 μg/ml ampicillin and grown overnight at 37°C. Grown culture was then transferred in 900 ml ampicillin containing LB media and was grown till OD_{600} reached 0.6. The culture was induced with 0.5 mM IPTG and was grown further for 3 hours. Cells were pelleted and resuspended in lysis buffer (20mMTris-Cl pH 7.4, 20% glycerol, 2mM β-mercaptoethanol, 0.2 mM EDTA, 2mM PMSF, 0.1% NP-40, 300mMKCl). Cell suspension was sonicated with 4 burst of 30 seconds at a setting of 32% amplitude (Sonics-Vibra cell). Lysate was incubated with 1% triton X-100 for 30 mins at 4 °C. The lysate was clarified by

centrifuging at 16,000 rpm for 30 mins at 4 °C. Supernatant was mixed with pre-equilibrated GST-bind resin and was kept on end to end rocker at 4 °C for 3 hours. Resin beads were washed 7-8 times with wash buffer (20 mMTris-Cl pH 7.4, 20% glycerol, 2mM β-mercaptoethanol, 0.2 mM EDTA, 2mM PMSF, 0.1% NP-40, 500mMKCl). Proteins bound to the beads were eluted with GST elution buffer (10 mM reduced glutathione and 50 mMTris-Cl, pH 8.0). Protein purification profile was visualised in CBB (coomassie brilliant blue) staining of 12% SDS PAGE (Figure 2.19A).Subsequently, activity of enzyme was verified by performing *in vitro* HDAC assay (Figure 2.19B, C).

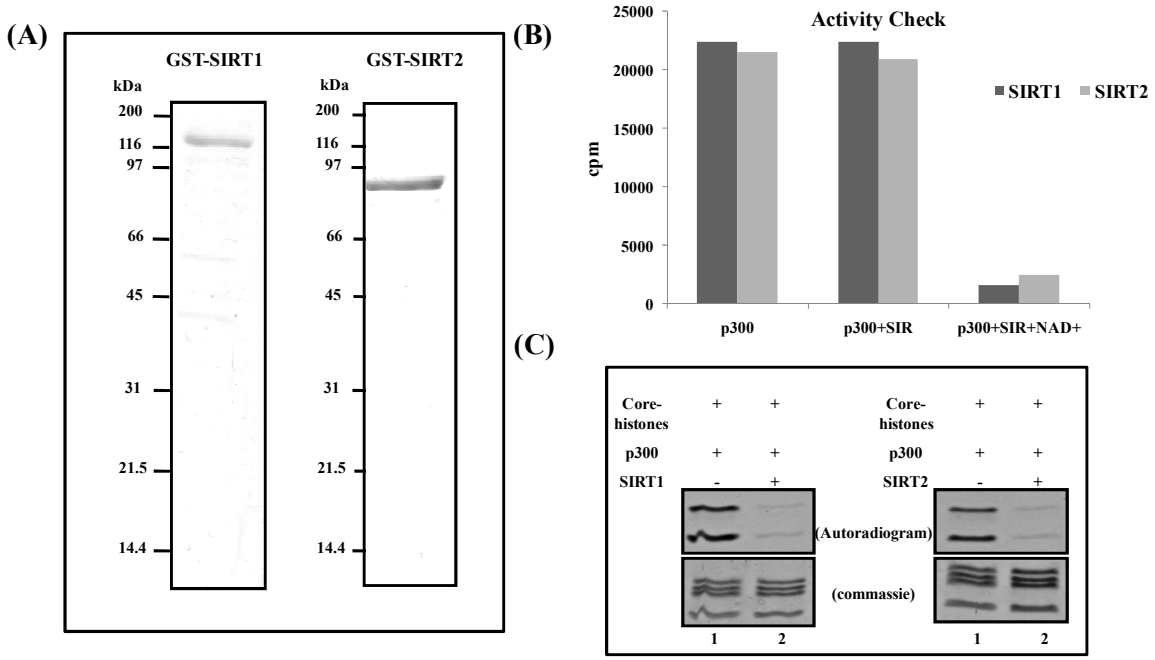


Figure 2.19 Purification of bacterially expressed GST tagged lysine deacetylases SIRT1 and SIRT2. (A) Purification profile of recombinant GST tagged SIRT1 and SIRT2. GST beads eluted proteins were loaded onto 12% SDS PAGE and stained with coommassie brilliant blue to visualise purity of purified proteins. Activity of these enzymes were checked by filter binding assay (B) and gel assay (C) using core-histones and ^{3}H-AcCoA as substrates in the presence of NAD+.

2.3.7 Purification of core-histones from HeLa nuclear pellet.

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 mMNaCl) and homogenized in a Dounce's homogenizer (Wheaton) with pestle B for 30 mins on ice. The suspension was centrifuged at 14000 rpm for 20mins at 4°C. Supernatant was incubated with hydroxyapatite biogel HTP (Biorad) presoaked in 10mM 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 beads were washed with buffer A with 630 mMNaCl 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 (Figure 2.20), dialysed in BC100, aliquoted and stored in -80°C.

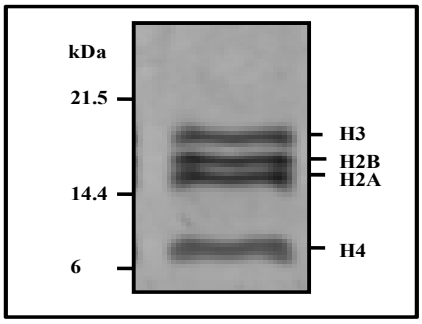


Figure 2.20Purification of core-histones from HeLa nuclear extract. Purified core-histones from HeLa nuclear extract were resolved onto 15% SDS PAGE followed by coomassie staining to visualise individual histones (H3, H2B, H2A and H4).

2.3.8 Purification of PC4 (Δ62-67) deletion mutant.

The C-terminal six histidinetagged PC4 (Δ62-67) (PC4(Δ62-67)-His_6) was purified using Ni-NTA agarose (Novagen). E. coli BL21 cells were transformed with PC4(Δ62-67)-His_6 expression vector and grown in 100 ml LB medium containing 50 μg/ml kanamycin for

overnight at 37°C. The overnight 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 mMTris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mMKCl, 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 eight times with the wash buffer (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 600 mMKCl, 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 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 250 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol), and analyzed in a 15% SDS PAGE (Figure 2.21). Fractions containing protein of interest were pooled and dialysed in BC100 buffer (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C.

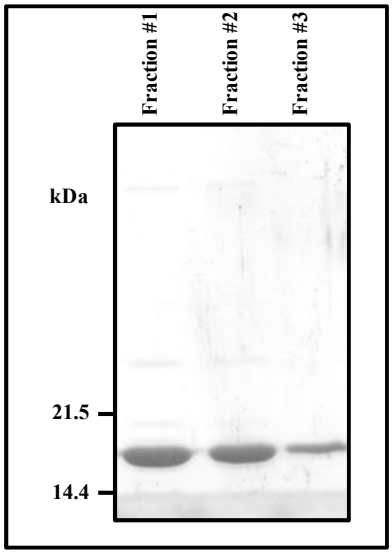


Figure 2.21 Purification of PC4 (Δ62-67) deletion mutant. Purification profile of PC4 (Δ62-67) deletion mutant resolved on 12% SDS PAGE.

2.3.9 Purification of bacterially expressed, recombinant lysine acetyltransferase domains of p300 (KAT3B) and TIP60(KAT5).

p300 (KAT3B) KAT domain purification: *E.coli* (DE3) cells were co-transformed with expression vectors expressing both p300 KAT domain and histone deacetylase SIRT2 and were grown on LB agar plates containing ampicillin and kanamycin. Single colony was inoculated in 100 ml of LB (ampicillin and kanamycin) and grown overnight in 37°C shaker incubator. Overnight grown culture was transferred to 900 ml LB (ampicillin and kanamycin) and was grown till O.D.600 reached 0.5. Culture was induced by 0.5 mM IPTG and culture was further allowed to grow for 3 hours. The culture was harvested, homogenized in BC300 (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2 mM EDTA, 300 mMKCl, 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 eight times with the wash buffer (20 mMTris-HCl, pH7.4, 20% Glycerol, 0.2 mM EDTA, 600 mMKCl, 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 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 250 mM Imidazole, 2 mM PMSF and 2mM β Mercaptoethanol), and analyzed in a 15% SDS PAGE (Figure 2.22B, I). Fractions containing protein of interest were pooled and dialysed in BC100 buffer (20 mMTris-HCl pH7.4, 20% Glycerol, 0.2mM EDTA, 100 mMKCl, 0.1% NP40, 2 mM PMSF and 2mM β Mercaptoethanol), aliquoted and stored in -80°C. Subsequently, activity of enzyme was verified by *in vitro* HAT assay (Figure 2.22B, II, III).

TIP60 (KAT5) KAT domain purification: TIP60 (KAT5) KAT domain expression vector harbouring *E.coli* (DE3) was inoculated 100 ml of LB containing appropriate antibiotic and grown overnight in 37°C shaker incubator. Overnight grown culture was transferred to 900

ml LB and was grown till OD_{600} reached 0.5. Culture was induced by 0.5 mM IPTG and culture was further allowed to grow for 3 hours. Subsequently cells were harvested and then resuspended in cold homogenization buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.1% NP40, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 500 mMNaCl, 15 mM Imidazole and 50 µg/ml of each of the protease inhibitors Leupeptin and Aprotinin). Cell suspension was sonicated to lyse the cells. The cell lysate was centrifuged 11000 rpm for 15 mins at 4°C. The supernatant was bound to Ni-NTAagarose (Novagen) beads for 3hrs at 4°C in an end-to-end shaker. The beads were washed 7-8 times in wash buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.2%NP40, 2 mM β Mercaptoethanol, 2 mM PMSF, 300 mMNaCl, 15 mM Imidazole). The beads were packed in a USB column (Catalogue No. 13928) and the protein was eluted with the elution buffer (10 mMTrisHCl pH 7.5, 10% Glycerol, 0.1% NP0, 2 mM β Mercaptoethanol, 0.2 mM PMSF, 200 mMNaCl, 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 (Figure 2.22C, I), aliquoted and stored at -80°C. Subsequently, activity of enzyme was verified by *in vitro* HAT assay (Figure 2.22C, II).

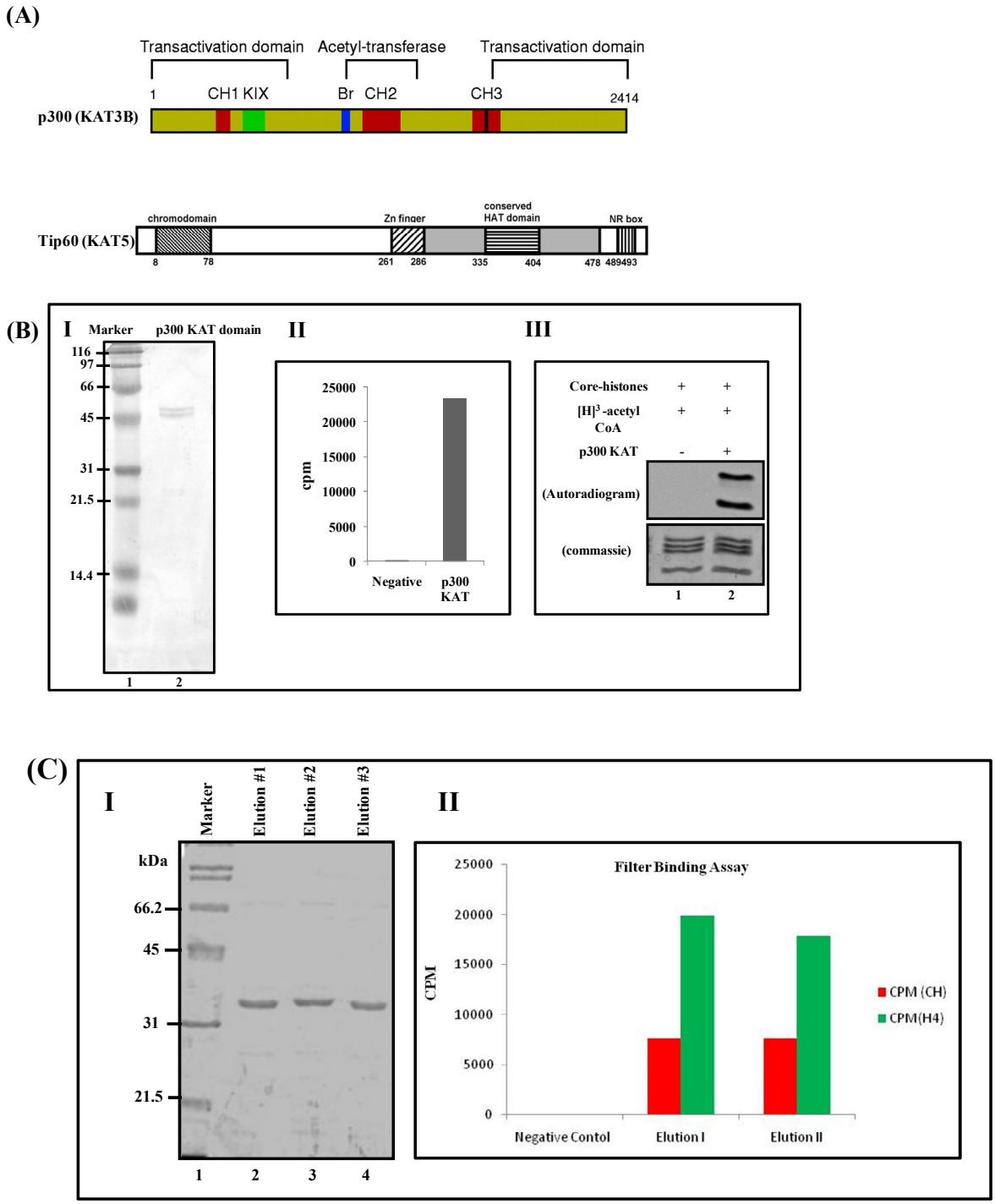


Figure 2.22 Purification of lysine acetyltransferase domains of p300 (KAT3B) and TIP60 (KAT5) from *E.coli.* (A) Schematic showing domain structure of p300 (KAT3B) and TIP60 (KAT5) respectively. (B) p300 HAT domain was purified till homogeneity from transformed bacterial culture (Panel I). Activity of the minimal HAT domain was examined by filter binding assay (Panel II) and gel assay (Panel III) using core-histones and ^{3}H-acetyl CoA as substrates. (B) Similarly, TIP60 KAT domain was purified following Ni-NTA affinity pull down protocol (Panel I). Activity of the TIP60

KAT domain was examined by filter binding assay (Panel II) using core-histones, H4 and ^{3}H-acetyl CoA as substrates.

2.4 Protocols for different assays and analysis

2.4.1 In vitro Histone acetyltransferase (HAT) assay

Filter binding assay: Approximately 600 ng of core histones was incubated with 1µl p300 enzyme (giving ~ 8000 Counts/ µl) at 30ºC for 30 mins in 2X HAT buffer (1X composition: 50 mMTris-HCl pH 8, 10% Glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA), 10 mM Na-butyrate and 1µl of 3.3 Ci/mmol of ^{3}H-acetyl CoA (NEN-PerkinElmer). 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: HAT assays were performed using 2.4 µg of highly purified HeLa core histones or 1 µg recombinant PC4, incubated in HAT assay buffer at 30ºC for 10 mins with or without baculovirus expressed recombinant p300 followed by addition of 1 µl of 3.6 Ci/mmol^{3}H-acetyl CoA (NEN-PerkinElmer) and further incubated for another 10 mins in a 30 µl reaction at 30ºC. For gel fluorography assays, proteins were TCA precipated using25% TCA. Precipitates were washed twice with acetone, dissolved in 2x SDS loading dye,heated for 5 minutes and separated using 15% SDS-PAGE. The gel was stained by coomassie to ascertain the presence of histones in equal amounts in each of the reaction and was later dehydrated in DMSO for 1hour. Later they were incubated in scintillation fluid (PPO solution in DMSO) for 45 minutes and rehydrated again in distilled water for 4 hours. The gel was later dried using a gel drier and exposed in an X-ray cassette using a film for 5 days in -80 degree cooler. The film would be developed later to get intensity profiles for each of the reaction.

2.4.2 In vitro Histone Deacetylase (HDAC) assay

Filter binding assay: Approximately 600 ng of core histones was incubated with the enzyme 1µl p300 (giving ~ 8000 Counts/ µl) at 30ºC/30 mins in 2X HAT buffer (1X composition: 50 mMTris-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. 1 µl of histone deacetylase is added and reaction is further incubated for 30 mins. 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: HDAC assays were performed using 2.4 µg of highly purified HeLa core histones or 1 µg recombinant PC4, incubated in HAT assay buffer at 30ºC for 10 mins with or without respective deacetylases followed by addition of 1 µl of 3.6 Ci/mmol ^{3}H-acetyl CoA (NEN-PerkinElmer). For deacetylase assays using class III HDACs, 500 µMNAD^{+} was added in the reaction as cofactor. Gel fluorography was done as described previously.

2.4.3 In vitro kinase assay

Approximately 1 µg of protein substrate (PC4) was incubated with the enzyme 1µl Casein Kinase II (20 mU) at 30ºC/30 mins in 2X Phospho buffer (1X composition: 50 mMHepes-KOH, pH-7.6, 125mM NaCl, 10 mM MgCl2, 6% Glycerol, 5 mM DTT, 0.5 mM PMSF) and [γ-^{32}P] ATP. To check for phosphorylation the reaction mixture was then loaded onto a 15% SDS PAGE, dried and exposed to phosphoimager or/and X-ray films to get intensity profiles.

2.4.4 In vitro acetylation of PC4

Gel assay was performed using 1 µg PC4 or PC4-His_6 as described above. For mass acetylation,1-1.5 µgPC4 or PC4-His_6were used in different assays.Reaction mixture containing proteins with 1µl p300 (giving ~ 8000 Counts/ µl), 2X HAT buffer, 10 mM Na-butyrate and 1.68 mMacetyl-CoA was incubated at 37ºC for 30 mins, followed by replenishment with the enzyme and acetyl-CoAthree times after every 1 hour. After final replenishment, an additional incubation for 6-8 hours was given for complete saturation of the reaction. As control, mock acetylation reactions were set with the same components except theenzyme p300, and similar protocol was followed. The confirmation of acetylation was done by western blotting analysis using anti- acetylated PC4 anti bodies (Figure 2.23A).

2.4.5 In vitro phosphorylation of PC4

1 µg PC4 or PC4-$His_6$was incubated with the 1µl Casein Kinase II enzyme (20 mU) at 30ºC for 30 mins in presence of 2X phospho buffer and [γ-^{32}P] ATP. To check for phosphorylation, reaction mixture was then loaded onto a 15% SDS PAGE, stained, dried and exposed to phosphoimager or/and X-ray films to get intensity profiles.

For mass phosphorylation of PC4 (or PC4-His_6),approximately, 1-1.5 µg protein was incubated 1µl Casein Kinase II (20 mU), 2X Phospho- buffer and 200 mM cold ATP at 37ºC for 30 mins, followed by thrice replenishment with the enzyme and ATP at one hour interval.Final incubation for 6-8 hours after last replenishment was given to ascertain completion of the reaction. As control, mock phosphorylation reactions were set with the same components except the enzyme CKII, and similar protocol was followed. The confirmation of phosphorylation was done by observing a mobility shift of phosphorylated PC4 in 12% SDS PAGE gel (Figure 2.23B).

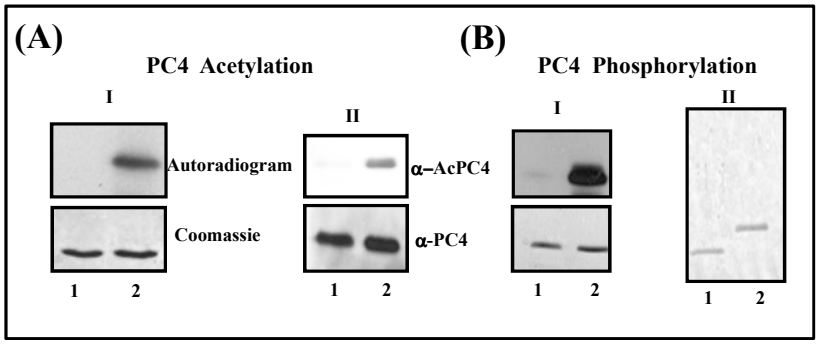


Figure 2.23*In vitro* acetylation and phosphorylation of PC4. (A) *In vitro* acetylation of recombinant PC4 was performed as described in the text. Acetylation was checked by scoring incorporation of ^{3}H-acetyl CoA in gel assay using PC4 as substrate and p300 as enzyme (Panel I). Mass acetylation of PC4 by cold acetyl-CoA was assessed by immunoblotting using anti Ac-PC4 antibody (Panel II). (B) PC4 phosphorylation was performed using [γ-^{32}P] ATP as phosphate group donor and incorporation of radioactivity was captured on to autoradiogram (Panel I).Mass phosphorylation of PC4 was performed using cold ATP and hyperphosphorylated form of PC4 showed lower mobility in 12% SDS PAGE (Panel II).

2.4.6 Transient transfection of plasmid DNA into mammalian cells

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 1:2 ratio of µg of DNA: µl of Lipofectamine 2000 (Invitrogen). 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.

2.4.7 Transient transfection of siRNAs

Approximately 85,000 cells per 30 mm dish were seeded 24 hours prior to transfection in antibiotic free media. At time of transfection medium was replaced with antibiotic and serum

free medium. For siRNA transfection in breast cancer cell lines opti-MEM media was used. Oligofectamine (invitrogen) was mixed with siRNAs according to manufacturer's protocol. For siRNAtrasfections, a non-specific scrambled siRNA was used as control.

2.4.8 Luciferase reporter assay

The mammalian expression constructs of FLAG WT PC4, acetylation and phosphorylation defective mutants M9 and MTP5were used in p53-responsive reporter assay. p53-responsive luciferase construct, pG13luc contains 13 p53 binding sites in tandem followed by the luciferase gene. The pCMV-β-galactosidaseconstruct was used as an internal control. H1299 cells were grown upto 80 %confluency in 30-mm dishes and transfected with different plasmid constructs usinglipofectamine (Invitrogen). The total amount of DNA was kept constant for eachtransfection. Luciferase and β-galactosidase activities were measured 24 hours after transfection according to the manufacturer's protocol (Promega, Madison, WI, USA).

Nearly eighty thousand cells were plated in a 30 mm dish or each well of a 6 well plate. 24 hours post plating cells were transfected with 0.5 µg of MMPs and fibronectin luciferase promoter reporters along with different concentrations of PC4 using Fugene HD transfection reagent in the ratio of 2:1 with DNA. 0.5 µg pRL construct carrying *Renillareniformis*luciferase gene was cotrasfected for normalization. Luciferase assays were performed utilising Dual Luciferase Assay System (Promega). Relative luciferase activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity

2.4.9 Stable cell line generation

PC4 knockdown cell line: For generation of PC4 knockdown stable cell lines, PC4 targeted shRNAs were obtained from the GIPZ lentiviralshRNAmir library (Open Biosystems).

Plasmid DNA was extracted from individual sh-clones. 10µg of sh-plasmid was mixed with helper plasmids (5 µg psPAX2, 1.5 µg pRSV-Rev, 3.5 µg pCMV-VSV-G) and DNA mix were cotransfected into HEK293T cells using calcium phosphate method(Figure 2.24). 48 hours post-transfection media containing assembled virus was collected and its titre was estimated. Desired cell line (here HEK293) was infected with 10^5 IU/ml virus. Infected cells were subjected to selection pressure 72 hours post-infection. Cells were grown in presence of 3 µg/ml puromycin for three passages to establish the cell line(Figure 2.25A, B).

PC4 overexpressing cell line: PC4 cDNA was cloned in pCDHlenticlone vector as described in section 2.2.6. Lenticlone containing FLAG epitope tagged PC4 was assembled in lentivirus using helper plasmids as discussed above. Lentivirus harbouring FLAG PC4 was used to infect HEK293 cells. Positive clones were screened by GFP fluroscence and were later confirmed with immunoblotting/immunofluroscence with anti PC4 and anti FLAG antibodies(Figure 2.25C).

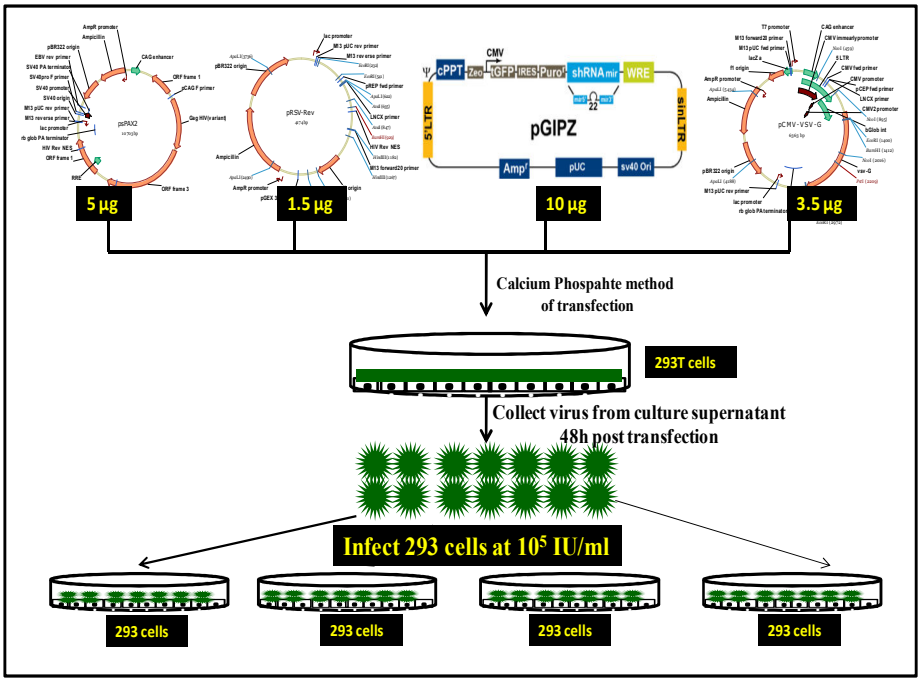


Figure 2.24 Scheme for generation of PC4 knockdown stable celllines. ShRNA containing plasmid and helper plasmids and were transfected in 293T cells. 48 hours post transfection culture supernatant

containing virus was collected and 293 cells were infected at 10^5 IU/ml. After 72 hours cells were subjected to antibiotic selection.

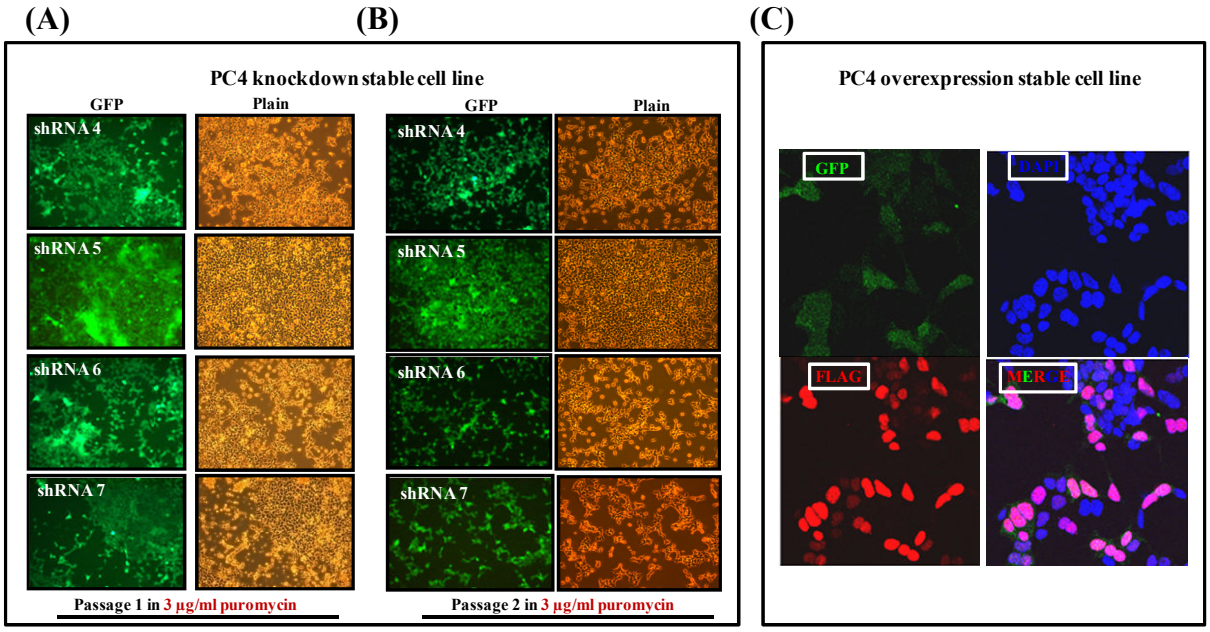


Fig 2.25Selection of stable pool of lentivirally transduced cells. (A) 72 hours post infection, cells were put for puromycin selection at final concentration of 3ug/ml. (B) After first passage cells became confluent, they were splitted and again put for puromycin selection at final concentration of 3ug/ml. (C)PC4 overexpression cell line were assessed by co-expression of GFP and FLAG epitope in the cell pool.

2.4.10 Cell cycle synchronization

HEK 293 cells were arrested in G0/G1 stage of cell cycle by serum starvation for a period of 72 hours, 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. Mitotic arrest was obtained by 500ng/mL nocodazole (Sigma) treatment, leading to a pre-metaphase arrest for a period of 16hours (Figure 2.27).

2.4.11 Immunofluroscence

Cells were grown in poly-lysine coated glass coverslips and fixed in 4% paraformaldehyde in PBS (172 mMNaCl, 2.7 mMKCl, 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 5% FBS at 37°C for 45 mins. Probing was done with different antibodies at appropriate dilutions at room temperature for 1 hour followed by corresponding secondary antibodies conjugated to alexaflurophores. Washes were performed three times after primary and secondary antibodies incubation with wash buffer (1% FBS in PBS).In order to visualize the DNA, the cells were stained with 0.1 µg /ml Hoechst 33258 in PBS. Fluorescence and Hoechst were visualized by using confocal microscope.

2.4.12 FACS analysis

HEK 293 cells were synchronised in different cell cycle stages andPropidium Iodide (PI) staining was done. Briefly, cells were harvested by mild trypsinization (0.25%) followed by centrifugation at 2000 rpm for 10 mins at 4°C. Cells were washed with cold PBS by centrifugation at 2000 rpm for 10 mins at 4°C. Cells were fixed in cold 70% Ethanol which was added dropwise along with mild vortexing. Samples were left for 12 hours or more at -20°C, after which Ethanol was removed followed by two washes in cold PBS. RNase (100 µg/ml) treatment was subsequently given at 37°C for 30 mins to ensure only DNA staining. 50 µg/ml propidiumiodide was added for staining (Figure 2.26).

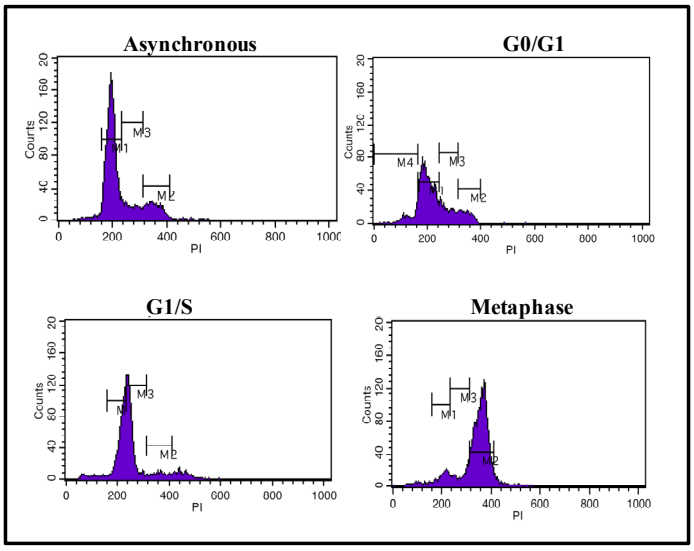


Figure 2.26 Cell cycle synchronization and FACS analysis. Histograms showing distribution of cells in different cell cycle stages as scored by intensity of DNA staining using propidium iodide.

2.4.13 M2-Agarose pull down of FLAG tagged proteins

Cells (stably or transiently expressing FLAG-tagged protein) were grown in 100 mm tissue culture dishes. After proper treatment cells were washed twice with ice cold 1X PBS. The cells were lysed by adding 1 ml of ice cold TNN (50 mMTris-HCl, pH 7.4; 100 mMNaCl, 5 mM EDTA, 0.5% NP-40, 1 µgpepstatin per ml, 200 µM PMSF, 0.5 mMdithiothreitol (DTT), 1 µg of leupeptin per ml) buffer. Cells were incubated in ice for 3 hours with regular mixing. Lysate was clarified by centrifuging at 10,000 rpm 4°C for 10 mins. Lysateswere used immediately or quick-frozen in liquid nitrogenand stored at -70 ºC until use. Nearly 500 µg lysate was incubated for 3 hours/overnight at 4ºC with 20 µl packed gel volume of protein G agarose affinity beads (pre-washed and equilibrated inlysis buffer) withmixing in end to end rocker. Thebeads were collected by centrifugation for 30 secondsat 8,000 x g and the supernatants were removed byaspiration. The pellets were washed three times with 1 mL of lysis buffer per wash and collected by centrifugation asdescribed. After aspirating the final wash supernatants,the affinity-bead pellets were each suspended in 25 µl oflysis buffer and

25 µl of 2X laemmli sample buffer and analyzed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and/or immunoblotting.

2.4.14 In vitro pull down assays

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 PC4-His_6 and 250 ng of recombinant (Xenopus) individual histone H3 or core-histones in a final volume of 200 µl in BC buffer (composition mentioned previously) containing 150 mMKCl supplemented with 30 mM imidazole at 4°C for 3.0 hours (Figure 2.27). The beads were washed three times (1 ml each) with the incubation buffers. The Ni-NTA agarose pull down complex was analyzed by western blotting using anti H3 polyclonal antibodies. Control experiments were performed with 5 µl of Ni-NTA beads incubated with 250 ng of histone H3 or core-histones in the same buffer. For interaction of linker histone variants and modified forms of PC4, 1 µg of C-terminal six histidine tagged H1 (H1-His_6) variants were incubated with 250 ng of different modified forms of PC4.

GST pull down assay: In order to investigate interaction of human SIRT2 with PC4, GST-pull down assays were performed. Briefly, interactions were set with 1 µg GST-SIRT2, 250 ng of unmodified and mass acetylated PC4 respectively in presence or absence of NAD^+at 150 mM salt concentration. Acetylation of PC4 was confirmed by immunoblotting using anti acetylated PC4 antibodies. For scoring the interaction, GST-pull down complex was probed with anti-PC4 antibodies.

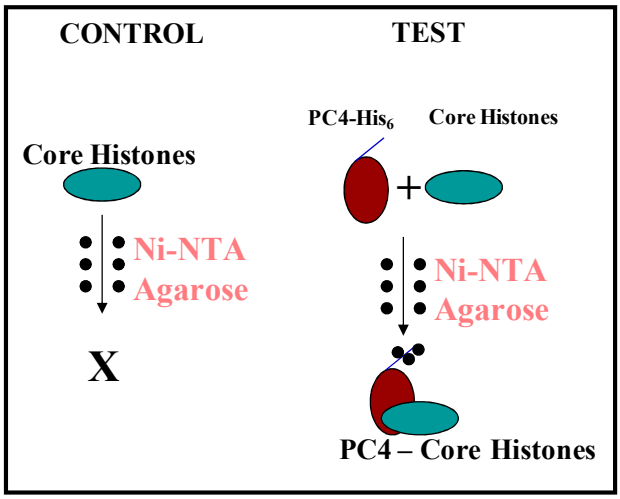


Figure 2.27Schematic illustrating Ni-NTA pull down assay utilising PC4-His_6 and core histones. Histidine tag has higher affinity for Ni-NTA agarose beads and thus interacting partner is pulled down along with it. As negative control of the assay, the protein lacking six histidine tag is incubated similarly with Ni-NTA agarose beads.

