MIXED LINEAGE LEUKEMIA HISTONE METHYLASES IN GENE REGULATION AND CELL CYCLE

by

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ABSTRACT

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IN GENE REGULATION AND CELL CYCLE

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Histone methyl-transferases (HMTs) are key enzymes that post-translationally methylate nuclear histone proteins and play critical roles in gene expression, epigenetic regulation and diseases in eukaryotic organisms. Mixed lineage leukemias (MLLs) are human HMTs that specifically methylate histone H3 at lysine-4 and regulate gene activation. MLLs are also well known to undergo rearrangement often in acute myeloid and lymphoid leukemias. Human DNA encodes several histone H3 lysine-4 (H3K4) specific methyl-transferases (HMTs) such as MLL1 (mixed lineage leukemia gene 1), MLL2, MLL3, hSet1 etc, which play critical roles in gene expression. These HMTs are present as distinct multi-protein complexes with several proteins in common. Herein, a human CpG dinucleotide binding protein (CGBP) has been affinity purified and characterized along with its interacting proteins from human cells. It was demonstrated that CGBP is co-purified with three H3K4 specific HMTs MLL1, MLL2, and hSet1.

MLL1 is the primary source of H3K4 specific HMT activity present in affinity purified CGBP associated proteins. In addition, CGBP is co-localized with MLL1, MLL2 and hSet1 *in vivo* and binds to the promoter of MLL target gene HoxA7. These results demonstrated that CGBP interacts with MLL1, MLL2 as well as hSet1 HMTs and is likely a common interacting component of these three HMTs.

As chromatin condensation, relaxation, and differential gene expression are keys to proper cell cycle progression, the dynamic association of MLL and H3K4-trimethylation at different stages of cell cycle was analyzed. Interestingly, MLL1 that is normally associated with transcriptionally active chromatins (G1 phase) dissociated from condensed mitotic chromatin and returned back at the end of telophase when the nucleus starts to relax. In contrast, H3K4-trimethyl marks, which are also normally associated with the euchromatins (in G1), remained associated even with condensed chromatin throughout the cell cycle. The global levels of MLL1 and H3K4-trimethylation are not affected during cell cycle, while the H3Ser28 phosphorylation was only observed during mitosis. Interestingly, MLL target Hox genes (HoxA5, A7 and A10) were differentially expressed during the cell cycle and the recruitment of MLL1 and H3K4-trimethylation levels were modulated in the promoter of those Hox genes as a function of their expression. In addition, down regulation of MLL1 resulted in cell cycle arrest at G2/M phase. The fluctuation of H3K4-trimethylation marks at specific promoters but not at the global level indicates that H3K4-trimethylation marks that are present in the G1-phase may not be the same marks in other phases of cell cycles, rather old marks are removed and new marks are introduced. In conclusion, our

studies demonstrated that MLL1 and H3K4-methylation have distinct dynamics during the cell cycle and play critical roles in differential expression of Hox genes associated with cell cycle regulation.

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LIST OF ABBREVIATIONS

MLL	Mixed lineage leukemia
HMT	Histone methyl transferase
CGBP	CpG dinucleotide binding protein
RNAP II	RNA polymerase II
SAM	S- adenosyl methionine
SAH	S- adenosyl homocysteine
SET	Su(var) 3-9, enhancer of zeste and trithorax
Hox	Homeobox domain containing gene
WDR5	WD repeats containing protein 5
Rbbp5	Retinoblastoma binding protein 5
Ash2	Absent, small or homeotic protein 2
Dpy 30	DPY 30 domain containing protein
HCF1	Host cell factor 1
ASC-2	Activating signal cointegator 2
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
RT-PCR	Reverse transcriptase polymerase chain reaction
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle's medium
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
LB medium	Lysogeny broth medium
IPTG	Isopropyl β-D-1-thiogalactopyranoside
DMP	Dimethyl pimelimidate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceticacid
PBS	Phosphate buffered saline
FITC	Fluorescein isothiocyanate
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
MMLV	Moloney murine leukemia virus
NP 40	Nonyl phenoxypolyethoxylethanol
Tris	Tris (hydroxymethyl) aminomethane
PMSF	Phenylmethanesulfonylfluoride
Trx	Trithorax
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leuke

PREFACE

This study "Mixed lineage leukemia histone methylases in gene regulation and cell cycle" is submitted by Bibhu Prasad Mishra to The University of Texas at Arlington for the degree of Doctor of Philosophy (Ph.D.) has the following kind contributions.

Chapter 2 was published as "Ansari KI, Mishra BP (equally contributing first author) and Mandal SS. Human CpG binding protein interacts with MLL1, MLL2 and hSet1 and regulates Hox gene expression, *Biochim Biophys Acta 1779*, 66-73 (2008)". In this study Dr Mandal kindly provided clones for CGBP and SSU72 in human expression vector p-Flag-CMV4. Dr Khairul Ansari carried out the studies for antisense mediated knockdown of CGBP and role of CGBP in HoxA7 expression. Figures 2.7 and 2.8 were kindly contributed by him.

Chapter 3 was published as "Mishra BP, Ansari KI and Mandal SS. Dynamic association of MLL1, H3K4 trimethylation with chromatin and Hox gene expression during the cell cycle. *FEBS J.* 276, 1629–1640, (2009)". In this study Dr Khairul Ansari designed the MLL1 antisense and carried out antisense mediated knockdown of MLL1 and FACS analysis of cells under MLL1 knockdown conditions and figures 3.10 (C) and 3.11 were kindly contributed by him.

CHAPTER 1

SIGNIFICANCE OF MLL HISTONE METHYLASES IN GENE EXPRESSION, AND CELL CYCLE

1.1 Introduction

In eukaryotes, DNA, the blueprint of life, is packaged inside the nucleus in the form of complex chromatin (1,2). The molecular mechanism by which the genetic information is packaged inside the chromatin, transcribed and regulated is poorly understood. Nucleosome, the repeating unit of chromatin, contains four histone proteins (two copies each), H2A, H2B, H3, and H4. These histones undergo various kinds of posttranslational modifications such as acetylation, methylation, phosphorylation, etc and determine the "active" or "silent" states of genes (3-5). Histone acetylation is generally associated with gene activation. Histone methylations, however based on the nature and position of the modification, are associated with both gene activation and silencing (4,6-12). For example, methylations of histone H3 at lysine-4 (K4) and K36 are found in active genes; while methylations at H3K9, K27, and H4K20 are associated with silent genes (13-15)(see Table 1.1 for different types of histone lysine methylations and their functions). The presence of various histone deacetylase and demethylases in cells makes these modification marks reversible (16,17). Although various histone-modifying enzymes have been discovered, their detail functions in gene regulation and disease are largely unknown and are just beginning to be revealed.

1.2. MLL, histone methylation and gene expression

1.2.1 H3K4 methylation in yeast and its roles on transcription

H3K4 methylation is an evolutionarily conserved mark closely associated with transcriptionally active chromatin (2). The function of H3K4 methylation is well studied in yeast (18). Set1 is the sole H3K4 specific HMT present in yeast (8,19,20) and a SET1 null mutation results in a slow-growth phenotype (19,21). In yeast, Set1 interacts physically with RNA polymerase II (RNAP II) during transcription and regulates gene expression (22,23). Set1 mediated trimethylation of H3K4 is associated with early phase of transcription and have been implicated with transcriptional memory (23). Set1 mediated histone methylation is functionally coupled with histone H2B monoubiquitination, Paf1 complex and other transcription factors involved in transcriptional regulation (22-26). In yeast Set1 is a component of a multi-protein complex (known as COMPASS) containing subunits Set1, Bre2, Spp1, Swd1, Swd2, Swd3, and Sdc1 and its HMT activity is fully active only in the context of a multi-subunit complex (Table 1.2). Recently, it has also been demonstrated that Set1 is required for methylation of conserved lysines in a kinetochore protein, Dam1, suggesting its important roles in mitosis (8).

1.2.2 MLLs are human histone H3 lysine 4 (H3K4) specific methyl-transferases

The Set1 HMT has diverged and became more complex in higher eukaryotes (18,27-34). In mammals, six different Set1 homologs have been characterized: MLL1, MLL2, MLL3, MLL4, Set1A and Set1B, collectively known as MLL family of HMTs

(Table 1)(15,35)**.** Like yeast Set1, each of these MLL family of HMTs possess H3K4-specific HMT activities and associated with gene activation (36-40) (Figure 1.1 A).

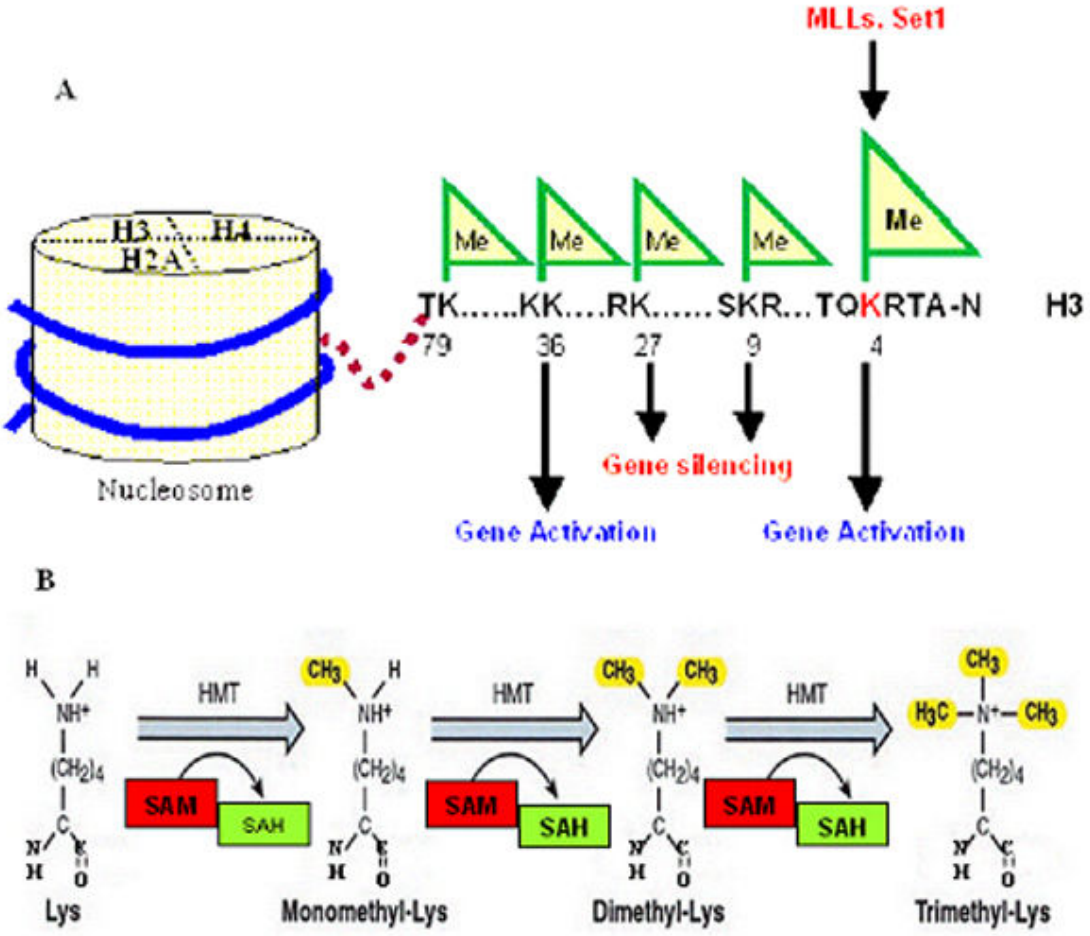

Figure 1.1 Methylation of lysine residues on histone H3 (**A**) Methylation of Histone H3 at lysine-4 (H3K4) by MLLs and Set1 is linked with gene activation. (**B**) Structure of Lysine, and mono-, di- and tri- methylation of lysine. HMTs add methyl group on ε-amino group of lysine. S-adenosyl methionine (SAM) is the methyl group donor, which is coverted to S-adenosyl homocysteine (SAH)(41).

In spite of their similar enzymatic activity (H3K4-methylation), sequence analysis demonstrates that these proteins do not have more than 30 % sequence homology. However, each of them contains a conserved SET domain that is responsible

for their HMT activity (27,33,42-44). The SET domain is an evolutionarily conserved motif of 130 aminoacids present in MLLs and responsible for its HMT activity. MLLs transfer the methyl group from S-adenosyl methionine to the ε-nitrogen atom on lysine (Figure 1.1 B).

Table 1.1 Histone lysine methylations and their functions(15)

H3K4	H3K9	H3K27	H3K36	H3K79	H4K20
MLL1, MLL2, MLL3, MLL4, Set1A, Set1B, Set9	G9A, Suv39H1, Suv39H2, EU-HMTase1, ESET, SETDB1,	EZH2	SETD2, HYPB, NSD1	Dot1L	Pr-SET7, SET8, SUV4-20
Transcriptional activation	Heterochromatin Formation, Transcriptional silencing	Gene silencing	Transcription Elongation, Chromatin maintenance	Telomere Silencing, meiotic check point damage response	Heterochromatin formation

In addition, they contain various protein-protein and protein-DNA interaction domains (Figure 1.2)(15). For example, MLL1 and MLL3 contain multiple DNA binding AT hook domains that may be responsible for targeting these HMTs into specific gene promoters. MLL1 and MLL4 contains CXXC Zn -finger domains that may also be involved in DNA binding and gene targeting. Each of the MLLs (MLL1-4) contains multiple PHD domains (plant homeodomain) that are known to be involved in protein-protein interaction and often found to be present in chromatin associated proteins (15). The PHD domains present in MLLs have been implicated in interactions with histone deacetylases (HDACs), polycomb group proteins (PcGs), and with

methylated lysines in histone tails (44,45). The FYDN and FYDC are two interesting domains that are present in MLL^N and MLL^C regions of MLL1 (46-48). These domains are involved in forming hetero-dimmers between MLL^N and MLL^C terminal fragments produced upon proteolysis of MLL1 by protease Taspase 1(46-48)**.** LXXLL domain (also known as NR box) present in MLLs make these proteins interesting for nuclear receptor mediated gene activation and hormone signaling (32,49-51). In fact, MLL2, MLL3, and MLL4 that contain multiple LXXLL domains are shown to interact with nuclear receptors and play important roles in hormone dependent gene activation**.** Although, further studies are needed to elucidate the detail function of different domains present in MLLs, their importance have addressed using deletion mutation experiments in mice models which showed that progressive deletion of MLL functional domains leads to progressively more severe phenotypes (52-54).

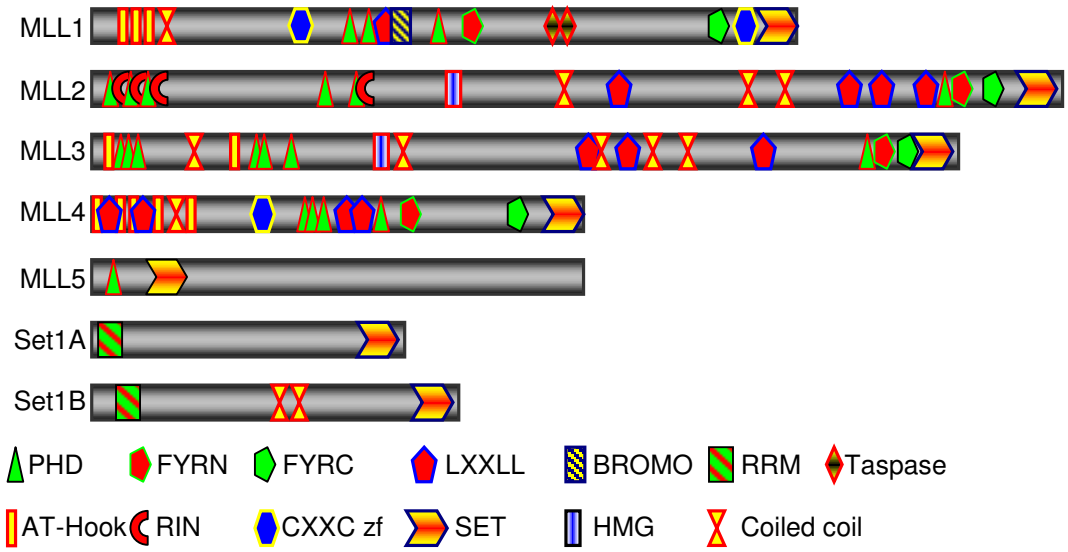

Figure 1.2 Domain structures of MLLs. PHD (plant homodomain) are usually involved in protein-protein interaction; FYRN and FYRC domains are involved in hetero-dimmerization between MLL^N and MLL^C terminal fragments. LXXLL domain (also known as NR box) is involved in interaction with nuclear receptor (NR). BROMO domains are involved in the recognition of acetylated lysine residues in histone tails. RRM is the RNA recognition motif. Taspase1 site is the proteolytic site for the protease Taspase1. AT-hook is a DNA binding domain. RING fingers are involved in protein-protein interactions. CXXC-zf is a Zn-finger domain that is involved in protein-protein interaction. SET is responsible for histone lysine methylation. HMG domains are involved in binding DNA with low sequence specificity. Coiled coil domains mediate homo-oligomerization (15).

Furthermore, the multiplicity of MLL family of HMTs in vertebrate genomes indicates that MLLs may have specialized functions likely in regulating differential expression of specific target genes or in methylation of distinct non-histone proteins for other functions. The distinguishable phenotypes of deletions or truncations in MLL1, MLL2 and MLL3 genes in mice suggests that MLL proteins are not redundant in their function but instead are specialized to deal with the regulatory complexity of vertebrate development (28,50,55). Like Drosophila Trx, mammalian MLL1 and MLL2 have a

role in long-term maintenance of Hox gene expression patterns that play important role in specifying cell fate during development and hematopoiesis (28,50,55).

In addition to the critical roles in histone methylation and gene regulations, rearrangements of the MLL1 are well known in a variety of aggressive human leukemias, including acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML)(1,2,21,53). These rearrangements take several forms. The most common are balanced translocations in which the genomic sequences encoding the N-terminal portion of MLL are fused to sequences encoding the C-terminus of another translocation partner. Notably, chromosomal breakpoints are found in a consistent region of MLL and more than 40 different MLL fusions have been described (23,56-58). When these genes are transcribed, spliced, and translated, an in-frame fusion protein with oncogenic activity is produced. The most frequent translocations in MLL are the t (4; 11) and t (11; 19) translocations, which are associated with the expression of MLL-AF4 and MLL-ENL, respectively, and a pro-B cell or mixed lineage phenotype (42,43). At least 50% of cases of infant ALL harbor the t (4; 11) translocation (42,43). The frequent occurrence of the t (4; 11) translocation in infant leukemia suggests that MLL-AF4 is unusually potent and less dependent on secondary genetic ‘hits’ for leukemia development. MLL2 and MLL3 are also frequently amplified in solid tumors, pancreatic carcinoma and glioblastomas (59,60).

1.2.3 MLLs are present as multi- protein complexes

Considering the importance in gene expression and disease, various researchers have isolated different MLL family of HMTs from human cells and characterized their protein composition and function (39,61-63). These studies demonstrated that MLL1, MLL2, MLL3, MLL4, Set1A, and Set1B are present in distinct multi-protein complexes inside the cells and each of these complexes share several common subunits which includes Ash2, Rbbp5 (retinoblastoma binding protein 5) and Wdr5 (WD40 repeat containing protein) along with several unique components (Table 1.2). By sequence analysis it is established that Ash2, Rbbp5, Wdr5 and CGBP (human CpG binding protein, also known as CFP1) are human homolog of yeast Set1 complex components Bre2, Swd1, Swd3, Sdc1 and Spp1 respectively (62). Recently, using an immuno affinity purification method, we have demonstrated that CGBP interacts with at least three different HMTs MLL1, MLL2, and Set1 and is a core component of these functionally active HMTs (64). Similarly, Cho *et al*. (2007) showed Dpy30 (a dosage compensation protein) as a common interacting subunit of MLL1, MLL2, MLL3, MLL4, and Set1 complexes (65). Although, these protein complexes have been isolated, the detail functions of MLLs and their interacting components in histone methylation, gene expressions, and diseases still remain elusive.

Table 1.2 Proteins associated with Set1 like HMT complexes in yeast and human (15)

	Yeast	**Human**					
Complexes	Set1	Set1	MLL1	MLL2	MLL3	MLL4	MLL5
Components	Set1	Set1A&B					
	Bre2	Ash2	Ash2	Ash2	Ash2	Ash2	
	Spp1	CGBP	CGBP	CGBP			
	SWd1	Rbbp5	Rbbp5	Rbbp5	Rbbp5	Rbbp5	
	Swd2	Swd2					
	Swd3	WDR5	WDR5	WDR5	WDR5	WDR5	
	Sdc1	Dpy30	Dpy30	Dpy30	Dpy30	Dpy30	
			Menin	Menin			
		HCF1	HCF1				
				ERα	ERα	ERα	
				ASC-2	ASC-2	ASC-2	
			MOF				

1.2.4 MLL interacting proteins play critical roles in histone methylation and MLL target gene expression

The findings that there are at least five core components (Ash2, Rbbp5, Wdr5, Dpy30 and CGBP) shared by several human H3K4 specific HMT complexes and these core components are evolutionarily conserved from yeast to humans suggest that they play important roles in regulating MLL-mediated histone methylation and target gene expression. Indeed, recently Wysocka *et al.* demonstrated that MLL interacting protein Wdr5 is required for histone H3K4 trimethylation (66). Wdr5 binds preferentially to dimethylated H3K4 and knock down of Wdr5 resulted in decreased expression of MLL1 target genes HoxA9 and HoxC8 without affecting binding of MLL1 complexes to these targets (66). Moreover independent knock down of Rbbp5 and Ash2 also

reduced expression of HoxA9 and HoxC8 (66). Dou *et al.* showed that MLL1 recruitment to either the HoxA9 or HoxC8 locus was not disturbed upon knock down of Wdr5, Rbbp5, or Ash2 (27,39). These observations indicated that core components Wdr5, Rbbp5 and Ash2 are not involved in MLL1 recruitment but regulate MLL1 associated histone methylation activity. Shilatifard and colleagues showed that Ash2 is also pivotal for trimethylation of MLL1 target gene promoters (38). The combined siRNA knock down results demonstrated that all three core components, Wdr5, Rbbp5 and Ash2, are needed for Hox gene expression and that this requirement lies in the regulation of H3K4 dimethylation and trimethylation (38). In contrast to Wdr5, Rbbp5, and Ash2, results from our laboratory demonstrated that antisense mediated knockdown of core component CGBP not only decreased the level of promoter H3K4 trimethylation and expression of MLL target HoxA7 gene but also abolished the recruitment of MLL1 into its promoter (64). Notably, CGBP has "AT-hook" and "CXXC" DNA binding motif and it can be hypothesized that these motifs play critical role in recruiting the MLL complexes into their specific target gene promoters (67,68). Although recent studies have provided important insights into how histone methylation is regulated by bringing together specific HMTs and histone tails via interacting components such as Wdr5, Ash2, CGBP etc, their detail implication in the gene expression, cell differentiation, and development are poorly understood.

1.2.5 MLLs are key regulators of Hox genes

Hox genes are a group of evolutionarily conserved homeobox containing genes that play crucial roles in embryonic development. Orchestrated expression of Hox genes

determines the positional tissue differentiation in higher organisms (69) (Figure 1.3). Hox genes also express throughout postnatal life and play important roles in hematopoietic cell differentiation (70-72). The Hox genes encode a complex network of transcription regulatory proteins whose precise targets remain poorly understood. The overall function of the network appears to be dictated by gene dosage. Human encodes 39 Hox genes located in four different clusters (HoxA: HoxA1-7, 9-13; HoxB: HoxB1-9; HoxC: HoxC4-13 and HoxD: HoxD1, 3, 4, 8-13) on chromosomes at 7p15, 17p21, 12q13 and 2q31, respectively. Several developmental disorders have been traced to mutations in different Hox genes and most of the Hox genes (except that in cluster D) are expressed in hematopoietic stem cells.

Genetic studies in both mice and flies established that MLLs regulate Hox and other homeotic genes expression (13,55). MLLs control the maintenance but not the initiation of expression of Hox genes during embryogenesis (73). Inactivation of the MLL gene in mice by homologous recombination (knock out) showed that MLL is not only a regulator of hematopoiesis but also is a master regulator in embryonic development and hence disruption of MLL expression results in embryonic lethality (42,54,55). Studies showed that knock down of MLL results in Hox gene expression abnormalities especially the expression of HoxA7, HoxA9, HoxA10, HoxC4, HoxC8, and HoxC9 in whole embryos, fetal liver or in cultured human cells (14,42,44,55,64,74). Analysis of the MLL super complex demonstrated direct binding of MLL1 to HoxA7, HoxA9 and HoxC8 promoters (14,64,74). Mice heterozygous for MLL-exon3LacZ allele display abnormal homeotic transformations with altered Hox

gene expression. Disruption of MLL-exon3LacZ cause haplo-insufficiency with bidirectional homeotic transformations and shift anterior boundaries of several Hox genes (55). Using $MLL^{-/-}$ deficient embryos and mouse embryonic fibroblasts it is demonstrated that MLL is required for the maintenance of selected Hox gene expression (55,73,75). Several studies targeting the mouse homologs of genes encoding fusion partners of MLL gave evidence that MLLs are regulators of cell differentiation. Reverse transcriptase PCR (RT-PCR) analysis of MPMP cell lines immortalized by five different MLL fusion proteins revealed a characteristic HoxA gene expression profile and found that all cell lines expressed HoxA7, HoxA9, HoxA10 and HoxA11 at the 5' end of the HoxA cluster. In contrast, 3' end HoxA genes were variably expressed with periodicity, as evidenced by low levels of HoxA1, higher levels of HoxA3 and HoxA5 and complete absence of HoxA2, HoxA4 and HoxA6 expression (76). Contrary to these findings the Taspase1 RNAi (Taspase1 is essential for cleavage of pre-MLL1 peptide to generate, functional of MLL1 protein) in HeLa cells led to diminished expression of selected Hox genes across the HoxA cluster (46-48,77). Knockdown of Taspase1 diminished the expression of the 3′ located and “earlier” expressed genes in the HoxA cluster (A1, A3, and A4), but not those genes located towards more 5′ and expressed “later” during embryonic development (A5, A9 and A10) (46-48,77). This selected attenuation contrasts with the global decrease in expression of most HoxA genes (A1 to A10) in cells with MLL knocked down. These data support the importance of Taspase1 in the correct expression of the early HoxA gene cluster (46-48,77).

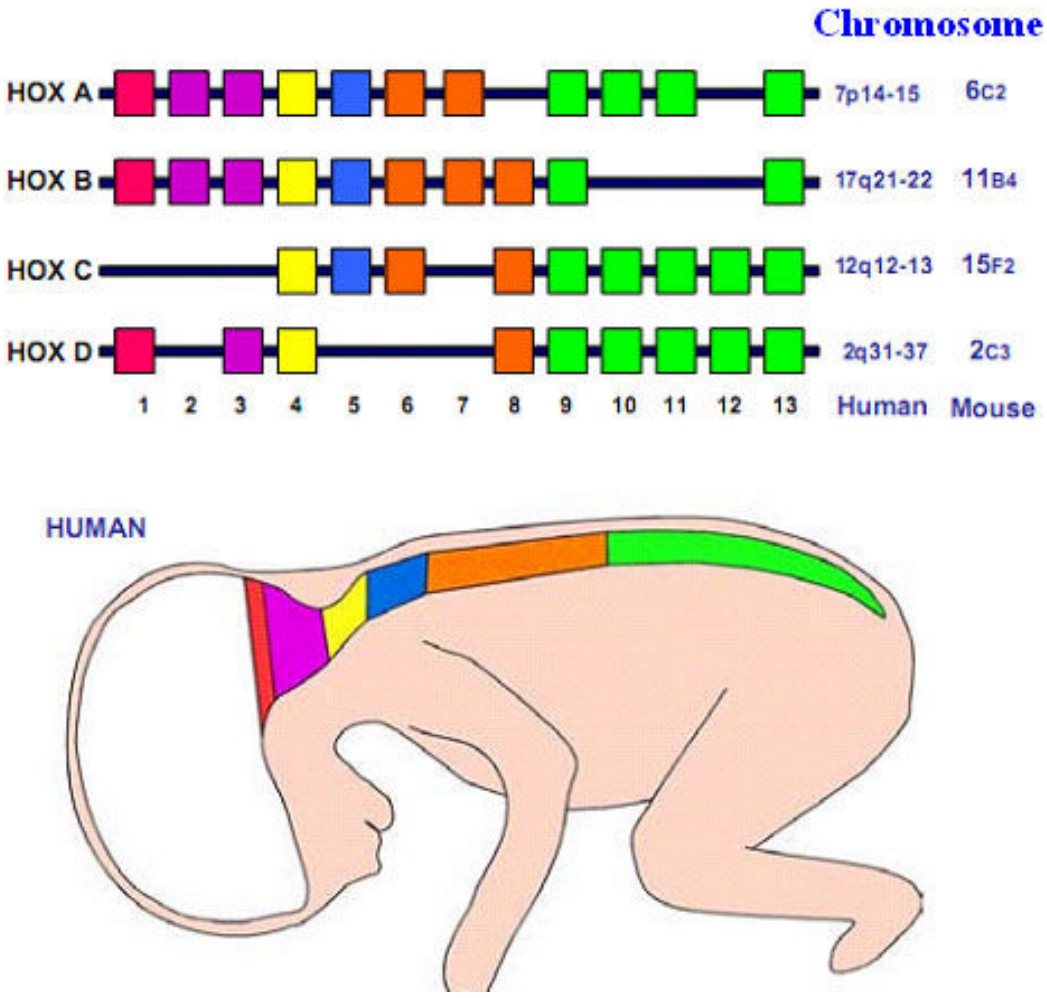

Figure 1.3 Hox genes in embryonic development. During embryonic development the Hox genes are expressed in a pattern that correlates with chromosomal positioning, depicted here for human and mouse (78).

In agreement with genetic studies and mutational analysis, direct interaction of MLL complex with the HoxA7, HoxA9 and HoxC8 genes promoters indicate important roles of MLL in Hox gene regulation (14,62,64,79). The distinctive role of MLLs in regulating different Hox genes is likely to be mediated through differential histone methylation and effects on chromatin structure (14,62,64,79).

1.3. MLL and cell cycle regulation

The cell cycle is a vital process by which cells divide into daughter cells. The cell cycle consists of four distinct phases: G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis) phases. M phase is composed of two tightly coupled processes: mitosis, in which the chromosomes are divided between the two daughter cells and cytokinesis, in which the cytoplasm divides forming distinct cells. Proper maintenance and progression of these stages are critical for normal cellular process (80). The Cyclin family of proteins which function as the regulatory subunit of cyclin dependent kinases (CDK) regulate the progression of cell cycle by varying their levels of gene expression. For example, cyclin D shows a continuous expression in all the different phases and thus controls the entry and progression throughout the cell cycle. Cyclin E is required for the transition from G1 to S phase while cyclin A helps the cell to move from S to G2 phase. Cyclin B is responsible for the cell to reach mitosis (80).

1.3.1 MLLs interact with cell cycle regulatory proteins

Much evidence indicates that MLLs play crucial role in cell cycle progression (39,46,48,81). The functional roles of MLLs in cell cycle regulation are supported by the observation that the knock out of Taspase-1 results in down-regulation of cyclin E, A and B and up-regulation of p16 (a S phase inhibitor) (48). Notably, Taspase-1, a threonine endopeptidase, cleaves MLL at conserved sites to generate N-terminal 320-kDa (MLL-N) and C-terminal 180 kDa (MLLC) fragments. Proteolysis of MLL family proteins by Taspase-1 is required to fully activate their HMT activity. Using chromatin immuno-precipitation (ChIP) experiments Takeda *et al.* (2006) demonstrated that MLL

protein is occupied in the promoters of cyclin E1 and E2 genes and there was a marked reduction in H3K4 trimethylation level as well as the MLL occupancy at the cyclin E1 and cyclin E2 promoters in Taspase-1 negative cells (48). Taspase-1 deficiency also resulted in upregulation of S-phase inhibitor protein p16 (48).

Similar to cyclins, E2F family proteins (E2F1-E2F6) are the key transcription factors that are responsible for the activation of genes essential for cell cycle progression including cyclins (39,46,48,81). Using immuno-precipitation experiments, Takeda *et al.* (2006) demonstrated that MLL1 directly interacts with several E2Fs (such as E2F2, E2F4 and E2F6), while MLL2 interacts with a different subset of E2Fs such as E2F2, E2F3, E2F5 and E2F6 (48). In an independent experiment, Dou *et al.* performed the immuno-purification of Wdr5 associated HMTs from human cells and demonstrated that Wdr5 interacts with MLLs along with E2F6 (39). Distinct interactions between E2Fs and MLLs suggest potential roles of MLL proteins in the cell cycle regulation (39). In addition to MLL1 and MLL2, over expression of MLL5 induced cell cycle arrest in G1 phase and knock down of MLL5 resulted in cell cycle arrest both at G2/M and G1 phase (82,83). These studies indicate more direct roles of MLL family of HMTs in cell cycle regulation.

In addition to MLLs, MLL interacting proteins are also shown linked with cell cycle regulation. Milne *et al* (2004) showed that Menin in a cooperative interaction with MLL proteins directly regulates the expression of CDK inhibitors like p27 and p18 (39). Menin activates transcription of p27 and p18 by recruiting MLL1 to their promoters and knock down of menin resulted in down regulation in p27 and p18 expression leading to

deregulated cell growth and tumorigenesis (74). HCF-1 (host cell factor 1) is another critical protein that interacts with MLLs and Set1 (63). Knock down of HCF-1 results in dinucleated cells and inhibits cell divisions by arresting the cells in G1 phase (81). HCF1 c-terminal domain has been linked with this dinucleated cells implicating its roles in cytokinesis (Figure 1.4). Furthermore, using ChIP experiments Tyagi *et al.* demonstrated that HCF-1 recruits MLL1 and Set1 to E2F responsive promoters during the G1 to S phase transition and induces histone methylation and transcriptional activation (81). Associations of HCF-1 and E2F are versatile and binding to E2F responsive promoters are cell cycle selective (Figure 1.4) (81). HCF-1 binds both to activator E2Fs like E2F1 and E2F3a and repressor E2Fs like E2F4. Thus, HCF-1 plays dual role both in induction and suppression of vital cell cycle regulatory proteins. HCF-1 selectively associates with "activator" H3K4 HMT complexes, when bound to E2F1, to activate transcription. It acts as a repressor in association with Sin3 HDAC complex when bound to E2F4 (81).

Recently, in our laboratory a defined dynamics of MLL histone methylases was demonstrated during cell cycle. MLL1, which is normally associated with transcriptionally active chromatins (in G1 phase), dissociated from condensed mitotic chromatin, migrated away to the cytoplasm and returned back at the end of telophase when the nucleus starts to relax (84). However, the global level of MLL1 does not get affected. In contrast, Liu *et al.* (2008) demonstrated a biphasic expression of MLL1 conferred by defined windows of degradation mediated by specialized cell cycle E3 ligases (47). They found that SCFSkp2 and APCCdc20 mark MLL for degradation at S

phase and late M phase, respectively. They also found that abolished peak expression of MLL1 incurs corresponding defects in G1/S transition and M-phase progression while over expression of MLL blocks S-phase progression (47). Their data indicated that a post-translational regulation of MLL by the cell cycle ubiquitin/proteasome system (UPS) assures the temporal necessity of MLL in coordinating cell cycle.

1.3.2 MLL target Hox genes in cell cycle regulation

The role of MLLs in cell cycle is further emphasized by the involvement of MLL regulated Hox genes in cell cycle. Recent studies demonstrated that HoxA5 activates p53, which is known to regulate the expression of p21, an inhibitor of CDK enzymes that is critical for cell cycle progression (85,86). Furthermore, Bromleigh and Freedman (2000) showed that Hox A10 directly upregulates the expression of p21 leading to cell cycle arrest at the G1 phase in both monocytic and fibroblast cell lines (86). It is interesting to note that both p21 and p53 plays vital role in cell cycle regulation. p21 is a CDK inhibitor which inhibits the protein kinase activity of cylin-CDK2 and cyclin-CDK4 complexes. Thus, p21 functions as a regulator of cell cycle progression at G1 phase. p53 is responsible for holding the cell at G1/S transition by regulating the expression of p21 and activating the DNA repair proteins when DNA damage is recognized in the cell. It is also responsible for initiating apoptosis if the DNA damage is found to be irreparable. Thus, we hypothesize that HoxA5, like HoxA10, regulates cell cycle through p53 and p21 channel (Figure 1.4). Also recently, Qian *et al.* showed that HoxA10 is responsible for differentiation of human endometrial stromal cells under progesterone treatment by regulating the expression of another G1

inhibitor p57 (87). They observed a lower expression of HoxA10 when the level of p57 is high under progesterone application. Lu *et al.* further reported that a higher percentage of cells arrested at the G2/M phase of cell cycle under the application of siRNA against HoxA10. While the level of apoptosis was insignificant, the number of cells arrested in G1 stage was considerably lower than the number of cells arrested in G2/M phase (88).

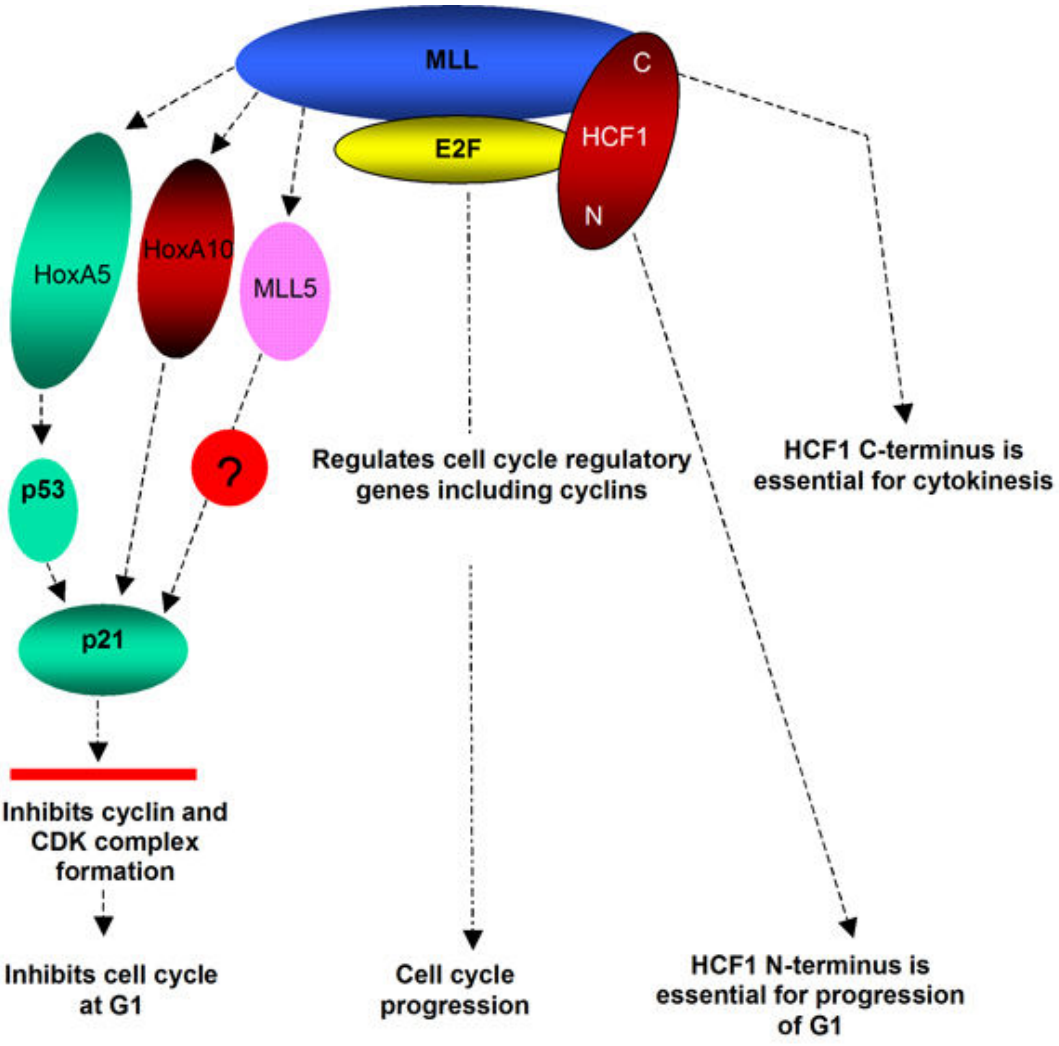

Figure 1.4 Roles of MLLs in cell cycle regulation. MLL target genes HoxA5 and HoxA10 inhibit cell cycle at G1 phase through upregulation of CDK inhibitor p21. MLL5 inhibits cyclin-CDK complex possibly involving p21. MLLs interact with E2Fs and regulate differential expression of cyclins that regulate proper cell cycle progression. HCF1, a MLL interacting protein, is involved in progression of G1. The C-terminal of HCF1 is essential for proper cytokinesis (15).

In our laboratory we synchronized cells in G1/S phase by the application of double thymidine treatment procedure and analyzed the expression pattern of HoxA10 at different stages of cell cycle and found that the level of expression for HoxA10 is very high in the S phase which goes significantly down in the G2/M phase and

completely absent in G1 phase (84). Most importantly down regulation of selected MLLs resulted in cell cycle arrest and inhibits cellular growth. Thus multiple lines of evidence indicate that MLL family of HMTs play critical role in regulation of critical cell cycle regulatory genes and hence implicated in cell cycle regulation.

1.4 Discussion

Understanding the functions of histone H3K4 methylation is crucial to uncover the complex gene regulatory network and underlying molecular mechanism of various human diseases. H3K4 methylation marks are well recognized as marks for transcriptionally active chromatins. The role of H3K4 methylation by Set1 in yeast is well studied, but the same is not very clear in higher eukaryotes due to the increased complexity of this enzyme (89). Set1 is the sole H3K4 specific HMT present in yeast. In contrast, human encodes several yeast Set1 homologs (such as MLL1, MLL2, MLL3, MLL4, Set1A and Set1B) suggesting increased complexity of these enzymes and their functions in regulating gene expression in higher eukaryotes (37,66). Although recent discoveries of MLL1 HMT activities and their interacting protein shed significant light in the complex function of MLL in gene regulation in general, the distinct function of different but very similar enzymes is still not clear. Increasing amount of evidence indicate that different MLL form distinct interactions with proteins involved in nuclear receptor mediated gene activation, cell cycle regulation or with chromatin remodeling. These suggest that different MLL have distinct functions inside the cell and still need lots of investigation to elucidate the complete MLL gene regulatory networks.

Although, at this point of time, significant efforts are being invested in understanding the functions of MLL proteins in target gene regulation and disease, little is known about their own regulation in normal/cancer cells or under stress. In fact, recent studies from our laboratory suggest that MLLs are mis-regulated under toxic and carcinogenic stress establishing a novel link between MLL, stress response and carcinogenesis (unpublished observation). With the present pace of investigations by different laboratories, we hope that within next decade or so we will have lot more understanding about the complex function of MLLs in gene regulation, stress response and disease that will help in developing novel therapy.

CHAPTER 2

HUMAN CpG BINDING PROTEIN INTERACTS WITH MLL1, MLL2 AND hSET1 AND REGULATES HOX GENE EXPRESSION

2.1 Introduction

Histone methylation plays critical role in gene expression, epigenetic regulation and disease (4,5,8,89-96). Methylation at histone H3-Lysine 4 (H3K4) is an evolutionary conserved mark and is closely associated with transcriptionally active chromatins and with various types of cancer (97,98). Set1 is the only H3K4 specific histone methyl-transferase (HMT) present in yeast and is directly involved in gene expression (99-102). In yeast, Set1 is present as a multi-protein complex (known as COMPASS), containing subunits Set1, Bre2, Spp1, Swd1, Swd2, Swd3, and Sdc1 (99,103,104).The Set1 family of HMTs has evolved into more complex systems in higher eukaryotes (18,28-34,39,55). In mammals, six Set1 family members have been characterized: Set1A, Set1B, and four MLL (mixed lineage leukemia)-family of HMTs. All these HMTs possess H3K4 specific HMT activity and play crucial roles in gene activation (36,38-40,66).