2.4.15 Immunohistochemistry

Prospective Samples: Breast cancer patient samples were collected at the Bangalore Institute of Oncology (BIO) at the time of surgery with prior consent from the patients. Tumor and adjacent normal tissues from patient samples were dehydrated, paraffin embedded, and sectioned with a microtome (Leica).Sections were baked overnight on dry bath at 56°C. Sections were subjected to antigen retrieval in sodium citrate buffer for 20 mins. Sections were blocked in 5% skimmed milk solution to avoid nonspecific binding. After blocking, sections were incubated with anti PC4 polyclonal antibodies. IHC analysis was performed with a Strept-Avidin Biotinkit (Dako).Immunoreactivity (brown precipitate) was developed in the chromogendiaminobenzidinetetrahydrochloride (DAB, Sigma), and counterstaining was donewith hematoxylin (Figure 2.28). The staining intensity of the images was evaluated by twoindependent pathologists and H-score was calculated.

Retrospective samples: Samples were collected from Healthcare Global Enterprises (HCG) biorepository in form of formaldehyde fixed paraffin embedded (FFPE) blocks. 3-5 µm thin sections were made and immunostaining with anti PC4 antibodies were followed as discussed previously.

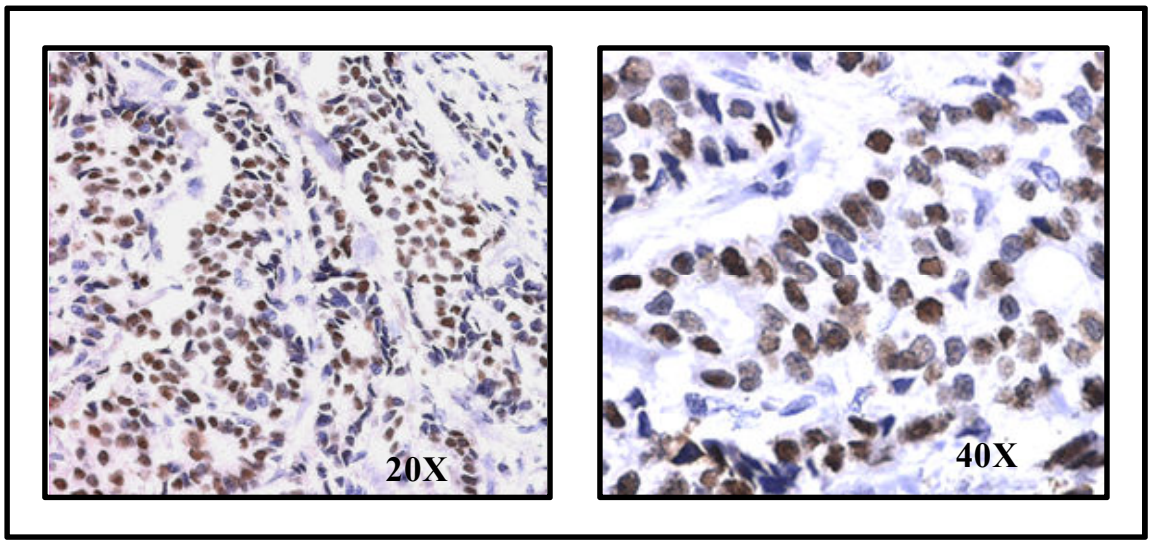


Figure2.28 Immunohistochemistry Immunohistochemical staining using polyclonal anti PC4 antibodies of breast tissue shown in two magnifications. Brown precipitate developed indicate nuclear positivity of PC4 in the tissue.

2.4.16 Electrophoretic mobility shift assay (EMSA)

The end labeled DNA fragments 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 mMNaCl and 0.1% NP40. The reaction mixtures were then resolved on a 4.5% native polyacrylamide gel at 130 V in TBE buffer (45 mMTris-Borate and 1 mM EDTA) for 3 hours. The gels were dried and autoradiogrammed.

2.4.17 Isothermal calorimetry (ITC)

ITC experiments were carried out in a MicroCal ITC200 instrument (MicroCal, Inc.) at 25 °C. Samples were centrifuged and degassed prior to titration. Titration of linker histone H1.1 against the protein, PC4/phospho-PC4 was carried out by injecting 1.5μMlinker histone H1.1 in 20 mMTris- HCl of pH 7.5, 0.2 mM EDTA, and 100 mMKCl buffer against 8.5 μMPC4/phospho-PC4. A 2 min interval was allowed between injectionsfor equilibration, sufficient for the return of the heat signal tobaseline. A total of 35 injections were carried out to ensurecomplete titration. The protein concentration was chosen toachieve sufficiently high heat signals with a minimum enthalpyof dilution. To minimize the error associated with diffusion fromthe syringe during baseline equilibration, the first injection wasonly 1 μL and the associated small heat change was notconsidered for data analysis.

For PC4 (and or mutant PC4) and core histone / H3 titration, 50 μM PC4 was injected from the syringe into the cell of 240 μL filled with 5 μM core histones or histone H3. Titrations were conducted in 20 mMTris-HCl (pH 7.5), 0.2 mM EDTA, and 100 mMKCl buffer at 25°C. Injections were made at intervals of 120 s, and the duration of each injection was 0.4 s. To ensure proper mixing after each injection, a constant stirring speed of 1000 rpm was maintained during the experiment. The heat change versus the molar ratio of the titrated products was plotted and analyzed using the manufacturer's software, which yielded the stoichiometry and the dissociation constant (Kd) of the reaction.

2.4.18 Cell Migration assay

One hundred and twenty thousand MCF-7 cells were plated in a 60 mm dish and grown to confluency. The cells were scratched with a sterile 200 μl pipette tip in four separate places and image of the scratch at 0 hour was acquired. Same area was examined after 18-24 hours

using phase contrast microscopy. Similar experiments were performed with HBL-100 cells. The data is representative of three independent experiments. For experiments with Mitomycin C, cells were treated with 10 mg/mL of Mitomycin C for 3 hours, then placed back in 10% serum containing media. For siRNA studies, MCF-7 and HBL-100 cells were plated at 50% confluency (eighty thousand cells) then transfected with siRNA targeting PC4 (Santa Cruz) or scrambled siRNA. Cells then grew for 48 hours before being scratched with a pipette tip.

2.4.19 Cell Invasion assay

Boyden Chamber assays were used to assess the invasive ability of MCF-7, and MDA-MB-231 cells upon PC4 silencing. The upper surface of the 6.5 mm filters (Corning) were coated with collagen (100 μg/filter) and Matrigel (BD Bioscience) (50 μg/filter). Twenty thousand cells (control and PC4 siRNA treated) were plated in the upper chamber with 0.1% bovine serum albumin (Sigma). Media containing 20% foetal bovine serum was placed in the lower well as chemo-attractant. After 18-24 hours, the cells that invaded through the filters were fixed in methanol and then stained with crystal violet stain. Quantification was done by counting three fields under 20× objective magnifications.

2.4.20Clonogenic assay

Clonogenic assay was performed with control and PC4 knockdown stable cell lines. 200-400 cells were seeded in 100 mm tissue culture dishes 12 hours prior to gamma irradiation. Cells were subjected to different doses of irradiation namely, 0, 2, 4, 8 Gy. Media was replaced with fresh complete media soon after the irradiation. Plate was kept in 37°C incubator with 5% CO2 for a week to monitor colony formation. Colonies were stained with giemsa stain, counted in both cases and survival fraction was calculated.

2.4.21 Gene expression microarray analysis

Whole genome gene expression analysis was done with control and PC4 knockdown cells to investigate the differential gene pathways upon PC4 silencing. AffymetrixPrimeView Human Gene Expression array was used for the purpose. Experiment was done using two biological repeats. Total RNA was extracted using RNA extraction kit and RNA Integrity and RIN number was documented using Agilent 2100 Bioanalyzer (Figure 2.29). Subsequently, labelling and hybridization was performed followed by scanning of array using Affymetrix Microarray Scanner. Basic Data Analysis which includes Data Processing, Normalization, Quality Control of Data, Statistical Analysis, Fold Chains Analysis and Clustering (Box Whisker Plot, Scatter Plot, Heat Map and Line Graph images) was performed by iLife Discoveries Pvt. Ltd.

(A) **(B)**

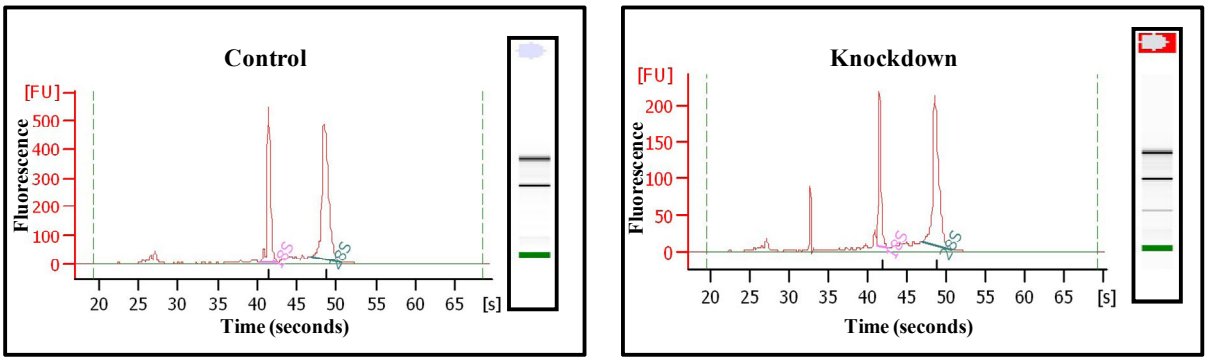


Figure 2.29Bioanalyser profile of total RNA isolated from control (A) and PC4 knockdown stable cell line (B).

2.4.22 Chromatin immunoprecipiation (ChIP)

Breast cancer cell line,MCF-7 was grown in Dulbecco modified Eagle medium and 10% foetal bovine serum upto 75% confluence. Cells were cross-linked using 1% formaldehyde followed by cell lysis in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mMTris–HCl, pH 8.0). Chromatin was sonicatedsix times for 10 s at 91% power setting. Following sonication, the pulldowns for ChIP assays were performed with anti-PC4 (Abcam) and anti-E2F1antibodies diluted in cold dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mMEDTA, 16.7mMTris–HCl, pH 8.0, 167 mMNaCl) at 4 °C overnight. Furthermore,preblocked protein G-Sepharose (Amersham Pharmacia) was added for binding for 2-3 hours at 4 °C. Beads were washed thrice with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mMTris–HCl, pH 8.0, 150 mMNaCl) followed by three times high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mMTris–HCl, pH 8.0, 500 mMNaCl), LiCl buffer (250 mMLiCl, 1% NP40, 1% NaDOC, 1 mM EDTA, 10 mMTris–HCl, pH 8.0) and TE (10 mMTris–HCl, pH 8.0, 1 mM EDTA) wash. Elution buffer (0.2% SDS, 100 mM NaHCO3) along with 200 mMNaCl was added to the washed beads, and the bead solution was kept overnight at 65 °C. Pulldowns were deproteinized using 0.1 mg/ml of proteinase K (Sigma) and 0.04 mg/ml of RNase A (Sigma) were added to the bead solution and the mixture was incubated for 2 h at 37°C. The immunoprecipitated samples were ethanol precipitated and used for PCR analysis using primers for fibronectin promoter region containing E2F1 binding sites.

2.4.23 Acid extraction of histones

Cells (3 million cells per 90-mm dish) were seeded overnight, and histones were extracted from the cells. Cells were harvested, washed in ice-cold buffer A (150 mMKCl, 20

mMHEPES, pH 7.9, 0.1 mM EDTA, and 2.5 mM $MgCl_2$) and lysed in buffer A containing 250 mM sucrose and 1% (v/v) Triton X-100. Nuclei were recovered by centrifugation, washed, and proteins were extracted (on ice) for 1 hour using 0.25 M HCl. Chromosomal proteins were precipitated with 25% (w/v) trichloroacetic acid and sequentially washed with ice-cold acidified acetone (20 µl of 12 N HCl in 100 ml of acetone), and acetone, air-dried, and dissolved in the sample buffer (5.8 M urea, 0.9 M glacial acetic acid, 16% glycerol, and 4.8% β-mercaptoethanol). The protein was quantified using a protein assay reagent (Bio-Rad). This was later used for immunoblotting analysis to assay the *in vivo* histone modification status.

2.4.24miRNA expression profiling of breast tissues

Total RNA was extracted from liquid nitrogen frozen tumor and adjacent normal tissues of breast cancer patient using BioradMicroBio Spin 6 columns(Cat # 732-6221). RNA was subjected miRNA expression analysis using Human miRNA 8x15k Arrays AMADID: 21827 (Agilent miRNA Hybridization kit Cat # 5190-0408). Agilent miRNAlabeling reagent and Hybridization Kit Cat # 5190-0408 was used to generate fluorescent miRNA by ligation of one cyanine3 –pCp molecule to the 3' end of RNA molecule with greater than 90% efficiency. Image analysis was done using Agilent Feature extraction software. The complete data for all miRNA's in the array based on intensities and the differentially regulated miRNA's for all the experiments were provided. The gene targets for the differentially regulated miRNA were identified using Genespring GX (Figure 2.30). (Target Scan database http://www.targetscan.org/ which is integrated in GeneSpring GX)

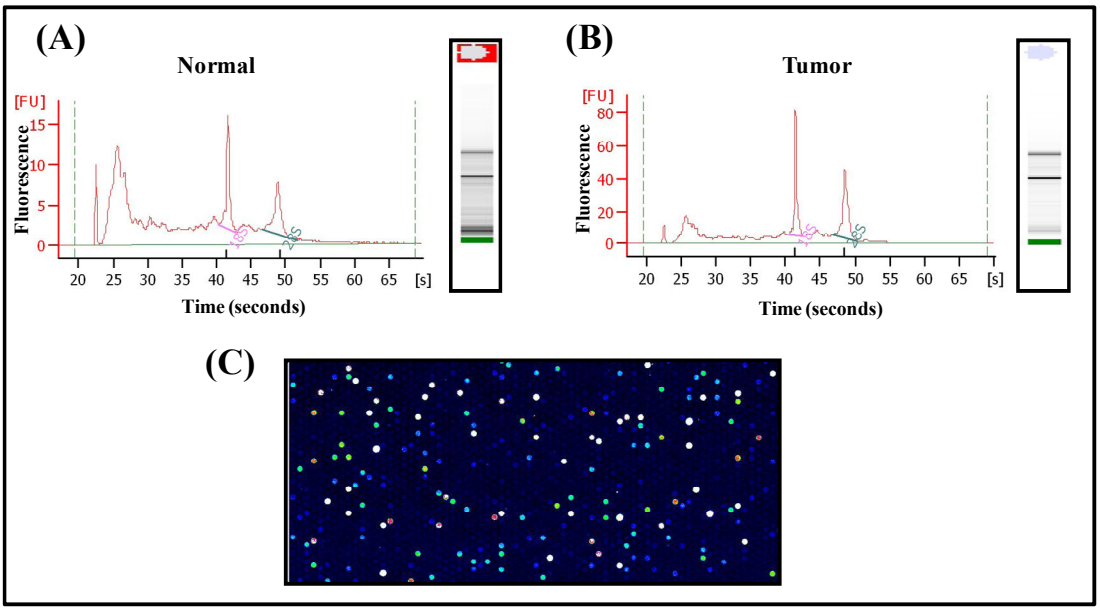


Figure 2.30miRNA expression profiling of breast tissues. Bioanalyzerprofile of total RNA purified from adjacent normal (A) and breast tumor (B) tissues respectively. (C) Representative image of human miRNA 8x15k Arrays slide following hybridization.

2.4.25 Polyclonal antibodies generation of anti acetylated PC4 in rabbit

A peptide containing two acetylated lysines corresponding to acetylated lysines 53 and 68 of PC4 protein was designed. KLH conjugated peptide (Peptide name: ACPC4; Peptide lot number: 8-17302) was obtained from PEPTRON CO., LTD., South Korea. Regular immunization schedules were followed to raise the antibody against acetylated PC4 in rabbit. Briefly,500 µgpeptide in 1X PBS (172 mMNaCl, 2.7 mMKCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4) 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 250µgof peptide 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 250 µgpeptide in PBS emulsified with equal volume of Freund's Incomplete Adjuvant injected subcutaneously. After 3 weeks of second booster major bleeding was done to obtain

immunized blood without killing the rabbit and serum was extracted from it. The antibody titre was checked after the first injection as well as subsequent booster doses. The rabbit polyclonal antibody was purified throughammonium sulphate precipitation followed by Protein–G Sepharose(Figure 2.31A, B) and used for the immunofluorescence experiment as well as other biochemical studies. Crude serum was also purified by peptide affinity purification and its affinity was verified through ELISA (Figure 2.31C, D).

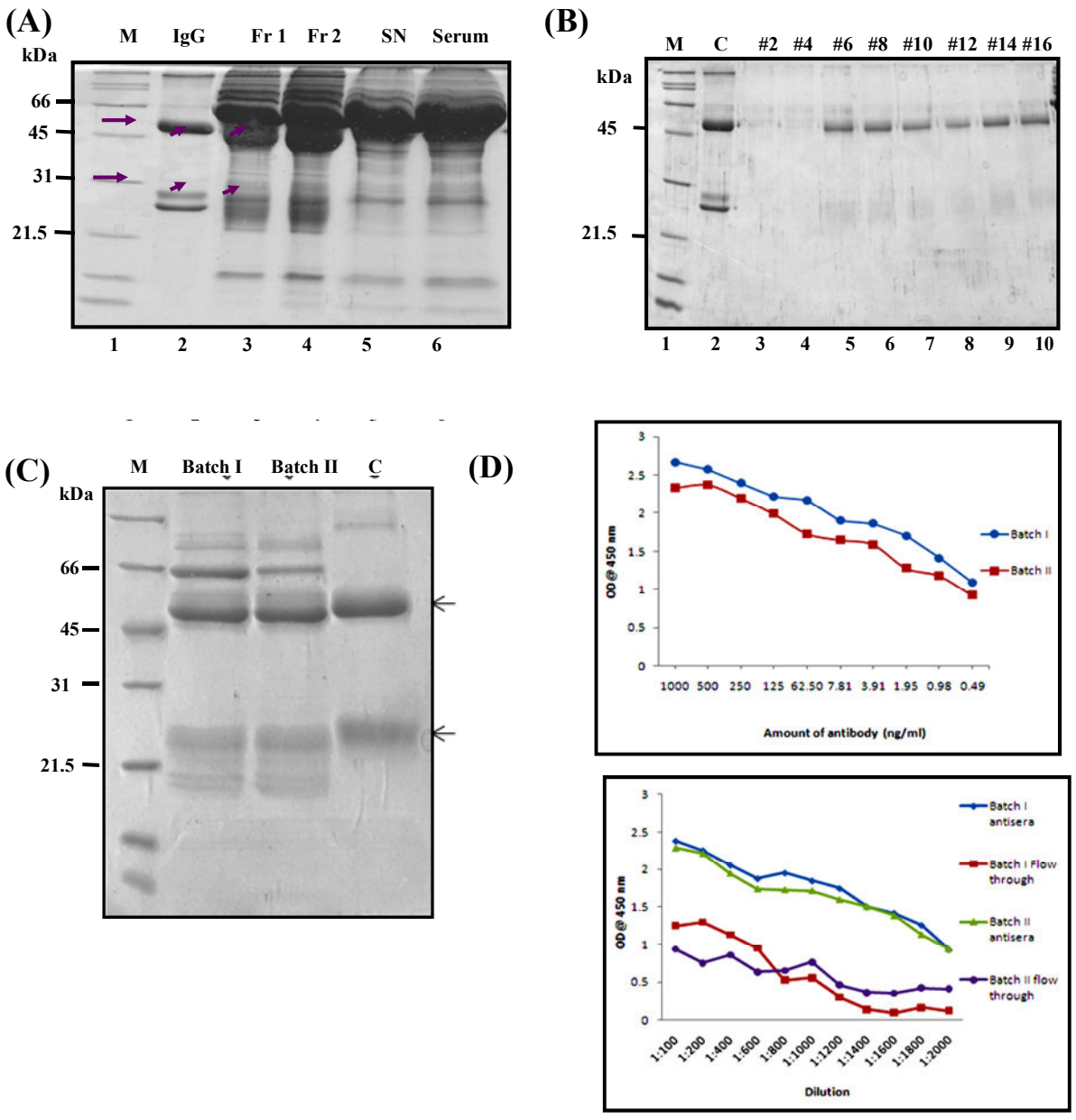


Figure 2.31 Purification of Anti AcPC4 antibody. (A) Ammonium sulphate purification of antibody. Crude antiserum was subjected to purification by mixing with ammonium sulphate. Enrichment of IGgs in purified fractions (lane 3-4) as compared to crude serum (lane 5) was visualised by coomassie brilliant blue stained 12% SDS PAGE gel. (B) Protein G purification of antibodies: Crude antiserum was subjected to purification by incubating with Protein G beads. Fractions eluted were loaded in the gel and was visualized by comassie brilliant blue staining. Fractions shows purified antibody (Lane 3-8) as compared to crude antiserum (Lane 9). (C) Crude serum was also purified by peptide affinity purification showing enrichment in heavy and light chains(arrows) in coomassie stained 12% SDS-PAGE gel. (D) Graphical representation of Indirect ELISA performed with anti-AcPC4 antisera, flow through and purified antibody. Various amounts of affinity purified antibody were used as the primary antibody (Left). Various dilutions of antisera and flow through was used as the primary antibody (Right).

Chapter 3

RESULTS

Chapter Outline:

Chapter 3.1 Identification and characterization of post-translational modifications of PC4

3.1.1 PC4 gets acetylated in cell cycle stage specific manner

3.1.2 Deacetylation of PC4

3.1.3 Casein kinase II is the bonafide kinase for PC4

3.1.4 Post-translational modifications of PC4 regulate its biochemical functions

3.2 PC4 is essential for the maintenance of structural competence of chromatin

3.2.1 Characterization of PC4 knockdown HEK 293 stable cell lines

3.2.2 Chromatin analysis of PC4 knockdown stable cell line

3.2.3 PC4 silencing induces tumorigenic potential in PC4 knockdown stable cells

3.2.4 Global gene expression and significant differential pathways analysis upon PC4 silencing

3.3 PC4 acts as a tumor suppressor in breast cancer

3.3.1 PC4 is significantly downregulated in breast cancer patient samples

3.3.2 PC4 plays a tumor suppressor role in breast carcinoma

3.4 Summary of the results

Chapter 3.1 Identification and characterization of post-translational modifications of PC4

PC4 has been reported to undergo post-translational modifications *in vitro*, especially acetylation and phosphorylation. The lysine acetyltransferase, p300 (KAT3B) was found to acetylate at least two lysine residues in PC4 as revealed by mass spectral analysis. Acetylation of PC4 was inhibited upon its prior phosphorylation by casein kinase II (CKII). Although, the ability of PC4 to get acetylated was observed *in vitro*, physiological evidence for acetylated PC4, the enzymes responsible and the functions of acetylation were unidentified. Phosphorylation of PC4, on the other hand has been studied extensively towards its negative influence on PC4 in its transcriptional coactivation function. It has been shown that in the mammalian cells about 95% of the total PC4 protein is present in the phosphorylated form. Upon phosphorylation by CKII, PC4 loses its double stranded DNA binding as well as ability to interact with different transcription factors thereby, phospho-PC4 cannot activate activator dependent RNA polymerase II driven transcription. Thus, the predominant form of PC4 (P-PC4) may be involved in functions other than transcriptional regulation.

3.1.1 PC4 gets acetylated in cell cycle stage specific manner

3.1.1.1 Identification of acetylation sites of PC4

To assess the existence of PC4 acetylation *in vivo*, it was essential to find out lysine residues getting acetylated in the cell. Therefore, FLAG tagged PC4 mammalian construct

was transfected transiently into HEK 293T cells and FLAG-PC4 was pulled down using M2-agarose beads. Bead bound proteins were resolved in SDS-PAGE and the band corresponding to PC4 was subjected to in gel tryptic digestion followed by MALDI-TOF analysis. Peptide mass fingerprinting data suggested that at least four lysine residues (K26, K28, K53, K68) in PC4 gets acetylated (Figure 3.1A; Batta K and Kundu TK, unpublished) with higher probability score for lysines 53 and 68. Later, acetylome data extending upto 1750 cellular proteins also confirmed lysine 68 as one of the acetylating residue of PC4 *in vivo* (Choudhary et al, 2009). Based on this result, a 23 amino acid peptide was designed spanning two acetylated lysines (corresponding to K53 and K68) (Figure 3.1B). Polyclonal antibodies were generated in rabbit using this peptide and were further checked for their affinity and specificity. Time course acetylation reaction using recombinant PC4 indicated that antibody was specific to acetylated form of PC4 as antibody failed to detect unmodified or mock acetylated PC4 (Figure 3.1C, Lanes 1, 2 versus 3-7). Antibody could also detect a major band from HeLa cell lysate corresponding to acetylated PC4 (Figure 3.1E, Lane 3). Specificity of the antibody was verified by probing it against increasing amount of unmodified PC4. Anti Ac-PC4 antibodies were found to recognize specifically the acetylated PC4 as these could not detect upto 4 μg of unmodified PC4 (Figure 3.1D, Lanes 1-3). Owing to a high turnover of acetylation known for other substrates like histones, we chose to prevent such an event from occurring within PC4 by using, broad spectrum histone deacetylase inhibitors, sodium butyrate (NaBu) and Trichostatin A (TSA) to enrich overall acetylation levels in the cell. HeLa cell lysate prepared after 12 hours of treatment with 500 μM NaBu and 1.3 μM TSA showed increase in PC4 acetylation as compared to similar treatment with solvent control (DMSO) when probed with anti Ac-PC4 antibodies (Figure 3.1F).

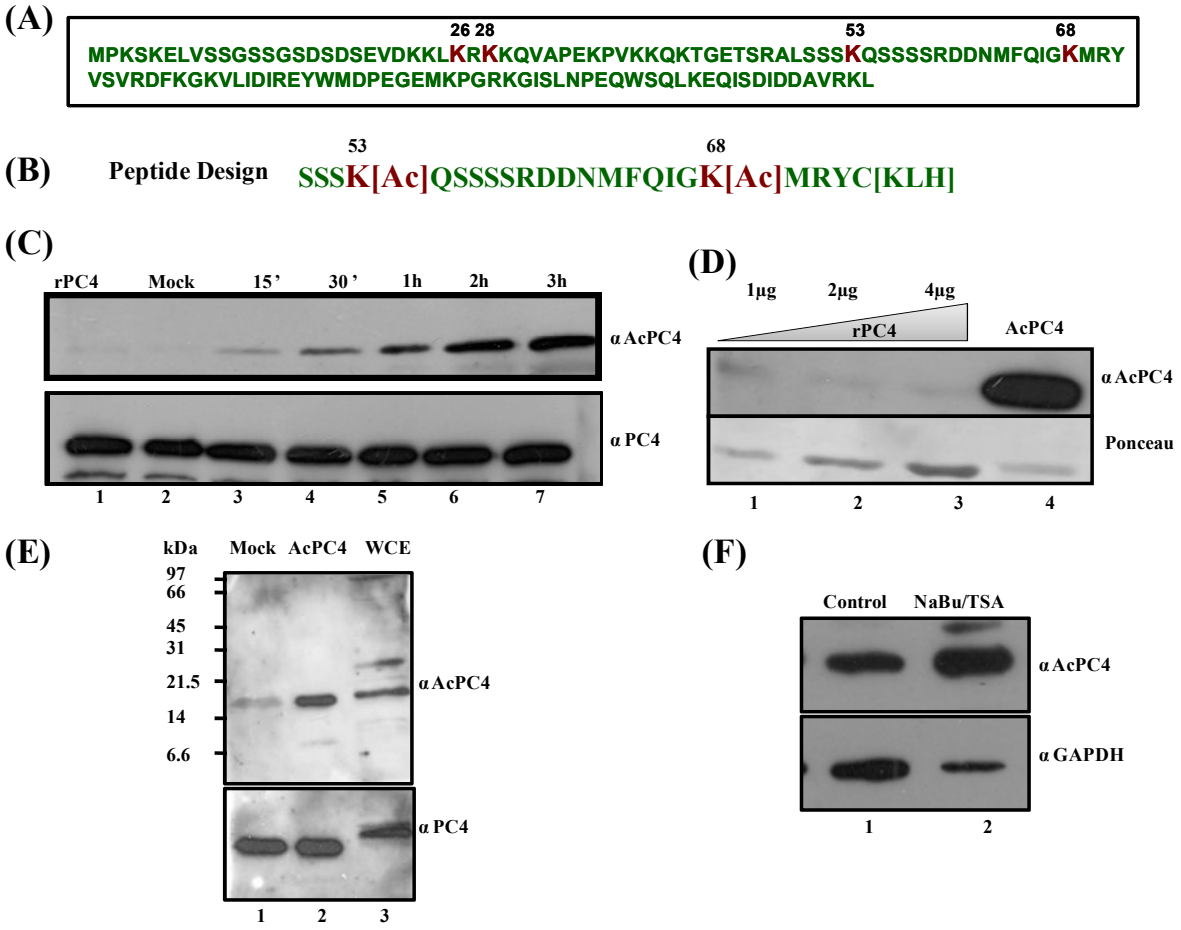


Figure 3.1 Characterization of Anti Acetylated PC4 Antibodies. (A) PC4 protein sequence indicating *in vivo* acetylation sites. (B) Peptide sequence used for raising polyclonal antibodies in rabbit. (C) Time course *in vitro* acetylation of PC4 was done for indicated time points and western blotting analysis was done using anti Ac-PC4 antibodies. Mock acetylated PC4 (Lane-2) and recombinant PC4 (Lane-1) was used as negative control. Protein loading was checked by reprobing the blot using anti PC4 antibodies (Lower panel). (D) Specificity of antibodies was further checked by probing it against increasing amount of unmodified recombinant PC4. (E) Acetylated PC4 was detected as one major band in HeLa whole cell extract (Lane 3) by anti acetylated PC4 antibodies. Reprobing with anti PC4 antibodies was done for loading control. (F) HeLa cells were treated with broad spectrum histone deacetylase inhibitors, sodium butyrate (NaBu) and Trichostatin A (TSA) followed by immunoblotting using anti Ac-PC4 antibodies. Blot was reprobed with anti GAPDH antibodies for the loading control.

3.1.1.2 Localization of acetylated PC4 during cell cycle stages

PC4 is a nuclear protein and has been found to be associated with chromatin throughout the different cell cycle stages. With the objective to examine localization of acetylated PC4 in the cell through different cell cycle stages, immunofluorescence was performed with anti Ac-PC4 antibodies. Asynchronous cell population of HEK 293 grown on glass coverslips were fixed with paraformaldehyde, stained with anti AcPC4 antibodies followed by fluorescent secondary antibodies. Acetylation of PC4 was found to be reduced during progression of mitosis and reappeared towards the end of mitotic cycle. Unlike PC4, acetylated PC4 was not uniformly present in chromatin during cell cycle (Figure 3.2).

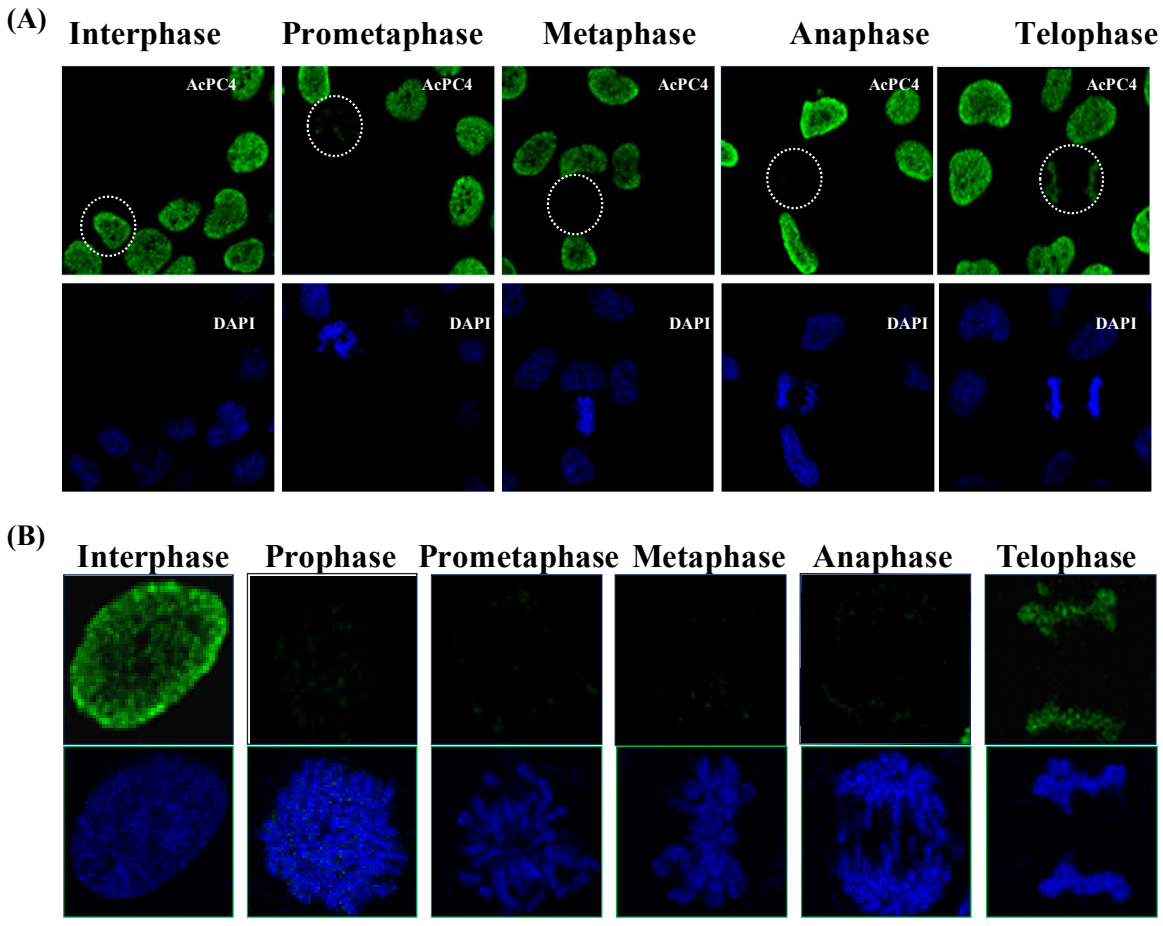


Figure 3.2 Localization of acetylated PC4 in different cell cycle stages. (A) Asnychronous HEK 293 cell population was grown on glass coverslips and immunostained using anti Ac-PC4 antibodies. Cells captured at indicated stage are shown by white circle. (B) Magnified images of cells stained

with anti Ac-PC4 antibodies at indicated cell cycle stages (Green; Upper panel). Cells were counterstained with DAPI to mark chromatin/ chromosome (Blue, Lower panel).

To further verify this finding, HeLa cells were synchronised in different cell cycle stages namely G0/G1, release of G0/G1 (upon serum stimulation for 3 hours), G1/S and G2/M. PC4 acetylation level was monitored in these different stages, where gradual reduction of acetylation was observed with the progression of mitosis (Figure 3.3A). The abundance of PC4 on the other hand, was found almost equal in similarly synchronised HeLa cell populations (Das et al, 2006). Since, there is significant decrease in transcription during entry to mitosis, association of acetylated PC4 with active transcriptional foci was examined by colocalization with phosphorylated RNA polymerase II (phosphorylated at serine 2 and serine 5 of CTD) antibodies which mark the active form of RNA polymerase . It was found that there was colocalization of acetylated PC4 and active RNA polymerase II at transcriptionally active foci (Figure 3.3B).