Similar to yeast Set1, MLL1, MLL2, MLL3 and human Set1 (hSet1) are also present in distinct multi-protein complexes sharing three common subunits Ash2, Rbbp5 (retinoblastoma binding protein 5), Wdr5 (WD40 repeat containing protein) along with several specific components (Table 1.2) (36,38,39,66). Sequence analysis

indicates that Ash2, Rbbp5, Wdr5, Dpy30, and CGBP (human CpG binding protein, also known as CFP1) are human homolog of yeast Bre2, Swd1, Swd3, Sdc1 and Spp1 respectively (Table 1.2). Importantly, Ash2, Wdr5 and Rbbp5 are reported to be the core components of MLL1, MLL2, MLL3 and hSet1 complexes, whereas, CGBP is shown to interact only with hSet1 (27). Recently, Dpy30, and UTX along with several novel proteins are also found to be associated with Set1 like complexes (Table 1.2) (31,65).

CGBP is a well-characterized protein, which contains a DNA binding "CXXC" Zn-finger domain (68). CGBP binds unmethylated CpG dinucleotides and plays critical roles in gene expression and mammalian development (68). CGBP co-localizes with acetylated histones in transcriptionally active chromatins (68). In addition, CGBP co-localizes with trithorax family of proteins like HRX (MLL1) in nucleus, suggesting it can be a partner of MLL1 histone methylase complex (67). Based on these observations and also on the sequence homology of CGBP with Spp1 (a component of yeast Set1 complex), it can be hypothesized that CGBP is likely an interacting partner of all the highly conserved human H3K4 specific HMTs such as MLL1, MLL2, MLL3 and hSet1 complexes.

To test this hypothesis, CGBP and associated proteins have been purified from human cell, their protein composition analyzed and histone methylation properties determined. CGBP was down regulated using CGBP specific antisense and its impact on functions of MLL mediated gene expression was analyzed. Also, it was demonstrated that CGBP interacts with MLL1, MLL2 and hSet1 HMT complexes and

plays critical role in transcriptional regulation of MLL target gene HoxA7.

2.2. Materials and methods

2.2.1 Establishing stable cell line and Flag affinity purification of CGBP and SSU72 (human)

Human CGBP (from a HeLa cDNA library) was cloned into a Flag tagged human expression vector p-Flag-CMV4 to develop the CGBP expression vector (carried out by Dr. Mandal previously and kindly provided by him) and transfected into HEK293 human cell line using Fugene 6 transformation reagent (Roche). The stably transfected cells expressing Flag-CGBP were selected using G418 (Sigma) antibiotic selection procedure (105). Dulbecco's modified Eagle's medium (DMEM) supplemented with heat inactivated fetal bovine serum (FBS, 10%), L-glutamine (1%) and penicillin/streptomycin (0.1%) was used as the growth medium as described previously (64,106,107). For antibiotic mediated selection of CGBP stable cell line, the transfected HEK293 cells were grown in growth medium containing 400 μg/ml G418. The cells surviving G418 antibiotic application because of the acquired G418 resistance gene present on the CGBP expression vector were allowed to grow and make colony for one week in G418 treated growth media. Then twelve colonies were randomly selected and allowed to grow in normal growth medium. 2×10^6 cells from each colony were used to prepare nuclear extract and analyzed by western blot technique with anti-Flag antibody (Sigma). Tryphan blue solution [0.8 mM in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4)] was used to stain the cells before counting. Cells were counted under light microscope (Vista Vision) after mounting them on a Hemocytometer (Hausser

Scientific). The colony showing highest CGBP expression was selected and used for future experiments as Flag-CGBP stable cell line. All the cells were cultured at 37 °C in a humified atmosphere of 5% CO_2 in an incubator (Nuaire IR autoflow). 2×10^6 cells were harvested from Flag-CGBP stable cell line and incubated in buffer A (20 mM Tris-HCl, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, 0.2 mM PMSF) on ice (10 min), and centrifuged at 3500×g for 5 min. The supernatant fluid was saved as cytoplasmic extracts and the pellet was used for preparation of nuclear extract. Nuclear extract was prepared from the pellet in buffer C [20 mM Tris-HCl, (pH 7.9), 5 mM $MgCl_2$, 0.42 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT] by resuspending the pellet in it and incubating for 20 min on ice before centrifuging at 12000×g for 10 min (105). The supernatant fluid was used as nuclear extract. The nuclear extract was subjected to anti-Flag immuno-precipitation by incubating with 10% (v/v) Flag-M2 agarose beads (Sigma) for overnight at 4 °C. Beads were washed thoroughly with buffer C containing 0.05% surfactant NP 40 (neutral surfactant to minimize non-specific interactions). The proteins bound to the anti-flag beads were eluted using buffer C containing 150 µg/ml of Flag peptide (Sigma). Herein, these Flag elution are named as Flag-CGBP-IP. Control Flag-IP was performed from nuclear extract of HEK293 cells which were not transfected with any plasmid. Human SSU72 was sub-cloned from EST clone (PNAS-120, Invitrogen) into p-Flag-CMV4 human expression construct, transfected into HEK293 cells (carried out by Dr Mandal previously and kindly provided by him). Stable cell line expressing human SSU72 was

isolated and subjected to anti-Flag immuno-precipitation (Flag-SSU72-IP) using similar protocol as described above for Flag-CGBP-IP.

2.2.2 Antibodies, western blot and silver staining

Protein concentration in nuclear extract and different IPs was measured spectrophotometrically using Bradford reagent (100 mg Coomassie Brilliant Blue (G-250) dissolved in 50 ml 95% ethanol and 100 ml 85% (w/v) phosphoric acid, and finally diluted to 1 liter when the dye has completely dissolved) (Sigma). Equivalent amounts of Flag-CGBP-IP, Flag-SSU72-IP, control-Flag-IP (control-IP) and CGBP cell nuclear extracts were electrophoresed by SDS-PAGE and subjected to western blot using anti-MLL1 (Bethyl lab), anti-MLL2 (Bethyl laboratory), anti-RNA polymerase II (Gal4-CTD antibody, kind gift from Dr. Danny Reinberg, UMDNJ, USA), anti-Ash2 (Bethyl Lab), anti-Set1 (Bethyl Lab), anti-Menin (Bethyl lab), anti-Rbbp5 (Bethyl lab), and anti-Wdr5 (Bethyl Lab) antibodies (68). Proteins from SDS-PAGE gel were transferred to nitrocellulose membrane at 0.8-mAmp/cm^2 constant current for 3 hours in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH), blocked with blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) to avoid non-specific binding of antibodies. Then the membranes were incubated in specific primary antibody solution (1:1000) in blocking buffer. After that, the membranes were washed 3 times, 10 min each time with intermediate shaking in wash buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20). Similarly, the membranes were again incubated with alkaline phosphatase-conjugated secondary antibodies and washed with wash buffer before developing with the alkaline phosphatase method. Western blots were developed

in 10 ml of alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris-Cl pH 9.5, and 50 mM $MgCl_2$) containing 35 µL of 0.452 mM BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine) and 70 µL of 0.432 mM NBT (nitro-blue tetrazolium chloride). Flag-CGBP and Flag-SSU72 were probed with anti-Flag antibody (Sigma).

Equivalent amounts of control-IP and Flag-CGBP-IP were electrophoresed by SDS-PAGE and subjected to silver staining. Here, the SDS-PAGE gel was fixed for 30 min in 50% methanol, 10% acetic acid on a shaker followed by washing in 10% methanol, 5% acetic acid for 30 min on a shaker. The gel was then treated with 30% glutaraldehyde for 1 hr followed by washing with water at least 6 times, 30 min each time. Then the gel was stained in a staining solution [solution of $AgNO_3$ (2 g/10ml) in water was added drop wise with agitation to an alkaline solution of 0.74 ml 5N NaOH, 5.6 ml 1N NH_4OH and 45 ml distilled water, and the volume was finally made up to 200 ml] for 40 min at RT on a shaker. The gel was then washed with water three times, 10 minutes each and developed in a developing solution (2.5 ml 1% citric acid, 0.25 ml formaldehyde and volume made up to 500 ml) until the bands were visible. The development process was stopped by addition of a stopping solution (50% methanol and 10% acetic acid).

2.2.3 MLL Immuno-depletion experiments

MLL1, MLL2, Set1 antibody (Upstate) were cross-linked with protein A-agrose beads. Before initiating the cross-linking reaction, beads were washed three times with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), centrifuged at 1,000 rpm to collect the beads

and washed again with 3X 0.2 M sodium borate (pH 9.0), finally resuspended in to 1000 μL of 0.2 M sodium borate. Then, solid DMP (dimethyl pimelimidate) was added as the cross-linking reagent in to the suspension to make a final concentration of 20 mM DMP. The reaction was finally stopped by washing several times with 0.2 M ethanolamine. The MLL1 cross-linked beads (10%) were then incubated with Flag-CGBP-IP for overnight at 4 °C. Beads were then separated from the supernatant using small disposable column (Qiagen) and washed with buffer C in presence of 0.05% NP40 (nonyl phenoxylpolyethoxylethanol, a neutral surfactant to minimize non-specific interaction). Immuno-depleted supernatant and the affinity column beads were then subjected to western blot analysis to detect the depletion of MLL1 and histone methylation assay to study HMT activities under MLL1 depletion.

2.2.4 Histone expression and purification, and histone methyltransferase (HMT) assay

BL21 (DE) pLysS cells were transfected with the pET-histone expression plasmid (histone expression plasmids and competent cells were kind gift from Dr. Danny Reinberg, UMDNJ, USA) and plated on LB/ampicillin (ISC Bioexpress), and incubated at 37 °C overnight. 5 ml of LB medium containing ampicillin was inoculated with single colonies at 37 °C overnight. About 5 different colonies were picked up for test of induction. After 12 hrs 5 ml of LB medium containing ampicillin was inoculated with 50 μL of the overnight culture and shaked until the OD_{600} is 0.5-0.7 and then induced with 0.2 mM IPTG, for 2 hrs. Level of induction was checked on a 12% SDS-PAGE, comparing with the uninduced sample, and the best-induced colony was

selected. Over night culture from the best-induced colony was used to inoculate 1L of LB medium containing ampicillin and shaken until OD_{600} is 0.5-0.7 with 0.2 mM IPTG, for 2 hrs. Cells were harvested by centrifugation at room temperature, resuspended to homogeneity in 100 ml wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM Na-EDTA, 1 mM benzamidine), and flash-freezed in liquid nitrogen. Then, the cell lysate was sonicated to reduce the viscosity and centrifuged for 20 min at 4 °C at 23000×g. The pellet contains inclusion bodies of corresponding histone proteins. The pellet was washed by completely resuspending in 100 ml wash buffer plus 1% Triton-X100, centrifuged for 10 min at 4 °C at 12000×g. This step was repeated once with wash buffer plus Triton and twice with wash buffer. After the last wash the drained pellet was resuspended with 30 ml unfolding buffer (7 M guanidinium HCl, 20 mM Tris-HCl, pH 7.5, 10 mM DTT) and stirred gently for 1 hr at room temperature. The undissolved materials were removed by centrifugation at 20 °C and 23000×g. Four histone proteins were mixed in equimolar ratio, dialyzed at 4 °C against at least 2 L refolding buffer (2M NaCl, 10 mM Tris-HCL, pH 7.5, 1 mM Na-EDTA, 5 mM 2-mercaptoethanol). The histone octamers were purified from aggregates and dimers/tetramers on a gel filtration column (Sephadex G100, Pharmacia) and analyzed by 12% SDS-PAGE. Then, the samples from sizing column containing histone octamer were pooled together and the concentration was determined spectrophotometrically using Bradford reagent before storing at 4 °C.

Histone methylation assay was performed as described before (108). HMT assays (25 µL total reaction volume) were performed by incubating 2 µg recombinant

histone octamers with 5 µL of Flag-CGBP-IP or crude nuclear extract in presence of radiolabeled *S*-adenosyl-L- [methyl-^{3}H] methionine ([^{3}H] SAM) in the HMT assay buffer [50 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, and 0.1 mM DTT] at 30 °C for 1 hr. The products were separated on 12% SDS-PAGE, transferred on to PVDF membrane (Millipore) and autoradiographed. For autoradiography, the membranes were exposed on to a film of photographic emulsion overnight and developed using developing reagents (Kodak). The photographic development was carried out in the dark room following manufacturer's instructions (Kodak). The membranes were later stained with Coomassie blue to observe the histone loading patterns. For Coomassie blue staining the membranes were stained in a staining solution [(0.1% (w/v) Coomassie blue, 20% (v/v) methanol and 10% (v/v) acetic acid in water] for 30 minutes on a shaker and then transferred to destaining solution [50% methanol in water with 10% acetic acid] and kept on a shaker until bands develop. Similar histone methylation experiments were performed with Flag-SSU72-IP and control-Flag-IP (control-IP).

2.2.5 Immuno-precipitation of MLL1, MLL2 and hSet1 complexes

In order to cross-verify the association of CGBP with MLL1, MLL2 and hSet1 complexes, the MLL1, MLL2 and hSet1 complexes were immuno-precipitated from HEK293 cell nuclear extract. In brief, the anti-MLL1, anti-MLL2 and anti-hSet1 antibodies were cross-linked with protein-G agarose and then incubated with HEK293 cell nuclear extract for overnight at 4 °C (as described in section 2.2.3). Beads were then separated and washed with buffer C in presence of 0.1% NP 40. The affinity bound

proteins were eluted from the beads using 0.2 M glycine, pH 2.9 and analyzed by Western blot for the presence of CGBP using anti-CGBP antibody (Imgenex).

2.2.6 Immuno-fluorescence studies

Flag-CGBP stable cells were seeded onto a cover glass for 24 hrs and subjected to immuno-staining (105). The cells were fixed with 4% paraformaldehyde in PBS for 20 min and washed twice with cold 1X PBS 10 min each time. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, and a blocking solution (PBS containing 5% normal goat serum and 0.2% Triton X-100) was added and incubated for 1 hr. Then the cells were incubated for 1 hr with primary antibodies (1:1000 dilutions), anti-Flag antibody in combination with either of anti-MLL1, anti-MLL2 and anti-hSet1. Secondary antibodies (raised in goat) conjugated with FITC and rhodamine (Upstate) were used for immuno-fluorescence detections. The cells were washed at least five times (10 min each time) with cold 1X PBS with 0.01% triton X-100 to remove nonspecific binding of the antibodies. Nuclear counterstaining was performed with DAPI (4', 6-diamidino-2-phenylindole) (Upstate). Immuno-stained cells were mounted and observed by using a fluorescence microscope (Nikon Eclipse TE2000-U, Japan).

2.2.7 Chromatin Immuno-precipitation (ChIP) experiment

The Flag CGBP stable cells cells in a 10 cm plate at 80% confluence were fixed with 1% formaldehyde at room temperature for 30 min and washed twice with ice-cold PBS before harvesting. Subsequently, the pellet was resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, 1 mg/ml protease inhibitors, 1 mM phenylmethylsulfonyl fluoride) and sonicated on ice until cross-linked chromatin was

sheared to an average DNA fragment length of 0.2–0.5 kbp. The fragmented DNA was subjected to ChIP assay using EZ Chip^{TM} Chromatin immuno-precipitation kit (Upstate) as described previously (109). Antibodies against RNAP II (RNA polymerase II), H3K4 trimethyl (upstate), MLL1 (bethyl lab) and CGBP (Imgenex) were used to carry out chromatin Immunoprecipitation (ChIP). Finally, the immuno precipitated DNA fragments were purified by phenol/chloroform method (please refer to section 2.2.9). Then the purified DNA obtained was used as template in PCR amplification using primer specific to promoter (forward: 5′-GAGCCTCCAGGTCTTTTTCC-3′ and reverse: 5′- ACACCCCCAGATTTACACCA-3′) and ORF (open reading frame) (forward: 5′-TTCCACTTCAACCGCTACCT-3′ and reverse: 5′-TTCATCATCGTCCTCCTCGT-3′) region of HoxA7 gene.

2.2.8 Antisense mediated down regulation of CGBP

The CGBP antisense (5'-GCCTCACACTCACCACACAT-3') modified with phosphorothioate linkages was commercially synthesized (Biosynthesis Inc). CGBP antisense oligonucleotides were transfected into HEK293 cells using Maxfect transfection reagent (MoleculA). In brief, HEK 293 cell were grown up to 60% confluence in normal DMEM supplemented with 10% FBS. Cells were washed twice with FBS free DMEM and then incubated with transfection reagent-antisense complex for 5 hrs in serum-free DMEM prior to the addition of complete growth medium (with 10% serum). The cells were then incubated for 72 hrs before harvesting for RNA, nuclear protein extraction or ChIP analysis. A scramble antisense without any sequence homology with CGBP (5'- CGTTTGTCCCTCCAGCATCT-3') was used as control.

2.2.9 Extraction of RNA and histone, and reverse transcription PCR

2×10^6 cells were harvested and pelleted by centrifugation at 3000g for 10 minutes. RNA was extracted from cell pellet as described previously (110). The cell pellet was resuspended in 250 µL of guanidium thiocyanate solution (4M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1% 2-mercaptoethanol) and vortexed until a clear viscous lysate is formed. The clear lysate was mixed without vortexing with 250 µL of 8M LiCl until it became cloudy. The suspension was cooled on ice for 1 hr and centrifuged at 13000×g for 20 min at 4 °C. The supernatant fluid was discarded and the pellet containing RNA was dissolved in 150 µL of DEPC (diethyl pyrocarbonate)-treated water. Then the RNA solution was subjected to phenol/chloroform purification in which equal volume of acidic phenol (pH 4.3) was added to the solution and vortexed vigorously before centrifuging at 13000×g to separate the organic and aqueous layers. The aqueous layer containing RNA was transferred into a fresh eppendorf tube and vortexed with equal volume of chloroform/isoamyl alcohol (24:1) before centrifuging at 13000×g for 10 min at 4 °C to separate the organic and aqueous layers. The aqeous layer was transferred into a new tube and mixed with 100% ethanol 2.5 times its volume and precipitated overnight at -80 °C. The RNA was precipitated by centrifugation at 13000×g for 30 min at 4 °C. The pellet containing RNA was washed with 70% ethanol and air dried before resuspending in DEPC-treated water. RNA was quantified spectrophotometrically and subjected to RT-PCR (reverse transcriptase PCR). Reverse transcription reactions were performed in a total volume of 25 µL containing 1 µg of total RNA, 2.4 µM of oligo-dT, 100 units of MMLV reverse transcriptase (Promega),

1X first strand buffer (Promega), 100 μM dNTP mix (dATP, dGTP, dCTP and dTTP), 1 mM dithiothreitol and 20 units of RNaseOut (Invitrogen). This cDNA (1 μL) was used for PCR with primer pairs specific to CGBP (forward: 5′-GCCACACGACTATTCTGTGA-3′, reverse: 5′- CAGTAATGGCGATTGCACTG-3′) and HoxA7 (forward: 5′- TTCCACTTCAACCGCTACCT-3′, reverse: 5″-TTCATACATCGTCCTCCTCGT-3′).

2×10^6 cells were used for histone extraction as described previously (111). The pellet after preparation of nuclear extract was resuspended in histone extraction buffer (0.4N H_2SO_4) and incubated for 2 hrs on ice before centrifuging at 10000×g for 20 min. The supernatant fluid carrying soluble histone proteins was used for western blot analysis with the help of anti-H3K4-trimethyl (Upstate Biotech).

2.3. Results

2.3.1 Affinity purification of CGBP and associated histone methyltransferases

HEK293 human cells were transfected with Flag-CGBP cDNA and isolated the stable transformed cell line expressing Flag-tagged CGBP using G418 antibiotic selection method. The Flag-CGBP was isolated by performing anti-Flag immuno-precipitation (Flag-CGBP-IP) from the nuclear extract of the Flag-CGBP stable cell line along with associated proteins. The immuno-precipitation of Flag-CGBP was confirmed by Western blot analysis of the Flag-CGBP-IP with anti-Flag antibody (Figure 2.1A). In order to determine the specificity of the H3 and H4 specific HMT activity associated with Flag-CGBP-IP, anti-Flag-IP (Flag-SSU72-IP) was performed from stable cell line over-expressing an unrelated protein, human homolog of yeast SSU72 (Figure 2.1B).

Notably, human SSU72 was cloned into the same expression vector, p-Flag-CMV4 and expressed in the human cell using the same procedure as described for CGBP. In parallel, a control Flag immuno-precipitation (control-IP) was performed from nuclear extract of HEK293 cells (that was not transfected with any plasmid).

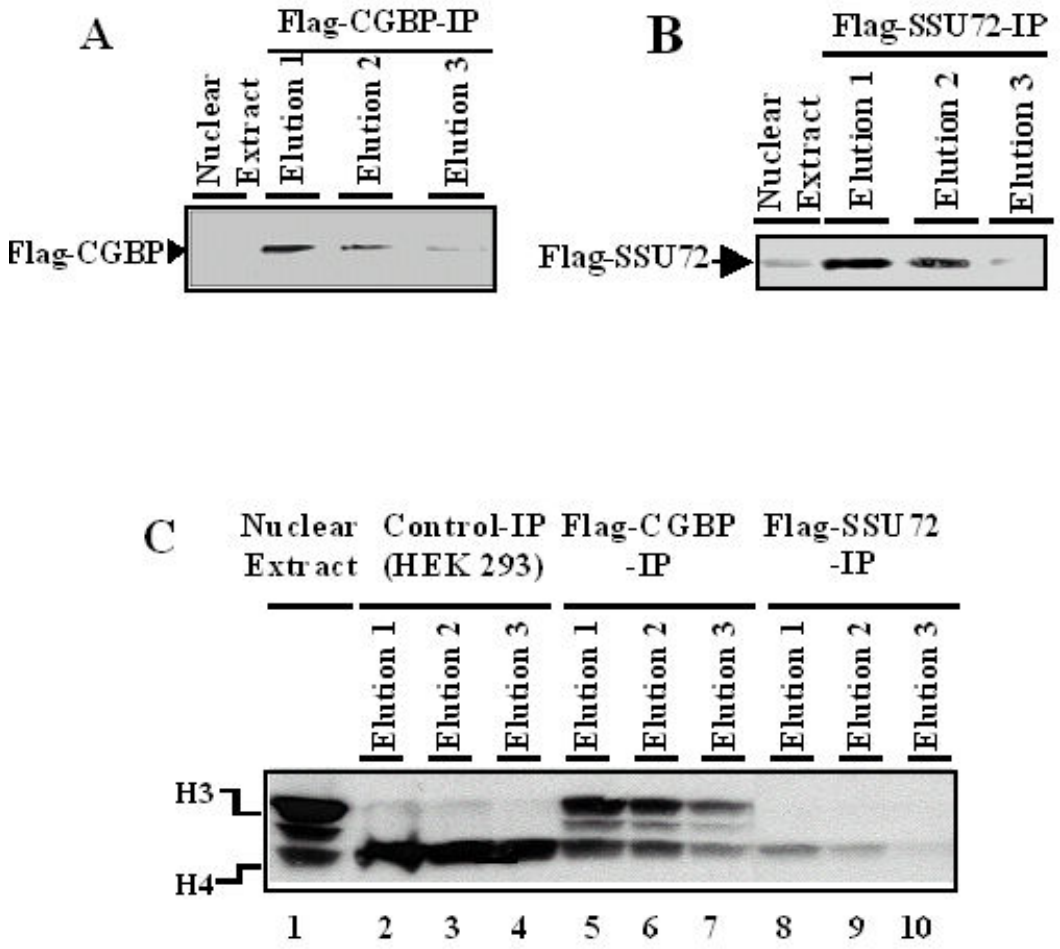

Figure 2.1 Affinity purification of Flag-CGBP and associated proteins. Nuclear extract of CGBP stable cell line was subjected to anti-Flag immuno-precipitation (Flag-CGBP-IP) that was analyzed by Western blot and HMT assay. (**A**) Western blot analysis of Flag-CGBP-IP by anti-Flag antibody. Flag-CGBP is indicated by arrow. (**B**) Western blot analysis of Flag-SSU72-IP elutions by anti-Flag antibody (sigma). The arrow indicates flag-SSU72. (**C**) Histone methylation assay was performed with Flag-CGBP-IP, Flag-SSU72-IP, and Control-IP (Flag-IP from nuclear extract of HEK293 cells that were not transfected with any plasmid) using wild type recombinant histone octamers.

In order to detect the presence of any histone methylases associated with Flag-CGBP-IP, we performed the HMT assay. Flag-CGBP-IP, Flag-SSU72-IP and control-IP elutions were incubated with wild type recombinant histone octamers (containing H2A, H2B, H3 and H4 wild type histones) in the presence of ^{3}H-SAM, and the products were analyzed by SDS-PAGE and autoradiography. HMT assays using wild type histone octamer (containing all four wild type histones) demonstrated that only Flag-CGBP-IP methylates specifically histones H3 (lanes 5-7, Figure 2.1C). Interestingly, control-IP, Flag-SSU72-IP and Flag-CGBP-IP, all contain H4 specific HMT activity (Figure 2.1C), whereas H3 specific HMT activity was only observed in Flag-CGBP-IP (Figure 2.1C). These data demonstrated that H4 activity is a non-specific HMT activity associated the anti-Flag-IP. In order to determine the site-specificity of the H3 specific HMT activity associated with CGBP-IP, HMT assay was performed using histone octamers that were mutated at either H3K4A (lysine at position 4 is mutated to alanine), or H3K4-K9-K27A (lysine at positions 4, 9 and 27 are all mutated to alanine) or H3K9-K27-K36A (lysine at positions 9, 27 and 36 are all mutated to alanine) (lanes 5, 6 and 7 respectively, Figure 2.2). Upon H3K4A mutation, the H3 specific HMT activity is reduced by 66.2% (compare lanes 2 with 5, Figure 2.2) demonstrating the presence of H3K4 specific HMT activity along with other possible H3 specific HMT activity present in the Flag-CGBP-IP. Moreover, upon mutation of all the lysines at H3K4, K9 and K27, the H3 specific HMT activity is completely abolished (lane 6, Figure 2.2) suggesting the presence of H3K4 specific HMT activity along with either H3K9 and/or H3K27 specific HMT activities associated with Flag-CGBP-IP. Mutation at H3K9, K27

and K36 to alanine and keeping the H3K4 intact resulted in retention of 71.9% of H3K4 specific HMT activity (compare lanes 7 with lane 2, Figure 2.2). The identity of the observed H3K9 and/or K27 specific activity is yet to be determined.

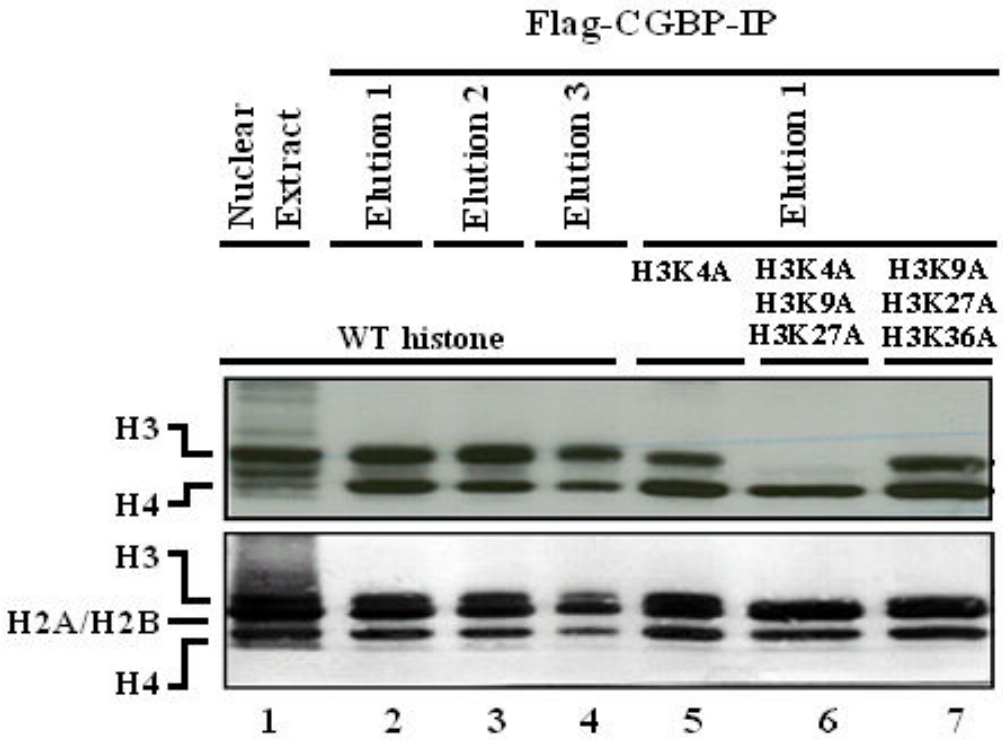

Figure 2.2 Histone methylation assay with Flag-CGBP-IP. Histone methylation assay was performed by incubating recombinant (wild type or mutated) histone octamers with Flag-CGBP-IP in presence of [3H] SAM and the products were analyzed by 12% SDS PAGE and autoradiography. Lane 1: Wild type (WT) histone octamer that was treated with crude nuclear extract. Lanes 2-4, WT histone octamers that was incubated with 5 µL of the Flag-CGBP-IP. Lanes 5, 6 and 7, are same as lane 2 except that WT histone octamer was replaced with mutant histone octamers, H3K4A (lysine at position 4 of H3 was substituted with alanine), H3K4, 9,27-A (lysines at positions 4, 9 and 27 of H3, were mutated to alanine) and H3K9, 27,36-A respectively. The upper panel is the autoradiograph of methylation assay and bottom panel is the coomassie blue stain of the same membrane.

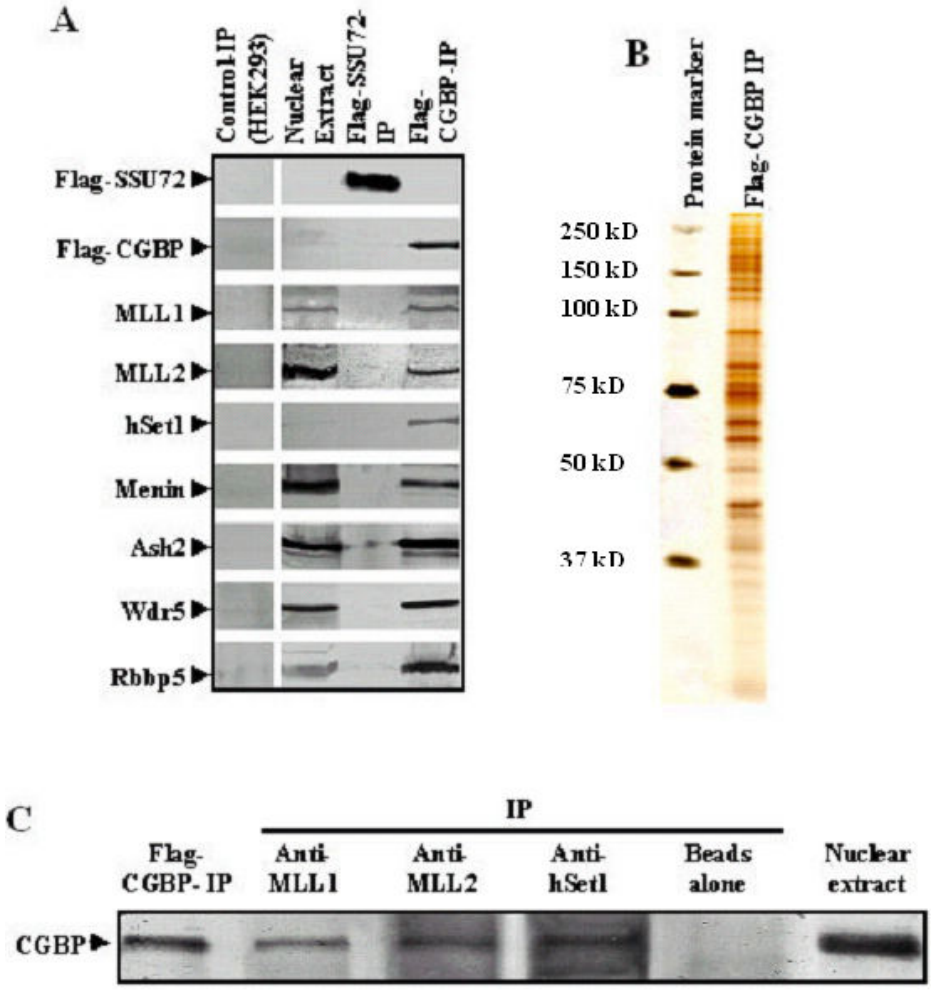

Figure 2.3 (**A**) Western blot analysis of Flag-CGBP-IP. The Flag-CGBP-IP, Flag-SSU72-IP, control Flag-IP and crude nuclear extract obtained from Flag-CGBP stable cell line were run on SDS PAGE and subjected to western blot analysis with different antibodies specific to MLL1, MLL2, hSet1, Menin, Ash2, Wdr5, and Rbbp5 (as indicated by arrows). CGBP and SSU72 were detected using anti-Flag antibody. (**B**) Silver stain of the Flag-CGBP-IP showing the purity of the IP. (**C**) Cross verification of the association of CGBP with MLL1, MLL2 and hSet1 complexes. MLL1, MLL2 and hSet1 HMT complexes were separately immuno-precipitated from HEK 293 cell nuclear extract by incubating with anti-MLL1, anti-MLL2 and anti-hSet1 attached protein-G agarose beads for over night at 4 °C. Immuno-precipitated protein bound beads were analyzed by western blot using anti-CGBP antibody. Control experiment was performed by the incubation of HEK293 nuclear extract with protein-G agarose beads alone.

2.3.2 MLL1, MLL2 and hSet1 are co-purified with CGBP

To determine the component that is responsible for HMT activity of the Flag-CGBP-IP, Western blot analysis was carried out with antibodies against different HMTs such as MLL1, MLL2, MLL3 and hSet1. Interestingly, it was found that, Flag-CGBP-IP contained Flag-CGBP along with three well known HMTs, MLL1, MLL2 and hSet1 (Figure 2.3 A). Neither of these proteins is present in the Flag-SSU72-IP or control-IP (Flag-IP from nuclear extract of HEK293 cells that were not transfected with any plasmid) (Figure 2.3 A). These data demonstrated that association of MLL1, MLL2 and hSet1 is specific to CGBP. In addition, the Flag-CGBP-IP also contained Ash2, Wdr5 and Rbbp5, which are known components of MLL1, MLL2 and hSet1 complexes (27). Moreover, Flag-CGBP-IP pulled down Menin, a tumor suppressor gene that is a known component of MLL1 and MLL2 complexes (Figure 2.3 A) (27). Presence of Menin further confirmed the presence of MLL1 and MLL2 in the Flag-CGBP-IP. Notably, any MLL3 could not be detected in Flag-CGBP-IP (data not shown). The purity of Flag-CGBP-IP is shown by silver staining (Figure 2.3 B). In order to cross verify the association of CGBP with MLL1, MLL2 and hSet1, independent immuno-precipitation experiments were performed for MLL1, MLL2 and hSet1 from HEK293 cell nuclear extract using antibodies against MLL1, MLL2 and hSet1 and the immuno-precipitates were analyzed separately by Western blot using anti-CGBP (Figure 2.3 C). In agreement with above observations, it was found that CGBP is present in each of the MLL1, MLL2 and hSet1 immuno-precipitates and is absent in the control-IP (beads alone, Figure 2.3 C) demonstrating further the association of CGBP with each of MLL1,

MLL2 and hSet1 HMT complexes. The Flag-CGBP-IP was further fractionated in a gel-filtration column (Sephadex G200; eluant: buffer C [20 mM Tris-HCl, (pH 7.9), 5 mM MgCl2, 0.42 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) along with 0.1% NP 40] to separate MLL1, MLL2 and hSet1 complexes present in the Flag-CGBP-IP (Figure 2.4). The gel-filtration column fractions were analyzed by using Western blot and histone methylation assay. As evidenced from the Western blot, the MLL1, MLL2 and hSet1 co-elute with CGBP along with other subunits (top panel, Figure 2.4). Histone methylation activities also co-eluted with the HMT containing fractions (middle and bottom panels, Figure 2.4). However, different HMT complexes did not separate into homogeneous fractions likely due to the similar size of the large multi-protein complexes. Further fractionations are needed to separate each of these complexes into homogeneity for detail characterizations. Overall, the co-purification of MLL1, MLL2 and hSet1 with CGBP in the Flag-CGBP-IP and the presence of CGBP in the independently immuno-precipitated MLL1, MLL2 and hSet1 complexes demonstrated that CGBP is an interacting partner of MLL1, MLL2 and hSet1 HMT complexes.

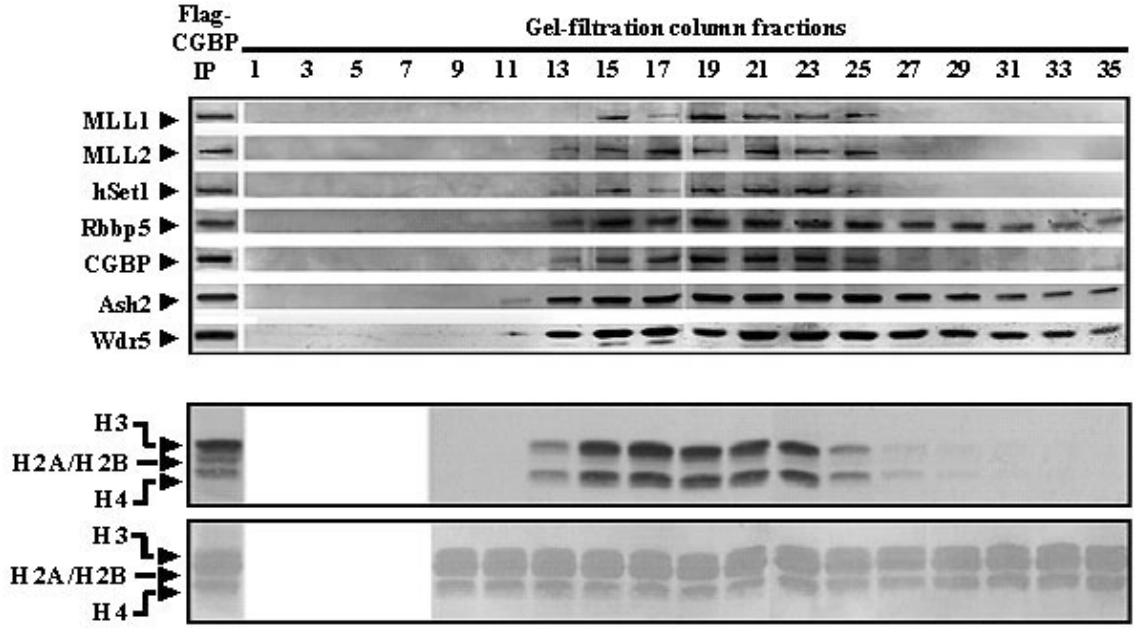

Figure 2.4 Fractionation of Flag-CGBP-IP by gel-filtration column (Sephadex G200). The Flag-CGBP-IP was fractionated on Sephadex G-200 column using buffer C along with 0.1% NP 40]. The fractions were analyzed by western blot with different antibodies (top panel) and histone methylation assays (middle panel: autoradiograph of the HMT assay; Botton panel: coomassie staining of the same gel as shown in the autoradiograph).

2.3.3 CGBP associated H3K4 specific HMT activity is due to the presence of MLL1, MLL2, and hSet1HMTs

Although, our above results demonstrated that the Flag-CGBP-IP contains at least three different H3K4 specific HMTs, this experiment does not reveal whether H3K4 specific HMT activity is due to hSet1, MLL1, MLL2 or all of them. In order to resolve this issue, MLL1 was initially immuno-depleted from the Flag-CGBP-IP. The Flag-CGBP-IP was incubated with anti-MLL1 antibody attached protein-G agarose beads overnight at 4 °C and then the beads were separated from the supernatant fluid. Western blot analysis demonstrated that MLL1 is almost completely depleted from the supernatant and bound to the beads (Figure 2.5A).

The immuno-depleted supernatants were analyzed by HMTase assay (Figure 2.5B). Interestingly, upon MLL1 depletion (supernatant), most of the H3K4 specific HMT activity of the Flag-CGBP-IP was depleted without affecting the H4 specific HMT activity (Figure 2.5B, compare lanes 2-5 with 6-9). This result (specifically compare lanes 5 with 9 where only H3K4 is intact, Figure 2.5B) demonstrates that one of the H3K4 specific HMT activities present in the Flag-CGBP-IP is certainly due to the association with MLL1. Notably, the affinity column beads that contain MLL1 were found to have H3K4 specific HMT activity (Figure 2.5C). This HMT activity was rather weak which might be due to the attachment of MLL1 to the beads.

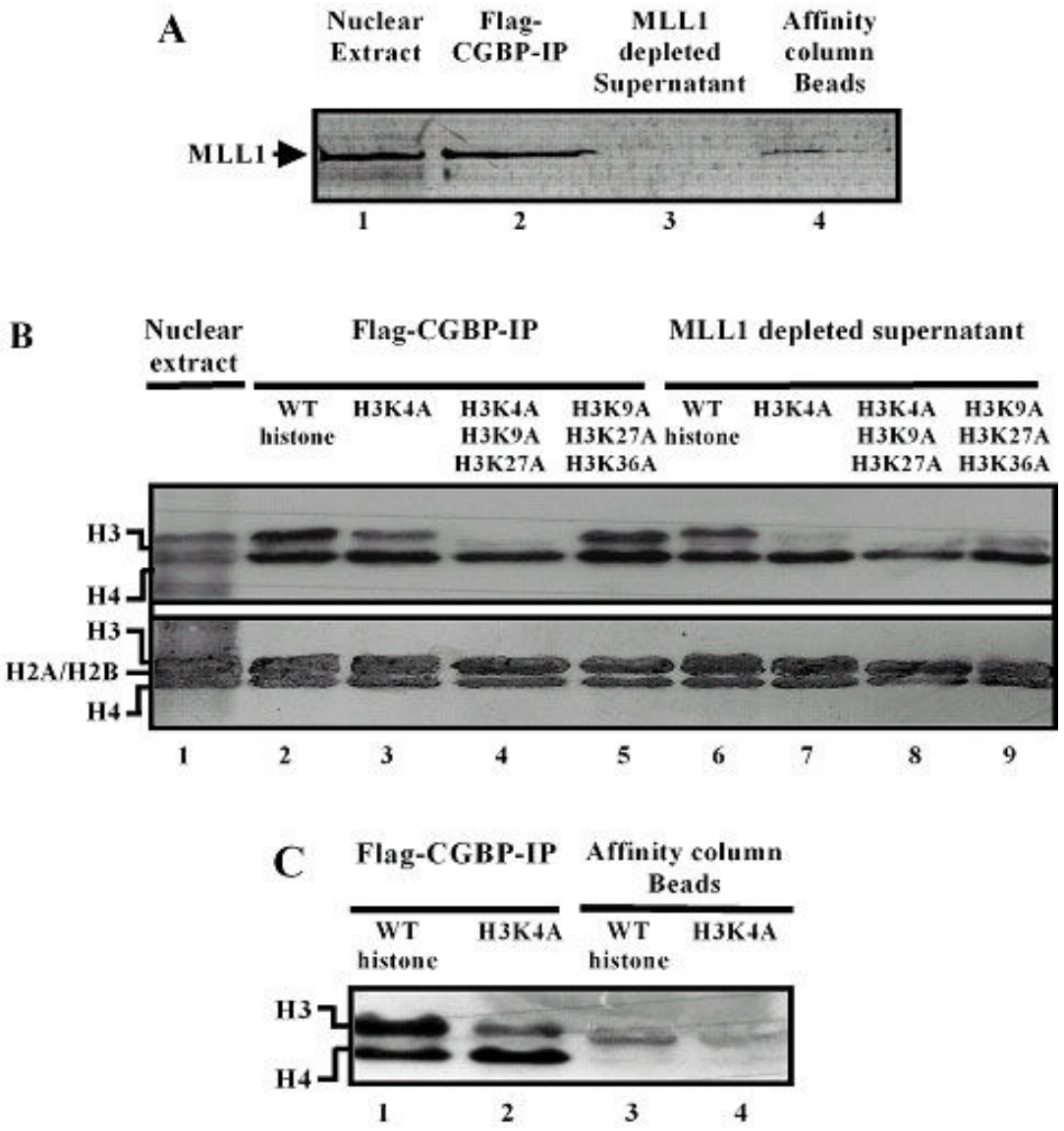

Figure 2.5 MLL1 immuno-depletion of Flag-CGBP-IP and histone methylation assay. MLL1 was immunodepled from Flag-CGBP-IP with anti- MLL1 (cross-linked protein G beads). (**A**) Western blot analysis of MLL1 immudepleted supernatant and MLL1 bound affinity column beads. (**B**) Histone methylation assay was performed with Flag-CGBP-IP before and after (MLL1 depleted supernatant fluid) immuno-depletion with wild type (WT) as well as the mutant histone octamers as substrates. The upper panel is the autoradiograph of methylation assay and bottom panel is the coomassie blue stain of the membrane. (**C**) HMT assays with the affinity column beads.