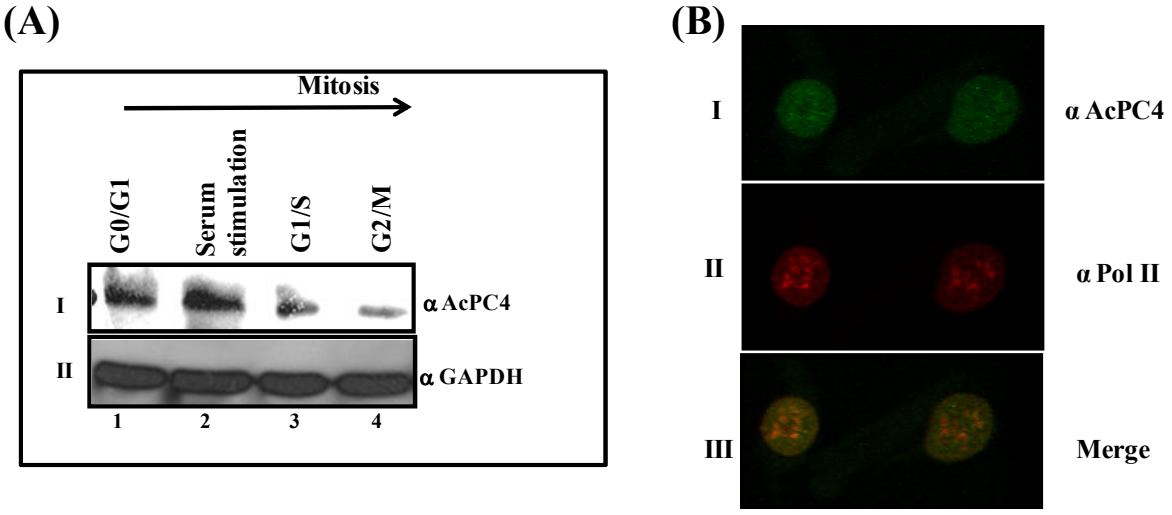


Figure 3.3 Expression level of acetylated PC4 in different cell cycle stages. (A) Distribution of acetylated PC4 throughout different stages of cell cycle. Relative amounts of acetylated PC4 present during Mitosis (lane 4), G1/S arrest (lane 3), release of G0/G1 arrest (upon serum stimulation) (lane 2) and G0/G1 arrest (lane 1), were assessed by probing with anti Ac-PC4 antibodies in western blotting analysis. As loading control, western blotting analysis was done with anti GAPDH antibodies

(panel II). (B) Co-immunofluroscence using anti Ac-PC4 and anti PolII antibodies was carried out in order to study colocolization of these proteins. Green, chromatin stained with AcPC4 antibodies (Panel I); Red, chromatin stained with Pol II antibody (Panel II). Panel III shows a merge of the two antibodies.

3.1.1.3 Screening lysine acetyltransferases acetylating PC4

Although, acetylation of PC4 by p300 (KAT3B) was reported *in vitro*, lysine acetyltransferases capable of acetylating PC4 *in vivo* was not known. We therefore, examined representative members of different lysine acetyltransferase families for their ability to acetylate PC4.

3.1.1.4 PC4 has multiple p300 (KAT3B) sites

Lysine acetyltransferase, p300 (KAT3B) is one of the most studied acetyltransferases amongst other KATs. p300/CBP family have a broad range of histone and nonhistone substrates and have been reported to be involved in important cellular functions like transcription, DNA replication and repair, apoptosis, differentiation etc. A consensus p300 binding site has been predicted by analysing sequences across different substrates that get acetylated. This consensus sequence indicates that there should be a Lys or Arg (positively charged) residue present either at -Lys-3/4 or +Lys-3/4 position or both from the primary acetylation site in question. Upon alignment of lysines present in PC4 protein according to the predicted p300 consensus protein sequence, we observed that there could be multiple lysines in PC4 that could be putative p300 substrate sites (Figure 3.4).

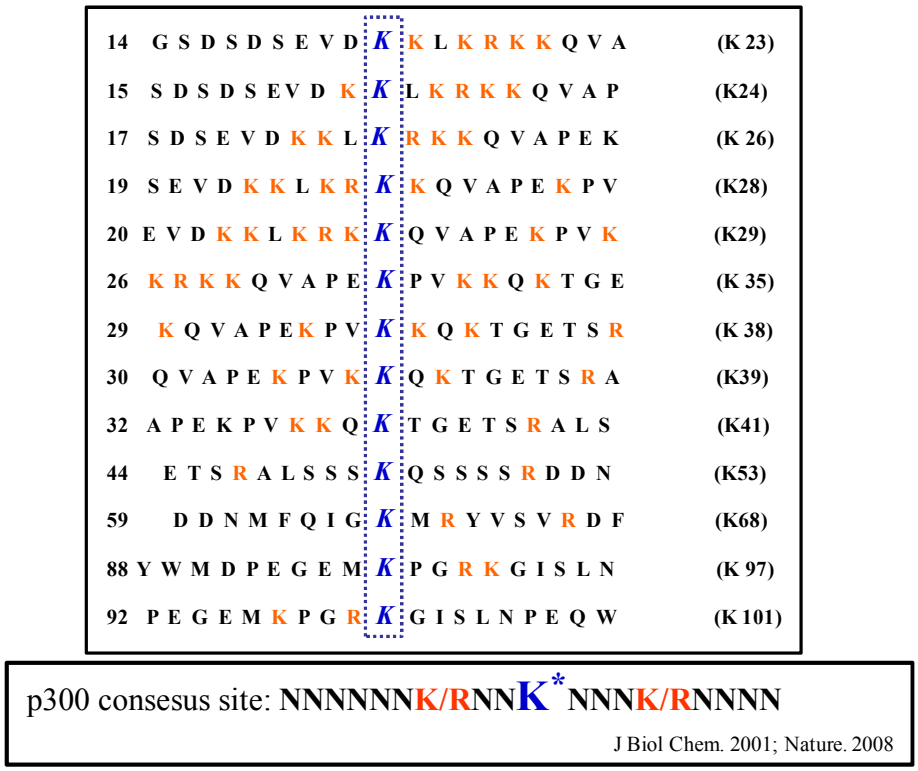


Figure 3.4 PC4 has multiple p300 (KAT3B) sites. Alignment of possible primary acetylation lysines in PC4 protein according to predicted p300 consensus site.

Furthermore, with the objective to explore enzymatic machinery responsible for PC4 acetylation *in vitro* and *in vivo*, we examined three major lysine acetyltransferase families, namely, p300/CBP, GNAT and MYST. Two representative members from each family were selected for *in vitro* acetylation reaction using PC4 as substrate. We found that except for GNAT family members PCAF (KAT2B) and GCN5 (KAT2A); other members of p300/CBP and MYST families (p300 (KAT3B), CBP (KAT3A); TIP60 (KAT5), MOZ (KAT6A) could acetylate PC4 (Figure 3.5). Specifically, minimal catalytic (KAT) domains of TIP60 (KAT5) and MOZ (KAT6A) were sufficient for the acetylation.

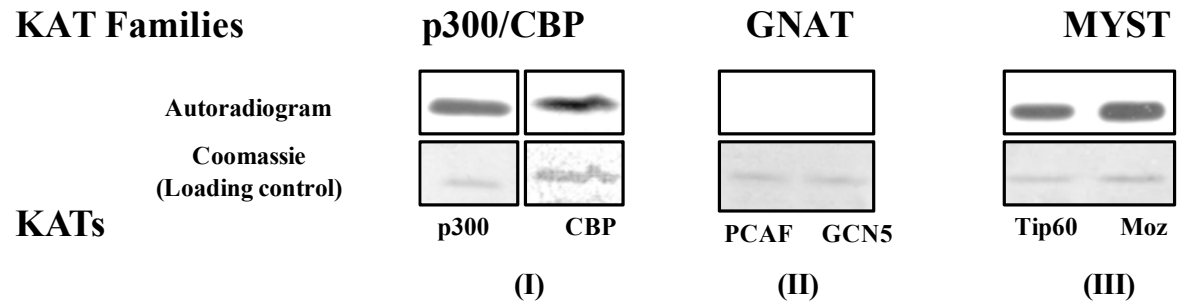


Figure 3.5 Acetylation of PC4 by different classes of KATs. *In vitr*o acetylation assay of PC4 with members of different classes of KATs, namely, p300/CBP (Full length p300 (KAT3B) and CBP (KAT3A) (I), GNAT (PCAF (KAT2B) and GCN5 (KAT2A) KAT domain) (II) and MYST (TIP60 (KAT5) and MOZ (KAT6A) KAT domain) (III) were carried out using ^{3}H-acetyl CoA. Incorporation of ^{3}H-acetyl CoA was detected by autoradiogram. Coomassie brilliant blue stained 12% SDS-PAGE gel was used as loading control (Bottom).

Lysine amino acid sites that were responsible for a major share of acetylation (critical lysines) of PC4 were analyzed using PC4 mutants, where lysine was replaced by alanine or arginine and the ability to get acetylated was tested by *in vitro* reactions. Sites that have been predicted to get acetylated *in vivo* (Lysines 26, 28, 53, 68) were either changed to arginine (an acetylation mimic) or alanine (acetylation defective residue). In this way, a series of lysine point mutations were created in PC4 protein (Figure 2.7). Mutations were not only created in single lysine sites but also in combinations of different lysines. The mutant proteins along with wild type proteins were tested *in vitro* for acetylation by p300. When all the four predicted lysines (K26, K28, K53 and K68) were mutated, acetylation was almost completely abolished. Both arginine and alanine substitutions (M11: K 26, 28, 53, 68 R and M12: K 26, 28, 53, 68 A) showed similar effects (Figure 3.6 A, B). It is important to note that mutants that were defective for two lysine residues at 53 and 68, (M9: K 53, 68 R and M10: K 53, 68 A) were found highly compromised in their ability for getting acetylated (Figure 3.6 C, D).Whereas, singly mutated lysine mutants did not show significant difference in their acetylation ability (data not shown).

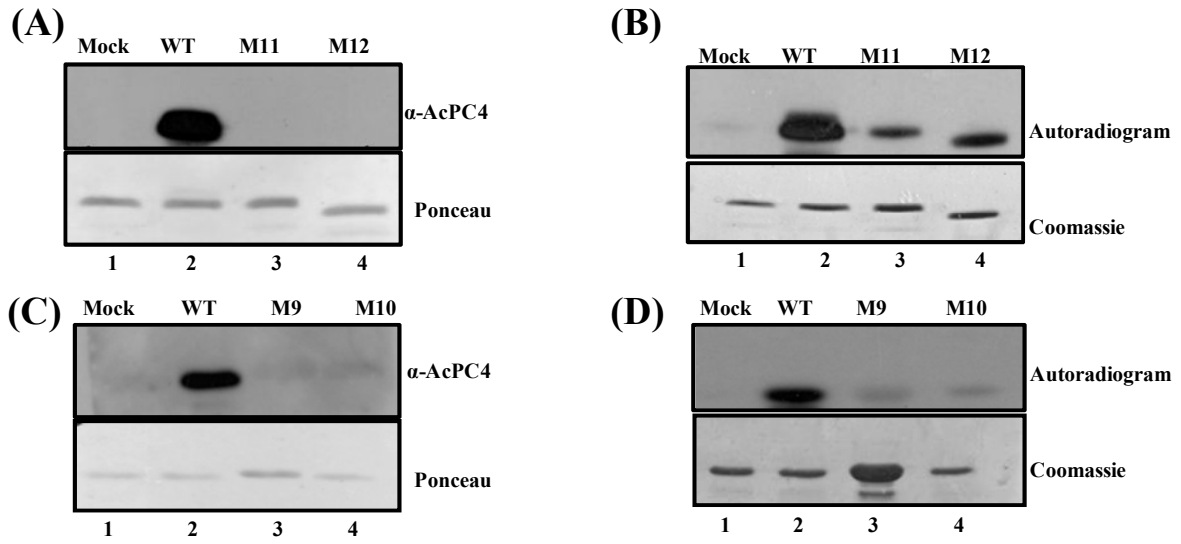


Figure 3.6 HAT assay using mutant PC4. *In vitro* HAT assay was performed with wild type and indicated mutant PC4 proteins with full length p300 followed by immunoblotting using anti Ac-PC4 antibodies (A, C; Top Panel) and gel assay using ^{3}H-acetyl CoA (B, D; Top Panel). The loading control of the proteins is shown by Ponceau staining of the Membrane (A, C; Bottom Panel) and coomassie stained 15% SDS-PAGE gel (B, D; Bottom Panel).

Following mapping of p300 mediated acetylation sites in PC4 protein, it was important to know whether these sites were common for other acetyltransferases as well. We chose a mutant M9 (K 53, 68R) which was found defective in p300 mediated acetylation, and subjected it to *in vitro* acetylation reaction using TIP60 (KAT5) KAT domain as enzyme source. We found that this mutant did not show any reduction in acetylation when compared to wild type emphasizing different target lysines for MYST family in PC4 (Figure 3.7B).

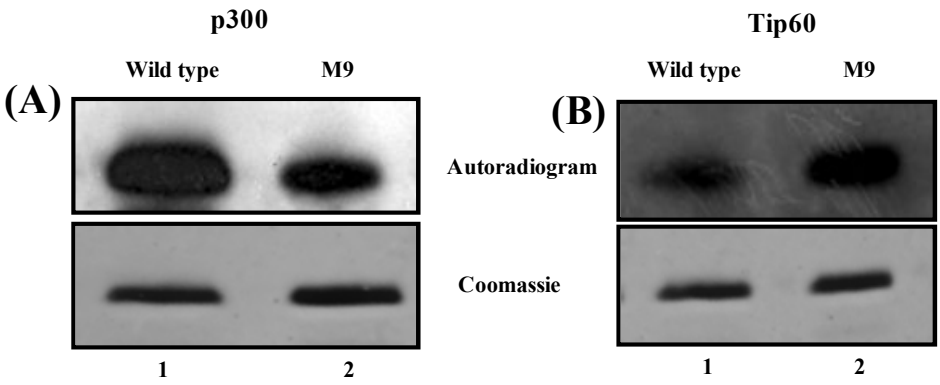


Figure 3.7 HAT assay using mutant PC4: *In vitro* acetyltransferase assay was performed with wild type (Lane 1) and mutant PC4 protein (Lane 2) with full length p300 (A) and TIP60 (KAT5) KAT domain (B) followed by gel assay using ^{3}H-acetyl CoA. The loading control of the proteins is shown by coomassie stained 15% SDS gel (Bottom). Lanes - WT recombinant PC4 (lane 1), M9 (K53, 68R) mutant recombinant PC4 (lane 2).

3.1.1.5 p300 (KAT3B) acetylates PC4 *in vivo*

In order to explore *in vivo* effect of acetyltransferase in PC4 acetylation, p300 (KAT3B) was silenced in HEK 293T cells utilizing p300 specific siRNAs (Sigma). siRNAs mediated silencing of p300 was standardized in this cell line to obtain approximately 2.5-3 fold repression (Figure 3.8A). Cells were treated with increasing concentrations of p300 siRNAs (10nM-50nM) and cell lysates were subjected to immunoblotting with anti Ac-PC4 antibodies. A gradual decrease in PC4 acetylation was observed with increase in p300 siRNA concentrations (Figure 3.8B). Reduction in PC4 acetylation was also visualized when siRNAs treated HEK 293T cells were stained with anti Ac-PC4 antibodies (Figure 3.8C). These findings suggest that KAT3B (p300) could be the major acetyltransferase for PC4 in normal physiological conditions.

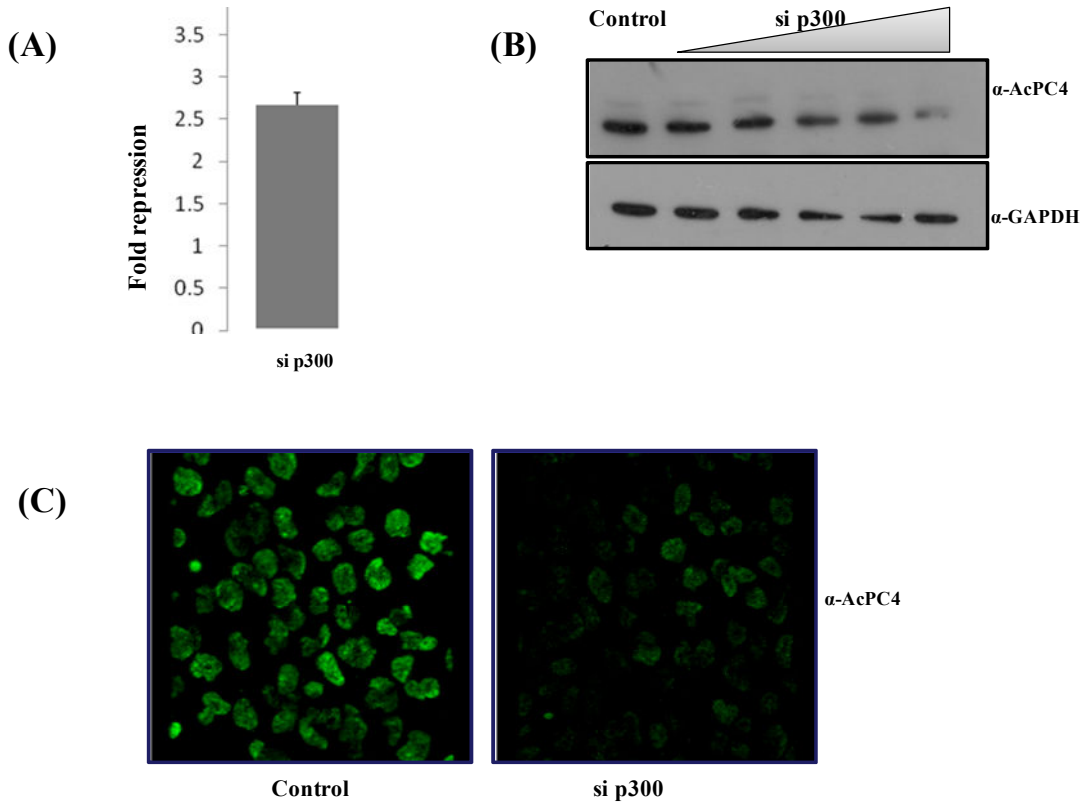


Figure 3.8 p300 acetylates PC4 *in vivo*. HEK 293T cells were transfected with p300 siRNAs for 48 hours and processed for cDNA synthesis for RT-PCRs or lysate preparation for western blotting. (A) Silencing of p300 was scored by performing real time RT-PCR using p300 gene specific primers. (B) Western blot analysis was done with anti-AcPC4 antibodies at 48 h posttransfection with increasing concentrations of p300 siRNAs (upper panel). A corresponding loading control was done with anti GAPDH antibodies (lower panel). (C) Immunofluroscence using anti Ac-PC4 antibodies was done with the cells transfected with scrammbled (Left) and 50 nM p300 siRNAs (Right) respectively.

3.1.2 Deacetylation of PC4

3.1.2.1 Screening of different classes of HDACs

Acetylation is a dynamic and reversible process. Deacetylases are a group of enzymes which revert the acetylation process by removing acetyl group. Thus, to investigate whether PC4 indeed gets deacetylated, we investigated members from different classes of histone deacetylases (HDAC class I and III). HDACI (Class I), SIRT1 and SIRT2 (Class III) were

purified till homogeneity and checked for their activity using core histones as substrates in a *in vitro* gel assay (Figure 3.9A, Lanes 1-7; Figure 3.9B Lanes 1-5). Active deacetylases, namely HDAC1, SIRT1 and SIRT2 were checked for their activity using preacetylated PC4. PC4 was acetylated in a p300 dependent acetyltransferase assay and the same reaction then was subjected to deacetylation. Both class I and III members used, facilitated PC4 deacetylation in a dose dependent manner *in vitro* (Figure 3.9A, Lanes 8-13; Figure 3.9B, Lanes 6-9 respectively). Class III deacetylases, SIRT1 and SIRT2 could revert p300 dependent acetylation of PC4 in NAD^+ dependent manner.

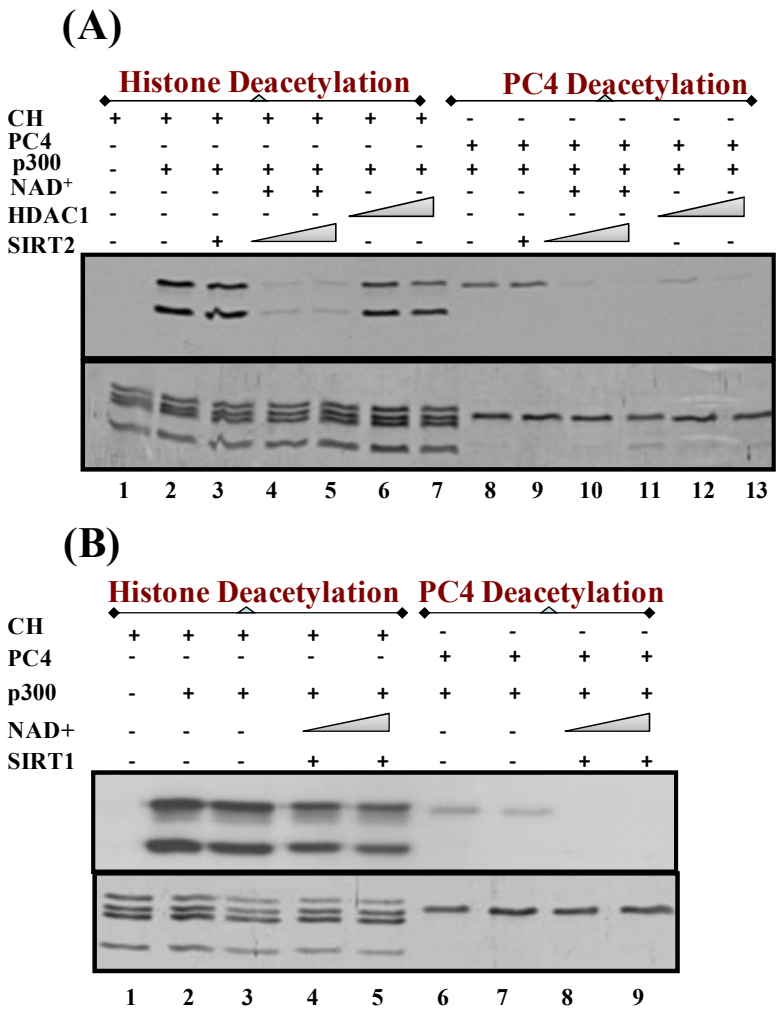


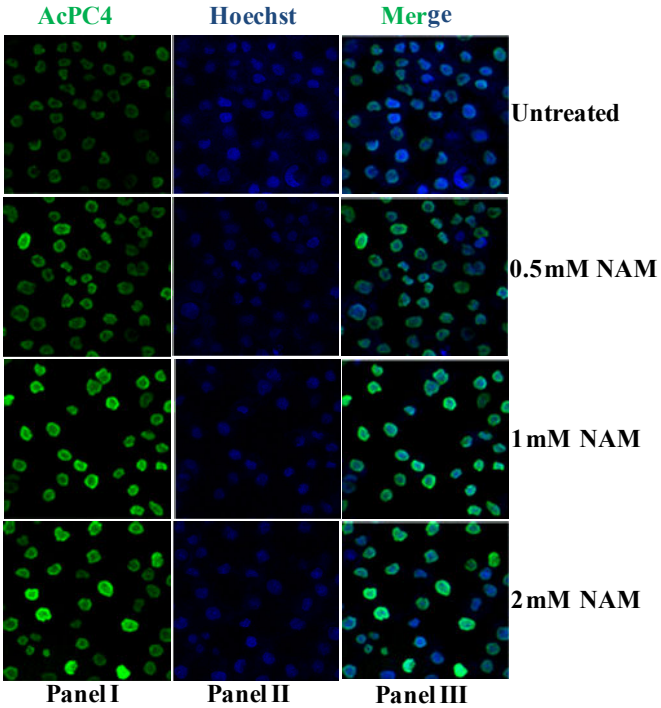
Figure 3.9 *In vitro* deacetylation of PC4: (A) Histone deacetylase assay was performed using core histones (lanes 1-7) and PC4 (lanes 8-13) as substrates for SIRT2 (lanes 4-5; lanes 10-11 respectively) and HDAC1 (lanes 6-7; lanes 12-13 respectively) deacetylases. p300 mediated acetylation was

performed prior to the deacetylase assay. Mock deacetylation reaction containing all components except for HDAC1 or SIRT2 (lane 2; lane 9 respectively) was used as negative control. Lane 1 contains core histones without any enzymes. (B) Similar deacetylase reaction was performed with SIRT1 deacetylase where core histones (lanes 1-5) and PC4 (lanes6-9) were used as substrates in presence of increasing concentrations of NAD+ (lanes4-5; lanes 8-9 respectively). Mock deacetylation reaction containing all components except for SIRT1 (lane 2; lane 6 respectively) was used as negative control.

3.1.2.2 HDAC class III deacetylases PC4 in the cell.

We took the inhibitor based approach (class specific histone deacetylase inhibitors) to specify enzymes deacetylating PC4 in the cell. HeLa cells were treated with increasing concentration of HDAC class III inhibitor, Nicotinamide. Cells were fixed and stained with anti Ac-PC4 antibodies for scoring PC4 acetylation in treated cells. Nicotinamide treated cells showed gradual increase in acetylated PC4 level (Figure 3.10A, Panel I) indicating class III deacetylase responsible for deacetylating PC4 *in vivo*.

(A)



(B)

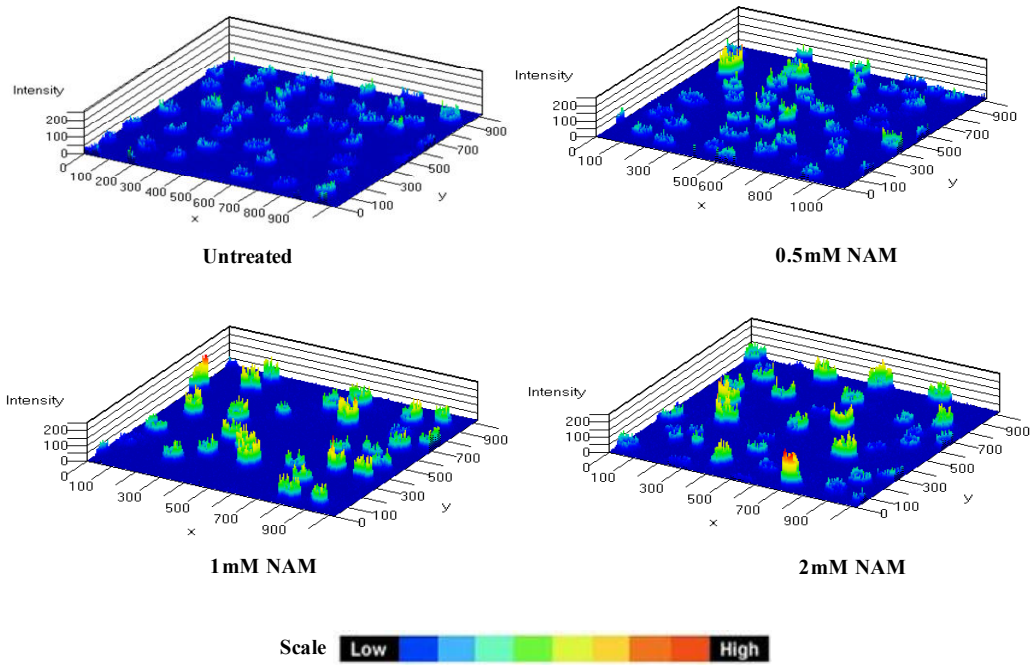


Figure 3.10 HDAC class III deacetylates PC4 in the cell. (A) Inhibition of deacetylation of PC4 upon a dose dependent treatment of HeLa cells with nicotinamide as visualised by confocal imaging. Panel I shows immunofluroscence of HeLa cells with anti Ac-PC4 antibodies. Panel II shows DNA staining by hoechst and merge of antibody and DNA staining is shown in Panel III. (B) Intensity plot representation of enhancement of acetylation of PC4 as visualised by confocal imaging. Warm colours (Yellow and red) represent higher intensity than cooler colour (cyan, green).

Increase in PC4 acetylation through inhibition of class III deacetylases was also verified by immunoblotting. Untreated and nicotinamide treated HeLa cells were lysed and the lysates were subjected to western blot analysis with anti Ac-PC4 antibodies. Nicotinamide treated cell lysates showed significant increase in PC4 acetylation in a dose dependent manner (Figure 3.11, Lanes 1-3 versus lane 4). These findings emphasize that PC4 could be substrate of at least the class III deacetylases.

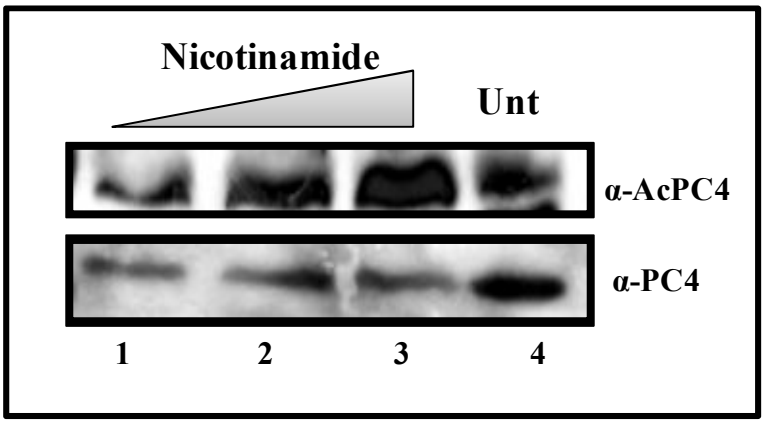


Figure 3.11 Nicotinamide treatment enhances acetylation of PC4 in a dose dependent manner Western blot analysis of nicotinamide treated (Lanes 1-3; 0.5mM, 1 mM, 2mM nicotinamide respectively)and untreated (lane 4) HeLa cells. Probing was done using anti Ac-PC4 antibodies. As loading control western blotting analysis was done with anti- PC4 antibodies (Lower panel).

3.1.2.3 Human SIRT2 specifically deacetylates PC4 *in vivo*

After observing HDAC class III as one of the classes to be involved in deacetylating PC4, determination of the member among class III deacetylases specifically deacetylating PC4 was the next step. We adopted the approach of overexpression of candidate enzymes in the cell and scoring their ability in terms of decrease in PC4 acetylation. For the purpose, mammalian constructs of human class III members, namely hSIRT1 and hSIRT2 were transfected for overexpression in HEK 293T cells. Transfected cells were first verified for overexpression of respective enzymes using antibodies against SIRT1 and histidine tag present in respective mammalian constructs (Figure 3.12A). When transfected cell lysates were checked for PC4 acetylation status, it was found that overexpression of SIRT2 enzyme could reduce PC4 acetylation in cells (Figure 3.12C) whereas overexpression of SIRT1 failed to do so. These findings suggest that although both SIRT1 and SIRT2 could deacetylate PC4 *in vitro*, there exists specificity in the cell. Involvement if SIRT2 in PC4 deacetylation was

also ascertained by overexpression of deacetylase defective SIRT2 mutant, hNDAC which failed to reduce PC4 acetylation (Figure 3.12D).

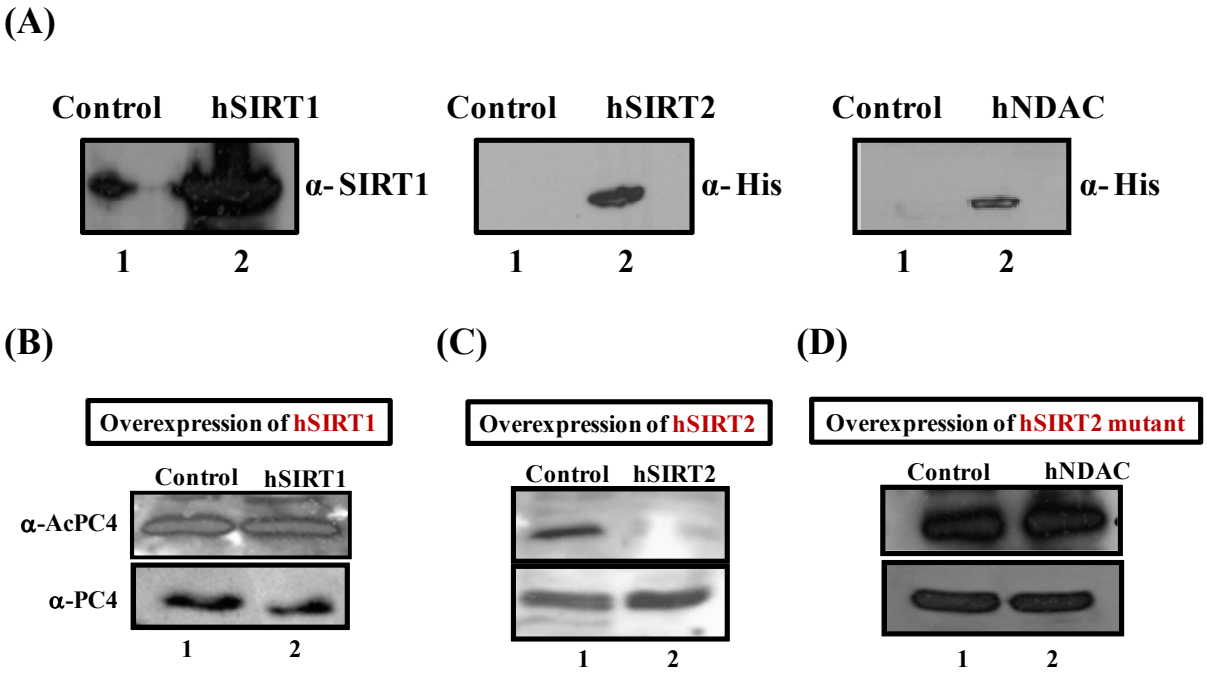


Figure 3.12 SIRT2 deacetylates PC4 *in vivo*. (A) HEK 293T cells were transfected with mammalian constructs of human SIRT1 (hSIRT1), SIRT2 (hSIRT2) and catalytic SIRT2 mutant (hNDAC). Untransfected (Lane 1) and transfected (Lane 2) cell lysates were checked for their overexpression using antibodies against SIRT1 and histidine tag present in respective mammalian constructs. Untransfected (Lane 1) and transfected (Lane 2) cells with hSIRT1 (B), hSIRT2 (C) and hNDAC (D) were subjected to western blot analysis using anti-AcPC4 antibodies (Upper panel). As loading control, western blotting analysis was done with anti PC4 antibodies (Lower panel).

Conversely, to ascertain if silencing of SIRT2 could revert the effect of SIRT2 mediated deacetylation, thereby enhancing PC4 acetylation, we transfected siRNAs against human SIRT2 in HEK 293 cells. Reduction in the transcript expression confirmed the silencing of SIRT2 in the cell (Figure 3.13A). Cells transfected with increasing concentrations of SIRT2 siRNAs (20nM, 30nM, 40nM) were immunostained with anti Ac-PC4 antibodies. SIRT2

silenced cells showed a dose dependent increase in PC4 acetylation when compared to control cells (Figure 3.13B) indicating PC4 is a bonafide substrate of SIRT2 *in vivo*.

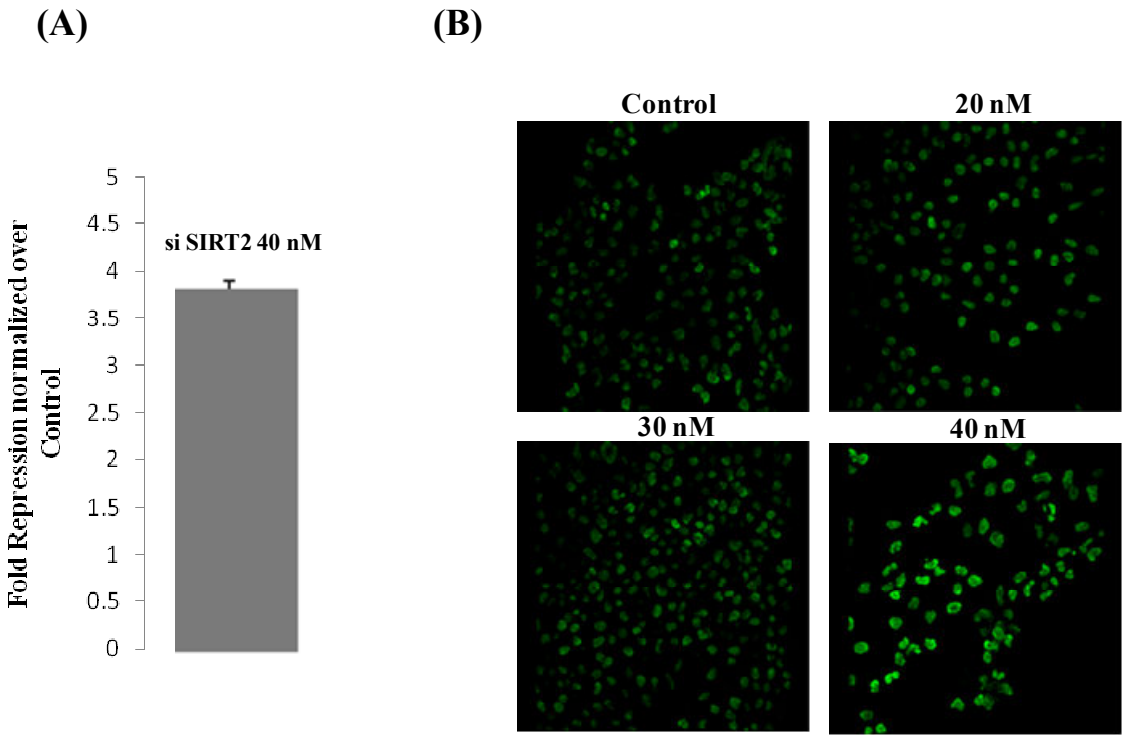


Figure 3.13 SIRT2 deacetylates PC4 *in vivo*. (A) HEK 293 cells were transfected with SIRT2 siRNAs for 48 hours. Silencing of SIRT2 was scored by performing real time RT-PCR using SIRT2 gene specific primers. (B) Immunofluroscence using using anti-AcPC4 antibody was done with the cells transfected with increasing concentrations of siRNAs (20, 30, 40 nM).