Similar immuno-depletion experiments were further performed for MLL2 and hSet1 separately from Flag-CGBP-IP using anti-MLL2 and anti-hSet1 and the immuno-depleted supernatant fluids and affinity protein bounds beads were analyzed by HMTase assay. Interestingly, similar to MLL1, depletion of either MLL2 or hSet1 resulted efficient depletion of H3 specific HMT activity (Figure 2.6A). The affinity protein bound beads were also found to contain H3K4 specific HMT activities (Figure 2.6B). These observations demonstrated that MLL2 and hSet1 along with MLL1 contribute to the overall H3K4 specific HMT activities present in Flag-CGBP-IP. Notably, it is not clear at this stage why depletion of any of these HMTs depleted more than 80% of the H3K4 specific HMT activities present in the Flag-CGBP-IP and this need further investigation.

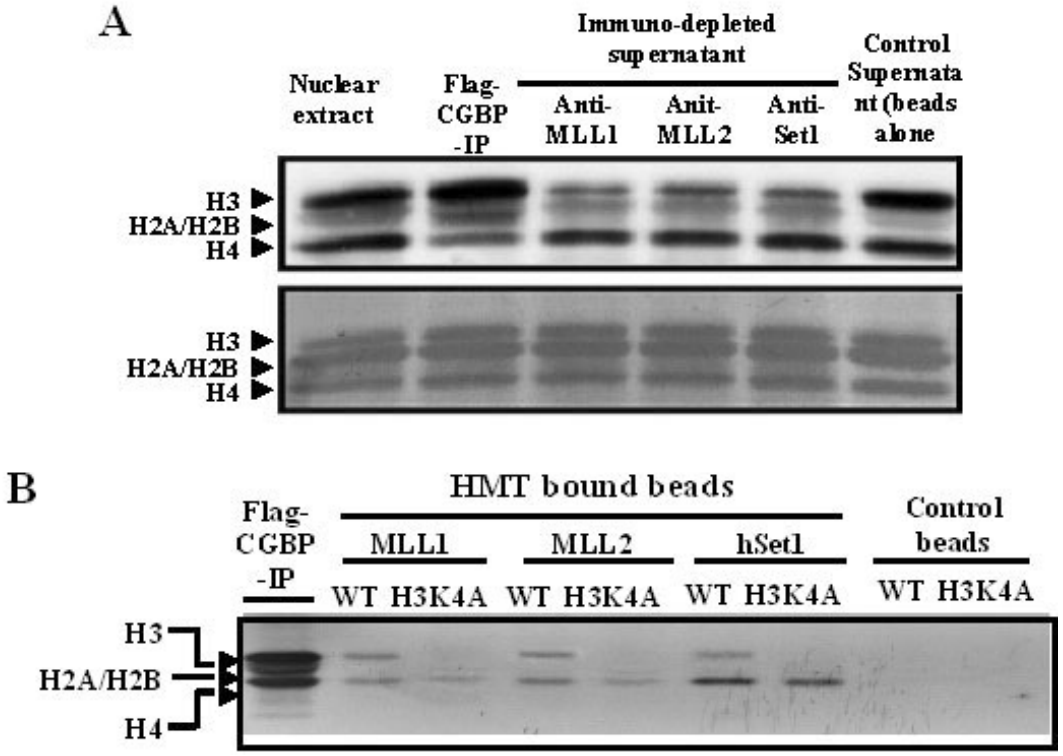

Figure 2.6 HMT assays with MLL1, MLL2, and hSet1 immuno-depleted Flag-CGBP-IP. (**A**) MLL1, MLL2 and hSet1 were immuno-depleted from Flag-CGBP-IP with anti-MLL1, anti-MLL2 and anti-hSet1 attached protein G agarose beads separately. The control experiment was performed with beads alone in the absence of any antibody. The immuno-depleted supernatants were analyzed by histone methylation assay using wild type (WT) recombinant histone octamers. The upper panel is the autoradiograph of methylation assay and bottom panel is the coomassie blue stain of the same membrane. (**B**) The affinity protein (HMT) bound beads were used for histone methylation assay using both wild (WT) type and histone H3-lysine 4 mutated (H3K4A) recombinant histones. The absence of H3 specific activity in H3K4A histones indicates that activity associated with HMT attached beads is H3K4 specific.

2.3.4 CGBP is associated with the promoter of MLL target genes and co-localized with MLL1, MLL2 and hSet1 in vivo.

In order to confirm the association of CGBP with MLLs in vivo, the recruitment of CGBP was analyzed in the promoter and open reading frame (ORF) of a transcriptionally active MLL target gene, HoxA7, using ChIP assay (43). The ChIP experiment was performed with anti-Flag (to detect Flag-CGBP), anti-H3K4 trimethyl

and anti-RNAP II (RNA polymerase II) antibodies using Flag-CGBP stable cell line (109). Interestingly, CGBP was found to be present in the promoter region of the HoxA7 gene (lanes 3 and 4, Figure 2.7A). RNAP II, as expected, is present both in the promoter and the coding region of HoxA7 gene (lanes 3 and 4, Figure 2.7A). H3K4 trimethylation, which is known to be involved in early phase of transcription, is present at the promoter region, which is in agreement with previous studies (62,101). These observations demonstrated that CGBP is recruited to the MLL target genes, indicating its possible interaction with MLL1 in Hox A7 promoter.

The interaction of CGBP with MLLs and hSet1 was further examined using immuno-fluorescence microscopy in live cells (Figure 2.7B). The Flag-CGBP stable cells were co-immuno-stained with anti-Flag antibody along with anti-MLL1, anti-MLL2 and anti-hSet1 antibodies separately. The cell nucleus was visualized by DAPI staining. Interestingly, nuclear speckles of CGBP were highly co-localized (shown by yellow color of merged panel) with nuclear speckles of MLL1 and MLL2 in the DAPI less intense euchromatic regions of the nucleus (Figure 2.7B). However, in the case of hSet1, although, CGBP and hSet1 are both localized in the DAPI less intense region of the nucleus, partial co-localization of the nuclear speckles was observed. The co-localization of CGBP with MLL1, MLL2, and hSet1 further support that CGBP is an interacting partner of MLL1, MLL2 and hSet1 HMTs in vivo. The partial colocalization may suggest possibility of alternate roles of each of these proteins in the cell.

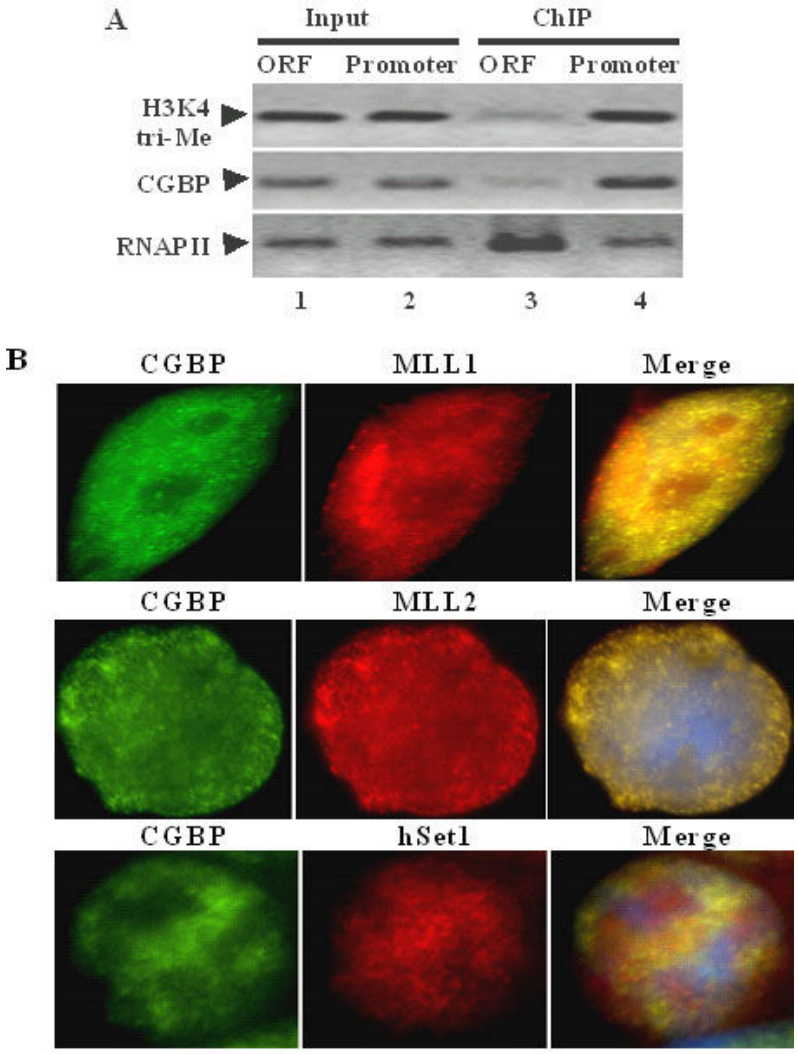

Figure 2.7 CGBP is associated with MLL in vivo (**A**) CGBP is associated with MLL target genes *in vivo* (ChIP assay). The ChIP analysis was performed with Flag-CGBP stable cells using anti-H3K4-tri-methyl (Upstate), anti-Flag (for Flag-CGBP) and anti-RNAP II antibodies. The immuno-precipitated DNA fragments were PCR amplified using primer specific to the promoter and open reading frame (ORF) of HoxA7 gene. Arrow indicated PCR product from respective ChIPs. (**B)** Immunofluorescence studies**.** CGBP stable cell lines were used for immuno-staining. The cell nucleus was visualized by DAPI (4',6 diamidino-2-phenyl indole) staining. Flag-CGBP cells were co-immuno-stained with anti-Flag antibody (for detecting CGBP) in combination with either anti-MLL1 or anti-MLL2 or anti- hSet1. CGBP was visualized by staining with FITC (green) conjugated secondary antibody and others by Rhodamine conjugated secondary antibody (red). The merge of the FITC and Rhodamine staining is also shown and the yellow colors indicate the co-localization of the red and green speckles.

2.3.5 Depletion of CGBP down- regulates MLL target gene HoxA7

In order to understand the functional significance of the interactions of CGBP with MLLs and hSet1, CGBP was knocked down from HEK293 cells using phosphorothioate antisense oligonucleotide specific to CGBP. The knock-down of CGBP upon antisense treatment was confirmed both in the protein (western blot of the whole cell extract) and RNA level (RT-PCR analysis) (Figure 2.8A and B, respectively). As seen in figure 2.8A, the antisense mediated knockdown of CGBP was concentration dependent and over 80% knockdown was obtained at 4 μg of CGBP antisense. Specificity of CGBP knock down was confirmed by using actin as control (Figure 2.8A). Based on these data, further antisense experiments were performed using 4 μg of CGBP antisense. The changes in global histone H3K4-methylation as well as the changes in H3K4 methylation in specific MLL target gene promoter HoxA7 were analyzed upon down regulation of CGBP. Notably, the knockdown of CGBP did not affect significantly the level of global H3K4 trimethylation (bottom panel, Figure 2.8A). However, the knock down of CGBP resulted in down-regulation of HoxA7 expression (Figure 2.8B). ChIP analysis showed that the recruitment of MLL1 and level of H3K4 trimethylation went down at the promoter region of HoxA7 gene upon down regulation of CGBP (Figure 2.8C). These observations demonstrate that CGBP is essential for the recruitment of MLL1 in the promoter region of HoxA7 gene that facilitate H3K4 tri-methylation and gene activation.

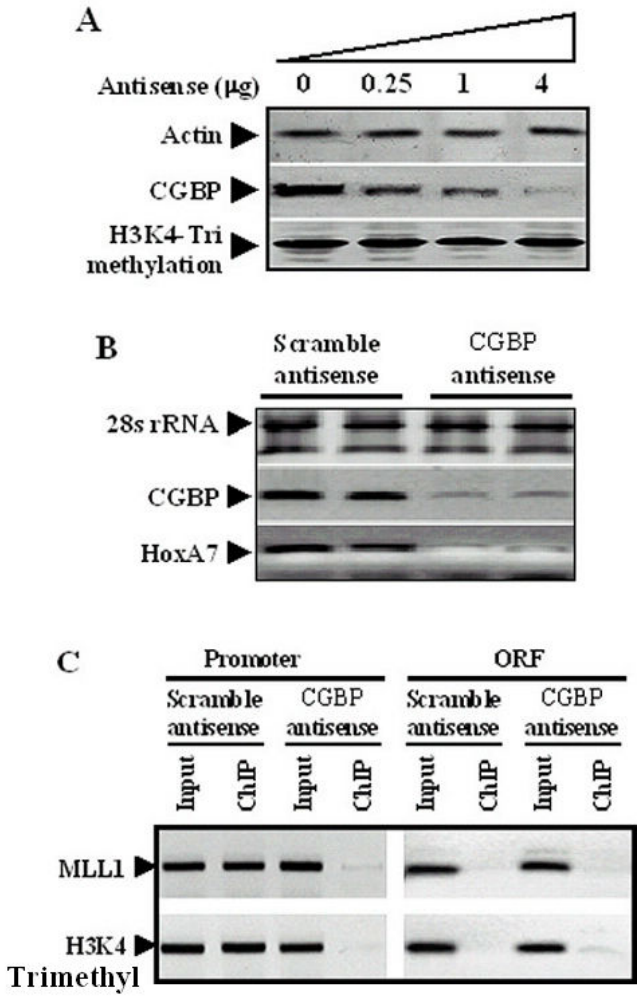

Figure 2.8 Knock down of CGBP using specific antisense. HEK 293 cells were treated with phosphorothioate antisense for 72h and then harvested for either RNA extraction or Chromatin immuno-precipitation (ChIP) assay. **A**. Western blot analysis of the nuclear extract using anti-Actin (as control) and anti-CGBP antibodies and histone protein with anti-H3K4 tri-methyl antibody. **B.** RT-PCR of RNA extract with primer specific to CGBP and HoxA7. Arrow indicates: 28s rRNA, quantitative control of RNA and PCr product of CGBP and HoxA7. **C**. ChIP analysis of HEK 293 cells treated with either with CGBP antisense or a scramble antisense using MLL1 and H3K4-trimethyl antibodies. The immuno-precipitated DNA fragments were PCR amplified using primer specific to the promoter and open reading frame (ORF) of HoxA7 gene. Arrow indicated PCR product from respective ChIPs. Notably, design of the CGBP antisense, scramble antisense, and knock down experiments, were carried out by Dr. Khairul Ansari under the supervision of Dr. Mandal and this figure is kindly provided by them.

2.4 Discussion

The H3K4 methylation is an evolutionarily conserved mark and plays critical roles in gene regulation and disease (6,38,42,76,112-115). Human encodes several H3K4 specific HMTs such as MLL1, MLL2, MLL3, MLL4, hSet1 etc. Notably, MLL1, MLL2, MLL3, and hSet1 are shown to be present as distinct multi-protein complexes with several highly conserved protein components. Each of these HMTs shares three protein subunits in common, which includes Ash2, Rbbp5, and Wdr5 (Table 1.2)(27). Recently, Dpy30, UTX and several other novel proteins are shown to present in Set1 like HMTs complexes (Table 1.2)(31,65). However, CGBP is only found to interact with hSet1 (68).

Herein, it was demonstrated that Flag-CGBP-IP pulled down MLL1, MLL2, and hSet1 HMTs along with their associated components Ash2, Wdr5, Rbbp5 and Menin. Absence of these proteins in Flag-SSU72-IP as well as in the control-IP further demonstrated that MLL1, MLL2 and hSet1 interactions are specific to CGBP. Notably, yeast homolog of SSU72 is shown to be RNA polymerase II specific CTD phosphatase(116). Moreover, the association of CGBP with independently immuno-precipitated MLL1, MLL2 and hSet1 complexes further confirmed the interaction of CGBP with each of the MLL1, MLL2 and hSet1 HMT complexes. In addition, the co-elution of the CGBP with MLL1, MLL2 and hSet1 complexes in the gel-filtration suggests the interaction of CGBP with each of these HMT complexes.

The immuno-depletion of MLL1, MLL2 and hSet1 separately from the Flag-CGBP-IP, efficiently depleted the H3K4 specific HMT activity from the supernatant

fluid. This data suggest that CGBP associated HMT activities are originated due to the presence of all of the MLL1, MLL2 and hSet1 complexes. However, it is not clear why depletion of either of these HMTs depletes more than 80% of the H3K4 specific HMT activities present in the Flag-CGBP-IP and is a subject of our further investigation.

It was also demonstrated that CGBP is co-localized with MLL1, MLL2 and hSet1 in vivo and is recruited to the promoter of transcriptionally active MLL target gene HoxA7.The co-purification and co-localization of MLL1, MLL2 and hSet1 with CGBP demonstrated that CGBP is an interacting partner of all the three HMTs. Notably, as MLL1, MLL2 and hSet1 exist as distinct multi-protein complexes in the cell, CGBP is most likely a common component of all of them. Furthermore, decrease in recruitment of MLL1 and down regulation of level of H3K4 trimethylation in the promoter of HoxA7 upon knockdown of CGBP suggest that CGBP plays critical roles in regulating functions of MLL1. This is also reflected in decreased expression of HoxA7 upon knockdown of CGBP. Notably, as knockdown of CGBP had little effects on global histone methylation, it is likely that some of the H3K4 HMTs are still functional even in the absence of CGBP. This suggests that CGBP is likely associated with sub-population of H3K4 specific HMTs that regulate expression of specific MLL target genes in human cell. Understanding the novel interactions and functions of each of the proteins associated with MLLs and Set1 are crucial for understanding the complex functions of H3K4 specific HMTs in vivo. Recent discoveries of novel MLL-interacting partners, including histone acetyl-transferases, nuclear hormone receptors etc, indicates diverse functions of H3K4 specific HMTs in gene expression and

signaling. The association of a "CXXC" DNA binding domain-containing protein, CGBP, with MLL1 and MLL2, as demonstrated here in (Table 1.2), opens new possibilities that CGBP may play roles in targeting MLLs into specific promoters. The detail function of CGBP and its different functional domains in regulating MLL mediated gene expression are subject of our future studies.

CHAPTER 3

DYNAMIC ASSOCIATION OF MLL1, H3K4 TRIMETHYLATION WITH CHROMATIN AND HOX GENE EXPRESSION DURING CELL CYCLE

3.1 Introduction

Mixed lineage leukemias (MLLs) are human HMTs that specifically methylate histone H3 at lysine-4 (H3K4) and are linked with gene activation (14,29,32,38,40,42,61,64,73). Notably, Set1 is the sole H3K4-specific HMT present in yeast (101,102,117). Human encodes six Set1 homologs such as MLL1, MLL2, MLL3, MLL4, Set1A, and Set1B (27,29,36,42,48,61,64,100). Each of these proteins exists as multi-protein complexes sharing several common subunits including Ash2, Wdr5, Rbbp5, human CpG binding protein (CGBP) and Dpy30 (27-29,31,36,38,42,48,61,64,65,100,109). MLLs are well known as the master regulator of Homeobox (Hox) genes that are critical for cell differentiation and development (18,42,78). Although, recent discoveries of HMT activities of MLLs have shed significant light into their complex function in gene regulation, their mechanism of action and distinct roles in different cellular events still remain elusive. The presence of multiple H3K4 specific HMTs in vertebrate genomes indicates that each of the MLLs may have specialized functions in regulating differential expression of specific target genes or in methylation of distinct non-histone proteins for other functions.

Recent studies indicate that MLL may play a crucial role in cell cycle progression. For example, knock out of Taspase1, a protease that specifically cleaves and activates MLL1, results in down-regulation of cell cycle regulatory cyclin genes by affecting H3K4-trimethylation in their promoters (48,118). Furthermore, MLLs directly interact with E2F family of transcription factors that are responsible for the activation of cyclins (48,81). MLL1 interacts with E2F2, E2F4, and E2F6 with different affinities, while MLL2 interacts with a different subset of E2Fs such as E2F2, E2F3, E2F5, and E2F6 (48,81). Distinct interactions between E2Fs and MLL suggest potential roles of MLL proteins in the cell cycle regulation. Similarly, independent studies showed that MLL interacting protein, menin, host cell factor-1 (HCF1) and CGBP are also implicated in cell cycle regulation (81). Menin directly regulates the expression of cyclin dependent kinase (CDK) inhibitors such as p27 and p18 (74,119). Knock down of HCF-1 results in cell cycle arrest at G1. Therefore, both physical and functional interactions of MLLs with cell cycle regulatory proteins indicate potential roles of MLLs in cell cycle regulation.

Notably, chromatin condensation, decondensation and differential expression of cell cycle associated proteins are critical for proper progression and maintenance of cell cycle. As MLLs and H3K4 specific methylations are well known to play critical roles in gene expression, herein, we analyzed the dynamics and functions of MLLs and H3K4 methylation during cell cycle progression. Our results demonstrated that MLL and H3K4-trimethylation have different dynamics during cell cycle progression. MLLs dissociate and re-associate with condensed and relaxed chromatin respectively while

H3K4-trimethylation remained associated with chromatins throughout cell cycle. In addition, although the global level of MLLs and histone H3K4-trimethylation does not get affected but is modulated at the promoters of specific genes over different phases of cell cycle.

3.2. Materials and methods

3.2.1 Cell culture and synchronization

HeLa cells were grown in growth media [Dulbecco's modified Eagle's media (DMEM) supplemented with heat inactivated fetal bovine serum (FBS, 10%), L-glutamine (1%) and penicillin/streptomycin (0.1%)] as described previously (64,106,107). Cells were synchronized at G1/S phase using double thymidine treatment as described previously (111,120). In brief, cells were grown in a 10 cm tissue culture plate up to 25% confluence, washed twice with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) and treated with 10 mM thymidine (Sigma) for 18 hr. Then the cells were washed twice with 1X PBS before releasing into fresh media for 9 hr, and blocked again by addition of 10 mM thymidine for additional 17 hr. Finally, the cells were released into fresh growth medium at G_1/S phase after washing twice with 1x PBS and analyzed at every 2.5 hr intervals. At each time point 4×10^5 cells were used for analysis.

3.2.2 Preparation of whole cell extract, histones and Western blotting

HeLa cells (4×10^5) were harvested, incubated with 200-µL whole cell extract buffer (50 mM Tris-HCI pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.2 mM PMSF, 1X protease inhibitors) on ice for 20 min and centrifuged (10000g for 10 min).

The supernatant fluid was used as whole cell extract and the pellet was used for histone purification as described previously (111) (please refer to section 2.2.9). The whole cell protein extracts and histones were analyzed by western blotting using anti-MLL1 (Bethyl lab), anti-Set1 (Bethyl lab), anti-Ash2 (Bethyl lab), anti-Rbbp5 (Bethyl lab), anti-CGBP (IMGENEX), anti-cyclin B (Santa Cruz), anti-cyclin E (Santa Cruz), anti-H3K4-trimethyl (Upstate Biotech), anti-H3S28-phosphoryl (Upstate Biotech) and anti-H3K9-dimethyl (Upstate Biotech) antibodies (as described in section 2.2.2).

3.2.3 RNA purification and reverse transcription-PCR (RT-PCR)

For the RNA purification, cells were resuspended in 200 µL diethylpyrocarbonate (DEPC) treated buffer A (20 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, 0.2 mM PMSF), incubated on ice (10 min) and centrifuged at 3500×g for 5 min. The supernatant fluid (cytoplasmic extracts) was subjected to phenol/chloroform extraction followed by ethanol precipitation to obtain the cytoplasmic mRNAs (please refer to section 2.2.9). The quality of RNA was checked by 1% agarose gel electrophoresis. The mRNA was washed with DEPC treated 70% ethanol, air dried, resuspended in DEPC treated water, quantified and subjected to RT-PCR. Reverse transcription reactions were performed in a total volume of 25 µL containing 1 µg of total RNA, 2.4 µM of oligo-dT, 100 U of MMLV reverse transcriptase (Promega), 1X first strand buffer (Promega), 100 µM dNTPs, 1 mM DTT and 20 U of RNaseOut (Invitrogen). This cDNA (1 µL) was PCR amplified with specific primer pairs listed in Table 3.1.

Table 3.1 Nucleotide sequence of primers used in PCR and ChIP analysis**.**

Transcript	Forward primer (5´→3´)	Reverse primer (5´→3´)
MLL1	GAGGACCCCGGATTAAACAT	GGAGCAAGAGGTTCAGCATC
Ash2	CCTGAAGCAGACTCCCCATA	AGCCCATGTCACTCATAGGG
Rbbp5	GCATCCATTTCCAGTGGAGT	TGGTGACATCCACTTCCTCA
CGBP	GCCACACGACTATTCTGTGA	CAGTAATGGCGATTGCACTG
Cyclin E	TTTCAGGGTATCAGTGGTGCGACA	ACAACATGGCTTTCTTTGCTCGGG
Cyclin A	AAGAAGCAGCCAGACATCACGGAA	AGCTGCAGTTTCCCTCTCAGAACA
Cyclin B	TTGATACTGCCTCTCCAAGCCCAA	TTGGTCTGACTGCTTGCTCTTCCT
HoxA5	GGCTACAATGGCATGGATCT	GCTGGAGTTGCTTAGGGAGTT
HoxA7	TTCCACTTCAACCGCTACCT	TTCATCATCGTCCTCCTCGT
HoxA10	CCATAGACCTGTGGCTAGACG	GAGACTTTGGGGCATTTGTC
p18	TGCGCTGCAGGTTATGAAACTTGG	AGGGCAGGTTCCCTTCATTATCCT
p19	TGCAGGTCATGATGTTTGGCAGCA	ACAGCAGTGTGACCCTCTTGAACT
p21	GACACCACTGGAGGGTGACT	CAGGTCCACATGGTCTTCCT
p27	ACTTGGAGAAGCACTGCAGAGACA	GTCGCTTCCTTATTCCTGCGCATT
HoxA5 (P) [a]	AGTAAGTCCCGAAGGGCATC	GAGAGACTGGGCTCTGTTGG
HoxA7 (P) [a]	GAGCCTCCAGGTCTTTTTCC	ACACCCCCAGATTTACACCA
HoxA10 (P) [a]	CTCCTGGCCCATCAATACAG	TAGCCCTTTCTGGCTGACAT
Actin	AGAGCTACGAGCTGCCTGAC	GTACTTGCGCTCAGGAGGAG

[a]Primer pairs specific to promoters of respective genes

3.2.4 Immunofluorescence studies

HeLa cells were grown on cover slips, synchronized, fixed in 4% p-formaldehyde, permeabilized with 0.2% Triton-X-100, blocked with goat serum, incubated (1 hr) with respective primary antibodies (MLL1, CGBP, Ash2, Rbbp5, H3K4-trimethyl and H3K9-dimethyl antibodies), washed and incubated with FITC or rhodamine (Jackson Immuno Research lab) conjugated secondary antibodies. Nuclear counter staining was performed with DAPI (see section 2.2.6). Immuno-stained cells were mounted and observed under fluorescence microscope (Nikon Eclipse TE2000-U).

3.2.5 Antisense mediated knock down of HoxA5, MLL1 and chromatin immuno-precipitation (ChIP) assay

HeLa cells were transfected with HoxA5 specific phosphorothioate antisense oligonucleotide (5′-TCAATCCTCCTTCTGCGGGTC-3′) using commercial Maxfect transfection reagent following manufacturer's instructions (MoleculA) (see section 2.2.8). Transfection was carried out at different concentrations of antisense like 2, 3 and 4 µg to achieve the maximum knockdown. For antisense mediated knockdown of MLL1 HeLa cells were transfected with MLL1 specific phosphorothioate antisense oligonucleotide (5′-TGCCAGTCGTTCCTCTCCAC-3′) (Notably, design of the MLL1 antisense, scramble antisense, and knock down experiments were carried out by Dr. Khairul Ansari under the supervision of Dr. Mandal). A scramble antisense without any sequence homology with MLL1 or HoxA5 (5′-CGTTTGTCCCTCCAGCATCT-3′) was used as control for both knockdown experiments. For ChIP assay, HeLa cells (collected at 0, 10 and 20 hrs after synchronization), were fixed with 1% formaldehyde at room temperature for 30 min and washed twice with ice-cold PBS before harvesting, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris.HCl, pH 8.0, 1X protease inhibitors and 0.2 mM phenylmethylsulfonyl fluoride), sonicated until chromatin was sheared to an average DNA fragment length of 0.2–0.5 kb, and then subjected to ChIP assay described previously (109). The length of chromatin fragments were analyzed using 1.5% agarose gel electrophoresis. Soluble cross-linked chromatin was diluted 1:3 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl and 16.7 mM Tris-HCl, pH 8.1), divided into aliquots, and stored at –80 °C. Before using for immunoprecipitation the chromatin preparations were

precleared by incubation with 50 µL protein G-agarose. The protein G-agarose was removed by centrifugation; the pre-cleared chromatin was immunoprecipitated by incubation with the specific antibody for 3 hrs at 4 °C. Antibodies against RNAP II, H3K4 trimethyl (upstate), MLL1 (bethyl lab) were used to carry out chromatin Immunoprecipitation (ChIP). Then, the immunoprecipitates were collected by incubation with protein G-agarose for 4 hrs and centrifuging at 1000g (10 min) at 4 °C. The protein G-agarose bound immunoprecipitates were washed sequentially, once with Low salt immune complex buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1), high salt immune complex buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris-HCl, pH 8.1), LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1 mM EDTA, 10 mM Tris, pH 8.1) and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Finally, the proteins G-agarose beads carrying immuno precipitate were centrifuged at 1000g (10 min) and the bead pellet was used for elution. The pellets were eluted twice with 200 µL of elution buffer (1% SDS, 0.1 M NaHCO3). The elutions were incubated with 20 µL 5M NaCl at 65 °C for 5 hours and then treated with 20 µL proteinase K [1 mg/ml proteinase K (Invitrogen), 1 M Tris HCl (pH 8.0) and 0.25 M EDTA (pH 8.0)] for 3 hrs for decross-linking of immunoprecipitated protein-DNA complexes. Finally, the DNA fragments were purified by phenol/chloroform method (please refer to section 2.9, Chapter 2). All the buffers used in ChIP assay were treated with protease inhibitor (Roche).

3.2.6 Flow cytometry analysis

HeLa cells were grown up to 60% confluency and transfected with MLL1 and scramble antisense oligonucleotides separately using Maxfect transfection (MoleculA) reagents and incubated for 24 hrs. Control and transfected cells were harvested, fixed in 70% ethanol for 2 hrs, washed twice with 1X PBS and then stained with propidium iodide (0.5 µg/ml final concentration in 1X PBS). Then cells were analyzed by flow cytometry (using Fusing Beckman Coulter Cytomics FC500 Flow Cytometry Analyzer).

3.3 Results

3.3.1 Dynamics of MLL1 and its interacting proteins during cell cycle

Prior to analyzing the dynamics of MLL and histone methylation, HeLa cells were synchronized at different phases of cell cycle using double thymidine treatment, as described previously (120). In brief, cells were treated with 10 mM thymidine (18 hr), released into fresh medium (9 hr), and then blocked again by addition of 10 mM thymidine (17 hr) and finally released into fresh medium at G_1/S boundary. Cyclins B and E were used as markers for cell cycle synchronization. In agreement with previous studies cyclin B was expressed prominently in the G2/M phase while cyclin E expression was high in S and G1 phase, but low in G2/M phase (Figure 3.1) (121).

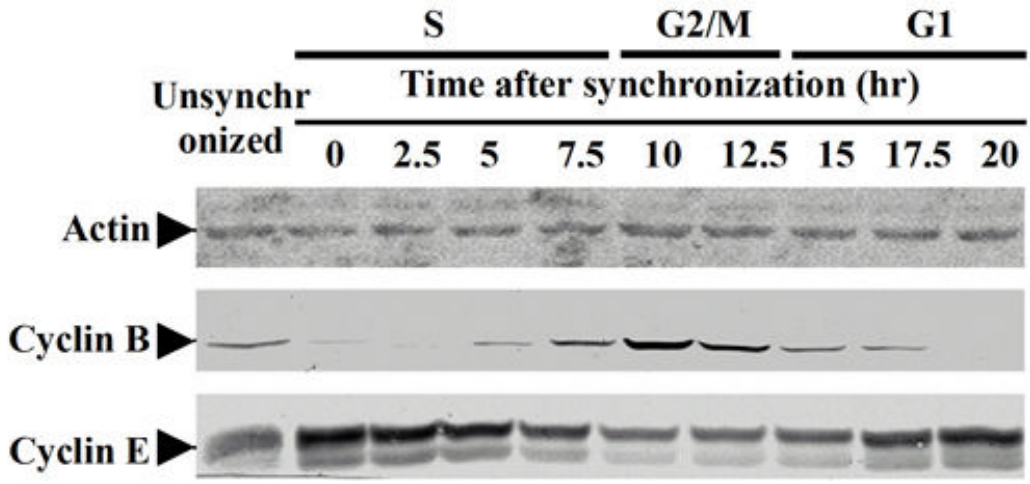

Figure 3.1 Synchronization of HeLa cells. HeLa cells were synchronized using double thymidine treatment and released into G1/S boundary as described previously. Cyclins B and E were used as marker for cell cycle synchronization. Proteins at different phases of cell cycles were analyzed by Western blotting using anti- cyclin E and B antibodies. Actin was used as loading control.

In order to understand the dynamics of MLL1 immunofluorescence staining was performed of the synchronized HeLa cells with anti-MLL1 antibody and visualized its localization using fluorescence microscope at different stages of cell cycle. In agreement with our previous studies, it was found that MLL1 was localized inside the euchromatic region (DAPI less intense) of nucleus at the G1 phase of the cells (G1-phase, panels 1-3, Figure 3.2). However, as the cell entered into mitosis and the chromatin was condensed, most of the MLL1 protein got dissociated from the chromatin and spread into the cytoplasm generating a distinct foot-step (gap) for the condensed chromatin (see metaphase, anaphase and early telophase stages, panels 1-3, Figure 3.2). Notably, the spreading of the MLL1 protein in the cytoplasm coincided with disappearance of the nuclear membrane at the beginning of mitosis (Figure 3.3). Interestingly, at the early telophase when cells were completely divided but the nucleus

of the nascent daughter cells are yet to relax into euchromatin, MLL1 was present in the cytoplasm (early telophase, panels 1-3, Figure 3.2). However, at later stage, MLL1 returned back into the condensed chromatin likely marking the initiation of chromatin relaxation (euchromatin formation) (late telophase, panels 1-3, Figure 3.2). To complement these observations, we studied the distribution of RNAP II in different stages of cell cycle, which shows similar dynamics as that of MLL1 (Figure 3.5).

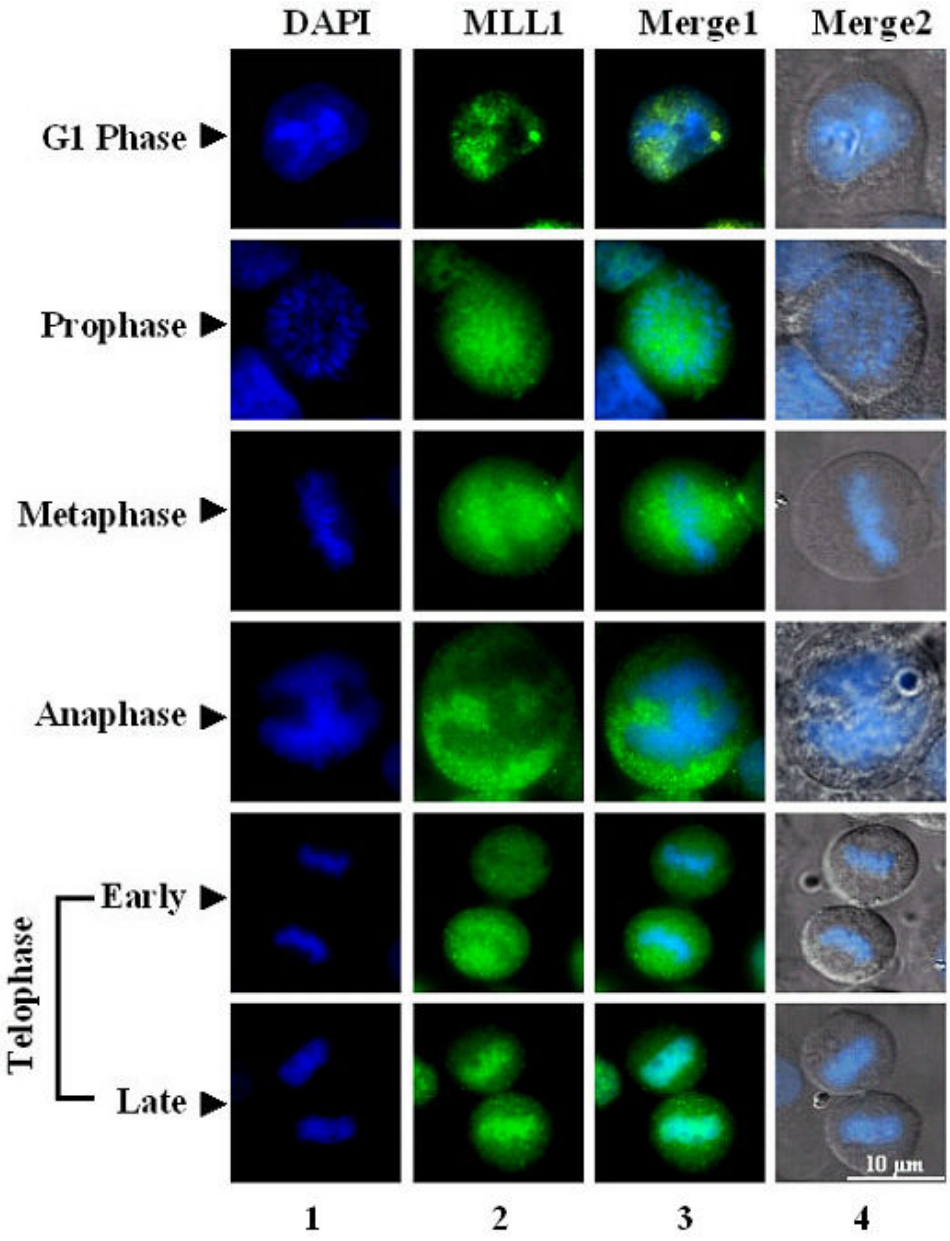

Figure 3.2 Dynamics of MLL1 during cell cycle. Synchronized HeLa cells (at different stages) were subjected to immunofluorescence staining with anti-MLL1 antibody and visualized by immuno-staining with either FITC (green) conjugated secondary antibodies. Cells were co-stained with DAPI to visualize the DNA. Merge 1 shows the merge between DAPI and MLL1 images. Merge 2 shows the merge between DAPI and DIC images (differential interference contrast) of the same cell.

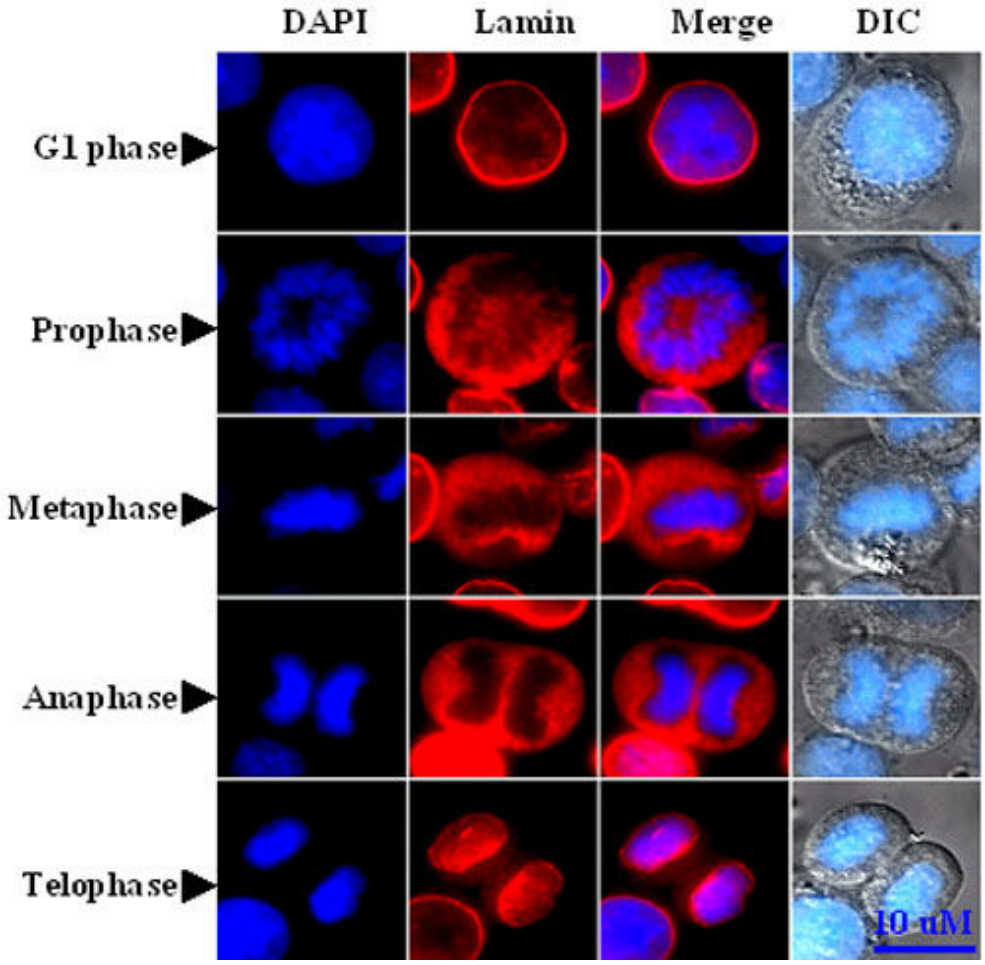

Figure 3.3 Localization of nuclear membrane during cell cycle. Synchronized *HeLa* cells were collected at different phases of cell cycle and immuno-stained with anti-lamin antibody and visualized by immuno-staining with rhodamine (red) conjugated secondary antibodies. Cells were co-stained with DAPI to visualize the DNA.

Recently, Liu *et al.* (2008) performed some immuno-staining experiments with anti-MLL1 antibodies using asynchronous HeLa cells (47). In contrast to our observations, they reported that MLL1 remains associated with condensed chromatins even during mitosis, although, it gets degraded at late M (mitosis) and S-phases. To address this apparent contradictory MLL1 distribution pattern in the mitotic cells, immuno-staining experiments were further performed with several MLL1 interacting

proteins such as CGBP, Ash2, and Rbbp5 using synchronized HeLa cells. Interestingly, each of these MLL interacting proteins (CGBP, Ash2 and Rbbp5) was dissociated from the mitotic chromatin leaving a distinct gap in the mitotic cells in a very similar fashion to MLL1 distribution (Figure 3.4). Notably, in our studies the presence of these distinct gaps for MLL1 and interacting proteins in the mitotic cells was also found in a population of asynchronous cells (data not shown). These results indicate that MLL1 and its interacting proteins dissociate from the mitotic chromatins, spreads into the cytoplasm and distributed in a similar fashion during cell cycle.