3.1.2.4 Human SIRT2 interacts with acetylated PC4 in presence of NAD+

A possible interaction of enzyme SIRT2 with its substrate PC4 was examined in an *in vitro* GST pull down assay. It was found that SIRT2 interacted with acetylated PC4 in presence of NAD^+ (Figure 3. 14A, Right panel, Lane 3) whereas unmodified PC4 was unable to show any interaction with SIRT2 (Figure 3. 14A, Left panel, Lane 3). SIRT2 being mainly a cytosolic enzyme, its interaction and deacetylation of PC4, a nuclear protein was puzzling.

However, cell cycle localization studies with SIRT2 and Ac-PC4 antibodies revealed that SIRT2 localized to chromatin during mitosis when acetylated PC4 level is greatly reduced (Figure 3.14B). A recent report also suggested the nucleocytoplasmic shuttling of SIRT2 during mitosis (Vaquero et al, 2006). It could be possible that the nuclear shuttling of SIRT2 also resulted in interaction and subsequent deacetylation of PC4. However, this possibility is yet untested.

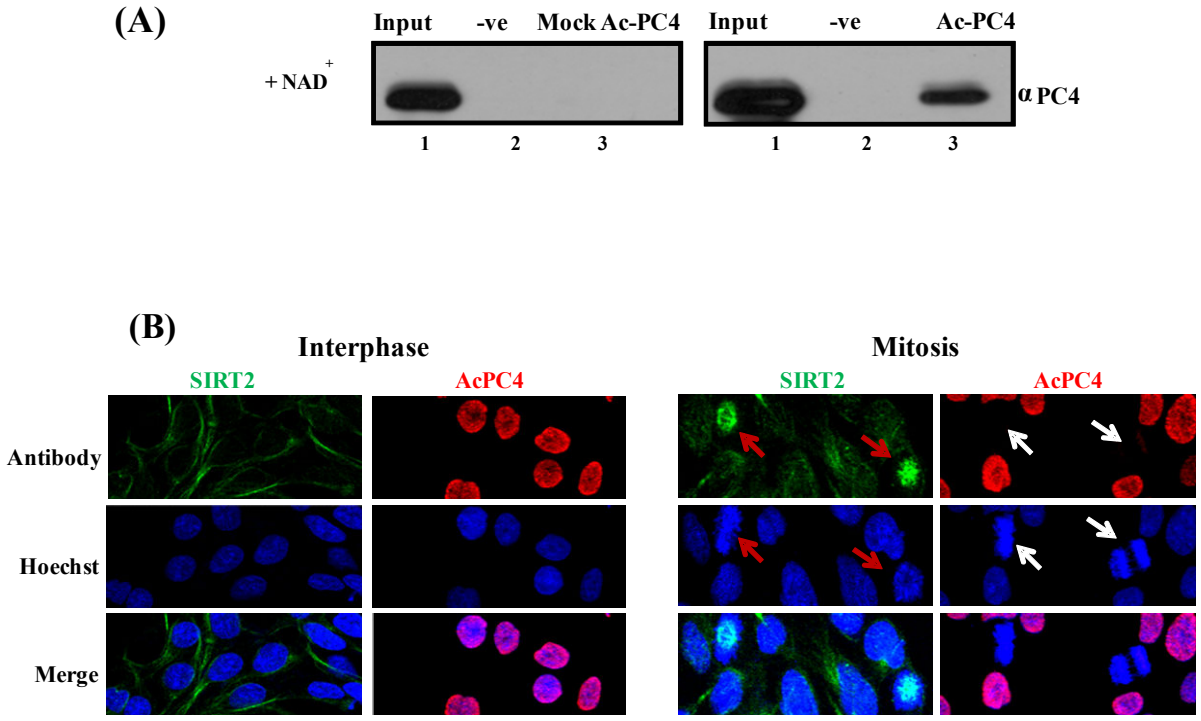


Figure 3.14 Human SIRT2 interacts with acetylated PC4. (A) GST pull down assay using GST SIRT2 with mock and acetylated PC4 in presence of NAD^+ at 150 mM salt concentration. (B) Immunofluorescence performed in HEK 293 cells utilizing anti SIRT2 (Green) and Ac-PC4 (Red) antibodies. Cells were counter stained with hoechst (Blue) dye to visualize DNA. Arrows indicate cells in mitosis.

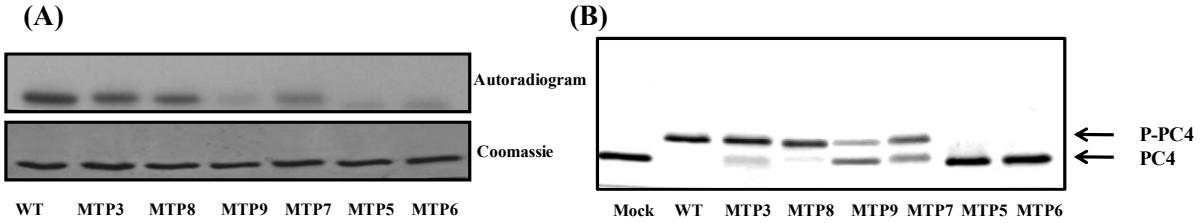
3.1.3 Casein kinase II is the bonafide kinase for PC4

The other known post-translational modification of PC4 is phosphorylation. PC4 has been reported to get phosphorylated by multiple kinases *in vitro*. Studies have indicated that PC4 is majorly phosphorylated by casein kinase II however; protein kinases (A and C) can also phosphorylate PC4 *in vitro*. Deletion mutant and mass spectrometric studies however, have indicated that *in vivo* hyperphosphorylation of PC4 could be mainly mediated by CKII (Ge et al, 1994). *In vitro* transcription assays testing PC4 as a coactivator have revealed that it gets phosphorylated at the time of preinitiation complex (PIC) formation by components of TFIID and TFIIH and subsequently gets released from promoter.

3.1.3.1 Mapping of critical residues in PC4 phosphorylated by casein kinase II

PC4 protein contains two serine rich acidic (SEAC) domains towards N-terminal, which are predicted to consist of phosphorylation sites. We resorted to mapping of critical phosphorylation residues with the help of point mutants. A series of point mutations were created in first SEAC region (Figure 2.10) and their phosphorylation ability was examined in CK II mediated reaction (Figure 3.15C). Serine residues 13, 15, 17 and 19 were mutated either individually or in combination. Serine 15 and 17 when mutated in combination (MTP5, MTP6, MTP9) showed significant reduction in phosphorylation as compared to wild type protein indicating their crucial involvement in CK II mediated phosphorylation of PC4. Serine 17 (MTP3) mutation did show minor reduction in radioactive ATP incorporation in CK II driven kinase reaction (Figure 3.15A). Mass phosphorylation (ensuring completion of the reaction by repeated replenishment of enzyme and ATP) of wild type and mutants using cold ATP and CK II enzyme were performed. Slower mobility of phosphorylated form of

proteins was visualized on coomassie blue stained 12% SDS-PAGE gel. Even in this case, mutation of serine 15 and 17 together, severely compromised phosphorylation as scored by decrease in slow moving form and an increase in intensity of the unmodified (thereby faster moving) form (Figure 3.15B). These results suggest that although, many serine residues get phosphorylated on PC4, the major sites that contribute to phosphorylation are serine 15 and serine 17.



(C)

Name	*Residues Mutated*	*Phosphorylation Ability*
MTP3	17	+
MTP6	13, 15, 17	-
MTP5	13, 15, 17, 19	-
MTP7	13, 15	+++
MTP8	13, 17	++
MTP9	15, 17	+/-
WT		++++

Figure 3.15 Mapping of Casein kinase II sites in PC4 protein. (A) Gel assay was performed followed by the phosphorylation reaction of wild type and PC4 serine mutants using [γ^{32}P] ATP and casein kinase II enzyme. (B) Coomassie staining of mass phosphorylated PC4 using cold ATP. Mock reactions contain all the components except for the CKII. (C) Table illustrating extent of phosphorylation ability of different serine mutants of PC4.

3.1.4 Post translational modifications of PC4 regulate its biochemical functions

PC4 is a multifunctional protein, shown to have involvement in transcription, replication, DNA repair, chromatin organization and tumor suppression. The mechanism behind multifunctionality of PC4 is largely unknown. It has been shown that the role of PC4 in activator dependent RNA polymerase II mediated transcription activation is modulated by its phosphorylation. The phosphorylation of PC4 negatively regulates its transcriptional activation property. PC4 binds to DNA (Kretzschmar et al, 1994; Figure 3.16B) and its DNA binding ability is crucial for its several functions like transcription, DNA replication and repair. Phosphorylated PC4 does not bind to ds DNA presumably due to the negative charge introduced by the phospho group (Kretzschmar et al, 1994; Figure 3.16A, C). Phosphorylation also affects the interaction of PC4 with activators thereby, preventing coactivation. Although, it is known that phosphorylation negatively influences transcriptional coactivation, the role of acetylation of PC4 is yet unknown in RNA polymerase II transcription. It could be possible that these modifications influence the function of the protein in a context dependent manner. It is known that the phosphorylated form does not get acetylated although, the acetylated form does get phosphorylated. So, such dynamics could indeed affect the functions of the protein. Indeed, studies have shown that acetylation of PC4 enhances its ds DNA binding ability (Kumar et al, 2001; Figure 3.16A, C) which is a prerequisite for PC4 mediated activator dependent transcription activation ability. Furthermore, acetylation of PC4 has been shown to bend ds DNA more effectively and the bent ds DNA in general, has been predicted to be a better docking site for activators and transcription factors at the promoter. PC4 mediated bending of ds DNA enhances p53 binding to it consensus sites (Batta et al, 2007). We therefore, hypothesised that the acetylated form of PC4 could act as a transcriptional coactivator for activator dependent RNA polymerase II

mediated transcription. We explored this possibility of acetylation influencing the PC4 mediated activation of p53 dependent transcription utilizing a p53 null cell line

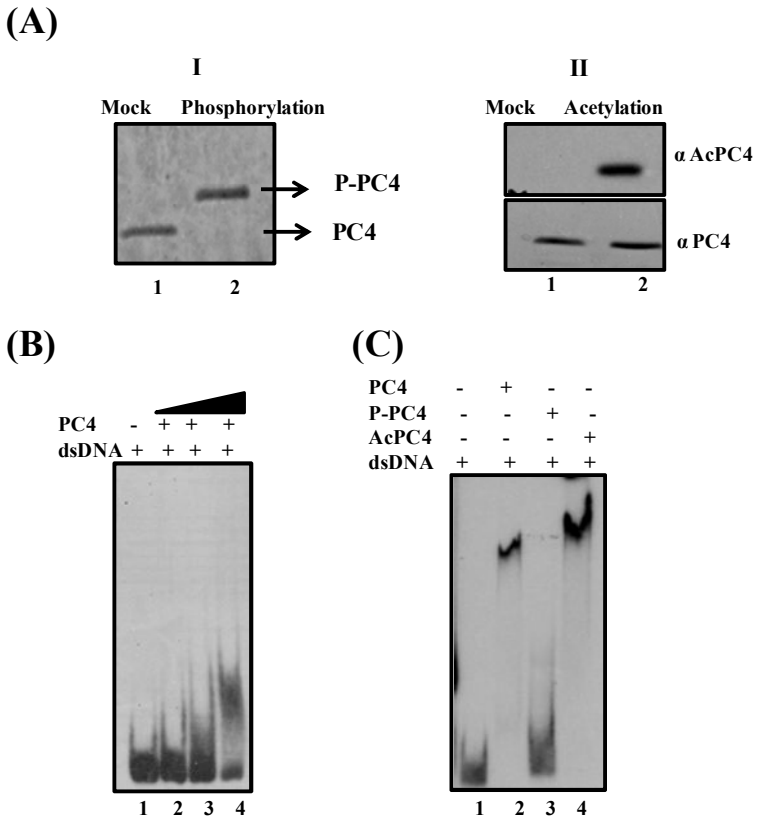


Figure 3.16 Acetylated and phosphorylated PC4 show differentialDNA binding ability. (A) Mass phosphorylation of PC4 was done using 5 mM cold ATP. For complete phosphorylation reaction mixture was replenished three times with ATP and CKII at one hour interval (Panel I). Mock (Lane-1) and phosphorylation reaction (Lane-2) was loaded in 12% SDS-PAGE gel and stained with coomassie brilliant blue. *In vitro* acetylation of recombinant PC4 was carried out in 30 µL reaction volume containing reaction buffer, cold acetyl coA with (Lane-2) or without (Lane-1) p300 enzyme (Panel II). Western blotting analysis was done by anti Ac-PC4 antibodies. Protein loading was checked using anti-PC4 antibodies (Bottom). (B) Three nanograms of a [γ^{32}P] labeled dsDNA oligonucleotide was incubated with increasing concentrations of PC4 (Lanes 2, 3 and 4) and EMSA was performed as described earlier. (C) EMSA was performed with equal amount of unmodified (Lane-2), phosphorylated (Lane-3) and acetylated (Lane-4) PC4 using double stranded labeled oligonucleotide (Lane 1).

3.1.4.1 Acetylation is crucial for p53 dependent transcriptional activation mediated by PC4

Role of PC4 in activating p53 dependent transcription and thereby, its downstream functions has been elegantly illustrated previously (Batta et al, 2007). The same system was explored to investigate a role for acetylation of PC4 in transcriptional activation. Promoter less luciferase gene construct cloned downstream to p53 responsive promoter element was transfected into p53 null cell line, H1299. Co-transfection of p53 expression vector could elicit luciferase expression as scored by increased luciferase activity (Figure 3.17, Lane 3). Transfection of PC4 and p53 expression vectors together, could further enhance luciferase activity by several folds as reported earlier (Figure 3.17, Lane 4). Expression of same concentration of acetylation defective mutant, M11 (K 26, 28, 53, 68 R), mutant for four critical lysine residues in the system showed lesser luciferase activity than wild type indicating acetylation of PC4 is important for driving p53 dependent transcription by PC4 (Figure 3.17, Lane 5). As expected, transfection of phosphorylation defective mutant of PC4, MTP5 (S13A S15G S17A S19G) did not show any significant change as compared to wild type again emphasizing that, phosphorylation probably plays no role in transcriptional activation *in vivo* as well (Figure 3.17, Lane 6).

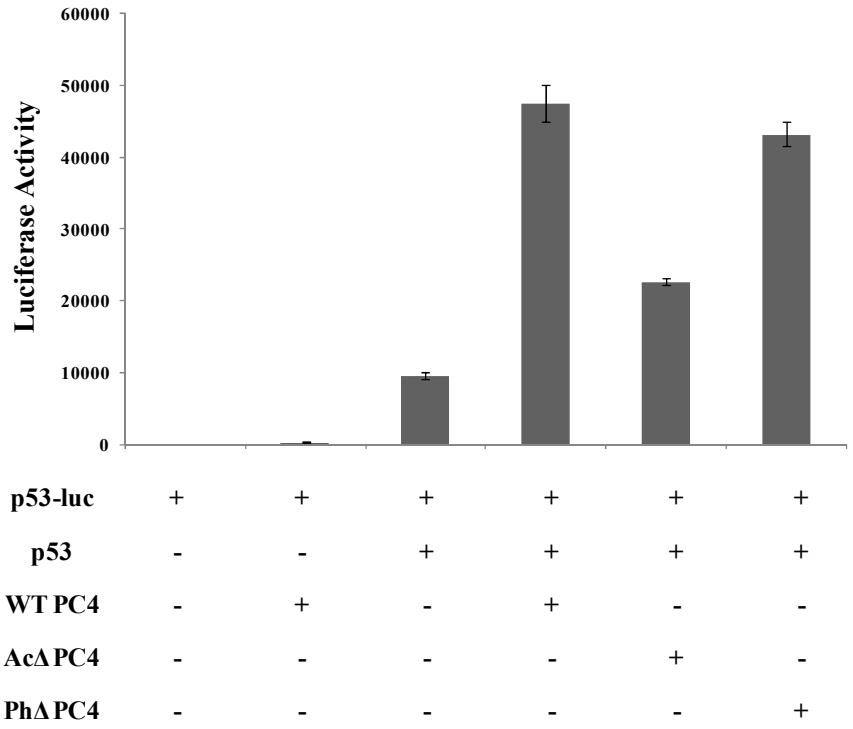


Figure 3.17 Acetylation is crucial for p53 dependent transcriptional activation mediated by PC4. A p53 responsive luciferase assay was performed with 1 μg of the Flag vector alone (Lane 1), Flag-tagged WT PC4 (Lane 4), or Flag-tagged acetylation defective mutant PC4, M11 (K 26, 28, 53, 68 R) (Ac ΔPC4) (Lane 5) and Flag-tagged phosphorylation defective mutant PC4, MTP5 (S13A S15G S17A S19G) (PhΔPC4) (lane 6) respectively in H1299 cells transiently transfected with p53-responsive luciferase construct, pG13luc (500 ng), p53 (100 ng), and pCMV-gal (500 ng). Cells were transfected with pG13luc and PC4 in absence of p53 as negative control (Lane 2). Transfection of p53 expression vector alone with pG13luc was performed to assess luciferase activity in this cell line (Lane 3). pCMV-gal was used as internal control for the transfection.

3.1.4.2 Post-translational modification alters core-histones interaction ability of PC4

Acetylation and phosphorylation of PC4 can be comprehended as exclusive for coactivation function; acetylation being essential for, whereas, phosphorylation completely abolishes activator dependent transcription. In addition of the earlier discovery of PC4 being a coactivator, we have also reported a chromatin compacting function for PC4. The ability of

PC4 to interact with core histones was found essential for the PC4 mediated chromatin compaction. *In vitro*, it preferentially interacts with the histones H3 and H2B (Das et al, 2006). A better understanding of the post translational modifications and their impact on chromatin compaction is essential to further dissect regulatory roles of these modifications in the context of chromatin organization. In this direction, isothermal titration calorimetry experiments were performed to have insights regarding the molecular nature of the interaction of PC4 with octameric core histones and individual histone H3. HeLa core histones were titrated against PC4 in the ratio 1:10 at 25°C in a MicroCal ITC200 instrument (MicroCal, Inc.). Similar experiments were repeated with individual histone H3. Core histones and histone H3 exhibited high binding affinity for PC4 with dissociation constant closer to 6 μM in both the cases. Binding pattern in both the cases seemed similar except for the binding stoichiometry between them. In summary, PC4 was found to have high binding affinities for both octameric core histones and individual histone H3 albeit with different stochiometry. There were two binding sites in core histones whereas for histone H3 there was just one site (Figure 3.18 B, C).

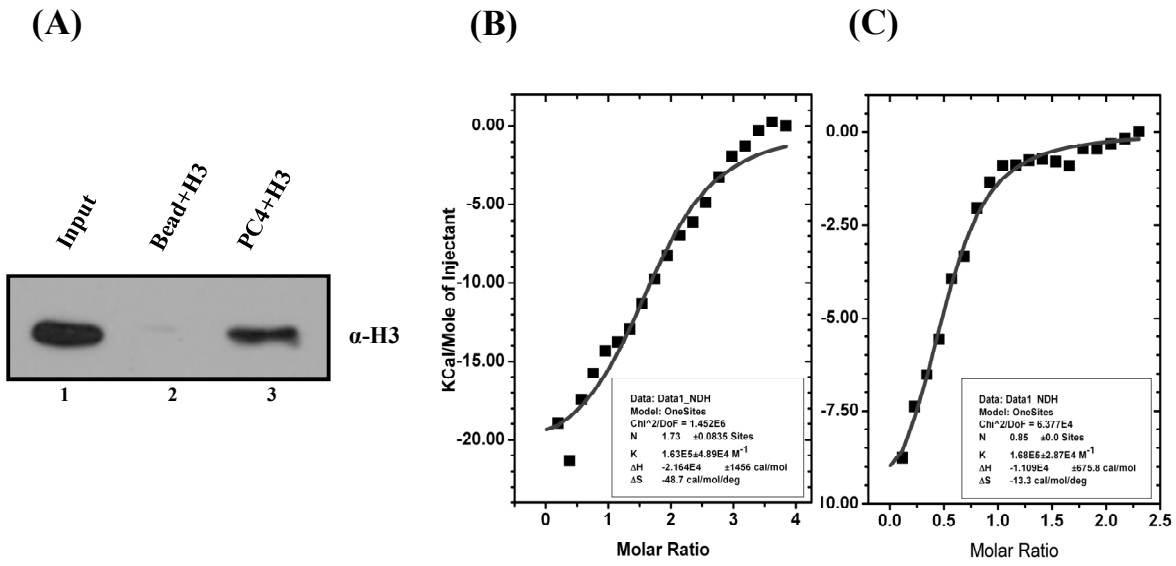


Figure 3.18 PC4 interacts with core histones and histone H3 in different stoichiometry. (A) The *in vitro* interaction of 1 μg of PC4- His_6 bound to Ni-NTA beads with 0.25 μg of H3 was assessed.

The complexes were pulled down and analyzed by western blotting using anti H3 antibody. Lane-1, recombinant H3 (input); Lane-2, H3 incubated with Ni-NTA agarose beads; Lane-3, H3 incubated with Ni-NTA agarose bound PC4- His_6 at 150 mM salt concentration. Isothermal calorimetry titration between HeLa core histones (B) and individual histone H3 (C) with PC4 was done at 25°C to assess enthalpy and stoichiometry of the interaction.

In order to map the domain in PC4, which interacts with histone H3, PC4 deletion mutant was generated. In our earlier report, we used various truncated proteins to indentify regions that possibly interacted with histones. By utilizing earlier information, an internal deletion mutant was created by removing 5 amino acid residues from the full length protein background (Figure 2.12). This mutant protein having five amino acids (62-67) deletion was assessed for its histone interaction ability through *in vitro* pull down assay using recombinant histone H3 and ITC. Mutant protein did not show any interaction with histone H3, unlike wild type PC4 (Figure 3.19 A Lane3 versus lane 4). Interaction inability of mutant PC4 protein was then verified by calculating enthalpy changes between histone H3 and mutant PC4 (1:10) in an ITC reaction. Titration did not furnish any valid thermodynamic parameters for a feasible reaction in case of PC4 (Δ62-67) mutant with histone H3 (Figure 3.19 C), unlike wild type PC4 with H3 (Figure 3.19B). Therefore, the stretch of residues from 62-67 was found critical for the interaction of PC4 with histone H3.

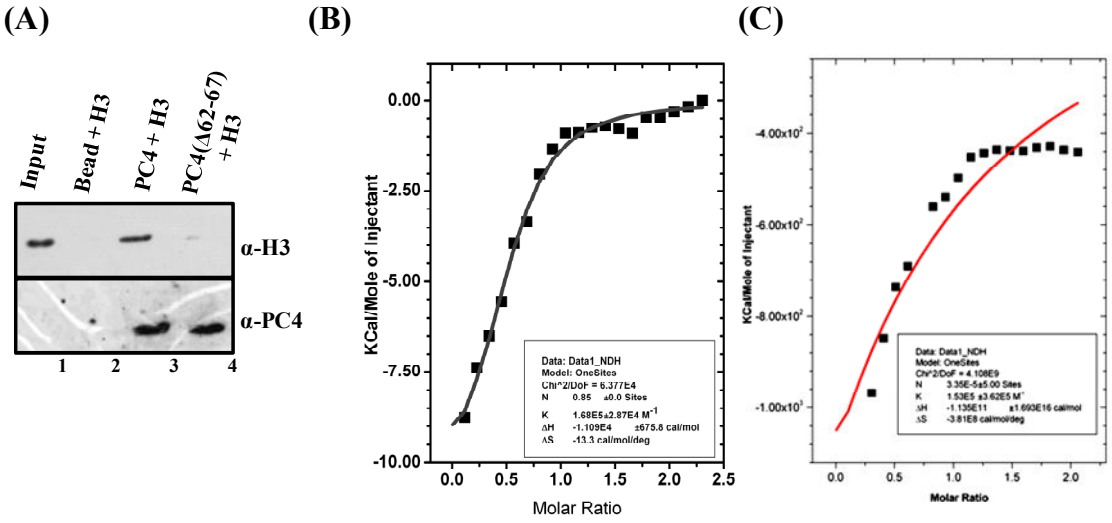


Figure 3.19 PC4 interacts with core histones with five amino acids containing minimal region. (A) Functional characterization of histone interacting region of PC4 was performed. Histone H3 interaction ability of mutant was checked by *in vitro* Ni-NTA pull down assay using both wild type (Lane-3) and mutant PC4(Δ62-67) (Lane-4). The interaction was also analyzed by isothermal calorimetry titration of histone H3 with wild type PC4 (B) and PC4 (Δ62-67) (C) in a MicroCal ITC200 instrument (MicroCal, Inc.) at 25°C.

PC4 is predominantly (~ 95%) present as phosphorylated form in the cell, the functional implications of which is unknown. We investigated role of phosphorylated PC4 in chromatin compaction. Histone interaction of PC4 is indispensable for its chromatin compaction ability. It has been shown that PC4 mutant defective in histone interaction could not condense *in vitro* reconstituted chromatin or partially digested HeLa chromatin (Das et al, 2006). Histone interaction analysis was carried out using phosphorylated PC4 and compared with unphosphorylated form. Phosphorylated PC4 showed enhanced interaction with core histones compared to unphosphorylated PC4 (Figure 3.20A) indicating phosphorylated PC4 could compact chromatin better than unphosphorylated PC4. Similar experiments were carried out with acetylated form of PC4 as well, and interestingly there was no significant change in histone interaction ability when compared to unacetylated PC4 (Figure 3.20B). This data possibly could have implications in differential ability of acetylated and phosphorylated PC4 in chromatin compaction. To further assess role of PC4 in chromatin compaction, we performed interaction with linker histones, a well known chromatin compacting protein.

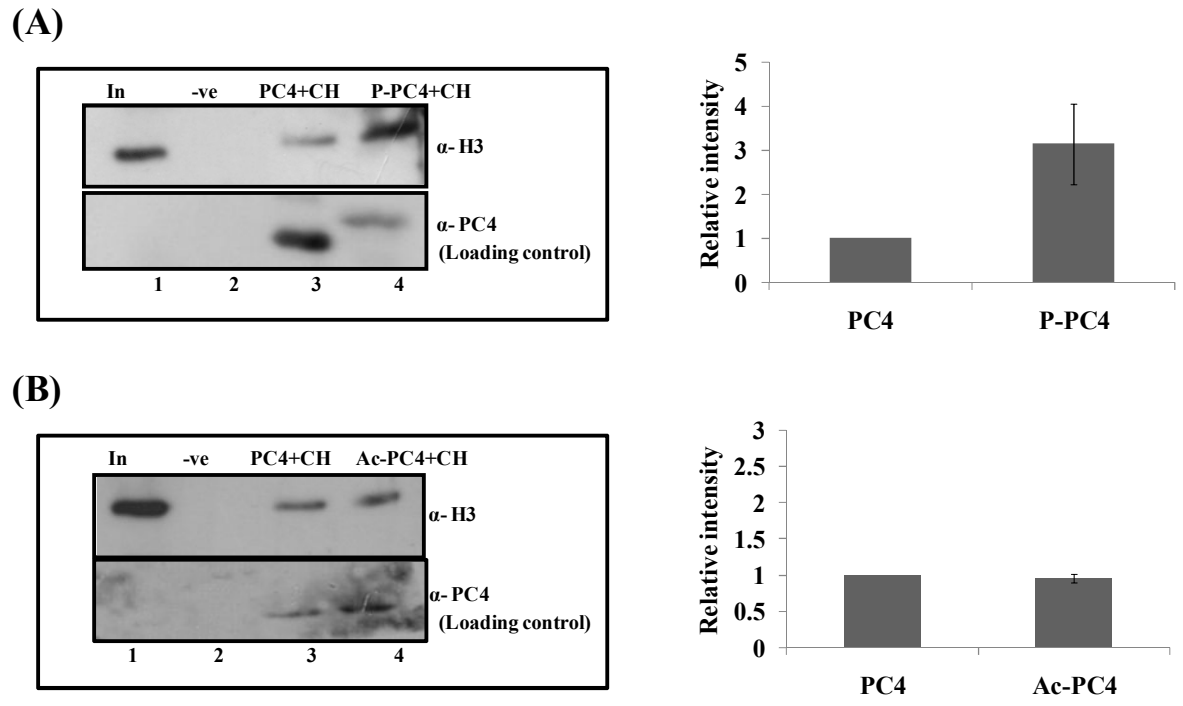


Figure 3.20 Phosphorylation enhances core histone interaction ability of PC4. *In vitro* interaction of phosphorylated (A) and acetylated PC4 (B) was carried out with core histones and its interaction ability was compared with that of unmodified PC4 (Lane-3). Lane-1, Core histones (input); Lane-2, core histones incubated with Ni-NTA agarose alone at 150 mM salt concentration; Lane-3, core histones incubated with Ni-NTA agarose bound PC4-His_6; Lane-4, core histones incubated with Ni-NTA agarose bound phosphorylated/acetylated PC4-His_6 respectively. Quantification of strength of interaction in terms of relative intensity (Lanes 3 and 4) is shown beside each western blot.

3.1.4.3 Phosphorylated PC4 interacts with linker histone H1

Chromatin condensation is an integrated event comprised of a series of DNA folding guided by different nonhistone proteins, chromatin remodellers, small RNAs etc. Nucleosome structure forms the fundamental unit of eukaryotic chromatin comprising of 1.6 turn of DNA wrapped around histone octamer. Where histones are elementary in the chromatin formation, different classes of nonhistone proteins act beyond nucleosomal level and add to the complexity of chromatin organization. Linker histones are one such group of proteins which play pivotal role in higher order chromatin folding. In order to explore the

possibility of PC4 involvement in higher order chromatin organization, its association with linker histones was examined. PC4 and its modified forms were subjected to an *in vitro* interaction assay using recombinant H1.1-His_6 at 150 mM salt concentration (Figure 3.21). PC4 was acetylated and phosphorylated in respective *in vitro* reactions. Equal concentrations of acetylated and phosphorylated PC4 were then subjected to Ni-NTA pull down assay with H1.1-His_6. Acetylated PC4 did not show any interaction like unmodified PC4 (Figure 3.21A, Panel I and II, Lane 3), whereas phosphorylated PC4 affirmed interaction with H1.1 under similar conditions (Figure 3.21A, Panel III, Lane 3). Unmodified PC4, later, was examined for interaction with other somatic variants of H1 (H1.1, H1.2, H1.3, H1.4, H1.5) in similar *in vitro* Ni-NTA pull down assay but no interaction was seen with any of the variants (Figure 3.23B). Phosphorylated PC4 on the other hand, showed interaction with all H1 somatic variants tested at 150 mM salt concentration (Figure 3.23C).

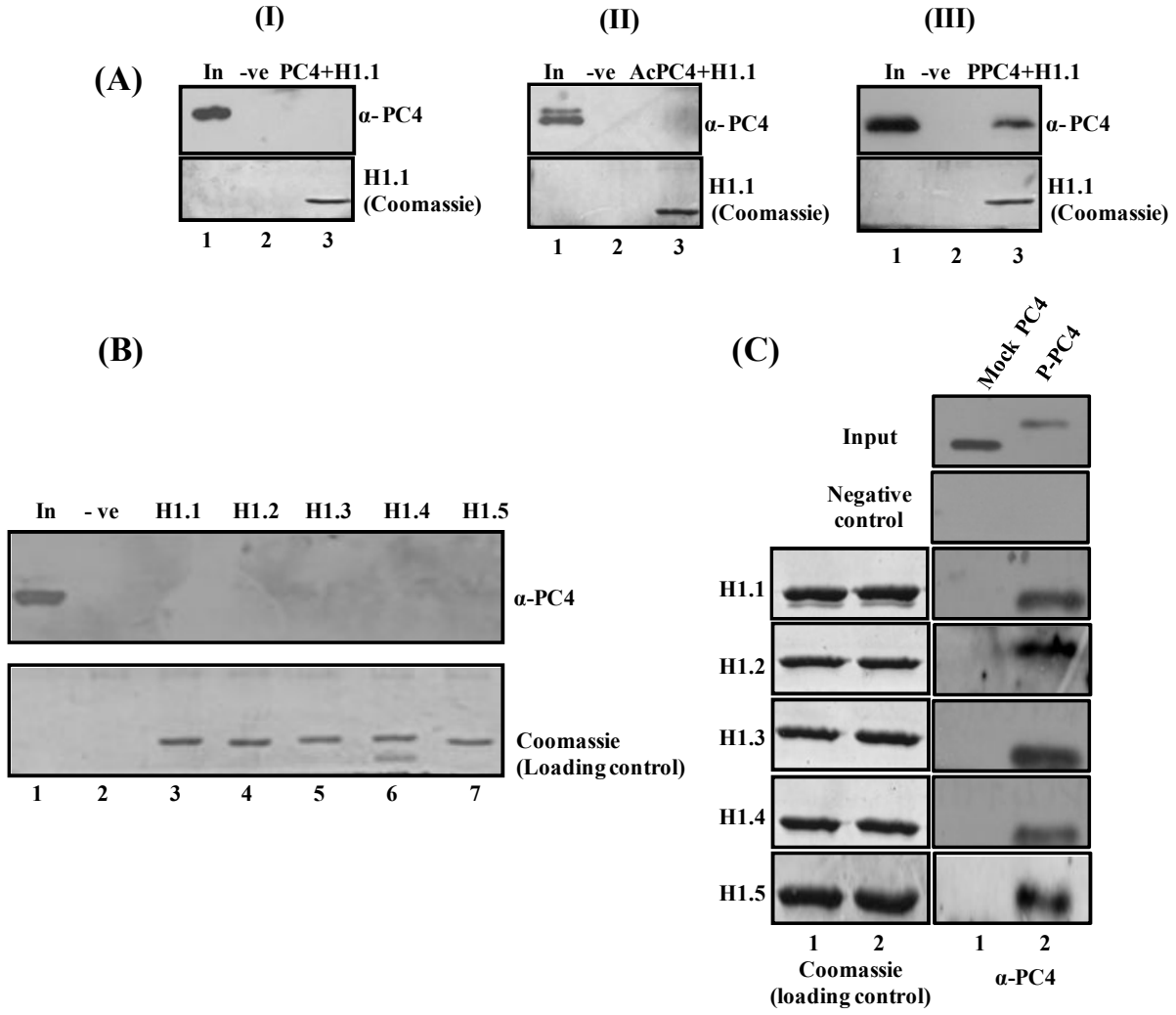


Figure 3.21 PC4 interacts with H1 variants in phosphorylation dependent manner. (A) Ni-NTA affinity pull down assays were performed with linker histone H1.1 and unmodified (I), acetylated (II) and phosphorylated (III) PC4. (B) The *in vitro* interaction of 1 μg of H1-His_6variants bound to Ni-NTA beads with 0.25 μg of PC4 was assessed. The complexes were pulled down and analyzed by western blotting using anti PC4 antibody. Lane 1, PC4 (input); lane 2, PC4 incubated with Ni-NTA agarose beads at 150 mM salt concentration. PC4 incubated with Ni-NTA agarose bound H1.1 (lane 3), H1.2 (lane 4), H1.3 (lane 5), H1.4 (lane 6) and H1.5 (lane 7) at 150 mM salt concentration. Lower panel shows coomassie stained SDS-PAGE gel of linker histone variants as loading control. (C) Similar pull downs were also performed with equal concentration of phosphorylated PC4 with different histone H1 variants (somatic) under similar conditions.