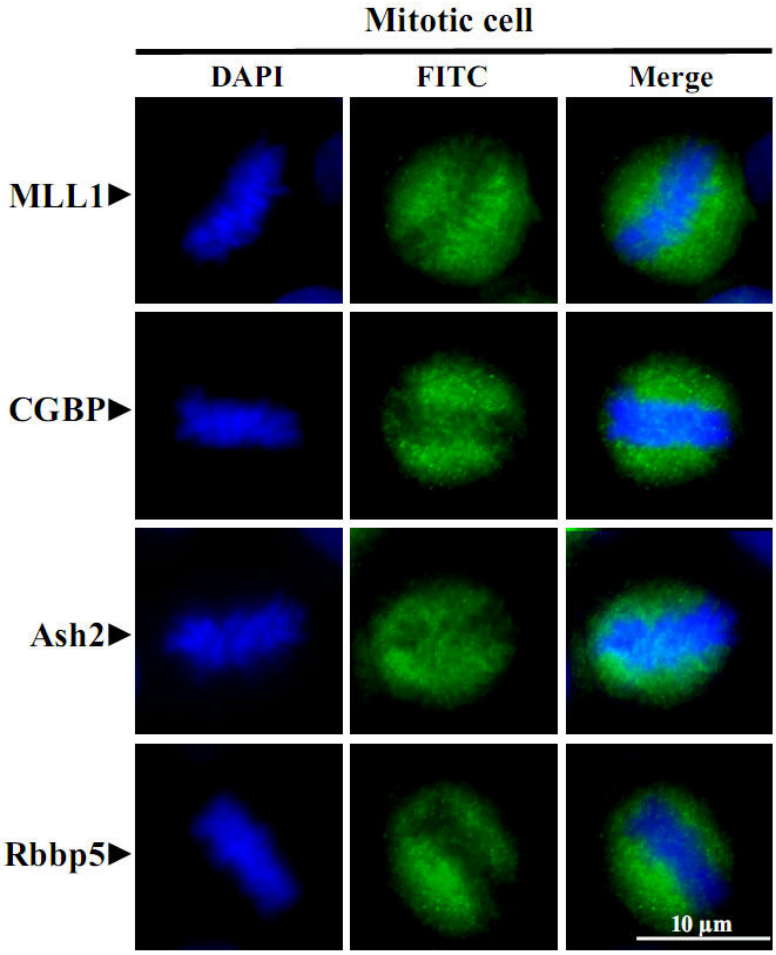

Figure 3.4 Dynamics of MLL associated proteins. Synchronized HeLa cells at metaphase stage (mitosis) were subjected to immunofluorescence staining with anti-MLL1, anti-CGBP, anti-Ash2 and anti-Rbbp5 antibodies and visualized by immuno-staining with FITC (green) conjugated secondary antibodies. Cells were co-stained with DAPI to visualize the DNA. Merge panel shows the overlay between DAPI and FITC images.

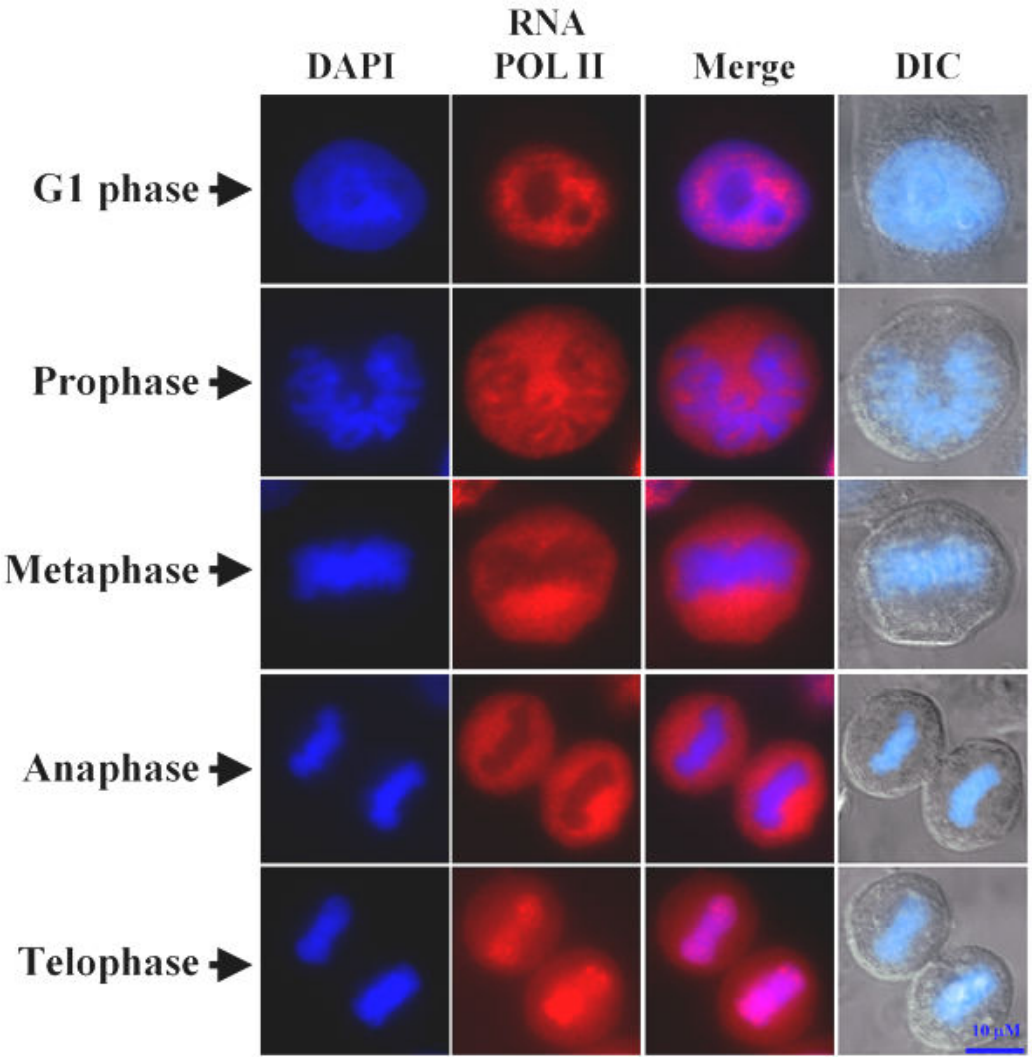

Figure 3.5 Localization of RNAP II during cell cycle. Synchronized *HeLa* cells were collected at different phases of cell cycle and immuno-stained with anti-RNAP II antibodies and visualized by immuno-staining with rhodamine (red) conjugated secondary antibodies. Cells were co-stained with DAPI to visualize the DNA.

3.3.2 H3K4-trimethylation marks are associated with mitotic chromatins

In contrast to MLL1 and their interacting proteins, the H3K4-trimethyl marks behave differently during cell cycle. Notably, like MLL1, H3K4 trimethylation is well known to be associated with transcriptionally active euchromatin (64,67). Therefore,

MLL1 and H3K4-trimethylation are shown (by our laboratory and others) co-localized in the euchromatic regions of the nucleus and this is likely because of their involvement in active gene expression (64,67). Herein, in order to understand the dynamic association of H3K4-trimethylation with chromatin during cell cycle, immunofluorescence staining of the HeLa cells was performed with anti-H3K4-trimethyl antibody at different stages of the cell cycle. Cell nucleus was counterstained and visualized by using DAPI staining. As expected, in the G1 phase, H3K4-trimethyl marks were localized in the DAPI less intense regions in the nucleus (that represents less condensed euchromatin) leaving gaps in the more intense DAPI stained regions (that represents more condensed heterochromatin) (G1-phase, panels 1-3, Figure 3.6). However, in contrast to MLL1, as the cells entered into mitosis and DNA is condensed, H3K4-trimethyl marks still remained associated with the condensed chromatin and remain so throughout the cell cycle (panels 1-2, Figure 3.6). As H3K4-trimethylation is well recognized as mark for active chromatins, the existence of these marks even in the highly condensed mitotic chromatin is unanticipated. The contradictory association of MLL1 and H3K4-trimethyl marks indicates at least two different possibilities. H3K4-trimethyl marks that are introduced in the transcriptionally active euchromatins at G1 phase are not removed from histones and carried over throughout the cell cycle. Secondly, even in the condensed chromatin during mitosis, some genes remained transcriptionally active and those are marked by the H3K4-trimethylation. Notably, the association of H3K4-trimethyl mark with the mitotic chromatin is also previously observed by Valls *et al.* (2005) (122). H3K9-dimethylation was also analyzed as the

mark of heterochromatin and as expected H3K9-methylation marks were found to be associated with heterochromatin throughout the cell cycle (panels 4-6, Figure 3.6).

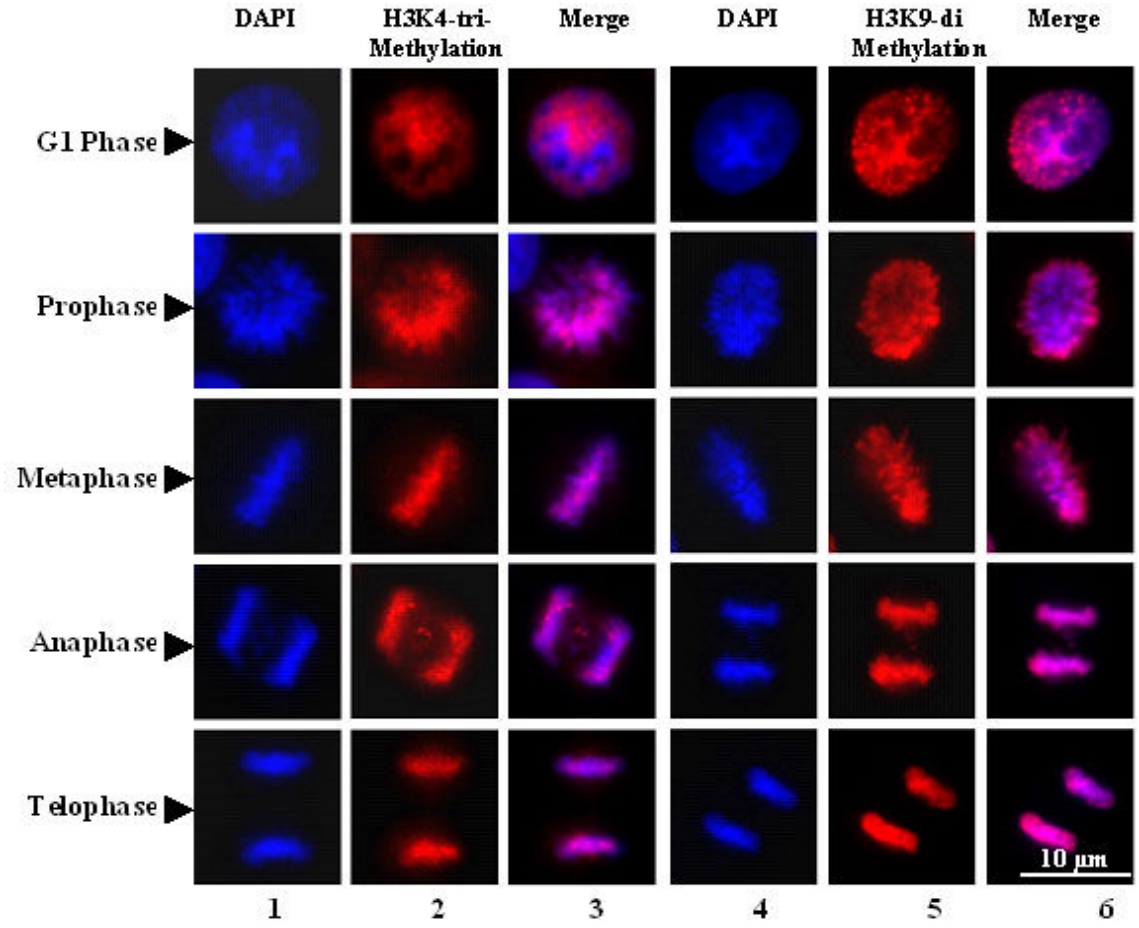

Figure 3.6 Dynamics of H3K4-trimethylation and H3K9-dimethylation during cell cycle. Synchronized HeLa cells (at different stages) were subjected to immunofluorescnce staining with H3K4-trimethyl and H3K9-dimethyl antibodies and visualized by immuno-staining with rhodamine (red) conjugated secondary antibodies. Cells were co-stained with DAPI to visualize the DNA. Merge panels show the overlay between DAPI and rhodamine stained images.

3.3.3 MLL1 and H3K4-trimethylation level remained unaffected while Hox genes were differentially expressed during cell cycle.

As MLL1 and H3K4-trimethylation showed distinct dynamics during cell cycle progression, the expression profiles of MLL1, CGBP, Ash2, and Rbbp5, along with cyclins E and B were analyzed as a function of cell cycle. Western blot analysis of the whole cell extract and histones from different stages of cell cycle demonstrated that the overall levels of MLL1 and H3K4 trimethylation do not get affected throughout the cell cycle (Figure 3.7). Similarly, MLL interacting proteins such as CGBP, Ash2 and Rbbp5 were not affected during cell cycle (data not shown).

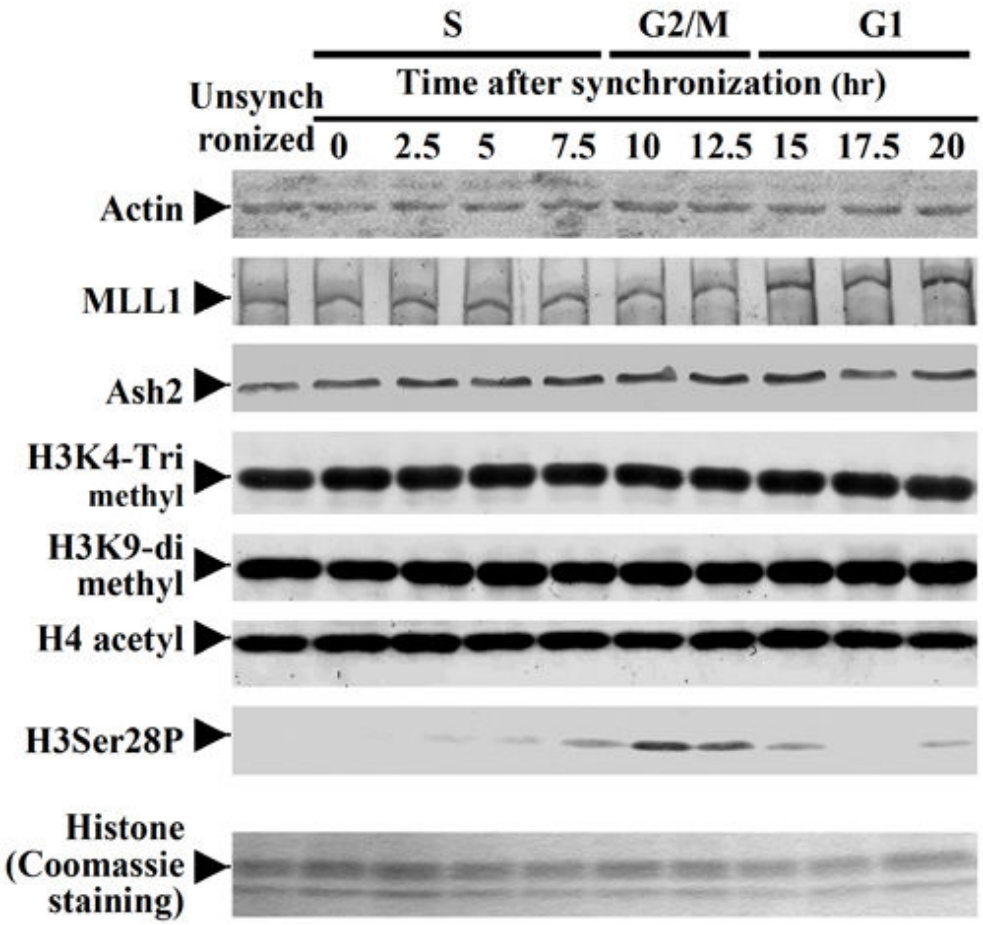

Figure 3.7 MLL1 expression and histone modifications during cell cycle. Synchronized Hela cells were collected every 2.5 hrs intervals after release at G1/S-boundary and subjected to whole cell protein extract and histone purification. The protein extracts were analyzed using Western blotting with antibodies specific to MLL1, Ash2 and CGBP. Actin was used as loading control. Histones were probed with anti-H3K4-trimethyl, H3K9-dimethyl, H4-acetylation and H3S28-phosphorylation antibodies. Cyclin-B, cyclin-E expression and H3S28 phosphorylation were used as marker for cell synchronization. Coomassie stain for histone was used as loading control.

Notably, again, our observations showing the unaffected global level of MLL1 (protein level) during cell cycle contradict with the observation by Liu *et al.* (2008), who demonstrated that MLL1 protein gets degraded during late M (mitosis) and S

phases (47). However, in agreement with Liu *et al*. (2008), using RT-PCR analysis, it was observed that the expression level of MLL1 at the mRNA level is increasing from G1/S towards G2/M (Figure 3.8). Furthermore, to confirm the cell synchronization, the change in phosphorylation level of H3Ser28 was analyzed, which is considered as the marker for mitotic cells. Indeed, in agreement with previous studies, it was found that H3Ser28 phosphorylation is only observed during mitosis indicating proper cell cycle progression and synchronization (Figure 3.7) (123,124). These observations further supported the fact that H3K4-trimethylation marks are maintained throughout cell cycle even in the mitotically condensed chromatins. As the levels of MLL1 protein remained unaffected, it can be conclude that MLL proteins are not degraded during mitosis rather moved away from the condensed chromatin towards the cytoplasm generating the MLL1 gaps present in the mitotic chromatin.

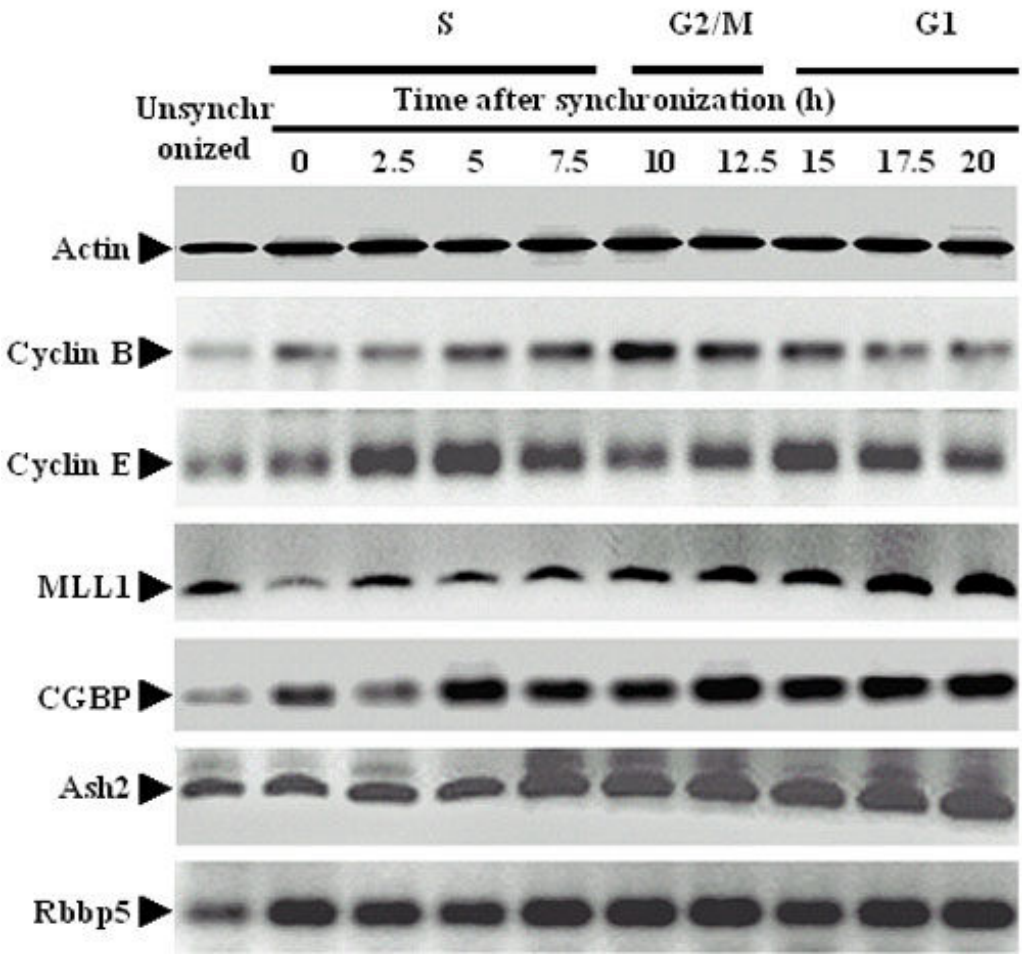

Figure 3.8 RT-PCR analysis of MLL1 and associated proteins in cell cycle. Synchronized HeLa cells were harvested for total RNA extraction. Total RNA was isolated and subjected to RT-PCR analysis using primers specific to MLL1, CGBP, Ash2, Rbbp5, Cyclin E and Cyclin B for detecting their expression in the different phase of cell cycle. This is a DNA gel and actin was used as quantitative control of RNA used in RT-PCR.

In contrast to MLL1 and H3K4-trimethylation levels, the MLL target Hox genes were differentially expressed during cell cycle. The expression profiles of three Hox genes were analyzed, HoxA5, A7 and A10. HoxA5 is expressed low at the beginning of S phase and increased by ~ 8 fold as the cell progressed from S to G2/M (0 to 10 hrs) and then decreased to almost the initial level and remained so through mitosis and G1 phase (Figure 3.9A and B). In contrast, HoxA7 expression was low in the beginning (S-

phase) and increased gradually all the way from S-G2/M-G1 phases (0 - 20 hrs) (Figure 3.9A and B). Interestingly however, HoxA10 is only expressed in the beginning of S phase and shut down almost completely for the remaining phases of cell cycle (Figure 3.9A and B). Cyclin B and E were used as marker and their expression patterns were in agreement with previous studies and results presented in Figure 3.1.

Recently several studies indicated that Hox gene might also be involved in cell cycle progression. For example, HoxA5 activates p53, which regulates the expression of P21, an inhibitor of cyclin dependent kinases (CDKs) that are critical for cell cycle progression. Furthermore, Bromleigh and Freedman (2000) showed that HoxA10 directly upregulates the expression of p21 leading to cell cycle arrest at the G1 phase (86). Both p21 and p53 plays vital role in cell cycle regulation. Thus, although, further studies are needed to elucidate the detail functions of different Hox genes in cell cycle regulation, our studies showing the differential expression of HoxA5, A7 and A10 at different phases of cell cycle indicate that these genes may have critical roles in cell cycle check point regulation likely via involvement of p53 and p21.

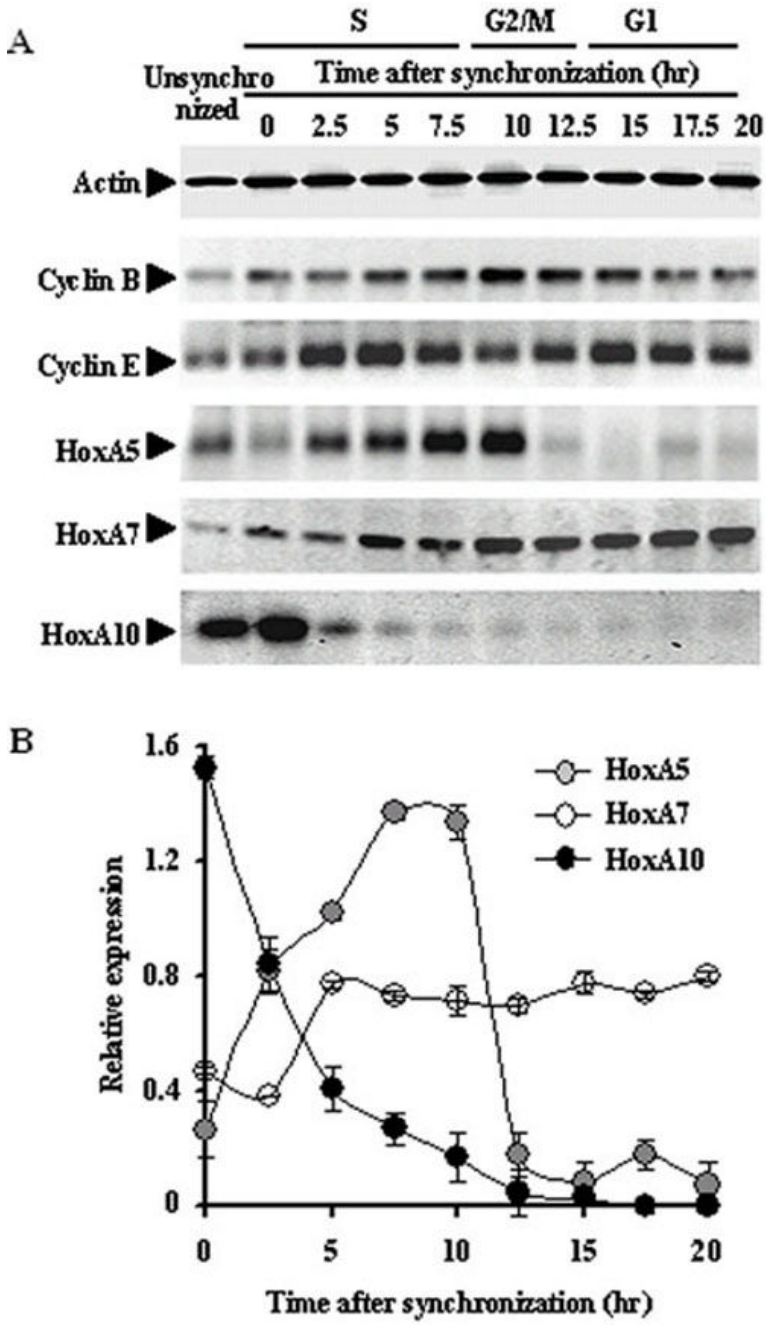

Figure 3.9 Hox gene expressions during cell cycle. (**A**) Total RNA was isolated from Hela cells at different phases of cell cycles and analyzed by RT-PCR using primers specific to cyclin E, cyclin B, HoxA5, HoxA7, and HoxA10. Actin was used as loading control. (**B**) PCR products of Hox genes in Fig A were quantified (ImageQuant TL, Amersham Biosciences) and plotted. Experiments were repeated thrice and bars indicate the standard errors on mean (SEM).

3.3.4 MLL1 and H3K4 trimethylation are critical for Hox gene regulation during cell cycle

In order to understand the molecular mechanism of differential regulation of Hox gene expression, the changes in histone H3K4 methylation was analyzed, recruitment of MLL1 and RNA polymerase II (RNAP II) at the Hox gene promoters at different phases of cell cycle using chromatin immuno-precipitation assay (ChIP) (64). ChIP analysis was performed using anti-RNAP II, anti-MLL1 and anti-H3K4-trimethyl antibodies at three different phases of cell cycle [0 hr (S), 10 hr (G2/M) and 20 hr (G1)] after synchronized cells were released at S phase. In case of HoxA5, recruitment of RNAP II and MLL1 as well as the level of H3K4 trimethylation in the promoter were low at the S phase (0 hr) and increased by 1.7 fold at G2/M (10 hr) and again decreased at G1 (20 hr) (Figure 3.10A and B). Notably the enrichment of RNAP II, MLL1 and H3K4 trimethylation at the HoxA5 gene promoter at G2/M phase is correlated with its expression profile (as shown in Figure 3.9A and B) indicating importance of MLL1 and H3K4-trimethylation in HoxA5 gene regulation during cell cycle progression. Notably, association of some amount of RNAP II with HoxA5 gene promoter at 20 hrs (although much lower in comparison to 10 hrs) indicates that some of amount of basal transcription is still continuing at this stage of cell cycle. Similar to HoxA5, the occupancy of RNAP II, MLL1 and H3K4-trimethylation in HoxA7 and HoxA10 gene promoters were also correlated with their respective expression profile (compare Figure 3.9A-B with 3.10A-B). In case of HoxA7 promoter, the recruitment of RNAP II, MLL1 and the level of H3K4 trimethylation were low at the beginning (S phase) and gradually increased as the cell progressed from S-G2/M-G1 reaching a maximum at G1 (20 hr)

(Figure 3.10A and B). In case of HoxA10 gene, a significantly higher level of RNAP II, MLL1 recruitment and H3K4 trimethyl marks were observed at the beginning of S phases (0 hr) and then these marks were attenuated for the rest of the cell cycle (10 and 20 hrs) correlating with the expression of the gene (Figure 3.9A and B). The correlation of promoter occupancy of MLL1, H3K4-trimethylation as well as RNAP II in Hox gene expression indicate critical roles of MLL1 and H3K4-trimethylation in differential Hox gene expression during cell cycle.

To further confirm the importance of MLL1 in regulation Hox A5, A7, and A10 genes and cell cycle progression, MLL1 was knocked down using specific antisense and analyzed the expression of Hox genes and cyclins. As seen in Figure 3.10C, the knock down of MLL1 down regulated the expression of HoxA5, A7, and A10 genes. Notably, HoxA5 expression is almost completely abrogated while HoxA7 and A10 are only partially down regulated. The partial down-regulations of HoxA7 and A10 upon knock down of MLL1 indicate that in addition to MLL1, alternate other factors may regulate their expression. Notably, cyclin B and E were also down regulated under MLL1 knocked down environment (data not shown).

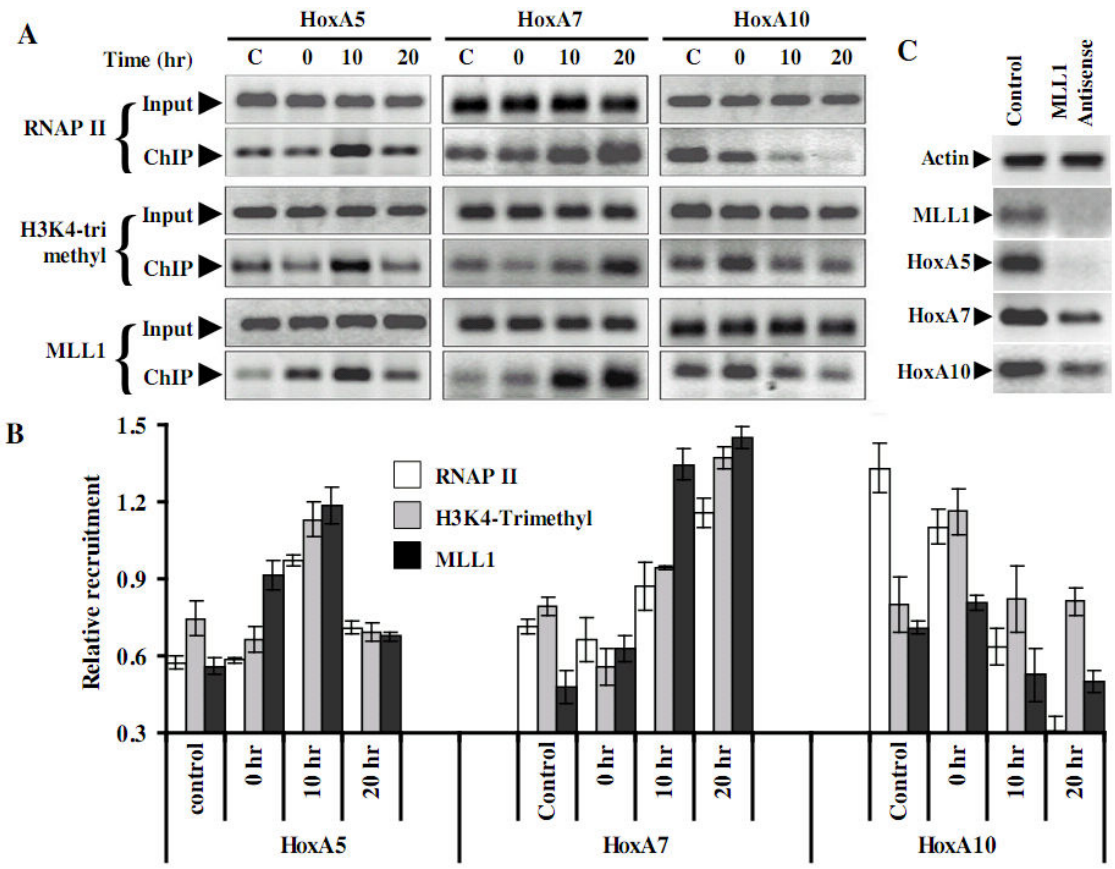

Figure 3.10 Chromatin Immuno-precipitation experiments (ChIP). (**A**) HeLa cells were collected at S (0 hr), M (10 hr) and G1 (20 hr) phases of cell cycle (after synchronization), fixed with formaldehyde, sonicated and analyzed by ChIP assay using antibodies against RNAP II, H3K4-trimethyl and MLL1. The immuno-precipitated DNAs were PCR amplified using primers specific to the promoters of HoxA5, A7, and A10 genes. (**B**) The PCR products in Fig. C were quantified and fold increase in the ChIP PCR products compared to control (input) were plotted for respective Hox genes. Bars indicate standard errors of mean. (**C**) Antisense mediated knock down of MLL1 and its effect on the expression of Hox genes. HeLa cells were transfected with MLL1 antisense or scramble phosphorothioate antisense for 48 h, then RNAs from the transfected cells were analyzed by RT-PCR using primers specific to MLL1, HoxA5, HoxA7, and HoxA10. Actin was used as leading control. Notably, design of the MLL1 antisense, scramble antisense, and knock down experiments were carried out by Dr. Khairul Ansari under the supervision of Dr. Mandal and figure 3.10 C is kindly provided by them.

To confirm further the roles of MLL1 in cell cycle regulation we examined the effects of knock down of MLL1 on cell cycle progression using flow cytometry

analysis. In brief, HeLa cells (at 60 % confluence) were transfected with MLL1 specific antisense for 24 hrs, stained with propidium iodide and then analyzed using flow cytometry analyzer. Interestingly, as seen in Figure 3.11, upon treatment with MLL1 antisense, cell population at G2/M phase increased from 3.5 % (control) to 19.7 % (antisense treated). Notably, application of the scramble antisense (with no homology to MLL1) also slightly increased the G2/M cell population (up to 7 %) in comparison to the control. The MLL1 antisense mediated increase in cell population at G2/M phase indicated that knock down of MLL1 resulted in cell cycle arrest at G2/M phase. These observations further confirmed the significant roles of MLL1 in cell cycle progression.

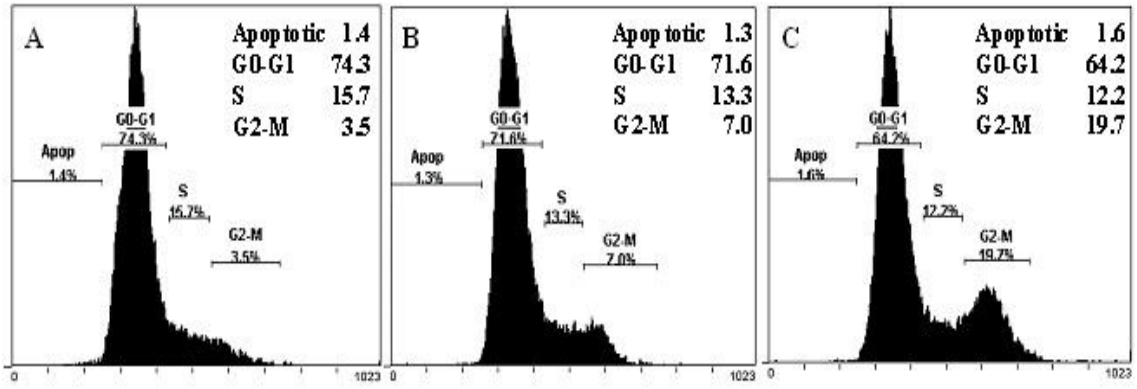

Figure 3.11 Knock down of MLL1 induces cell cycle arrest at G2/M phase. HeLa cells were treated with MLL1 and scramble antisense separately for 24 hrs and subjected to flow cytometry analysis. (**A**) Control cells treated with no antisense; (**B**) Cells treated with phosphorothioate scramble antisense (no homology to MLL1); (**C**) Cells treated with MLL1 specific antisense. The cell populations at different stages of the cell cycles are shown inside the respective panels. Notably, the flow cytometry experiments were carried out by Dr. Khairul Ansari under the supervision of Dr. Mandal and and this figure is kindly provided by them.

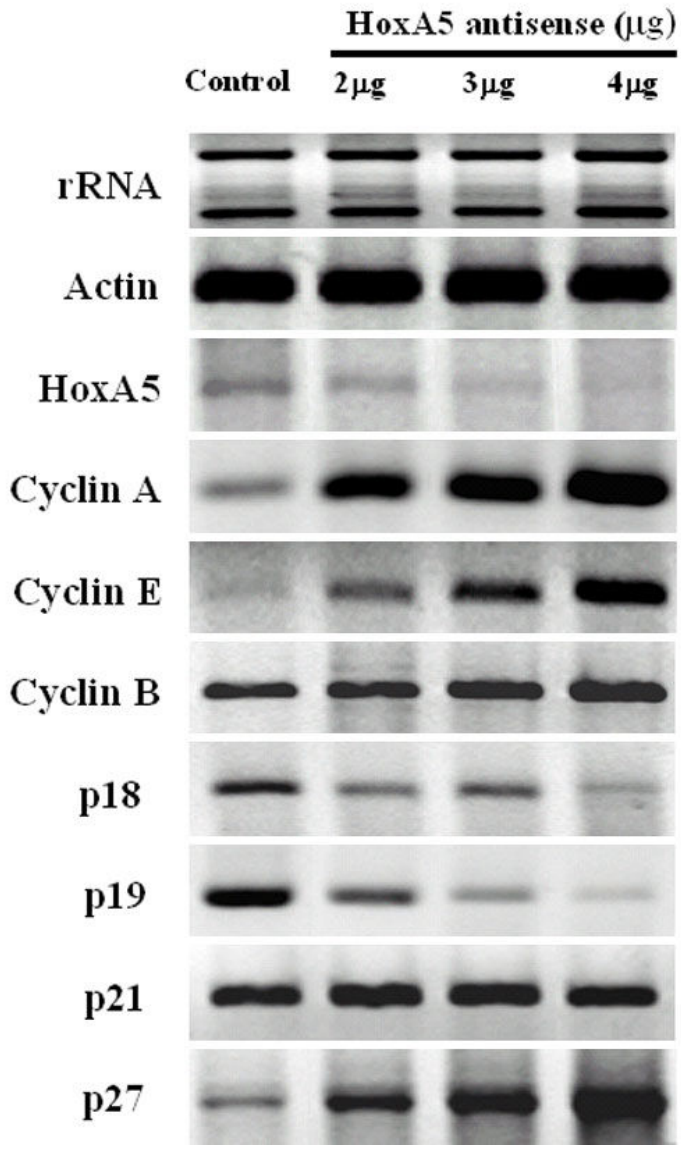

Figure 3.12 Antisense-mediated knock down of HoxA5. Different concentration of antisense (μg) was used to achieve the maximum level of HoxA5 kncokdown. A scramble antisense without any specificity with HoxA5 was used as control. Expression level of different cell cycle regulatory proteins was analyzed by RT-PCR using specific primers under HoxA5 knockdown condition. Actin was used as control. Level of rRNA was analyzed to monitor the quality of total RNA.

3.3.5 Role of MLL regulated Hox genes in cell cycle regulation

To further understand the role of MLL1 in cell cycle regulation, it is important to study the critical role of MLL1 regulated Hox genes in cell cycle. Furthermore, Bromleigh and Freedman (2000) showed that Hox A10 arrests cells at the G1 phase by directly upregulating the expression of p21 in both monocytic and fibroblast cell lines (86). This encouraging study makes it interesting to understand role of HoxA5 in cell cycle regulation. In order to understand that, antisense mediated knockdown was carried out using antisense specific to HoxA5 and expression level of different cell cycle regulatory proteins was studied under HoxA5 knockdown condition. In brief, HeLa cells at 60% confluence were treated with different concentration of HoxA5 antisense to achieve maximum knockdown and total RNA was purified. Knock down of HoxA5 was confirmed by RT-PCR (Figure 3.12). RT-PCR analysis was also carried out for other cell cycle regulatory proteins using primer specific to them under HoxA5 knock down condition. Cyclin B, required by cells to progress through mitosis, and p21, a well known G1 inhibitor, were observed to be unaffected (Figure 3.12), while proteins like cyclin A and E, responsible for G1/S progression were observed to be upregulated. Interestingly, p18 and p19, G1 specific cell cycle inhibitors, showed reduced expression with the knock down of HoxA5. In contrast to the observation for p18 and p19, p27 was observed to be up regulated under HoxA5 knock down condition. p18, p19, p21and p27 are responsible for arresting cell cycle at G1 phase by their inhibitory effect on different cyclin dependent Kinases (48). As the cell cycle regulatory proteins function inside cell as part of different multiprotein complexes, HoxA5 based on their requirement for the

different phases of cell cycle possibly regulates them differentially. These observations strongly indicate a vital role played by HoxA5 in cell cycle regulation, though exact role of HoxA5 in regulation of G1 inhibitors requires detail investigation.

3.4 Discussion

Results from this study demonstrated that MLL1 and H3K4-trimethylation have different dynamics during cell cycle. MLL1, which is well known for transcription activation, remained associated with transcriptionally active chromatin (euchromatin), dissociated from condensed mitotic chromatin and returned back at the end of telophase when likely the nucleus starts to relax. In contrast, H3K4-trimethyl mark, which is also recognized as mark for gene activation, remained associated with euchromatin in the G1-phase and stayed on even with condensed chromatin throughout the cell cycle. The global levels of MLL1 protein and H3K4-trimethylation do not get degraded or removed from the cells during mitosis, while the H3Ser28 phosphorylation was only observed during mitosis. However, the recruitment of MLL1 and level of H3K4-trimethylation were modulated in the promoter of specific Hox genes as a function of their expression. Importantly, as H3K4-trimethylation fluctuates at the specific gene promoters, it can be hypothesized that the H3K4-trimethylation marks that are present in the S-phase may not be the same marks in other phases of cell cycles (as seen by immunofluorescence staining and Western blotting), rather old marks are removed and new marks are introduced at least in some of the promoters. Furthermore, even though distinct gaps were observed for MLL1 (as well as their interacting proteins) in the immunofluorescence staining experiments in the region of mitotically condensed

chromatin, ChIP experiments demonstrated that MLL1 is still bound with the promoters of active Hox genes even during mitosis. These observations indicate that some amounts of MLL1 proteins are still associated with the chromatin even during mitosis, although most of the proteins are migrated away from the chromatin.

These studies also demonstrated that Hox genes (HoxA5, A7 and A10) are differentially regulated during cell cycle and MLL1 occupancy at the Hox genes promoter fluctuates as a function of the Hox gene expression. Notably, HoxA5 is shown to activate p53, which regulates the expression of cyclin dependent kinase inhibitor p21 (85). Similarly, HoxA10 is known to up regulate p21 leading to cell cycle arrest at G1 phases in both monocytic and fibroblast cell lines (86). Thus, it is possible that HoxA5, similar to HoxA10, regulates the cell cycle via P53 and p21 channel. In order to understand that we carried out antisense mediated knockdown of HoxA5 and observed a differential regulation of different G1 inhibitors like p18, p19, p21 and p27. Similar to Hox gene, MLLs are also shown to interact with E2F family of proteins and regulate cell cycle regulatory genes including cyclins (48). Thus, our results and independent observations from other different laboratories indicate that both MLL1 and Hox genes are critical players in cell cycle progression. Although further studies are needed to understand the detail roles of MLLs and different Hox genes in cell cycle regulation, our studies demonstrated distinct dynamics and importance of MLL1, H3K4-methylation and selected Hox genes during cell cycle progression.

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BIOGRAPHICAL INFORMATION

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