PC4 interaction with linker histone in phosphorylation dependent manner was further verified and strength of interaction was quantified by isothermal calorimetry titration. The assay was

carried out in presence of PC4 (unmodified and phosphorylated) and linker histone variant H1.1 in the ratio 6:1. No valid enthalpy change was witnessed with PC4 and H1.1 reaction (Figure 3.22, Left panel), whereas phosphorylated PC4 showed interation with H1.1 having dissociation constant of 5 nM (Figure 3.22, Right panel). The one-site binding model of the isotherm was observed.

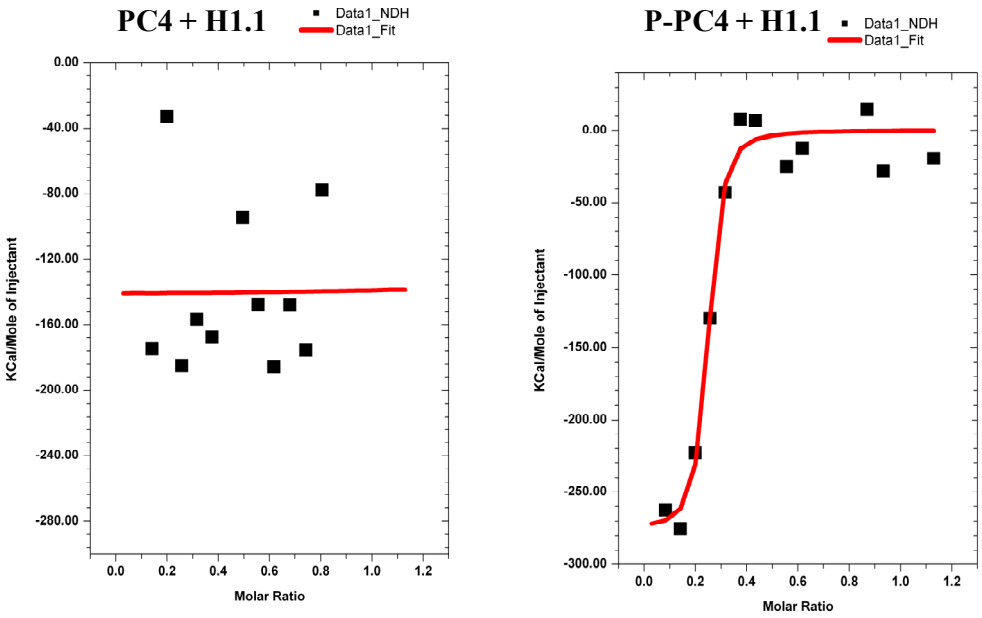


Figure 3.22 Isothermal calorimetry titration. Isothermal calorimetry titration of PC4:H1 (Left) and phosphorylated PC4:H1 (Right) were performed to acquire enthalpy change and stoichiometry of interaction. The one-site binding model of the isotherm is shown.

3.1.4.4 Phosphorylated PC4 interacts with C-terminal domain of H1

With the objective to identify regions in linker histone H1 interacting with phosphorylated PC4, truncated proteins of variants H1.1 and H1.3 were employed. Proteins deleted in their N-terminal (GC: Globular + C-terminal domain) and C-terminal (NG: N-terminal + Globular domain) (Figure 3.23A) were incubated with phosphorylated PC4 and

complex was pulled down using Ni-NTA agarose beads. Pulled down complex was resolved in 12% SDS-PAGE following immunoblotting with anti PC4 antibodies. Apart from full length proteins, GC proteins of H1.1 (Figure 3.23B) and H1.3 (Figure 3.23C) could pull down PC4 indicating C-terminal of linker histones being involved in the physical interaction with PC4.

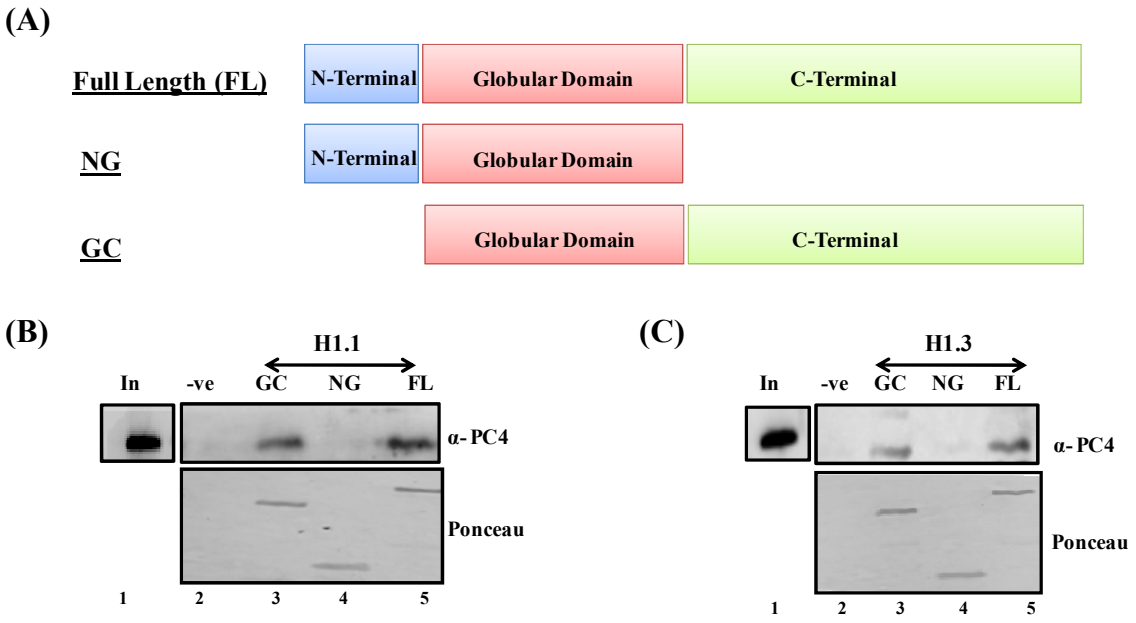


Figure 3.23 Phosphorylated PC4 interacts with C-terminal domain of H1. (A) Schematic shows the domain diagram of linker histone H1, FL (full length) and its deletion mutants, NG (N-terminal+Globular), GC (Globular + C-terminal). Full length (Lane 5) and deleted proteins (Lanes 3 and 4) of H1.1 (B) and H1.3 (C) were used for *in vitro* Ni-NTA affinity pull down assay with phosphorylated PC4 protein. Bead bound PC4 protein was detected by immunoblotting using anti PC4 antibodies (Upper panel). Interaction of Ni-NTA beads with phosphorylated PC4 protein was done as negative control (-ve) (Lane 2). 10% of phosphorylated PC4 was used as input for the reaction (Lane 1). Equal amount of recombinant full length and deleted proteins were used in the reaction as visualised by ponceau stain (Lower Panel).

3.1.4.5 Serine 17 phosphorylation is crucial for PC4 interation with linker histone.

Since PC4 interacts with linker histone variants in a phosphorylation dependent manner, it was essential to determine critical residues of PC4 involved in the interaction. We have identified S15 and 17 as critical residues for phosphorylation itself but would they have any implications in this scenario? To test the possibility, serine defective mutants of PC4 were employed for interactions with H1 variants. Mutants defective in two or more than two serine residues (MTP5: S13A S15G S17A S19G, MTP7: S13A S15G, MTP8: S13A S17A, MTP9: S15GS17A) were phosphorylated in an *in vitro* kinase assay in the presence of casein kinase II enzyme. These mutants were found to get phosphorylated with different extents, MTP5 being minimum followed by MTP9, then MTP8 and maximum for MTP7 (Figure 3.15C). Equal concentrations (approx. 250 ng) of phosphorylated (Figure 3.24, Lanes 2-6) and mock phosphorylated (Figure 3.24, Lane 1) PC4 proteins were incubated and subsequently pulled down with Ni-NTA agarose beads bound H1.1 protein. Immunoblotting with anti PC4 antibodies revealed that mutants defective in serine 17 phosphorylation, lost their ability to interact with linker histone (Figure 3.24, Lanes 3, 5, 6). Presence of just one phosphorylation site S17 was enough to rescue interaction to a major extent (Figure 3.24, Lane 4) as compared to wild type protein (Figure 3.24, Lane 2).

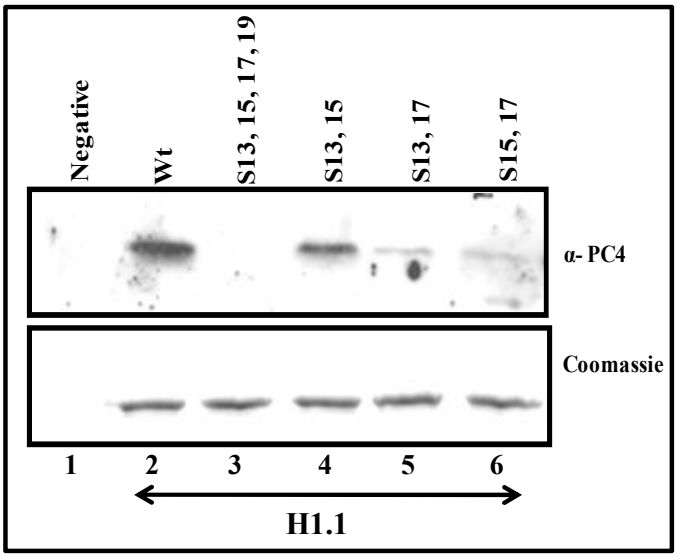


Figure 3.24 Serine 17 phosphorylation is crucial for PC4 interation with linker histone. Interaction ability of serine defective PC4 point mutants was verified by performing *in vitro* Ni-NTA affinity pull down assay with recombinant linker histone variant H1.1-His_6. Wild type (Lane 2) and mutant proteins (Lanes 3-6) were phosphorylated in presence of casein kinase II enzyme and then subjected to interaction assay. Pulled down proteins were detected by immunoblotting using anti PC4 antibodies (Upper panel). Interaction of Ni-NTA beads with phosphorylated PC4 protein was done as negative control (Lane 1). Coomassie staining showing amount of linker histone used in each reaction (Lower panel).

In vivo association of PC4 with linker histones was also examined utilizing PC4 stable cell line. FLAG-tagged PC4 stable HEK 293 cell line was generated (Section 2.4.9) and cell lysate was subjected for M2-agarose based pull down experiment followed by mass spectral analysis of the complex. The linker histone H1 was identified as one of the interacting partner of *in vivo* PC4 (data not shown).

The observations made through these experiments suggest that post translational modifications of PC4, specifically acetylation and phosphorylation modulate its functions apparently in opposite fashion. We have studied two known modifications of PC4 and their impact on two very important cellular processes transcriptional activation and chromatin

organization mediated by PC4. Acetylation was found to be very crucial in PC4 mediated p53 dependent transactivation as transactivation was compromised in case of acetylation defective mutant PC4. Phosphorylated PC4, on the other hand, is inert as coactivator. Thus, PC4 in its acetylated form seems to act as transcriptional coactivator. Chromatin organising ability in the form of *in vitro* chromatin compaction mediated by PC4 is governed by its interaction with core histones. Loss in core histone interaction ability severely compromises *in vitro* chromatin compaction mediated by PC4. Phosphorylation of PC4 enhances its core histones interaction and thereby chromatin compaction ability *in vitro* (Das C and Kundu TK, unpublished data). These findings present two major functions of PC4 transcription activation and chromatin compaction being driven by two different modifications, acetylation and phosphorylation of PC4 respectively. The present study discovers a novel association of PC4 phosphorylation with linker histones predicting involvement of PC4 beyond nucleosomal level of chromatin folding. Nevertheless, the association needs an extensive investigation to establish role of PC4 in higher order chromatin folding. Phosphorylation being a predominant modification, PC4 possesses, chromatin organization could be the major function of PC4. The next level question is how does PC4 chromatin organizing ability translated into physiology? It is essential to investigate implications of PC4 in the cellular system. In this direction, PC4 knockdown stable cell lines were generated and resulting phenotypes were investigated.

3.2 PC4 is essential for the maintenance of structural competence of chromatin

With the objective to explore significance of PC4 in cellular context, PC4 knockdown stable cell lines were generated through lentivirus mediated PC4 targeting shRNAs delivery system (Section 2.4.9). Genomic integration of these shRNAs were stabilized and ensured by puromycin antibiotic selection through several generations. Cell lines with constitutively expressing shRNAs targeting PC4 coding regions were established and characterized.

3.2.1 Characterization of PC4 knockdown HEK 293 stable cell lines

3.2.1.1 RNA and protein expression analysis of different PC4 knockdown stable cell lines

PC4 knockdown stable cell lines were verified for their knockdown efficiencies. Different cell lines derived from different shRNA clones were examined for PC4 expression. All four different cell lines showed lower expression of PC4 than their parent control cell line HEK 293 cells when cells were immunostained with anti PC4 antibodies (Figure 3.25A). Cell lysates prepared from control and shRNA stable cell lines were also used for immunoblotting with PC4 antibodies. Results confirmed that all shRNA harbouring cell lines have lower PC4 protein expression as compared to control cell line (Figure 3.25B). Among four stable cell lines, one (# 5) showed maximum silencing of PC4 and was used for subsequent experiments. PC4 transcript expression was also compared to the control cells and was found reduced in shRNA stable cells as expected (Figure 3.25C).

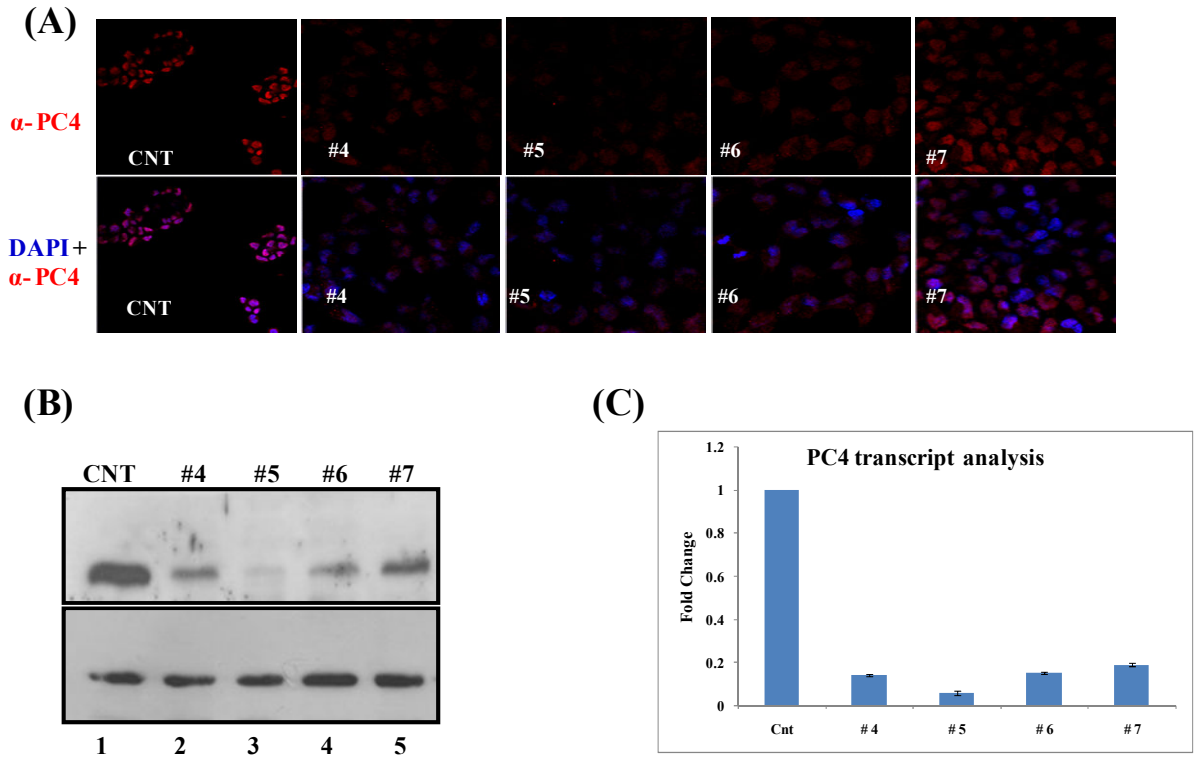


Figure 3.25 Characterization of sh-PC4 stable cell lines. Efficiency of PC4 protein knockdown was analyzed by performing immunofluroscence (A) and subsequently western blot analysis (B) of control 293 cells (Lane-1) and stable cell lines derived using different shRNA clones targeting PC4 as indicated (Lanes-2-5) using anti PC4 antibodies. Anti GAPDH antibodies were used for protein loading control. (C) Real time PCR analysis was performed for checking PC4 transcript expression levels in control and stable knockdown cells.

3.2.1.2 Validation of PC4 knockdown by checking expression of its target genes

Further validation of these stable knockdown cells were performed by analyzing expression status of PC4 target genes in these cell lines. The neural genes, namely, GAD1, M4, SCN2 expression was found to be upregulated in HeLa and HEK 293T cells upon transient silencing of PC4 (Das et al, 2010). Furthermore, it was found that PC4 was a part of the REST-CoREST repressive complex controlling expression of neural genes in non neuronal cell lines like HeLa and HEK 293T. In order to investigate susceptibility of these

genes expression in PC4 stably silenced background, expression analysis was performed using gene specific primers. As compared to control cells, PC4 knockdown cells showed upregulation of neural gene expression as seen in cells with transiently silenced PC4 (Figure 3.26).

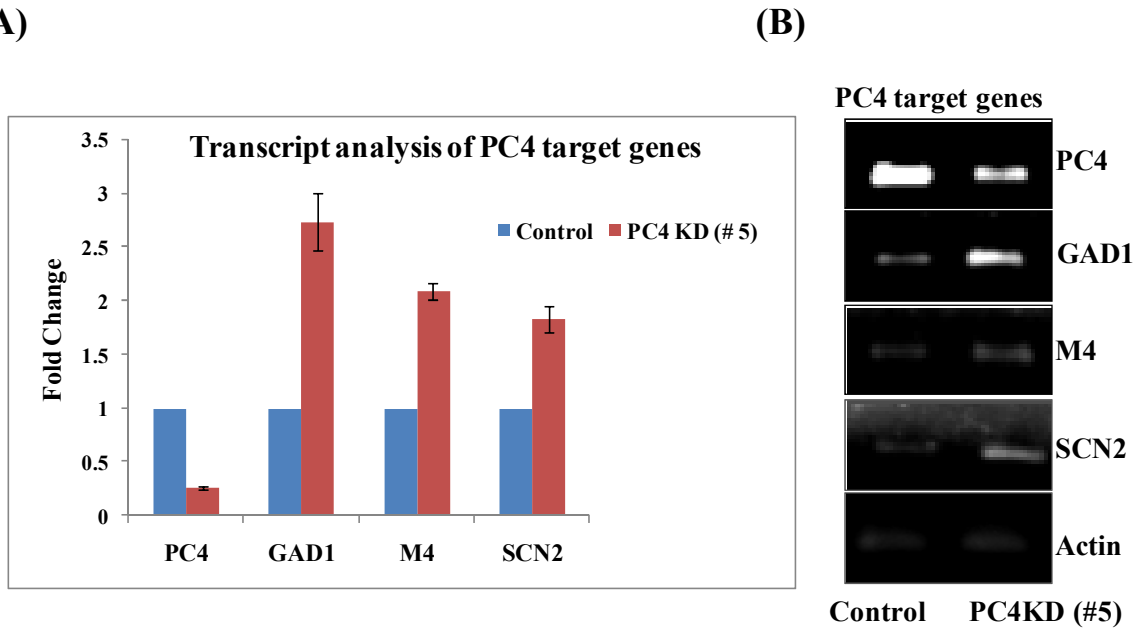


Figure 3.26 Neural gene expressions in PC4 sh-5 stable cell lines. (A) Transcript level of PC4, GAD1, M4, SCN2 were analyzed in control and PC4 knockdown (PC4 KD #5) cells by performing real time PCR using gene specific primers. (B) Agarose gel image representation of real time PCR amplicons generated by specific primers of genes indicated.

3.2.2 Chromatin analysis of PC4 knockdown stable cell line

PC4 being a chromatin organizing protein, it was tempting to explore chromatin properties in the absence of PC4. Different approaches were employed to study chromatin structure in these cells. As described earlier, appropriate organization of chromatin is very essential for proper functioning of the cell and several pathological conditions are associated with altered chromatin structure and thereby functions.

3.2.2.1 PC4 knockdown stable cell line exhibits increased transcriptional activation associated histone modification marks not affecting repressive marks expression

PC4 is an important chromatin compacting protein thus, it is expected that in its absence or limited availability cells could undergo significant changes in terms of chromatin structure and function. It was important to measure the changes qualitatively and if possible, quantitatively. As indicated previously, transient silencing of PC4 could alter chromatin structure and its epigenetic state. We wanted to observe the changes happening in the PC4 silenced stable cell line model and score the chromatin events. PC4 knockdown cells were analysed for transcriptional activation associated histone modifications and they were found elevated as compared to control cells. Cell lysates of both control and PC4 knockdown cell lines were probed by immunoblotting with different histone modification marks, namely acetylation marks of H3K9, H2AK5 and H4K16 and trimethylation of H3K4 (Figure 3.27 A). All the transcriptional activation associated histone modification marks analyzed showed enhancement in PC4 knockdown cell lines. Control and PC4 knockdown cell lines were also stained with above mentioned antibodies to visualize their expression. Consistent increase in antibody staining was observed in PC4 knockdown cells as compared to control cells (Figure 3.27B).

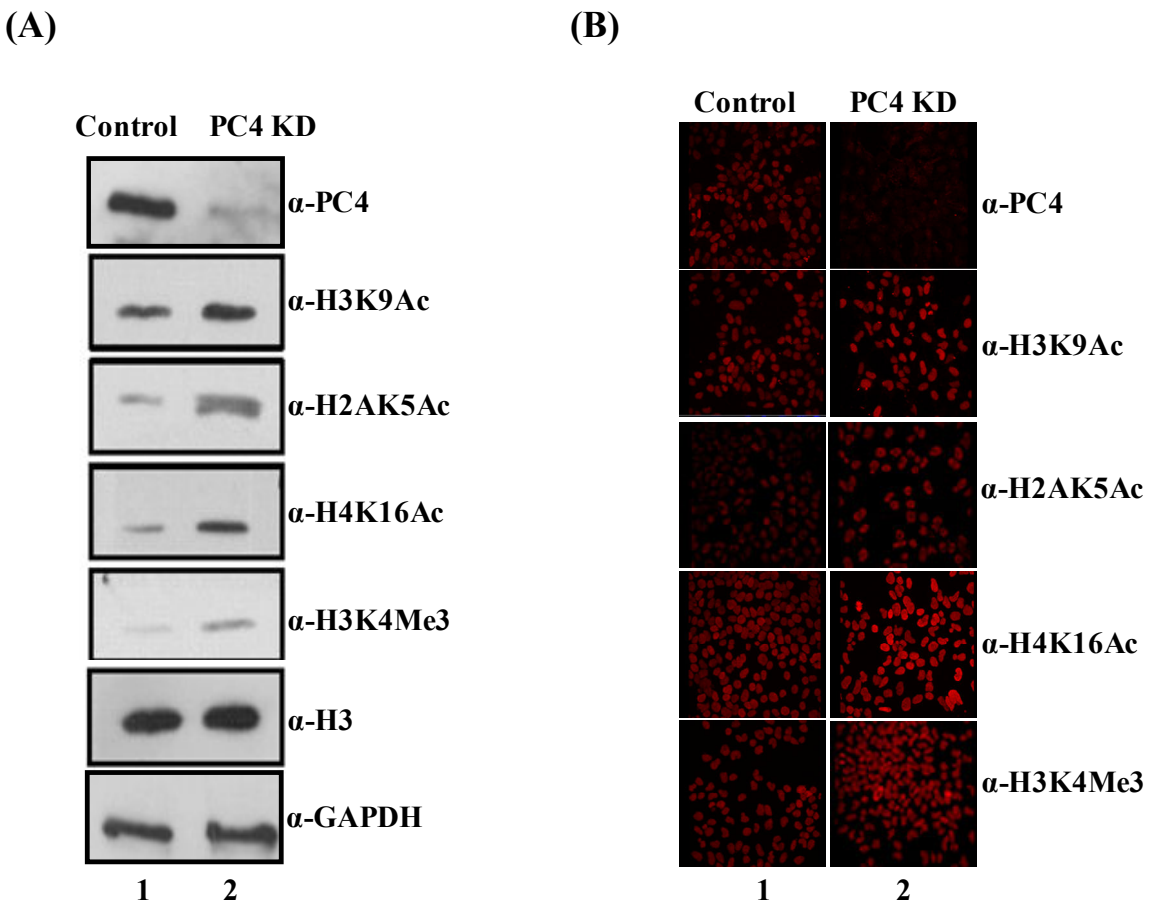


Figure 3.27 PC4 knockdown stable cell line exhibits increased transcriptional activation associated histone modifications. (A) Transcriptional activation associated histone modification status was analyzed in control HEK 293 (Control, Lane 1) and PC4sh-5 knockdown (KD, Lane 2) cells by immunoblotting using specific antibodies as indicated. Reprobing with anti H3 and anti GAPDH antibodies were performed to ensure equal loading. (B) Immunofluorscence experiments using same set of antibodies were done on paraformaldehyde fixed control HEK 293 (Control, Lane 1) and PC4sh-5 knockdown (KD, Lane 2) cells grown on glass coverslips.

The histone modifications like methylation of H3K9 represent transcriptionally silenced or closed chromatin. When PC4 knockdown cells were examined for H3K9 dimethylation and trimethylation modifications, they did not show any significant alteration in their expression level as compared to control HEK 293 cells (Figure 3.28).

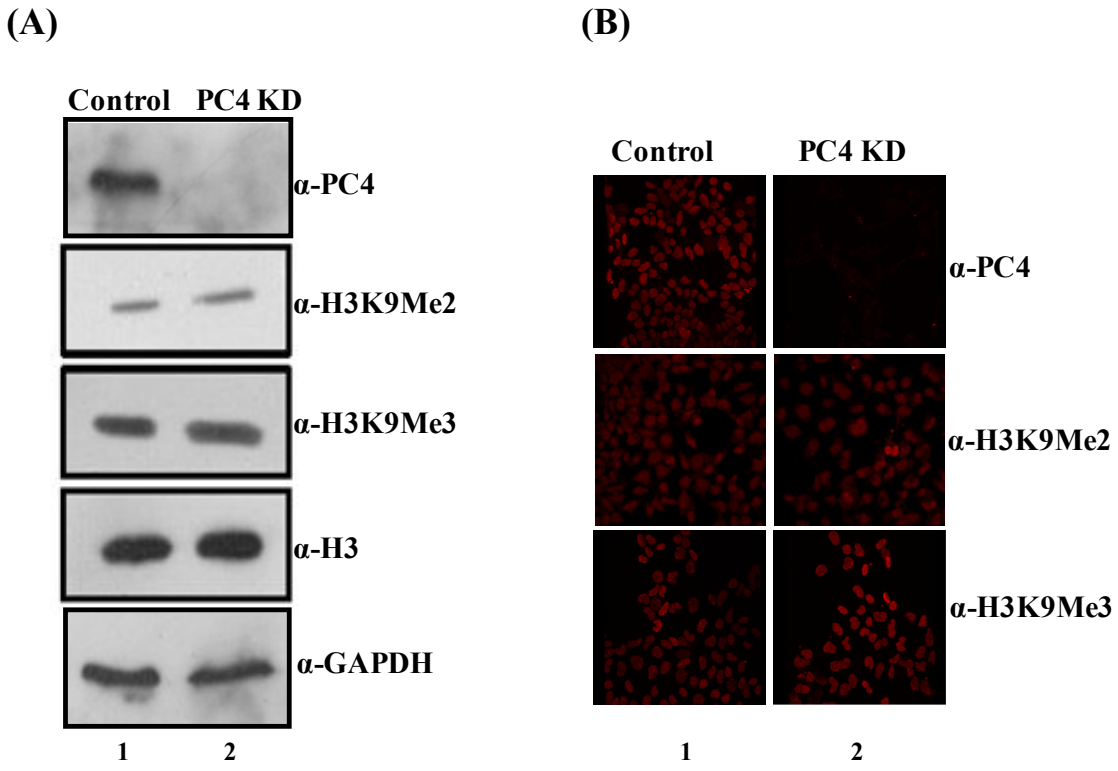


Figure 3.28 PC4 knockdown stable cell line does not affect histone modifications associated with transcription repression. (A) Immunoblotting analysis was performed in control HEK 293 (Control, Lane 1) and PC4sh-5 knockdown (KD, Lane 2) cells using transcription repression associated histone modifications specific antibodies (H3K9Me2, H3K9Me3). Reprobing with anti H3 and anti GAPDH antibodies were performed to ensure equal loading. (B) Immunofluorscence experiments using same set of antibodies were done on paraformaldehyde fixed control HEK 293 (Control, Lane 1) and PC4sh-5 knockdown (KD, Lane 2) cells grown on glass coverslips.

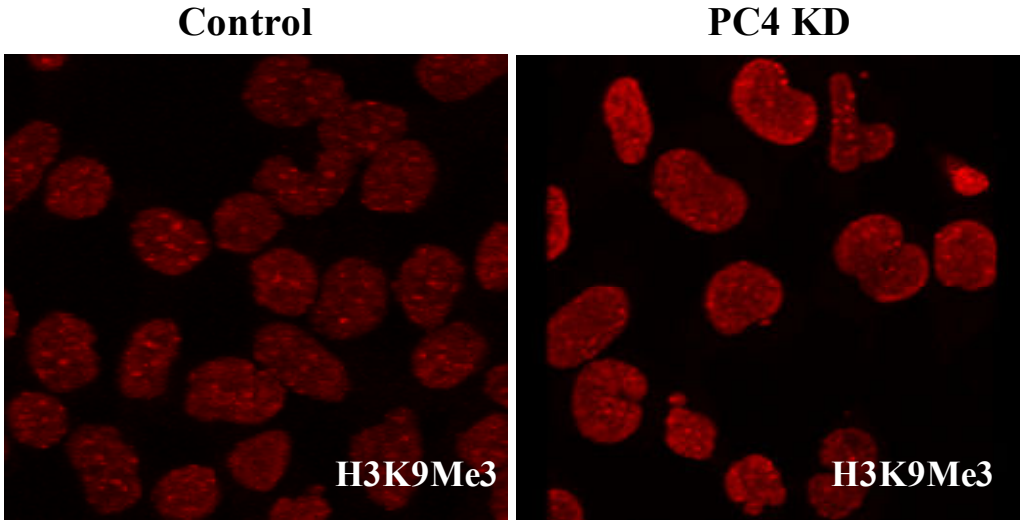
Epigenetic histone modifications dictate the chromatin and transcription state of a cell. Most of the acetylation (H3K9, H3K14, H2AK5, H4K16) and H3K4 trimethylation modifications mark for more open chromatin, where transcription or gene expression is active and rapid. Closed and transcriptionally repressed chromatin on the other hand, is characterized by hyper DNA methylation, and histone H3K9, H3K27, H4K20 methylation. Upregulation of transcription associated histone modification marks upon PC4 silencing suggest that PC4 overall has repressive impact on chromatin probably, through condensing the chromatin,

which can be rescued by downregulating PC4. In the absence of PC4, repressive constrain is withdrawn and chromatin is rendered accessible and unfolded.

3.2.2.2 Loss of distinct heterochromatin foci was observed upon PC4 knock down

Although no significant reduction in repressive histone modifications was observed, H3K9 trimethylation, a constitutive heterochromatin marker showed differential pattern in PC4 knockdown cell. There was lesser number of heterochromatin foci in PC4 silenced cell than control HEK 293 cells. Also, diffused and/ or less distinct foci were observed in silenced cell suggesting rearrangement of repressive form of chromatin (Figure 3.29A). Heterochromatin foci were also visualized by DAPI staining of the nucleus which also suggested loss of condensed chromatin regions in absence of PC4 (Figure 3.29B). Observations indicate rearrangement of chromatin towards some level of decondensation.

(A)



(B)

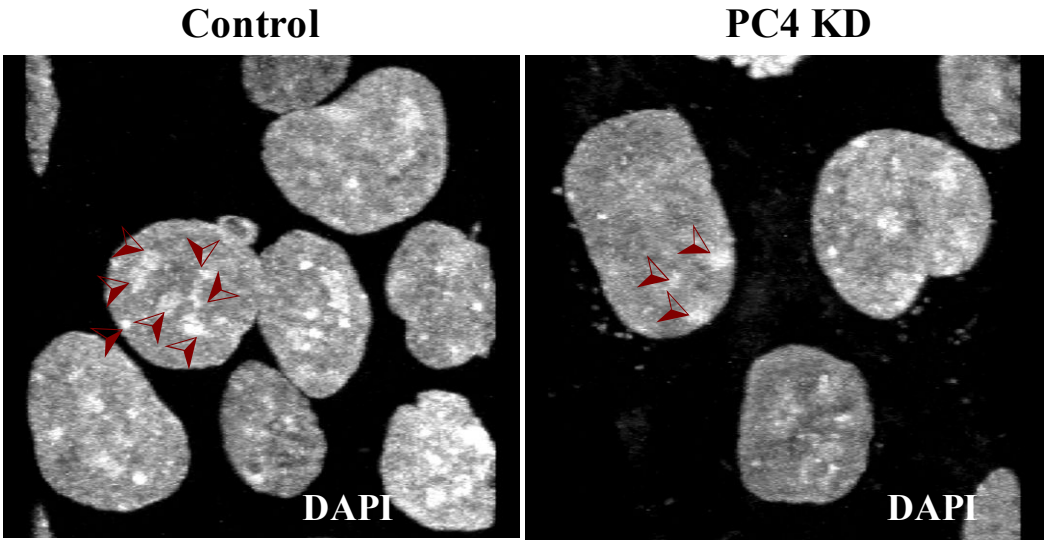


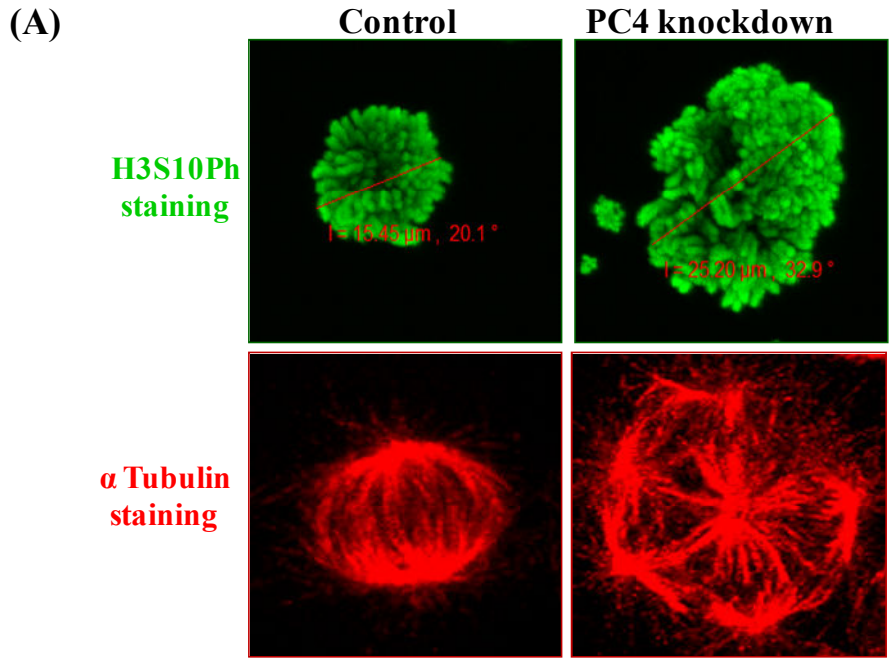
Figure 3.29 Loss of distinct heterochromatin foci was observed upon PC4 knock down. Control HEK 293 and PC4 knockdown cells were stained with constitutive heterochromatin marker H3K9Me3 (A) and DNA binding dye, DAPI (B) upon cross linking cells using paraformaldehyde to visualize heterochromatin foci in the cell. Heterochromatin foci are indicated with arrows.

3.2.3 PC4 silencing induces tumorigenic potential in PC4 knockdown stable cells

Chromatin organization not only influences transcription state of the cell, it is also detrimental for cellular life cycle. Chromatin undergoes cyclic condensation and decondensation during cell division. This process is accompanied by several biochemical and cellular changes. Several architectural proteins show dynamic association with the chromatin in cells undergoing cell division. With the view to anticipate the process of chromatin condensation-decondensation during mitosis in the absence of PC4, cell cycle based analyses were performed in PC4 stable cells.

3.2.3.1 PC4 knockdown results in accumulation of abnormal chromatin and induces cell cycle defects

Dividing cells undergo gradual chromatin condensation with the onset of mitosis. Visually, condensation can be spotted with the commencement of prometaphase through telophase which is followed by cytokinesis and gradual decondensation of chromatin. We aim to look into chromatin at mitotic and postmitotic stages in PC4 knockdown cell lines. Probing asynchronous cell population with mitotic marker H3S10 phosphorylation, a large number of abnormal mitotic chromatin was observed (Figure 3.30A). Abnormalities observed were diverse in nature including bigger sized chromosomes, chromosomal breakage, multiple metaphase plates, increased ploidy etc. There were defects in spindle organization and orientation as well as visualized by antibodies against α tubulin, the major constituents of mitotic spindle fibres (Figure 3.30A). Extending the study in large number of cells, it was found that there was approximately 25-30% increase in mitotic defects in PC4 knockdown cells as compared to control HEK 293 cells (Figure 3.30B).



(B)

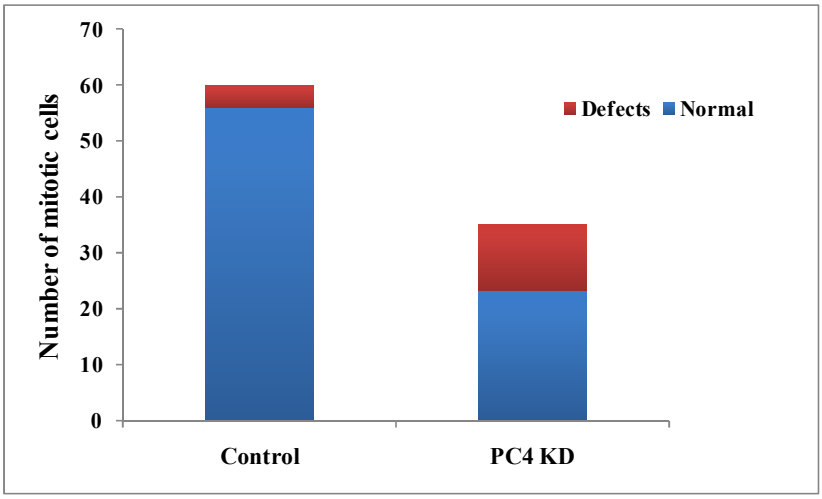
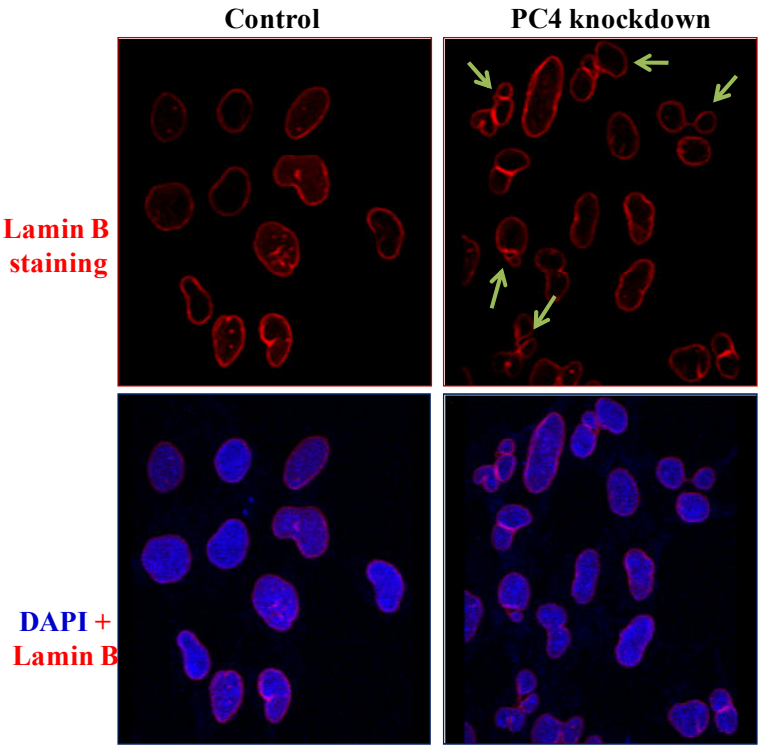


Figure 3.30 PC4 knockdown results in accumulation of abnormal chromatin and induces cell cycle defects. (A) Asynchronous population of control HEK293 and PC4 knockdown stable cell lines were grown on glass cover slips till approximately 60% confluency and immunostained with mitotic marker, H3S10 phosphorylation (Green, Upper panel) and microtubule marker, alpha tubulin (Red, Lower Panel) to identify cells undergoing mitosis. (B) Statistical analysis done for the accumulation of defective mitotic chromosomes upon PC4 knockdown.

During mitosis cells undergo karyokinesis followed by cytokinesis to ensure equal distribution of genetic material and formation of identical daughter cells. Control and PC4 knockdown cells were stained with anti lamin B antibodies to demarcate the nuclear boundary. The analysis indicated postmitotic abnormalities in absence of PC4 as well. Cells with PC4 knockdown showed both karyokinesis and cytokinesis defects. It was found that cell division was incomplete in these cells after nuclear division. Occasionally, large mass of cells were seen with distinct nuclei but attached together by septa (Figure 3.31A). In some cases, unequal nuclear division were seen (shown by arrow). Statistical analysis done with large number of cells showed significant increase (approximately 13%) in postmitotic defects (Figure 3.31B).

(A)



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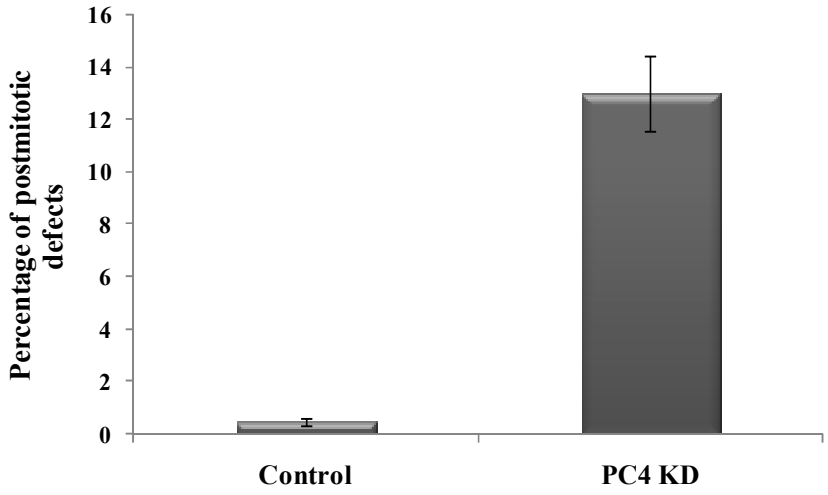


Figure 3.31 PC4 knockdown induces postmitotic defects. (A) Asynchronous population of control HEK293 and PC4 knockdown stable cell lines were grown on glass cover slips, and immunostained with anti Lamin B antibodies to visualize nuclear periphery. (B) Statistical analysis done for the defect in postmitotic events upon PC4 knockdown.

Cells suffer several of cell cycle defects in the absence of PC4. Apart from its role in chromatin organization, PC4 could have direct role to play during cell division, the possibility of which remains untested yet. Accumulation of defective chromatin or acquiring any genomic defect would forbid cells to undergo cell division further thereby stopping proliferation subsequently.

3.2.3.2 PC4 knockdown stable cells exhibit high proliferative rate

Proliferation rate of PC4 knockdown cells were compared with control HEK 293 cells by performing wound healing assay. Cells were grown to 80% confluency and equal amount of wound or scratch was created using a sterile tip. Healing of the wound was monitored at regular interval. It was found that healing was much faster in case of PC4 knockdown cells when compared to control cells, probably due to increased proliferation in absence of PC4 (Figure 3.32).

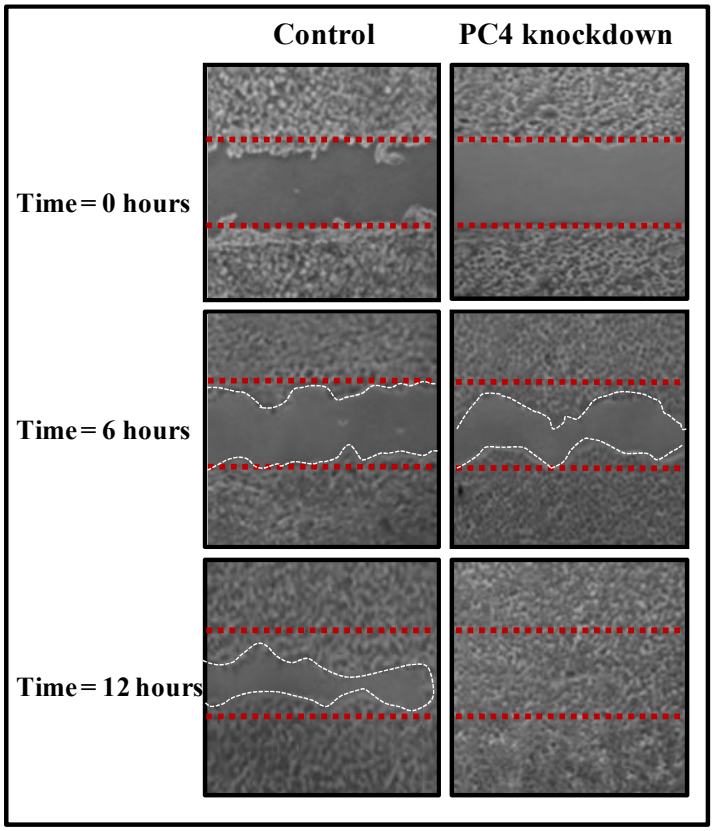
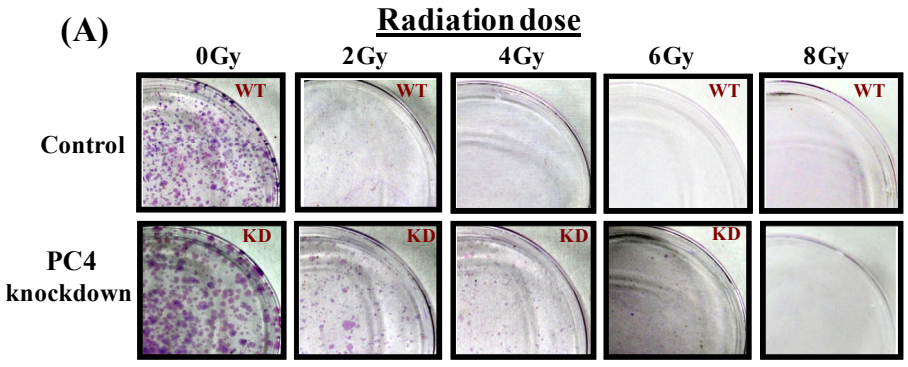


Figure 3.32 PC4 knockdown stable cells exhibit high proliferative rate. Representative images of the scratch assay performed in control HEK 293 and derived PC4 knockdown stable cell line. Equal wound was created in both cell lines and healing was monitored through the indicated time periods.

3.2.3.3 PC4 silencing increases cell survival upon gamma irradiation

Increased proliferation in PC4 knockdown cells inspite of several defects was puzzling. We ought to induce DNA damage further and then compare proliferation rate in control and PC4 knockdown cells. In this direction, we performed clonogenic assay in presence of different doses of gamma irradiation. 200-400 control and knockdown cells were plated in 100 mm dish 24 hours before irradiation. Cells were exposed to increasing doses of gamma irradiation and left for colony formation for a week. Colonies were stained with giemsa and counted (Figure 3.33A). Survival fraction was compared and greater survival was seen in case of PC4 knockdown upon increased gamma radiation as compared to control cells. Surviving fraction fell to almost 1/10 for lowest dose (2 Gy) for control cells whereas, PC4 knockdown showed significant resistance for irradiation mediated cytotoxicity (Figure 3.33B).



(B)

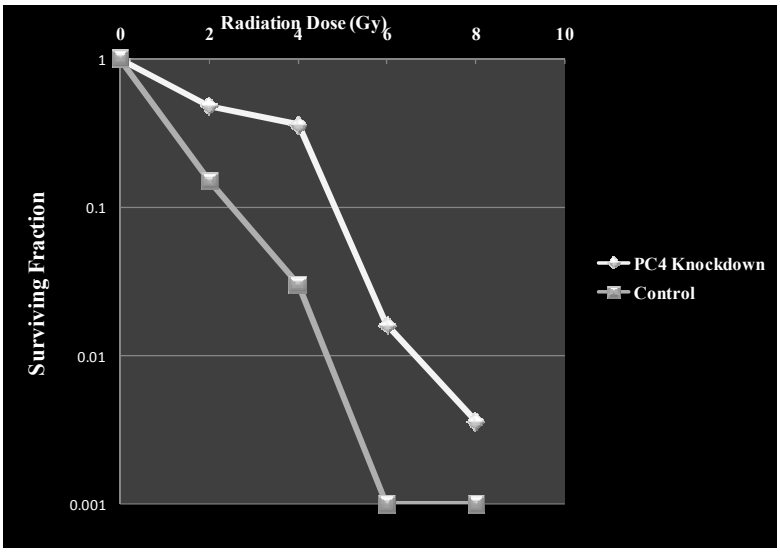


Figure 3.33 PC4 silencing increases cell survival upon gamma irradiation. (A) Images of the clonogenic assay performed with control HEK 293 (WT) and PC4 knockdown (KD) cells upon increasing doses of gamma irradiation as indicated. (B) Graph representing surviving fraction calculation of both cell lines plotted against each radiation dose.

Stably silencing of PC4 showed multiple phenotypes, some of which could be explained by virtue of its chromatin organising property. In absence of PC4, cells show altered nuclear architecture inclined towards more decondensed chromatin, emphasizing chromatin compaction as one of the major function of PC4 *in vivo*. Altered chromatin organization was also witnessed during the process of cell division. Overall, these results suggest pioneer role of PC4 in the maintenance of structural competence of the chromatin. However, observations like increased proliferation of PC4 silenced cells and enhanced resistance towards gamma irradiation mediated cytotoxicity point towards some unrevealed roles of PC4, specially during cell division. To have insight about association of PC4 with machineries involved in different cellular pathways, global gene expression analysis was performed using human whole genome affymetrix chip in PC4 silenced background.

3.2.4 Global gene expression and significant differential pathways analysis upon PC4 silencing

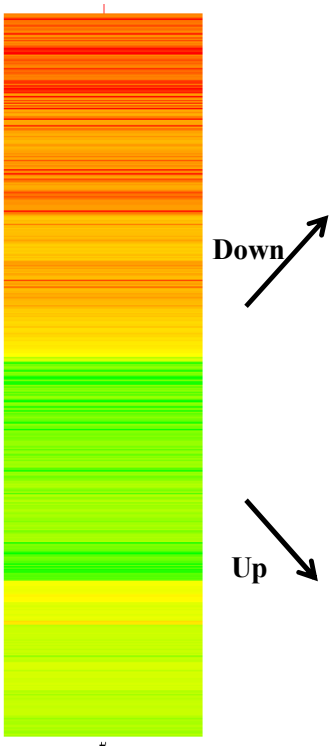
PC4 is a multifunctional protein involved in several cellular pathways. In order to anticipate and delineate consequences cells meet in absence of PC4, whole genome gene expression analysis was performed. The approach expects to reveal significant pathways and gene ontologies directly or indirectly influenced by PC4 downregulation. The relevant genes would be further validated for their dependence on PC4 in the regulation of particular pathway.

3.2.4.1 Alteration of important members of significant pathways related to cell cycle, and cancer upon PC4 silencing

To investigate the effect of PC4 knockdown on the global gene expression, genome wide differential expression analysis in control and PC4 knockdown stable cell line was carried out on human whole genome affymetrix microarray chip. The expression profile analysis revealed up-regulation of 262 genes and 290 down-regulated genes in response to PC4 knockdown, when a cut off of 1.5 fold was chosen (P-Value 0.05). Differential fold change was normalized with control cell expression. The depletion of PC4 resulted in differential regulation of several genes, which have wide ranging functions in the cell.

Interestingly, several genes belonging to gene ontologies (DAVID) chromatin organization, cell cycle, mitotic cell cycle checkpoint showed reduced expression, whereas, genes involved in microtubule organization, cell migration, angiogenesis and cell motion were significantly

upregulated. Additionally, expression of some of the genes involved in apoptosis and cell death were decreased (Figure 3.34).



Gene Ontology	Pathway	Genes
GO:0051276	Chromosome Organization	SUPT3H, NBN, KDM6A, HP1BP3, CBX2, NR3C1, DMAP1, CBX5, EPC2, CHD7, DKC1, PRMT5, MLL3, USP16, BCOR, BUB3, SHPRH, SMYD3, CENPF, LIG4, PHF17, EYA4, TEX15, BPTF, FANCD2, RNF2, PHF21A, SETD2
GO:0006325	Chromatin Organization	SUPT3H, KDM6A, SHPRH, HP1BP3, SMYD3, CBX2, NR3C1, DMAP1, CBX5, EYA4, PHF17, EPC2, CHD7, BPTF, RNF2, PRMT5, PHF21A, BCOR, USP16, SETD2, MLL3
GO:0051301	Cell Division	CDK19, CEP110, LIG1, CENPF, CDK6, CDC16, LIG4, FSD1, TOP1, NEK9, POU3F3, HAUS7, CHFR, USP16, NEK4, NUP43, STAG1
GO:0000278	Mitotic Cell Cycle	PPP2R3B, POLA1, CENPF, CDK6, CHEK1, CDC16, FSD1, CUL4A, RNF2, PSMD3, NEK9, HAUS7, CHFR, USP16, NEK4, NUP43, BUB3, STAG1, PPP5C
GO:0007093	Mitotic Cell Cycle Checkpoint	NBN, CENPF, CHFR, TPR, BUB3

Gene Ontology	Pathway	Genes
GO:0016477	Cell Migration	HIF1A, OVOL2, CKLF, ABI2, SEMA3C, MMP14, ITGB1, SLIT2
GO:0006928	Cell Motion	CD9, HIF1A, OVOL2, CKLF, ANXA1, ABI2, SEMA3C, MMP14, ITGB1, SLIT2
GO:0001525	Angiogenesis	HIF1A, HMOX1, MMP14, SLIT2, ANXA2
GO:0070507	Regulation of microtubule cytoskeleton organization	CAV1, MAP2, MID1
GO:0045767	Regulation of anti-apoptosis	CAV1, HMOX1, SLC9A1

Figure 3.34 Alteration of important members of significant pathways related to cell cycle, and cancer upon PC4 silencing. Hierarchical clustering of the gene expression profiling data obtained by cDNA microarray analysis of control HEK 293 and PC4 knockdown stable cell line. Table depicting differential regulation of genes belonging to cellular pathways upon PC4 knockdown.

3.2.4.2 Validation of differential expressed genes obtained in microarray gene expression analysis by real time PCR.

In order to validate the microarray data, two candidate genes were chosen and their expression levels were compared by Real Time PCR analysis in control and PC4 knockdown cells. It was found that, as compared to control HEK 293 cells there was an enhancement in the expression of NFEM gene in PC4 knockdown cells (Figure 3.35). On the other hand, IL8 gene expression was reduced in PC4 knockdown cells (Figure 3.35). These results were in agreement with the microarray data.

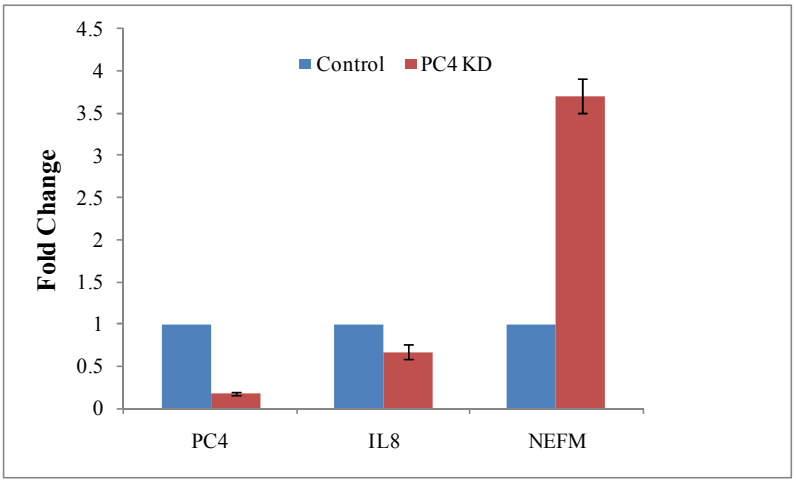


Figure 3.35 Validation of differential expressed genes obtained in microarray gene expression analysis by real time PCR. Real Time PCR analysis of the down regulated and up regulated candidate genes, IL8 and NFEM respectively upon knocking down of PC4 expression was performed in order to validate the microarray data.

As evident from the microarray data, several genes of chromosome and chromatin organization get disturbed in absence of PC4, indicating regulatory or buffering role of PC4 in the process. Probably, PC4 is a component of different complexes involved in chromatin architectural function. *In vivo* pull down experiment followed by mass spectral analysis have

also identified few chromatin structural proteins as PC4 interacting partners (unpublished data). Induction of several defects during cell division in PC4 knockdown cell line can be explained by altered expression of several cell cycle related genes as revealed in microarray data. These possibilities need extensive investigation in the context dependent manner. Many classes of cancer specific genes like matrix metalloproteases (MMPs) and EMT markers (Fibronectin) were found upregulated in PC4 knockdown cells. These genes were further studied in the context of breast cancer in the next section.

3.3 PC4 acts as a tumor suppressor in breast cancer

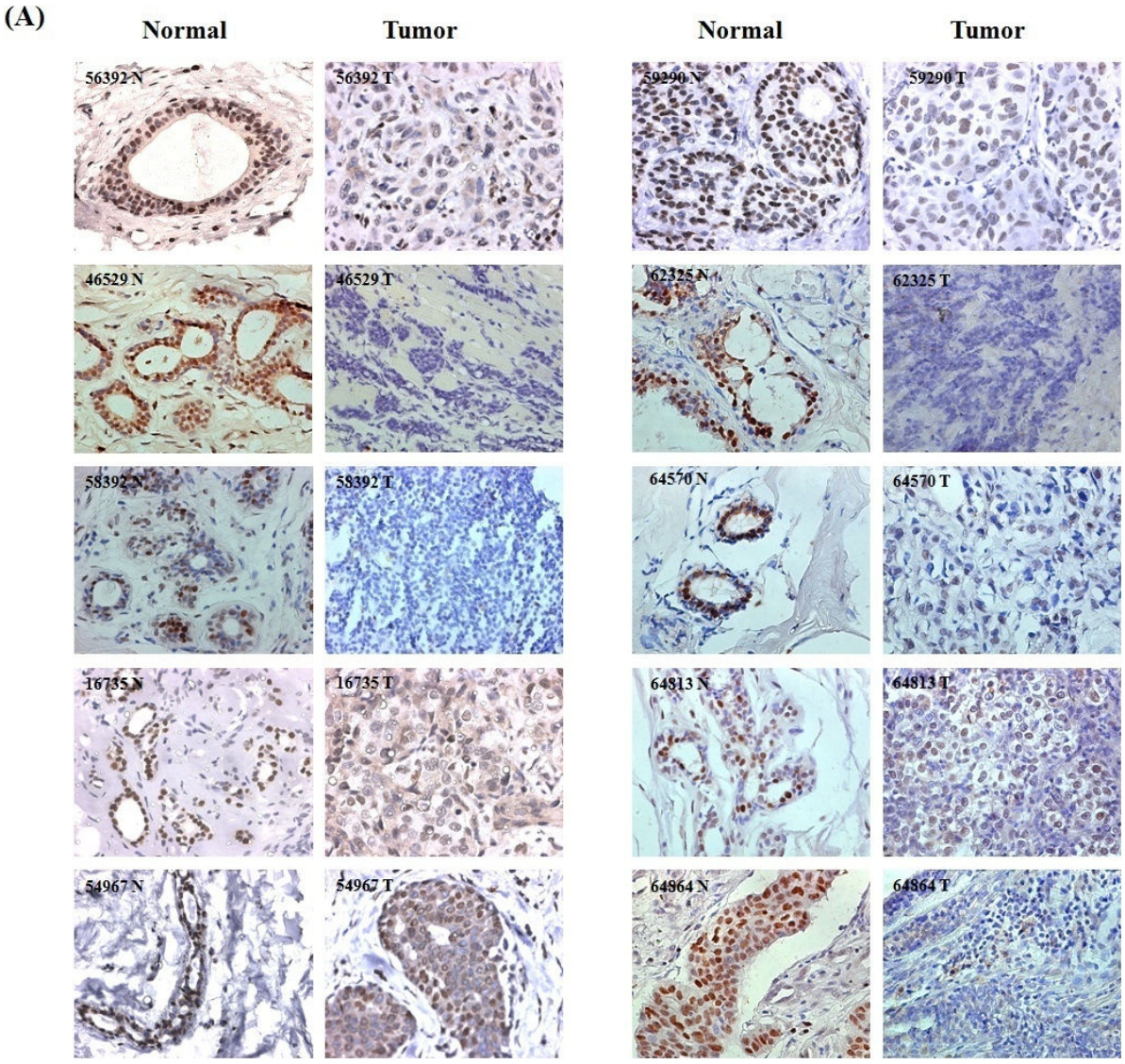
Cell based studies conducted in the background of PC4 depletion revealed several altered phenoptypes, especially with respect to chromatin. In the absence of PC4, cells acquired an altered nuclear architecture. To be specific, cells appeared to be composed of less condensed chromatin as reavealed by an increase in histone modifications for open form of chromatin. There were also fewer heterochromatin foci and some foci also seemed less compact and disrupted. Cells seemed to override cell cycle checkpoints and grow faster thereby accumulating large number of cell cycle defects like polyploidy, incomplete cytokinesis and abnormally sized nucleus. PC4 knockdown cells were also found more resistant to gamma irradiation mediated cytotoxicity. In other words, PC4 knockdown enhances genomic instability in the cell. We hypothesised that PC4 downregulation could potentiate genome instability and therefore could play a role in tumorogenicity. Many chromatin associated proteins have been implicated as drivers of tumorogenic behaviours in various cancers. Till date nothing is known in terms of PC4 with respect to tumorogenicity except for very recent report of its upregulation in lung cancer (Peng et al, 2012). To test this hypothesis of ours, we decided to choose breast cancer as a model.

3.3.1 PC4 is significantly downregulated in breast cancer patient samples

Cancer cell possess a very abnormal nuclear architecture with distinct chromatin texture and distribution. Breast cancer is one of the rapidly increasing cancer in India and world. Breast cancer is highly heterogenous in nature and its progression is often associated with increasing genomic instabilities (Chin et al, 2004). We initially began to study with the pathological samples from breast cancers of all grades and types in an effort to understand the status of regulation of PC4.

3.3.1.1 PC4 expression is greatly reduced in breast cancer patient samples

The studies were initiated to analyze the expression levels of PC4 in cancerous patient samples with respect to adjacent normal breast tissue from the same patient using the Immunohistochemistry (IHC) technique. The study was extended to 50 patient samples. A chromogenic substrate was used and therefore appearance of intense brown colour suggested expression of PC4 while the absence of the brown colour meant decreased expression levels. The nuclei have been counterstained with hematoxylin for better visualization of the difference. A drastic difference was observed in the levels of PC4 expression in cancer samples as compared to normal counterpart (Figure 3.36A, B). The normal sections show an intense brown precipitate in the nucleus of the cells lining the lactiferous ducts. However, this was either found absent or very faint in the cancerous samples which could be easily made out by the rich hematoxylin (blue) counter stain. It is important to note that the normal tissue architecture is disrupted in cancerous specimens and therefore sheets of cells with big nuclei are to be considered for comparison with the normal ductules. Approximately 64% of breast cancer samples analyzed showed significant down regulation of PC4 protein (Table 3.1).


(A)
Normal
Tumor
Normal
Tumor
56392 N
56392 T
59290 N
59290 T
46529 N
46529 T
62325 N
62325 T
58392 N
58392 T
64570 N
64570 T
16735 N
16735 T
64813 N
64813 T
54967 N
54967 T
64864 N
64864 T

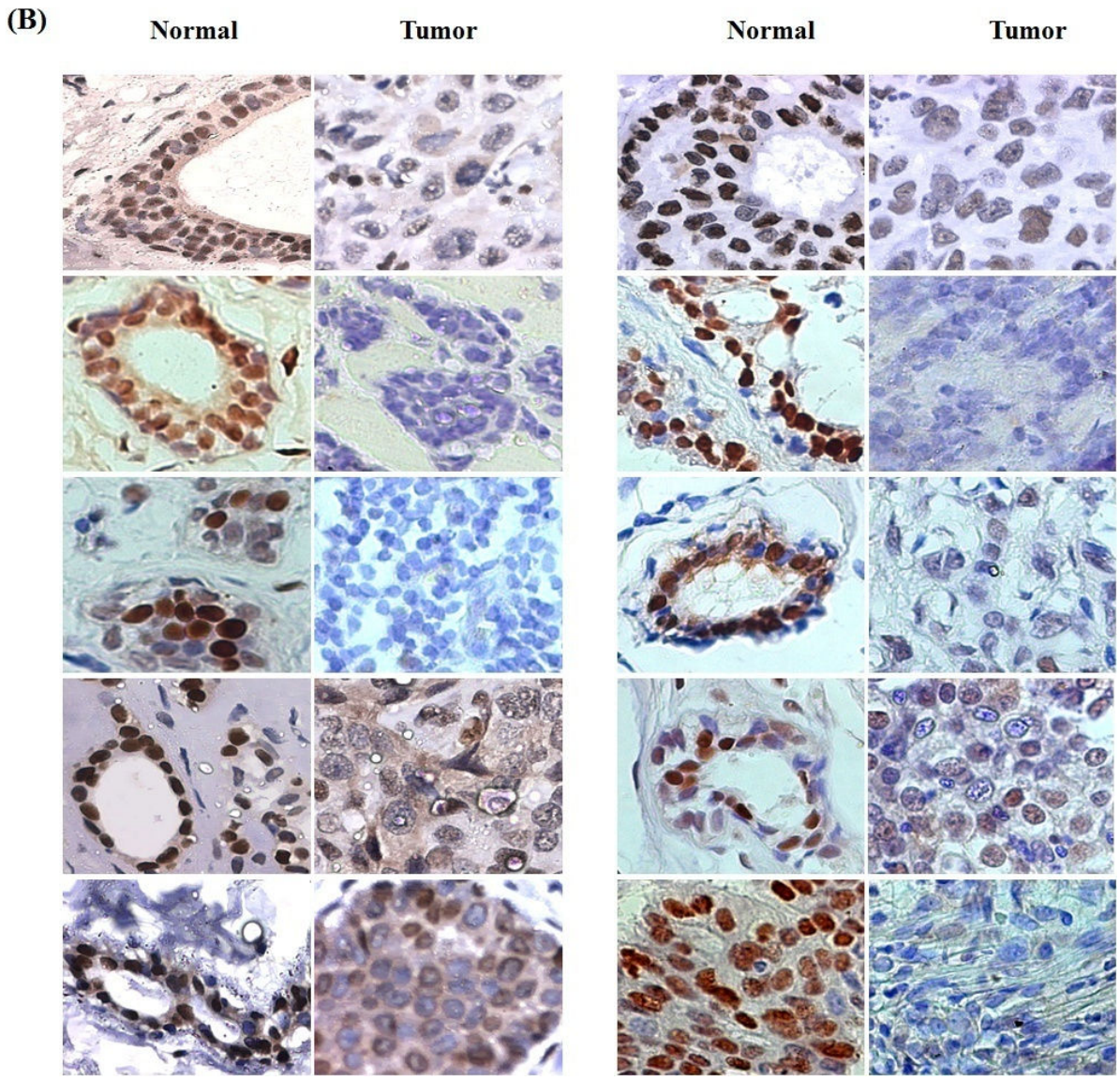


Figure 3.36 PC4 expression is greatly reduced in breast cancer patient samples (A) Representative images of immunohistochemistry (IHC) analysis performed in breast tissues using anti PC4 antibodies. IHC was performed in tumor and adjacent normal samples from the same patient as indicated by the patient ID for each case. (B) Magnified images of the stained breast tumor and normal tissues.

Sr. no:	Patients ID	Expression profile of PC4
1	148233	Downregulation
2	151405	Downregulation
3	16735	Downregulation
4	176488	Downregulation
5	42754	Downregulation
6	46529	Downregulation
7	49412	Downregulation
8	54466	Up regulation
9	54584	Up regulation
10	54967	Downregulation
11	55255	Downregulation
12	55281	No change
13	55681	Downregulation
14	55795	Downregulation
15	56110	Downregulation
16	56325	Downregulation
17	56392	Downregulation
18	56879	No change
19	56919	Downregulation
20	57341	Up regulation
21	57864	Downregulation
22	57875	Downregulation
23	58299	Up regulation
24	58336	Downregulation
25	58392	Downregulation
26	58490	No change
27	58739	No change
28	59106	Up regulation
29	59290	Downregulation
30	59296	Up regulation
31	59303	No change
32	61153	Up regulation
33	62325	Downregulation
34	62386	Downregulation
35	62908	Downregulation
36	62988	No change
37	64171	Downregulation
38	64405	Downregulation
39	64570	Downregulation
40	64813	Downregulation
41	64864	Downregulation
42	66269	Up regulation
43	66413	Downregulation
44	66455	Up regulation
45	66671	Downregulation
46	67486	Up regulation
47	67709	No change
48	68132	Downregulation
49	68424	No change
50	69541	Downregulation

Table 3.1 Table showing PC4 expression profile in the patient samples analyzed.

Immunohistochemistry only gives an indication about the abundance of a particular protein. It does not however, give any information about the expression levels. Therefore in order to ascertain the actual expression status of PC4 in breast cancer, real-time RT-PCR analysis of PC4 using RNA isolated from paraffin embedded patient samples was done. It is important to note that due to histological complexities like excessive parenchyma and fatty tissue in human mammary glands, care was taken to select the lactiferous sinuses and ducts from the paraffin fixed sections. Care was also taken to select tumour sections having 70% or more cancerous cells. Upon isolation of RNA from the paraffin sections, corresponding cDNAs

were normalized for three house-keeping genes with different degree of abundance in the cell (Beta actin, HKG-RPL 13A and HPRT) before actually calculating the expression for PC4. Such a rigorous analysis showed lower transcript levels of PC4 in breast cancer tissues compared to the adjacent normal tissue (Figure 3.37).

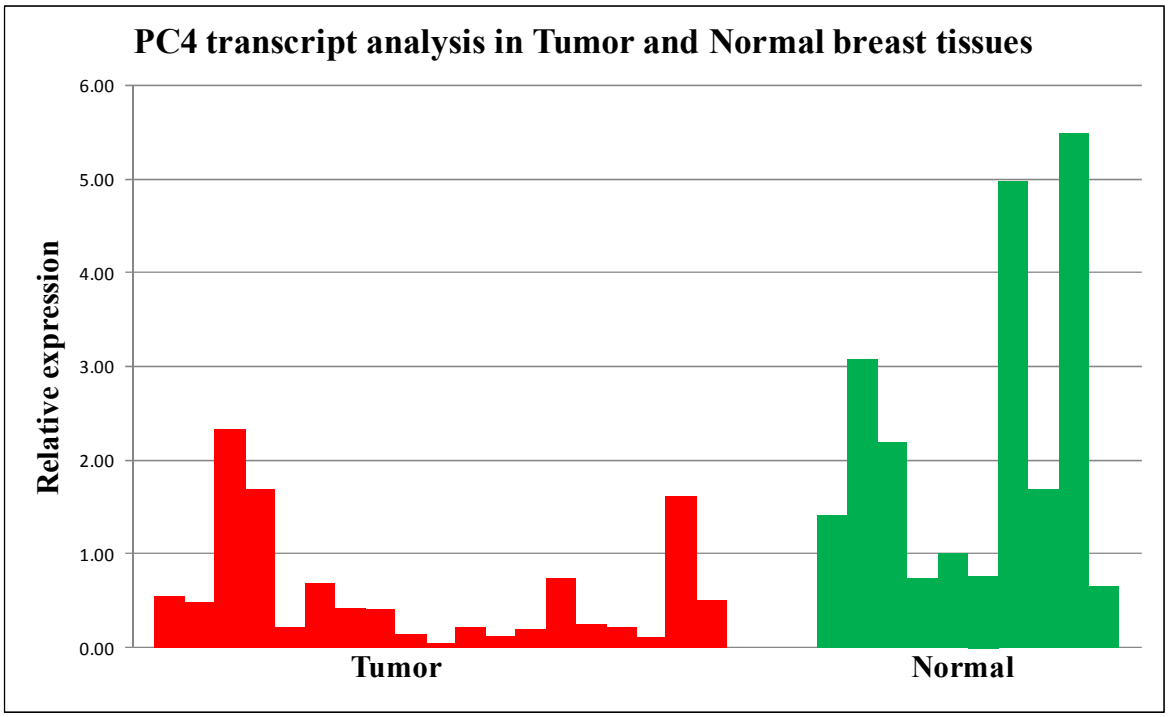


Figure 3.37 PC4 transcript is downregulated in breast cancer patient samples. Total RNA was extracted from formalin fixed paraffin embedded breast tissues and real time PCR was performed using gene primers specific to PC4. Graph shows normalised relative expression of PC4 gene in tumor and normal samples. Each bar represents relative expression of individual patient sample of respective category (cancerous and normal).

3.3.1.2 Downregulation of PC4 level was seen irrespective of grades, stages and receptor status tested

Although, we initially performed the analysis with prospective samples without having any prior knowledge of the exact classification of the cancer at the molecular level

(receptor status), we resorted to more detailed analysis using retrospective breast cancer samples with diverse histopathology and clinical scenarios. These samples were selected from biorepository. Samples were in form of formaldehyde fixed paraffin embedded tissues. Thin sections from the respective paraffin blocks were cut and stained with anti PC4 antibodies. Reduction in PC4 staining was observed in all samples. No strict correlation could be found between breast cancer grades and reduction in PC4 expression. In some of the advanced grade samples (Grade III), intense cytoplasmic staining of PC4 was observed. The extent of nuclear immunorectivity of anti PC4 antibodies in tissue sections was further quantified employing H-score system. Scores for each stained tissue section were furnished by the pathologists. The H-score estimates the percentage of cells (0—100%) in each intensity category (0—3+) and calculates a final score, giving a range of 0 to 300 (Table 3.2).

Sl No.	Ref No.	Age	Receptor Status (Allred Score) (Normal parenchyma positive)	Grade	LN Status	H-Score (Comments)
1	147531	62	ER+/PR+/Her2-	II	Positive	200 (Cyto>Nuc)
2	147721	61	ER-/PR-/Her2+	III	Positive	180 (Nuc>Cyto); **Post neo adjuvant chemotherapy**
3	148233	33	ER+/PR+/Her2-	III	Negative	0 (Nuclear staining); Cyto 120
4	150016	59	NA	II		160 (Nuc>Cyto); **Lumpectomy**
5	151405	48	ER+/PR+/Her2-	II	Negative	80 (Nuc>Cyto)
6	178114	52	ER+/PR+/Her2-	III	Positive	Cyto>Nuc; Cyto 200, Nuc 50; **Metastatic; Normal parenchyma within has very strong staining**
7	181270	75	NA	III	Negative	100 (Nuc>Cyto); **Normal parenchyma within has very strong staining**
8	181658	59	ER+/PR+/Her2-	III	Positive	0 (Nuclear staining); Cyto 300; **Papillary carcinoma**
9	176488	65	ER+/PR+/Her2-		Positive	100 (Nuc>Cyto); **Lobular;Low grade; Metastatic**
10	176781	50	NA	II	Negative	200 (Nuc>Cyto)
11	176763	80	ER+/PR-/ Her2-	III	Negative	0 (Nuclear staining); Cyto 200; **Necrosis seen in tumor cells**
12	176820	58	NA	II		200 (Nuc>Cyto); **Normal parenchyma within has very strong staining; Lumpectomy**
13	177352	52	ER+/PR+/Her2-	II	Negative	200 (Nuc>Cyto)

Table 3.2 Retrospective breast tissue analyses. Table showing patient medical history and H-scoring of the corresponding IHC staining of tumor samples. Normal tissue score was set as 300.

Breast cancer is heterogenous in nature and exists in various subtypes. Breast cancer can be classified based on tissue of origin and availability of receptors. Breast cancer is classified as endocrine receptor (estrogen or progesterone receptor) positive, HER2 (amplification of HER2 gene) positive, Triple negative (not positive to receptors for estrogen, progesterone, or HER2), and Triple positive (positive for estrogen receptors, progesterone receptors and HER2). We verified PC4 expression in two subtypes of breast cancer as well. Ten cases each of ER positive and triple negative breast cancer were selected for PC4 expression analysis. Normal breast tissue was used as control. Immunohistochemistry done with anti PC4 antibodies showed drastic downregulation in both ER positive and triple negative breast cancer (Figure 3.38). Similar extent of reduction in PC4 expression was found in ER positive as well as triple negative breast cancer. H-score was assigned for each tumor samples belonging to ER positive and triple negative subtypes as described previously. Normal tissue staining score was set at 300. All the tumor samples tested showed lower immunoreactivity for PC4 antibodies as evident from <300 score in them (Table 3.3).

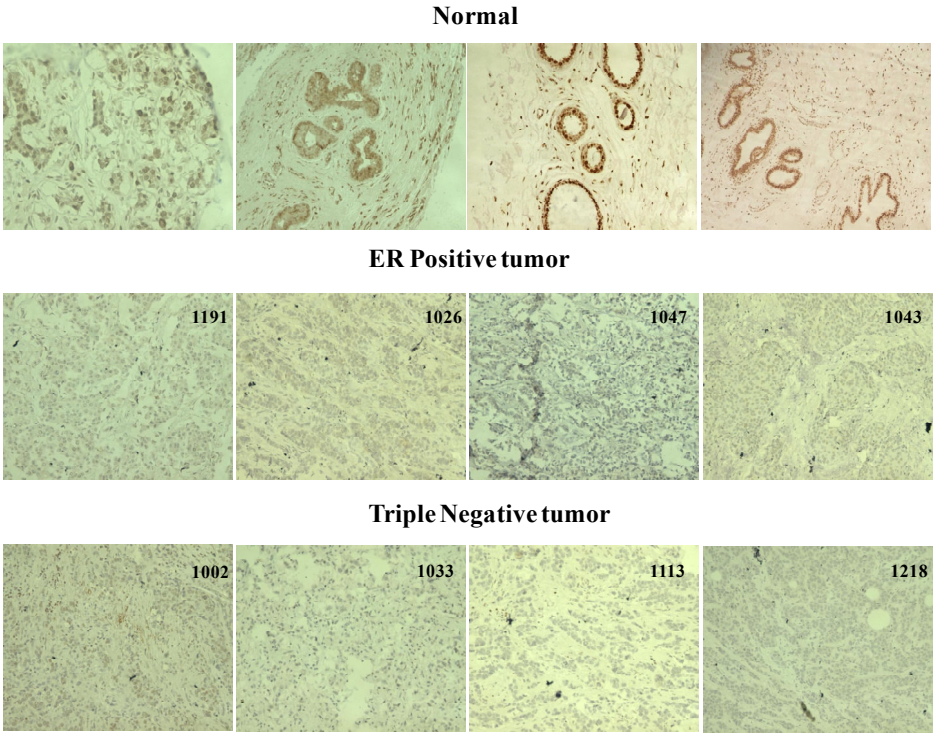


Figure 3.38 PC4 expression analysis in ER positive and triple negative breast cancer patient samples. (A) Representative IHC images of ER (Estrogen receptor) positive and triple negative patient samples stained with anti PC4 antibodies. Normal breast tissue staining was performed under similar conditions as control.

	I.D	AGE	MENOP STAT	LN STATUS	STAGE	GRADE	HISTO	Intensity	Percentage	H-score
TPN										
	1002	49	pre	POS	III	2	IDC	2,1	25,25	75
	1003	60	post	NEG	II	2	METAPLASTIC CA	1	20	20
	1004	46	pre	NEG	II	3	MEDULLARY CA	0	0	0
	1033	46	post	POS	III	2	IDC	0	0	0
	1035	48	post	NEG	I	2	IDC	0	0	0
	1040	54	post	POS	IV	3	IDC	0	0	0
	1041	56	post	NEG	I	3	IDC	0	0	0
	1218	40	Pre	POS	III	3	PLEOMORPHIC LOBULAR Ca	0	0	0
	1219	54	post	POS	III	2	IDC	1	70	70
	1113	49	post	NEG	I	3	IDC	0	0	0
ER Pos										
	1014	60	Post	NEG	I	1	LOBULAR CA	1	20	20
	1023	40	Pre	NEG	II	3	IDC	0	0	0
	1026	42	Pre	NEG	I	2	IDC	0	0	0
	1043	52	Post	NEG	II	1	IDC	0	0	0
	1047	74	Post	POS	II	1	IDC	0	0	0
	1073	62	post	POS	III	3	IDC	2	50	100
	1179	67	post	POS	II	2	IDC	0	0	0
	1191	58	post	POS	III	3	IDC	1	30	30
	1105	48	Pre	POS	III	3	IDC	0	0	0
	1107	48	pre	POS	III	3	IDC	3,2	10,10	50

Table 3.3 Table showing patient medical history and H-scoring of the corresponding IHC staining of tumor samples. Normal tissue scoring was set as 300.

Breast cancer samples screened for PC4 expression level revealed reduced transcript and protein as compared to normal tissue counterpart. Sample size of the study was close to 150 also including breast cancer tissue microarrays (data not shown). Although, majority of tumor samples tested showed drastic reduction in PC4, some samples (Grade III) showed intense cytoplasmic PC4 localization in tumor cells, mechanism of which is largely unknown. In order to determine implications of PC4 downregulation in breast cancer, some tumorigenic potential based functional assays were performed utilizing breast cancer cell lines.

3.3.2 PC4 plays a tumor suppressor role in breast carcinoma

3.3.2.1 PC4 silencing enhances the migratory and invasive ability of breast cancer cell lines

The epithelial breast cancer cell lines, HBL-100 MCF-7 and MDA-MB-231 (increasing degree of invasiveness) were grown in respective media and subsequently used for assays. PC4 expression was transiently silenced by treating HBL-100 and MCF-7 breast cancer cell lines with siRNAs against PC4 and scrambled siRNAs respectively (Figure 3.39 A). Cells were subjected to wound healing assay with a minor modification. Earlier with HEK 293 and PC4 stable knockdown cells, we did not employ any method to restrict cell proliferation. Therefore, the healing of the wound could be due to both cell proliferation and enhanced migration (Figure 3.32). However, HEK 293 cells are non-tumorigenic and not known to migrate, unlike cancerous cells. While repeating the assay with breast cancer cell

lines we used mitomycin to eliminate contribution of cell proliferation. It is also important to note that the microarray results upon PC4 depletion suggested overexpression of genes required for cell migration. Therefore, the wound healing assay here measured cell migration. The assay revealed that the migration of cells into the wound was more in PC4 silenced cells as compared to control HBL-100 (Figure 3.39B) and MCF-7cells (Figure 3.39C) at appropriate time points.

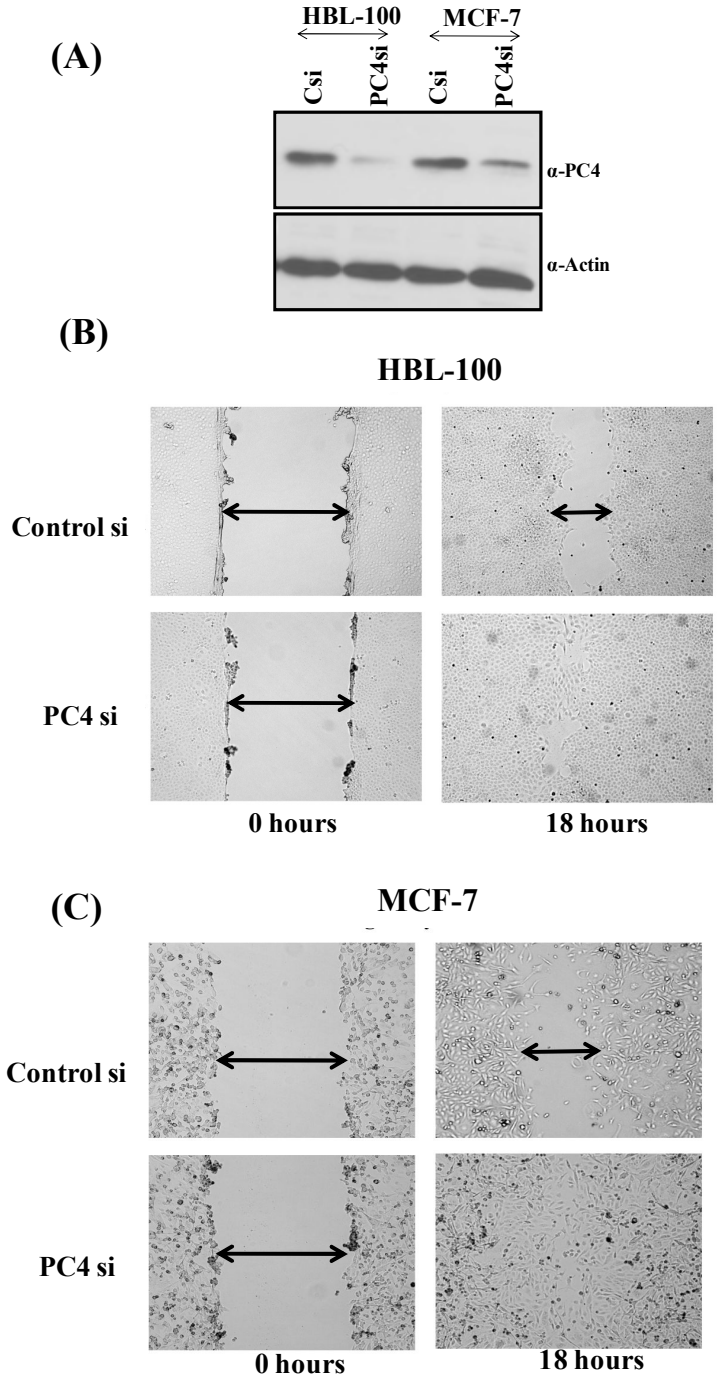


Figure 3.39 PC4 silencing enhances the migratory ability in breast cancer cell lines. (A) PC4 siRNAs were transfected in two breast cancer cell line HBL-100 and MCF-7 for 48 hours and silencing was confirmed by immunoblotting using anti PC4 antibodies. Blot was reprobed with anti actin antibodies to ensure equal loading. Equal wound was created (Left panels) in control siRNA treated and PC4 siRNA treated HBL-100 (B) and MCF-7 (C) cell lines in presence of mitomycin C. Healing was captured after 24 hours (Right panels).

Cancer cells tend to invade through basement membrane which is the first step in cancer metastasis in glandular tumors. Therefore, since PC4 depleted cells migrated more, role of PC4 in cancer cell invasion was examined. Breast cancer cell lines MDA-MB-231 and MCF-7 were treated with scrambled and PC4 siRNAs respectively (Figure 3.40A) and subjected to Boyden chamber invasion assay. Briefly, Boyden chamber assay evaluates invasive ability of cells through basement membrane mimic against a chemoattractant (mostly serum). Cells in question are resuspended in serum free media and are placed in upper chamber which is separated from serum containing lower chamber by matrigel based membrane. Cells move out through the matrigel and their invasive ability is scored by the number of cells on the outer surface of matrigel. Breast cancer cells transfected with PC4 siRNAs could invade more efficiently through matrigel than scrambled siRNA transfected cells (Figure 3.40B, C). Invasion was enhanced by 2.5 fold in MDA-MB-231cells and close to 1.5 fold in MCF-7 cells with PC4 silencing (Figure 3.40 D).

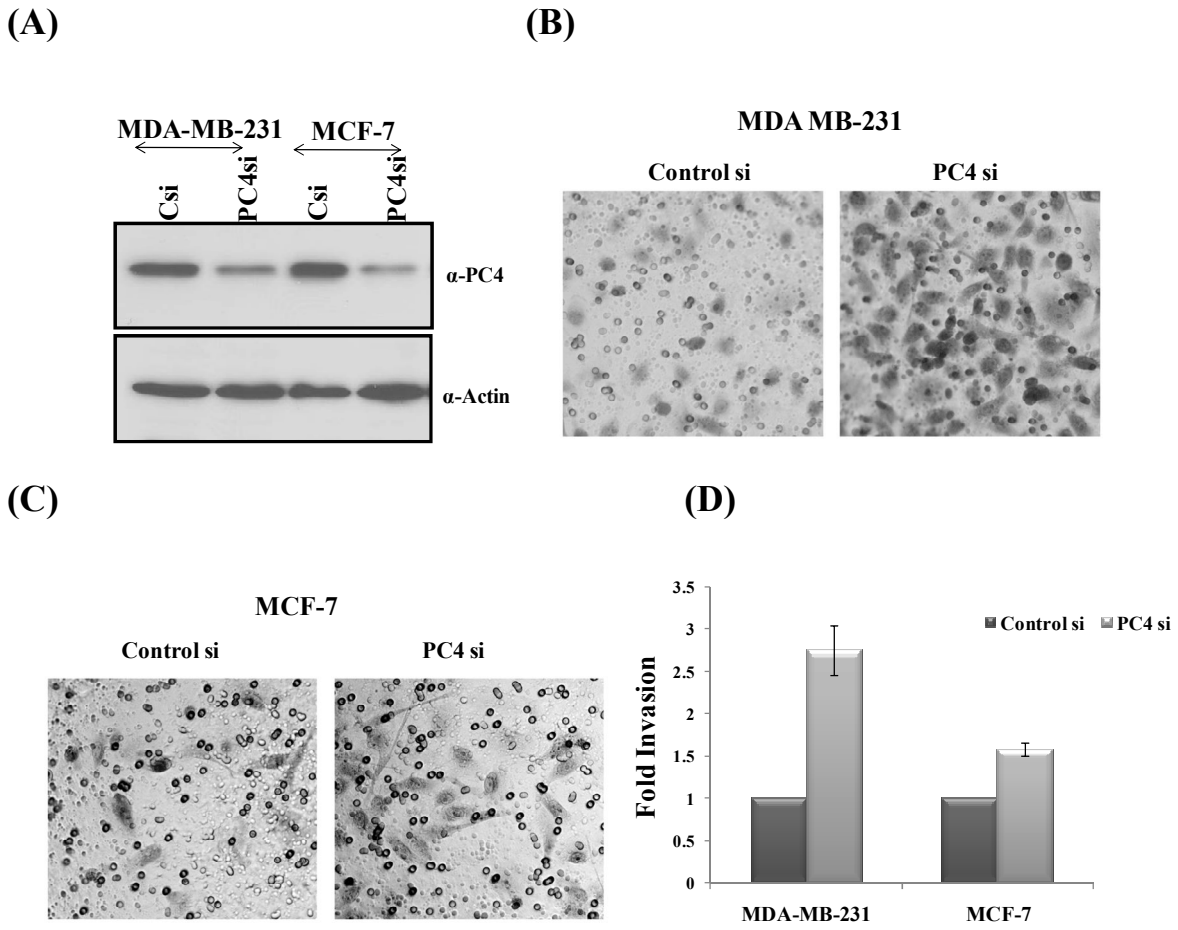


Figure 3.40 PC4 depletion enhances cell invasion ability in breast cancer cell lines. (A) MDA MB- 231 and MCF-7 breast cancer cell lines were transfected with control and PC4 siRNAs for 48 hours. Silencing was confirmed by immunoblotting using anti PC4 antibodies. Blot was reprobed with anti actin antibodies to ensure equal loading. Control and PC4 siRNAs treated MDA-MB-231 (B) and MCF-7 cells were subjected to Boyden chamber invasion assay using matrigel as basement membrane mimic. After 24 hours, invaded cells were fixed, stained and imaged. (D) Graph depicting fold invasion in these cell lines upon PC4 silencing.

3.3.2.2 PC4 regulates expression of matrix metalloproteinases and fibronectin in breast cancer cell lines

Tumor progression involves a series of events which includes epithelial to mesenchymal transition (EMT) of the tumor cells, breakdown of extracellular matrix (ECM)

and basement membrane, and subsequent migration and invasion of tumor cells from the primary site. Some of the key molecular candidates involved in these events are matrix metalloproteinases expressed by tumor cells which degrades ECM and mesenchymal markers like fibronectin and vimentin which are upregulated in tumor cells during metastasis. Gene expression analysis performed using PC4 knockdown stable cell line has shown upregulation of few of the MMPs and fibronectin gene expression. Effect of PC4 silencing in regulation of these genes in cancerous cells remained untested. Since, PC4 silencing could improve the migration and invasion ability in breast cancer cell lines, we investigated expression pattern of different MMPs, mesenchymal markers, fibronectin, vimentin and regulators of EMT, Zeb1/2 upon PC4 silencing. PC4 siRNAs and control scrambled siRNAs were transfected in two breast cancer cell lines, MCF-7 and MDA-MB-231 for 48 hours followed by total RNA extraction. Corresponding cDNAs were used in PCR using gene specific primers for different matrix metalloproteinases like MMP 2, MMP 9, MMP 14, MMP 15; EMT markers, fibronectin, and vimentin; and EMT regulators like, Zeb1 and Zeb2. All the MMPs analyzed and fibronectin showed higher expression when PC4 is downregulated both in MCF-7 and MDA-MB-231 cell lines. MMP 14, MMP 15 and fibronectin showed higher upregulation in both the cell lines than the other MMPs. Zeb1 and Zeb2 genes showed marginal upregulation in MDA-MB-231 cells upon PC4 silencing but not in MCF-7 cells (Figure 3.41).

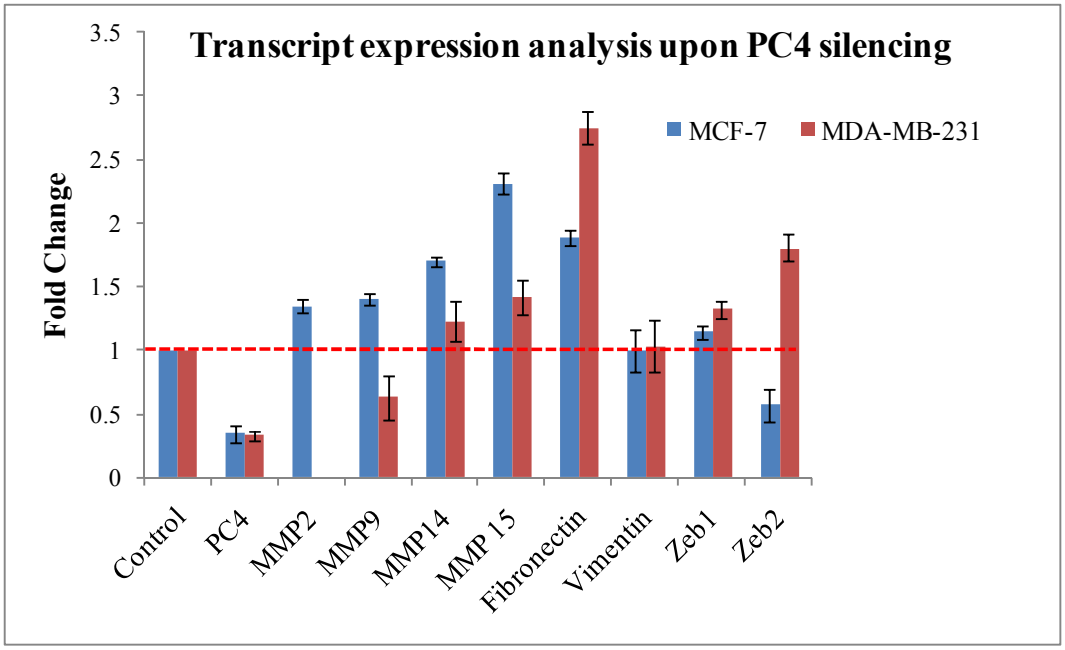
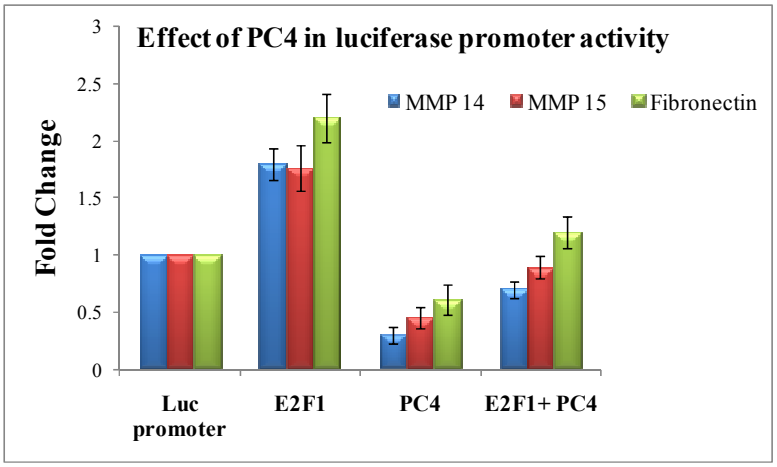


Figure 3.41 PC4 silencing induces MMPs and mesenchymal markers. MCF-7 (Blue bar) and MDA-MB-231 (Red bar) breast cancer cell lines were transfected with scrambled and PC4 siRNAs followed by whole RNA extraction. Reverse transcriptase PCR were performed with corresponding cDNAs using different gene primers as indicated. Fold change was calculated using ΔΔCt method. GAPDH gene primers were used for normalisation.

Higher gene expression of MMPs and fibronectin upon PC4 downregulation suggest PC4 could have negative role in regulating their expression in normal cells which is rescued in the absence of PC4. It could also be possible that upregulation of these genes is just secondary effect of PC4 downregulation. To implicate PC4's regulatory function, luciferase based promoter activity of target genes were examined and PC4 occupancy on the fibronectin promoter was investigated. The direct effect of PC4 in the expression of MMPs and fibronectin, at the promoter level was ascertained by transfecting luciferase gene containing promoter constructs of MMP 14, MMP 15 and fibronectin in MCF-7 cell lines with or without PC4 cDNA expression contruct. The entire promoter constructs used consisted of one or multiple E2F1 binding sites as E2F1 is the known positive regulator of these genes. Co-transfection of E2F1 expression constructs along with luciferase promoter constructs

increased the luciferase activity more than 1.5 folds for each (Figure 3.42A, Lane 2). Expression of PC4 could significantly decrease the basal luciferase activity of the promoter constructs (Figure 3.42A, Lane 3). Co-expression of both E2F1 and PC4 could rescue PC4 mediated repression of luciferase activity to some extent (Figure 3.42A, Lane 4). These results indicate that PC4 have a repressive impact on the transcription of MMP14, MMP15 and fibronectin. To further verify involvement of PC4 in the expression of fibronectin, a mesenchymal marker, ChIP analysis in MCF-7 cells was performed to examine localization of PC4 at its promoter. Chromatin pull down was performed with anti PC4 and anti E2F1 antibodies and their localization was ascertained by PCR using fibronectin promoter specific primers set (Figure 3.42B). PC4 occupancy was observed on fibronectin promoter which manifests its direct involvement in the repression of fibronectin gene expression under normal conditions.

(A)



(B)

ChIP: Fibronectin promoter

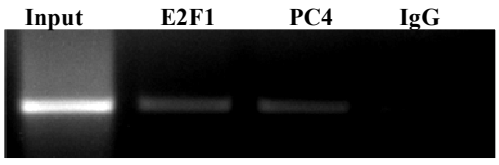


Figure 3.42 PC4 represses MMP 14, MMP 15 and Fibronectin reporter promoter. (A) MCF-7 cells were transfected with 0.5 ug of indicated MMPs and fibronectin luciferase reporters along with different concentrations of PC4 using Fugene HD transfection reagent in the ratio of 2:1 with DNA. pRL construct carrying *Renilla reniformis* luciferase gene was cotrasfected for normalisation. (B) ChIP analysis was performed on fibronectin promoter having E2F1 binding sites and PC4 occupancy was explored by pulling down the complex using anti PC4 antibodies (Lane 3). Pull down using anti E2F1 (Lane 2) and nonspecific IgG (Lane 4) antibodies served as positive and negative controls respectively.

This section deals with expression analysis of PC4 in breast cancer patient samples and its probable tumor suppressive role in breast cancer manifestation. PC4 expression was observed drastically reduced in majority of the samples analyzed irrespective of the different grades or subtypes of breast cancer. Reduced PC4 could enhance migratory and invasive ability of breast cancer cell lines tested. PC4 downregulation was found to cause de-repression of few MMPs and mesenchymal marker, fibronectin. Luciferase based promoter activity experiments indicate PC4 act at transcriptional level to repress their expression and occupancy of PC4 was also uncovered at fibronectin promoter. These findings indicate that PC4 acts as a tumor suppressor, whose loss potentiates enhanced tumorigenic potential in breast cancer cell lines.

3.4 Summary of the results

Human transcriptional coactivators, PC4 is a highly abundant nuclear protein shown to participate in multiple chromatin templated phenomena. The present study investigates the post-translational modifications specifically, acetylation and phosphorylation of PC4 and their possible involvement in transcriptional coactivation and chromatin organization mediated by PC4. Attempts have been made to understand significance of PC4 in physiology and pathobiology. PC4 was found to get acetylated by p300 (KAT3B) and deacetylated by SIRT2, a sirtuin class of deacetylase in the cell. Interestingly, deacetylation of acetylated PC4 is a cell cycle stage specific event where acetylation is reduced significantly during the onset of mitosis. Acetylation of PC4 is crucial for p53 dependent transcription activation. Acetylation and phosphorylation of PC4 can be comprehended as exclusive for coactivation function; acetylation being essential for, whereas, phosphorylation completely abolishes activator dependent transcription. PC4 binds with high affinity to both octameric core histone and individual histone H3 albeit with different stochiometry. There are two binding sites in core histones whereas for histone H3 there is just one site. PC4 interacting region to histone H3 is mapped and the stretch of residues from 62 to 67 was found to be critical for the interaction. PC4 interacts to somatic variants of linker histone in phosphorylation dependent manner with serine 17 in PC4 being crucial residue for the interaction. C-terminal of linker histones participates in the interaction with phosphorylated PC4 predicting involvement of PC4 beyond nucleosomal level of chromatin folding. To explore significance of PC4 in cellular context, PC4 knockdown stable cell lines were generated through lentivirus mediated PC4 targeting shRNAs delivery system. The genomic integration of these shRNAs were stabilized and ensured by puromycin antibiotic selection through several generations. PC4 knockdown stable cell line exhibits altered epigenetic readouts with enhanced transcription associated histone modifications (H3K9Ac, H2AK5Ac, H4K16Ac, H3K4Me3). These cells

also possess lesser compact chromatin with fewer number of heterochromatin foci resulting in overall altered nuclear architecture. PC4 stable knockdown resulted in the accumulation of abnormal chromatin and induced several cell cycle related defects. PC4 knockdown cells have a higher proliferation rate and higher resistance to gamma irradiation mediated cytotoxicity. Global gene expression analysis done with PC4 knockdown cell line reveals several dysregulated genes belonging to diverse pathways like cell cycle, chromosome organization, cell migration, microtubule organization etc. Expression status of PC4 when analyzed in breast cancer patient samples, evidenced reduction of PC4 both at protein and transcript level in tumors when compared to adjacent normal samples. Possibility of involvement of PC4 in potentiating tumorigenicity was verified utilizing breast cancer cell lines. Transient silencing of PC4 in breast cancer cell lines increases their migratory and invasive ability probably through regulating few MMPs (MMP2, 9, 14, 15) and fibronectin gene expression. PC4 was found to negatively regulate promoter activity of MMP 14, MMP15 and fibronectin in a luciferase based experiment. PC4 was also localized at fibronectin promoter as evident from the ChIP experiment done with PC4 antibodies in MCF-7 cells. The significance of these findings and future perspectives has been discussed in the next chapter.

Chapter 4

DISCUSSION AND FUTURE PERSPECTIVE

Chapter Outline:

4.1 Post-translational modifications and functions of PC4

4.2 PC4 in the maintenance of chromatin architecture

4.3 PC4 and cell cycle progression

4.4 PC4 as a tumor suppressor in breast cancer

4.1 Post-translational modifications and functions of PC4:

Human positive coactivator 4 (PC4) is a multifunctional, nuclear protein which is present in high abundance in the cell. PC4 was functionally characterized as a general coactivator of activator dependent transcription mediated by RNA polymerase II (Ge and Roeder, 1994; Kretzschmar et al, 1994). Coactivation ability of PC4 is largely dependent on its ds DNA and diverse group of activators binding property. PC4 through its interaction with activators and general transcription factors at promoter region acts as a mediator and bring about the activation. Although, PC4 has been projected as a general coactivator, it is also known to behave as a repressor of transcription. PC4, in the absence of activators, is a potent repressor of basic transcription (Malik et al, 1995). PC4 has also been reported to be present in a repressor complex, silencing specific set of genes; for e.g., PC4 is associated with REST-

CoREST complex along with HP1α to suppress the expression of neuronal genes in non neuronal cells like HeLa and HEK 293T (Das et al, 2010). Also, the overall repressive impact of PC4 on chromatin structure and thereby transcription, raises a questions about the mechanism behind the coactivation property. PC4 is a chromatin associated protein which compacts the chromatin and thus, renders chromatin inaccessible for transcription machineries. The same protein seemed to be involved in antagonistic function. It was hypothesised that the key to this mystery was in the different post-translationally modified forms of this protein. PC4 has been shown to get modified by two modifications namely, acetylation and phosphorylation. PC4 exhibits different biochemical properties upon its acetylation and phosphorylation (Table 4.1). As evident from the table, ds DNA binding, which is pre-requisite for the transcriptional coactivation by PC4, is totally abolished upon its phosphorylation. Also, PC4 upon phosphorylation cannot bind to activators rendering phosphorylated PC4 (P-PC4) inactive as a transcriptional coactivator. On the other hand, acetylated PC4 (Ac-PC4) has better DNA binding and bending ability and is found essential for p53 dependent transcription (Batta et al, 2007). Therefore, phosphorylation and acetylation act as distinct signals for modulating the functions of PC4 in transcriptional activation. Phosphorylated PC4 interacts better with core histones (**Figure 3.20)**. Phosphorylation also bestows the ability for exclusive interaction with linker histone H1 **(Figure 3.21)**, which possibly assists PC4 to hook on to the chromatin and therefore, contributing directly to its chromatin organizing ability. Majority of PC4 in the cell is in its phosphorylated form (95%), which implies chromatin condensation could be the major function of PC4, whereas transcription activation could be carried out by a small subset of acetylated PC4 in a context dependent manner. This assumption is supported by the fact that silencing of PC4 results in decompaction of chromatin with increase in transcriptional activation associated histone modifications **(Figure 3.27)**. PC4 mediated transcriptional

activation through its acetylation seems to be cell cycle dependent. Acetylated PC4 colocalizes with phosphorylated RNA polymerase II at transcriptionally active foci during interphase and gets deacetylated during mitosis **(Figure 3.2, 3.3)**. Association of acetylated PC4 with active transcriptional foci during interphase, points towards transcriptional activation ability of acetylated form of PC4. Such correlation is strengthened by the observation of reduction in PC4 acetylation during mitosis, when transcriptional competency of the cell is largely decreased. Basic importance of acetylation of PC4 in coactivation is exemplified using p53 dependent transcription system. In the lack of acetylating lysines, PC4 was not able to activate p53 dependent luciferase expression **(Figure 3.17)**. Although, it has been shown that acetylation of PC4 is important for p53 dependent transcription, the mechanism by which acetylated PC4 enhances transcription is yet to be resolved. For this, it is necessary to examine level of transcriptional activation with acetylated PC4 in an *in vitro* reconstituted transcription system. Verification of interaction ability of acetylated PC4 with general transcription factors (GTFs) and different activators would be insightful for the study. Figure 4.1 illustrates a model proposing phosphorylation and acetylation as functional switch between chromatin organization and transcriptional coativation mediated by PC4.

Properties	Unmodified PC4	Acetylation of PC4	Phosphorylation of PC4
ds DNA binding	+	++	-
ss DNA binding	+	NA	+
Core-histone interaction	+	+	++
Linker-histone interaction	-	-	+
Interaction with activators	+	NA	-
Interaction with GTFs	+	NA	-
Dimerization	+	++	-

Table 4.1 List of biochemical properties of unmodified, acetylated and phosphorylated PC4. Abbreviations- +: Present; -: Absent; ++: Enhancement; NA: Data not available.

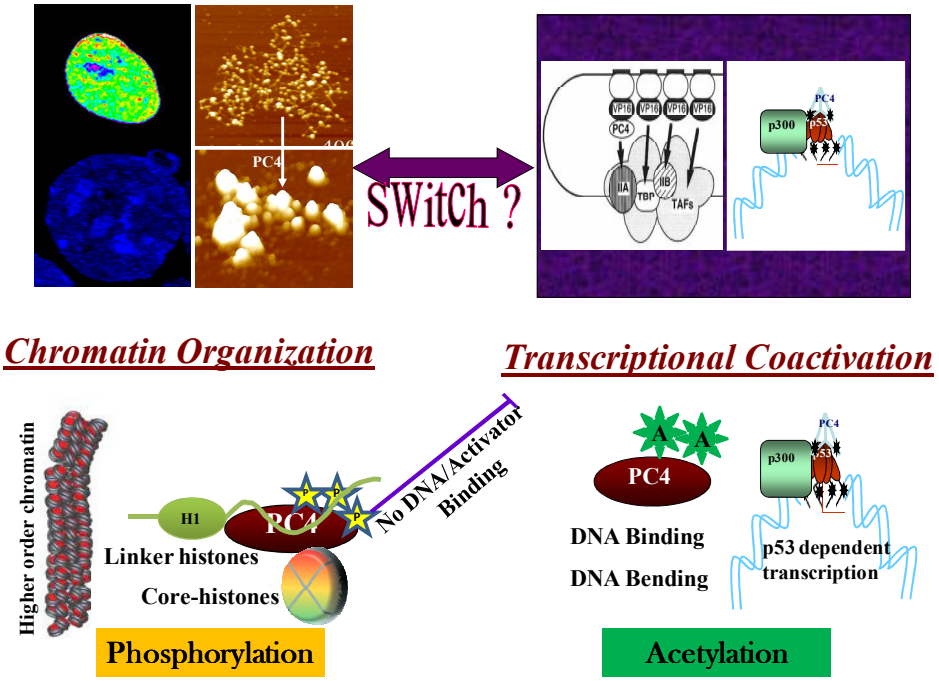


Figure 4.1 A model illustrating phosphorylation and acetylation as functional switch between chromatin organization and transcriptional coativation mediated by PC4. PC4 is involved in two main cellular functions, namely chromatin organization and transcriptional coactivation. Phosphorylation of PC4 enables it to interact with linker histones and provides better association with core-histones, thereby helping it to organize chromatin. Due to lack of its ability to bind to ds DNA and activators upon phosphorylation, phosphorylated PC4 is unable to activate transcription. On the other hand, acetylated PC4 shows enhanced DNA binding and DNA bending and increases p53 dependent transcription coactivation.

Acetylation and Phosphorylation of PC4 has been investigated individually in the present study. The cross talk between these two modifications needs to be addressed. It needed to be ascertained whether these two modifications of PC4 were exclusive to each other or interdependent *in-vivo*. It has been shown that PC4 upon phosphorylation cannot undergo acetylation but acetylated PC4 can get phosphorylated *in vitro* (Kumar et al, 2001). In the cellular context, existence of bimodified form of PC4 (acetylated and phosphorylated) still

remains hypothetical. Acetylation of PC4 specifically gets reduced presumably, by deacetylation during the onset of mitosis. HDAC class III enzyme, SIRT2 has been identified as one of the deacetylases of PC4 *in vivo*. SIRT2 localizes to chromatin during mitosis and presumably, through interaction with acetylated PC4 deacetylates it. On the contrary, high throughput mass spectrometric data of the mitotic phosphoproteome in HeLa cell lysates synchronised at G1 and M-phase (Dephoure et al, 2008), suggests that PC4 gets heavily phosphorylated during mitosis. Phosphorylated PC4 is hypothesised as a chromatin organizer in interphase chromatin in the present study. Nevertheless, role of phosphorylation of PC4 in the organization of mitotic chromosome is an open question. PC4 has been shown to be present throughout the arms of chromosomes. Also, newer set of serine residues get phosphorylated during mitosis including serine 17. In the present study, serine 17 has been shown as the critical residue, whose phosphorylation is indispensable for interaction with linker histone H1. It needs to be ascertained whether mitotic phosphorylation of PC4 has any added advantage in organization of chromosomes. Since, deacetylation of PC4 is accomplished with the onset of mitosis, it is possible that mitotic phosphorylation could trigger deacetylation of PC4 during mitosis. A proposed model for the dynamicity of post-translational modifications of PC4 in the context of cell cycle is shown in Figure 4.2.

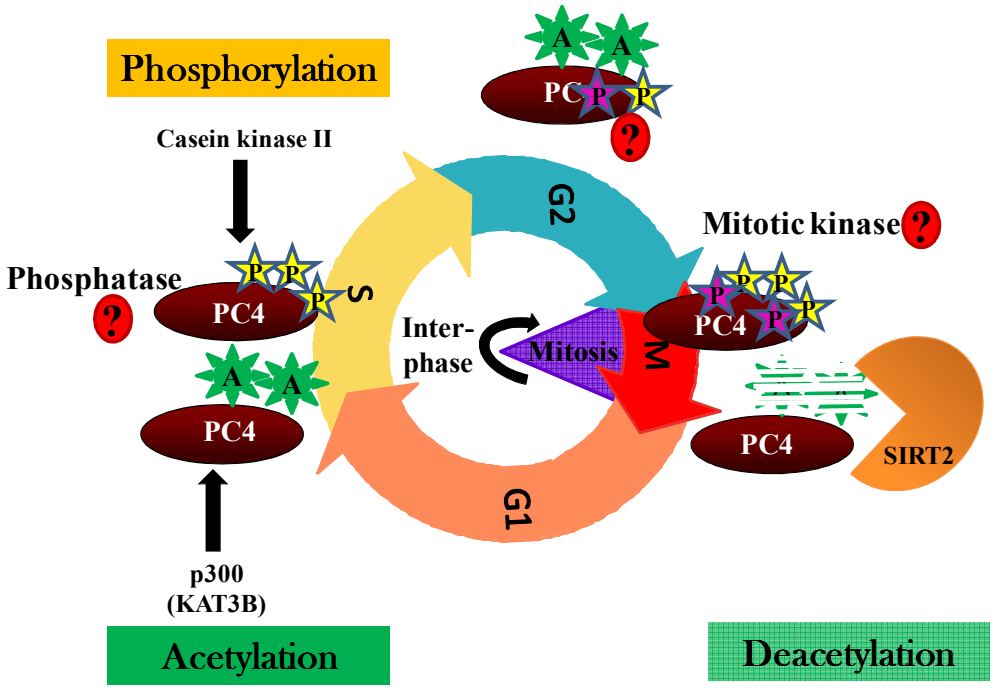


Figure 4.2 Dynamicity of post-translational modifications of PC4 in the context of cell cycle. A model showing distribution of modified PC4 in different cell cycle stages and known enzymatic machineries regulating these modifications. The unrevealed events are shown by interrogative sign (?).

The post-translational modifications of PC4 have a huge impact on its mode of functioning. The present study has identified acetylation of PC4 mediated by MYST family of lysine acetyltransferases, apart from already reported p300/CBP family **(Figure 3.5)**. This observation might open up newer prospects for PC4 functions. MYST family of acetyltransferases have been implicated in DNA double strand break responses through studies involving different organisms. Many DNA repair related proteins have been found to be substrates of TIP60. ATM kinase, the DNA damage sensor protein gets acetylated by TIP60 which is required for efficient ATM autophosphorylation and enhancement of ATM kinase activity (Sun et al, 2005). The catalytic activity of TIP60 itself is stimulated in response to damage and is recruited to damage site and affects acetylation of histone H4 at lysine 16. This acetylation probably opens up the chromatin structure to facilitate the

accessibility of the DNA repair machinery (Tamburini and Tyler, 2005). PC4 is a damage sensing protein and known to get accumulated at damaged site (Mortusewicz et al, 2008). Our own data suggests that PC4 has ds strand break repair activity (Batta et al, 2008). With the new found information that MYST family KATs acetylate PC4 on residues different from the p300/CBP, there is a strong possibility of differentially acetylated PC4 being involved in DNA damage response. Also, single stranded DNA binding ability of PC4 has been demonstrated essential for its recruitment to damage site as well as DNA repair property (Wang et al, 2004; Mortusewicz et al, 2008; Batta et al, 2008). Acetylation of PC4 also enhances its DNA binding ability. Put together, there are strong clues for the involvement of PC4 in DNA damage repair and therefore needs to be explored further.

PC4 in the maintenance of chromatin architecture:

Global alteration of nuclear architecture as seen by loss of heterochromatin foci rendering nucleus composed more of open chromatin in the absence of PC4 **(Figure 3.29)**, establishes it as one of the important chromatin architectural proteins. Such loss of function approach has highlighted PC4 as one of the major determinants for the structural competence of chromatin. Although, the exact mechanism of PC4 mediated structuring of chromatin is not fully understood, its interaction with both core histones and linker histones suggests that it might act at different levels. The positively charged C-terminal domain of linker histones has been implicated as a major determinant of H1 mediated chromatin condensation through its binding to linker DNA (Hendzel et al, 2004). Binding of PC4 to the C-terminal region of linker histones might be required for recruitment of H1 to the dyad axis, although direct proof for this hypothesis remains inert. The other possibility is that PC4 could get bound to the dyad axis by H1 and thereby increases compaction. PC4 bound to core histones will further

enhance overall condensation thereby bringing about such high compaction levels. PC4 interacts with all somatic variants of linker histone H1 (H1.1-H1.5) *in vitro* **(Figure 3.21)**. Linker histone variants show sequence variability and are thought to share redundancy in their functions. Nevertheless, several *in vitro* studies have suggested that H1 variants have different degrees of affinity for chromatin and thus, impact on chromatin condensation (Th'ng et al, 2005; Clausell et al, 2009). It would be interesting to examine if PC4 exhibits any variant specificity in the cell. Linker histones are highly mobile in the nucleus and the major determinants of the chromatin residence time of H1 in living cells include (i) the structure of the H1 molecule, (ii) post-translational modifications of the H1 molecule or its chromatin binding site, and (iii) competition for chromatin binding sites (Catez et al, 2006). Studies have shown cells treated with histone deacetylase inhibitor, Trichostatin A cause decreased binding of H1 with the chromatin and thus increased mobility. This increase in H1 mobility could be attributed to either altered docking of H1 to nucleosomes containing acetylated histone tails or to acetylation-induced alterations in chromatin structure (Misteli et al, 2000). PC4 being one of the determinants of epigenetic readout of the chromatin, it would be interesting to investigate influence of PC4 (synergistic or antagonistic) in H1 association with chromatin. It is known that phosphorylation of H1, which creates a patch of negative charges, retard its binding to the chromatin (Dou and Gorovsky, 2000; Dou et al, 2002). Cell cycle–related chromatin decondensation and other cellular events like chromatin decondensation and remodeling, replication and transcription closely correlates with reversible phosphorylation of H1 and its intranuclear mobility. Post-translational modifications of linker histones have been demonstrated to modulate its interaction with other nuclear proteins. The heterochromatin protein 1, HP1 binds specifically to methylated histone H1.4 at lysine 26, whereas serine 27 phosphorylation blocks HP1 binding (Daujat et al, 2005). In this direction, association of PC4 with context dependent modified H1 would be

interesting to explore. A repertoire of chromatin binding proteins has been demonstrated to compete with linker histones for the chromatin binding sites. Importantly, non-site-specific proteins (e.g. HMGs) affect chromatin binding of a larger fraction of the cellular H1s. The different families of HMGs showed synergistic effects in competing with H1 binding to the chromatin probably because each type of HMG competes for a distinct set of H1 binding sites (Catez et al, 2004). PC4, which is a highly abundant non specific DNA binding protein, through its interaction with linker histones might modulate its binding to chromatin and aids in the chromatin dynamics. It would be interesting to monitor mobility and chromatin residence of linker histones in PC4 knockdown cells.

PC4 and cell cycle progression

The present study utilizing PC4 knockdown stable cell line demonstrated disruption of several cell cycle related events in the absence of PC4 **(Figure 3.30)**. These cell lines exhibited accumulation of chromatin with altered structure and composition. It is possible that abnormalities related to chromatin architecture caused in the absence of PC4 also influences chromatin condensation processes during cell division. PC4 knockdown cell lines exhibited mitotic and post-mitotic defects indicating participation of PC4 in different cell cycle related machineries **(Figure 3.31)**. Microarray analysis suggests that several cell cycle related genes are affected in the absence of PC4 **(Figure 3.34)**. Extensive investigation of these genes is required to delineate role of PC4 during onset of mitosis (prometaphase-metaphase) and post-mitotic events. Interacting partners of PC4 in different cell cycle stages could give new insights. Studies indicate that there is profound impact of cell cycle on PC4 post-translational modifications. Phospho proteome analysis shows different sets of phosphorylation sites in PC4 during mitosis as compared to interphase. Therefore, extensive

analysis of these newer sites in direction of cell cycle stages is required and cell cycle specific kinase for PC4 is hypothesized. Our studies have also shown that acetylation of PC4 gradually diminishes with the progression of mitosis. These observations pose the question: Do these post-translational modification combinations of PC4 have any role in the process of cell division or are these just consequences?

PC4 has been shown as a substrate specific inhibitor of RNA polymerase II phosphorylation (Schang et al, 2000). PC4 inhibits TFIIH- or trimeric cdk-7 kinase complex mediated phosphorylation of RNA polymerase II which is one of the proposed mechanisms for PC4 mediated transcriptional repression. The cdk inhibitory activity of PC4 is independent of its transcriptional coactivation and DNA binding. A dual role for cdk-7 has been implicated namely, in transcription as part of TFIIH (transcription repair factor IIH) and in the control of the cell cycle as the trimeric CAK (cyclin-dependent kinase-activating kinase) complex (Feaver et al, 1994; Shiekhattar et al, 1995; Fesquet et al, 1993; Solomon et al, 1993; Poon et al, 1993). The ability of PC4 to inhibit cdk-7 kinase activity might play an important role in modulating the dynamic regulation of transcription and cell-cycle progression.

PC4 as a tumor suppressor in breast cancer

The tumor suppressive ability of PC4 has been previously demonstrated in *ras* oncogene induced tumorigenesis. Stable expression of PC4 in *ras* oncogene transformed human teratocarcinoma cell line, PA-1 shows growth suppressive effects and abolishes *ras* oncogene induced tumorigenicity with diminished growth rate, loss of anchorage independent growth, and lack of tumor induction in nude mice. It was found that *ras* oncogene transformation caused overexpression of AP-2 in PA-1 cells, which in turn caused AP-2 self interference and tumorigenic properties in these cells (Kannan et al, 1999). Overexpression of AP-2 in non-

tumorigenic human teratocarcinoma cell line, PA-1 cells induces tumorigenicity similar to that of caused by *ras* transformation (Kannan et al, 1999). This phenomenon is explained as transcriptional interference i.e. overexpression of a transcriptional factor results in inhibition of itself or other transcriptionn factor. It was shown that PC4 through its direct interaction with AP-2, relieves AP-2 transcriptional self-interference, thereby exhibiting tumor suppressive properties. The study emphasizes that the expression level of PC4 being so critical in the mechanism of *ras* transformation that controlling the level of PC4 could revert *ras*-transformed cells. Such mechanism could be extrapolated to other transcription factors that require PC4 as a coactivator. Possible approach investigating PC4-AP-2 connection in other cancers which often suffer from AP-2 overexpression and/ or *ras* oncogene transformation could provide further evidences where PC4 behave as a tumor suppressor at least in a subset of cancers.

Previous studies ascertained tumor suppressive ability of PC4 in *ras* induced tumors by virtue of its coactivation ability, our present study however, describe global effects of PC4 downregulation and its possible implications in breast cancer manifestation. PC4 protein and transcript expression is drastically reduced in breast cancer samples irrespective of sample stage, grade or receptor status **(Figure 3.38; Table 3.2)**. The observations suggests reduction of PC4 being beneficial for cancer cells in some way. Studies performed with PC4 knockdown cells could be interpreted as gain of tumorigenic potential in non-tumorigenic cell line upon depletion of PC4. Decreased amount of PC4 in the cell caused altered chromatin architecture towards more open and transcriptionally competent chromatin, induced several cell cycle defects leading to genomic instability and enhanced proliferation rate probably through violating few cell cycle checkpoints. These phenotypes observed in PC4 knockdown cell typically mimics pathological state of a cancer cell. We have tried to look for specific implications of depletion of PC4 in the course of tumorigenesis. Our studies suggest that PC4

negatively regulate expression of few matrix metalloproteinases and mesenchymal marker genes in the normal cell **(Figure 3.42)**, which is enhanced in cancer cells where PC4 level is low. Consistent with this observation, loss of PC4 caused increased migratory and invasive ability in breast cancer cells **(Figure 3.39, 40)**. Reduction in PC4 level also increased expression of different MMPs and fibronectin genes both in breast cancer and non-tumorigenic HEK 293 cell lines **(Figure 3.41)**. These findings emphasize that loss of PC4 potentiates tumorigenicity in non-tumorigenic cells. Such an accomplishment is attained by regulating expression of cancer related genes like MMPs and fibronectin. Nevertheless, downregulation of PC4 in breast cancer could also be seen beyond its ability to regulate MMPs functions, as a general genome organizer. PC4 is also a DNA repair protein and downregulation of DNA repair proteins is one of the common strategies found in cancer cells, in order to bypass the barrier to accumulate and maintain genomic mutations and instabilities. PC4 has been shown detrimental for the integrity of DNA as cells with loss of PC4 suffer from accumulation of defective chromatin. Cells with large sized genome and abnormally high proliferative rate have been detected where PC4 expression is low. PC4 might pose a check on genomic instability and its reduction therefore, could help tumors to acquire unique characteristics for its survival and growth.

The mechanism of downregulation of PC4 in breast cancer is unknown. Hypothetically, it could be due to genetic or epigenetic factors. Genomic instability is a common characteristic of a cancer cell, where particular genome loci suffers copy number variations in different tumors. The genomic locus where PC4 resides is very unstable as found in several cancers. PC4 maps to human chromosome 5p13, which is a location frequently associated with loss of heterozygosity in several cancers (Bohm et al, 1997; Wieland et al, 1996). Further experiments in order to analyze change in copy number of PC4 gene in breast cancer samples have been designed and are in progress. Furthermore, PC4 expression could also be in control

of epigenetic factors like DNMTs, microRNAs which are also perturbed in cancerous state. DNA hypermethylation in the promoter regions of tumor suppressor genes, thereby silencing their expression is a common event found in various cancers. Similar possibility is being verified for PC4 promoter in the context of breast cancer. PC4 promoter contains approximately 762 bp long CpG island spanning the first exon (Figure 4.3). The number of CG dinucleotide in this island is 89. Methylation status of these CpGs is now being investigated to correlate expression of PC4 in breast cancer and corresponding normal tissues.

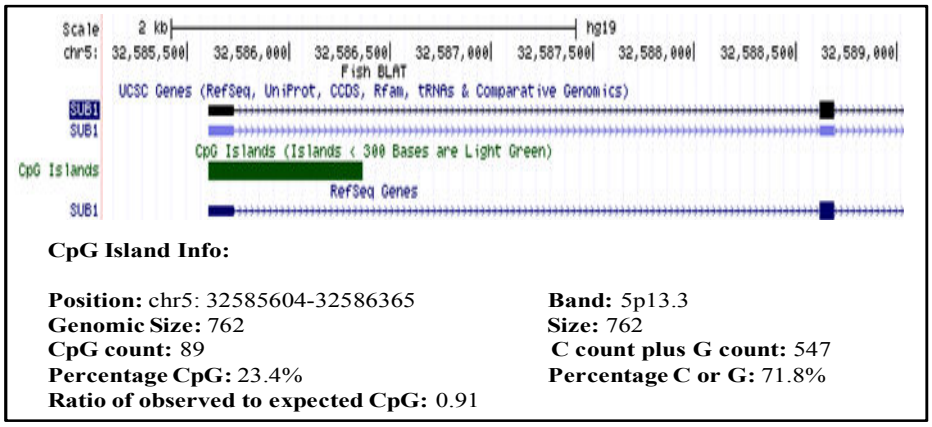


Figure 4.3 UCSC genome browser image of PC4 (SUB1) CpG island and flanking regions.

Cancer type	Upregulated	Downregulated
Breast cancer	miR-10b, miR-21, miR-22, miR-27a, miR-155, miR-210, miR-221, miR-222, miR-328, miR-373, miR-520c	let-7, miR-7, miR-9-1, miR-17/miR-20, miR-31, miR-125a, miR-125b, miR-146, miR-200 family, miR-205, miR-206, miR-335
CLL	miR-21, miR-155	miR-15, miR-16, miR-29b, miR-29c, miR-34a, miR-143, miR-145, miR-181b, miR-223
Lung cancer	miR-17-92 cluster, miR-21, miR-1061, miR-155	miR-1, miR-7,let-7 family, miR-15a/miR-16, miR-29 family
Lymphoma	miR-17-92 cluster, miR-155	miR-143, miR-145
Prostate cancer	miR-221, miR-222	miR-15a-miR-16-1 cluster, miR-101, miR-127, miR-449a
Glioblastoma	miR-21, miR-221, miR-222	miR-7
Hepatocellular carcinoma	miR-17-92 cluster, miR-21, miR-143, miR-224	miR-1, miR-101, miR-122a
Colorectal carcinoma	miR-17-92 cluster, miR-21	miR-34a, miR-34b/c, miR-127, miR-143, miR-145, miR-342
Gastric cencer	miR-21, miR-27a	miR-143, miR-145
Ovarian cancer	miR-214	miR-34b/c, miR-200 family
Melanoma	miR-221, miR-222	let-7a, miR-34a
Head and neck squamous cell carcinoma	miR-21	let-7d, miR-138, miR-205

Table 4.2 Table showing list of miRNAs upregulated (oncomiRNAs) and downregulated (Tumor suppressor miRNAs) in different cancers (Reviewed in Mengfeng et al, 2010).

Recent evidences have implicated miRNAs as critical regulators in human cancers by virtue of their involvement in diverse biological processes. It has been shown that mutations or mis-expression of miRNA correlate with various human cancers and can function as tumor suppressors and oncogenes (Table 4.2). miRNAs modulate several tumor suppressor and oncogenic pathways. These are found to regulate the expression of proteins involved in diverse signalling pathways critical for cancer development and progression. The tumor suppressor protein retinoblastoma, which is frequently altered in cancer cells is targeted by miR-106a, which is found elevated in several cancers (Volinia et al, 2006). Similarly, miRNAs target other key regulatory proteins and thus, have complicated molecular networks, which control cell cycle, proliferation and DNA repair. Table 4.3 shows the list of the miRNAs and their target mRNAs in a wide variety of cancers.

miRNA	Target gene	Note (cancer type, etc)
let-7 family	RAS, CCND2, CDK6, CDC25A, HMGA2, c-Myc, k-RAS, NF2	Lung cancer, ovarian cancer, liver tumors, cancer cell lines (A549, HepG2, HeLa) and cholangiocarcinoma cell lines
miR-9, miR-125a, miR-125b	Tropomyosin-related kinase C (trkC)	Neuroblastoma
miR-10b	HOXD10	Breast cancer
miR-16-1, miR-15a	Bcl2	Chronic lymphocytic leukemia
miR-17-5p, miR-20	AIB1, Transforming growth factor-β receptor type II (TβRII)	Breast cancer
miR-18	CTGF (connective tissue growth factor)	Colon cancer model of angiogenesis
miR-19	Thrombospondin-1	Colon cancer model of angiogenesis
miR-20a	E2F1,2,3	
miR-21	Pdcd4, PTEN, Tropomyosin 1 (TPM1),	Colorectal cancer, hepatocellular cancer, cholangiocarcinoma
miR-27b	CYP1B1	Breast cancer
miR-29	DNMT3A, DNMT3B, Mcl-1, TCL1	Non small cell lung cancer, cholangiocarcinoma cell line
miR-34a, b, c	E2F3, cyclin E2, hepatocyte growth factor receptor (MET), Bcl2	Neuroblastoma, non small cell lung cancer
miR-106a cluster	Mylip (myosin regulatory light chain interacting protein), Hipk3, Rbp1-like (retinoblastoma-binding protein 1-like)	T- cell leukemia
miR-122a	Cyclin G1	Hepatocellular cancer
miR-124a	Cdk6	Colon cancer, lung cancer
miR-125a, b	ERBB2, ERBB3	Breast cancer
miR-127	Bcl6	Bladder cencer
miR-206	ER alpha	Breast cancer
miR-221,222	P27 (Kip)	Glioblastoma and prostate cancer
miR-372,373, 378	LATAS2, Sufu, Fus-1	Testicular germ cell tumor, U87 glioblastoma cell line
BART 16, 17-5p	LMP1	Nasopharyngeal carcinoma
KSHV miRNAs	Thrombospondin 1	

Table 4.3 The list of the miRNAs and their target mRNAs in diverse cancers and cancer cell lines (Reviewed in Lee and Dutta, 2009).

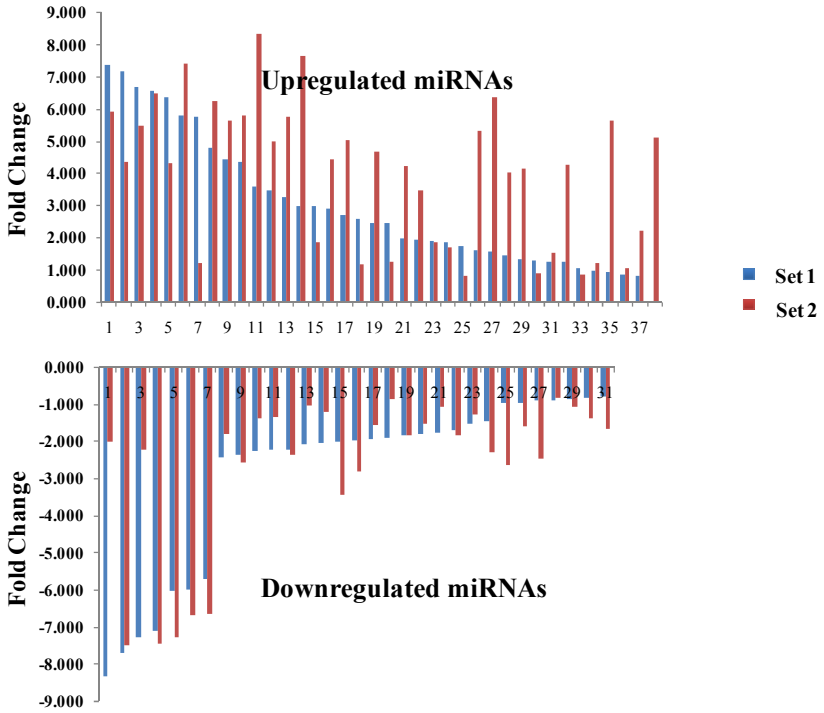


Figure 4.4 Differential expression of miRNAs in two sets of breast cancer when compared to adjacent normal tissues.

S. No.	Upregulated miRNAs	Downregulated miRNAs
1	hsa-miR-301a	hsa-miR-630
2	hsa-miR-181d	hsa-miR-378
3	hsa-miR-183	hsa-miR-139-5p
4	hsa-miR-96	hsa-miR-187
5	hsa-miR-454	hsa-miR-652
6	hsa-miR-449a	hsa-miR-452
7	hsa-miR-885-5p	hsa-miR-378
8	Has-let-7d	hcmv-miR-UL70-3p
9	hsa-miR-629	hsa-miR-551b
10	hsa-miR-200a	hsa-miR-134

Table 4.4 Table showing commonly up and downregulated miRNAs in two sets of breast cancer samples. Fold change is set upto two folds and more.

miRNAs have been shown to repress the expression of important tumor suppressor genes in the cancer cell. In order to investigate the possibility of regulation of PC4 by miRNAs in breast cancer, miRNA expression profiling was performed in two sets of breast samples (normal and tumor tissues). Both the tumor samples showed several up and downregulated miRNAs when compared to corresponding normal tissues (Figure 4.4). Table 4.4 shows list of miRNAs commonly up and down regulated in these samples. Such differential expression of miRNAs would be extended to a large number of breast samples to fish out miRNAs which are significantly altered in majority of the tumor samples. Such miRNAs would be further examined for their ability to regulate expression of PC4. Figure 4.5 illustrates our current understanding about role of PC4 in breast cancer manifestation.

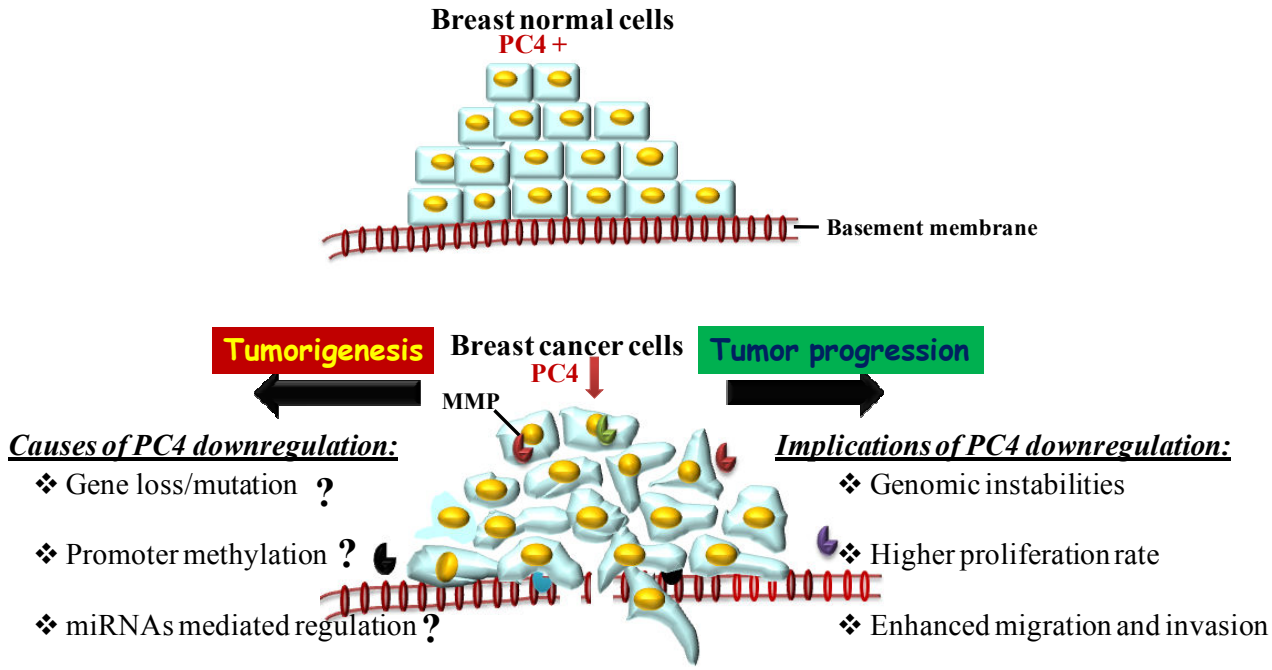


Figure 4.5 The proposed model for role of PC4 in breast cancer manifestation. PC4 expression is drastically reduced in breast cancer cells when compared to normal cells of breast. The mechanism behind the downregulation of PC4 is largely unknown (?) but some genetic and epigenetic phenomena have been proposed. Loss of PC4 potentiates several tumorigenic events in the cells, thereby favouring the tumor progression.

PC4 participates in several diverse biological functions and thereby qualifies itself as a multifunctional protein. Alteration of its expression, post-translational modifications and interacting partners, all add further complexities to its functions. Depletion of PC4, as observed in knockdown cells jeopardizes normal functioning of the cell in more than one way which included aberrant organization of chromatin and a defective cell cycle. Versatility of the functions seems to be achieved to some extent atleast through dynamic post-translational modifications of PC4 under normal physiological conditions. Phosphorylation of PC4 was found to be essential for compaction of chromatin while the existence of acetylation has been elucidated which possibly will enrich the complexity. In a context dependent manner, different interacting partners of PC4 could also direct its functions. For example, chromatin organising protein histone H1 could interact specifically and exclusively interact with the phosphorylated form of PC4. It is expected that such a specific interaction would futher aid the compaction of chromatin.

The magnitude of its role in the cell in maintaining cellular homeostasis is reflected by its loss of function. PC4 knockdown in a non tumors cell line results in the cells mimicing the properties of cancerous cells which include rapid proliferation and migration at the cellular level, aneuploidy, chromosome instability and aberrant chromatin at subcellular levels. Enhanced expression of metalloproteases upon silencing of PC4, as observed in breast cancer cell lines is possibly one such factor that aids increased cellular migration. A drastic reduction of PC4 was also observed in clinical samples of breast cancers. Loss of PC4 in breast cancer cells might contribute to the abnormal genome which ultimately boycotts the cell cycle checkpoints and possibly other surveillance systems of the cells that are dedicated for maintainence of cellular homeostasis.

Although PC4 is a chromatin associated protein and owing to the general property of cancers like increased nucleocytoplasmic ratio and multinucleated cells, it could be expected that PC4 is expressed more in cancers. However, the contrary is true, at least in various types of breast cancers. Does that mean that the absence of PC4 is partially responsible for tumorigenicity? Is the absence of PC4 a cause or an effect of tumor formation? How critical is the lack of PC4 for invasiveness of the cancer cells? Could the absence of PC4 aid in metastasis? Is the reduced expression unique for breast cancer or is it a general molecular signature across tumors? Does PC4 qualify as a tumor suppressor protein? Many such questions arise. Therefore a proper understanding of PC4 functions would not only elucidate its cellular functions but also have broader implications in our perspectives regarding pathobiology of cancers in general.

Abbreviations

µg:	microgram
µl:	microlitre
AFM:	Atomic Force Microscopy
bp:	base pair
cDNA:	Complementary Deoxyribonucleic acid
CPM:	Counts per minute
DNA:	Deoxyribonucleic acid
DTT:	Dithiothreitol
DEPC:	Diethyl pyrocarbonate
EDTA:	Ethylene diamine tetraacetic acid
kDa:	kilo Dalton
mins:	minute(s)
ml:	milliliter
mM:	milli Molar
pmol:	pico mole
nmol:	nano mole
PBS:	Phosphate buffered saline
ng:	nanogram
nM:	nano Molar
PAGE:	Polyacrylamide Gel Electrophoresis.
rpm:	Revolutions per minute
RNA:	Ribonucleic acid
SDS:	Sodium Dodecyl Sulphate
TE:	Tris EDTA
TEMED:	N,N,N'N'-Tetramethyl-ethylene diamine
UV:	Ultraviolet
CTD:	C-terminal domain
ER:	Estrogen receptor
PR:	Progestrone receptor
PVDF:	Polyvinylidene difluoride
BSA:	Bovine serum albumin
CBB:	Coomassie Brilliant Blue

List of Publications

1. **Kumari S**, Swaminathan A, Chatterjee S, Senapati P, Boopathi R and Kundu TK(2012). Chromatin Organization, Epigenetics and Differentiation: An evolutionary perspective. ***SubcellBiochem*. Epigenetics Development and Disease *(Springer)* 61**, **(In Press).**

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3. Mohankrishna DV, Mizar P, **Kumari S**, Siddhanta S, Ravindra KC, NaryanaCandKundu TK (2012). Multiple mechanisms of Lysine Acetyltransferase KAT3B/p300 inhibition by 1,4-Napthoquinones: Basis for nontoxic inhibitor synthesis. J. Biol. Chem. **(Under Revision).**

4. **Kumari S**, Mohankrishna DV, Prabhu SP, Bachu M, Bhat A, Kumar M, Sridhar TS, Gopinath KS and Kundu TK (2012). Role of human PC4 in maintenance of structural competence of chromatin and breast cancer manifestation, **(Manuscript under preparation).**

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